Bacillus cereus: epidemiology, virulence factors and host-pathogen interactions

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19 **ABSTRACT**

20 The toxin-producing bacterium, *Bacillus cereus*, is an important and neglected human
21 pathogen and a common cause of food poisoning. Several toxins have been
22 implicated in disease, including the pore-forming toxins hemolysin BL (HBL) and non-
23 hemolytic enterotoxin (NHE). Recent work revealed that HBL binds to the mammalian
24 surface receptors LITAF and CDIP1, and that both HBL and NHE induce potassium
25 efflux and activate the NLRP3 inflammasome, leading to pyroptosis. These
26 mammalian receptors, in part, contribute to inflammation and pathology. Other
27 putative virulence factors of *B. cereus* include cytotoxin K, cereulide,
28 metalloproteases, sphingomyelinase and phospholipases. In this review, we highlight
29 the latest progress in our understanding of *B. cereus* biology, epidemiology and
30 pathogenesis, and discuss potential new directions for the research field.

31 **An overview of *Bacillus cereus***

32 *Bacillus cereus sensu stricto* (herein referred to as *B. cereus*) is an important cause of
33 food poisoning in humans. Isolated in 1887, *B. cereus* was considered a harmless
34 contaminant for almost 80 years before it was widely accepted as a pathogen [1, 2].
35 Since the 1960s, *B. cereus* has gained notoriety as the etiological agent for a variety
36 of intestinal and extra-intestinal diseases [3]. These clinical manifestations include
37 gastroenteritis, vomiting, **endophthalmitis** (see Glossary), respiratory tract infections
38 or infections similar to **gas-gangrene** [4] (**Fig. 1**).

39

40 *B. cereus* belongs to a bacterial group known as *B. cereus sensu lato* which also
41 includes *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B.*
42 *weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis,* as well as several newly
43 described members identified through more recent genetic taxonomic analyses, such
44 as *B. gaemokensis*, *B. manliponensis*, and *B. bingmayongensis* [5-9]. These bacteria
45 have a high level of genetic similarity [5, 10, 11], yet can be classified into unique
46 species based on their morphological and physiological features, and the clinical
47 manifestations resulting from these infections [11]. Of these bacteria, *B. anthracis* is
48 recognized for its ability to cause a lethal **anthrax** disease in humans [12]. Although
49 *B. cereus* is considered a neglected pathogen, studies are now beginning to unravel
50 its biology and host-pathogen interactions.

51

52 In this review, we explore the emerging research themes within the *B. cereus* field,
53 including the fundamental biology of virulence factors, their mammalian host receptors
54 and the characterization of immune pathways associated with *B. cereus* infection. We
55 also discuss the current gaps in our understanding of *B. cereus* pathogenesis,

56 including the functions of several poorly characterized virulence factors and the
57 mechanisms mediating their ability to initiate or contribute to disease in the host.
58
59 **General microbiology of *B. cereus***
60 *B. cereus* is a Gram-positive, rod-shaped and spore-forming **facultative anaerobe**
61 [11] (**Fig. 2**) . *B. cereus* strains possess **peritrichous flagella** [13] (**Fig. 2**), which are
62 involved in locomotion and/or toxin secretion [13, 14]. Further, some strains are
63 encased with a crystalline **surface glycoprotein layer** (**S-layer**), covering the cell wall
64 [15, 16]. Proteins within the S-layer mediate adhesion of *B. cereus* to host cells, and
65 provide resistance to gamma-ray radiation [17].
66
67 *B. cereus* strains can vary in their growth and survival characteristics. They are divided
68 into two groups: **psychrotrophic** and **mesophilic**. Psychrotrophic strains grow well
69 at temperatures below 10 °C, but grow poorly at 37 °C [18]. Psychrotrophic *B. cereus*
70 are generally found in chilled foods and, in some cases, fresh foods [19-21]. In
71 contrast, mesophilic strains grow well at 37 °C and can survive at temperatures below
72 10°C [18].
73
74 *B. cereus* can persist and survive in harsh environmental conditions by the production
75 of endospores and formation of biofilms [22-24]. *B. cereus* spores are elongated,
76 characterized by a core surrounded by an inner membrane, peptidoglycan cortex,
77 inner coat and outer coat. The bacterial spores have no metabolic activity and are
78 resistant to heating, freezing, drying, and gamma-ray and ultraviolet radiation [17, 25].
79 These spores are extremely resistant to environmental assaults that would normally
80 kill vegetative bacteria, thereby facilitating persistence in the environment until more

81 favorable conditions return. They also facilitate adhesion to human epithelial cells [26,
82 27]. Biofilms of *B. cereus* form on abiotic surfaces and living tissue, but also persist as
83 floating **pellicles** [24]. These biofilms have been found on central venous catheters
84 and are associated with nosocomial **bacteremia** [28, 29] (**Fig. 1**). Importantly, *B.*
85 *cereus* in biofilms produce higher amounts of secondary metabolites, such as
86 **catalase** and **superoxide dismutases**, compared to **planktonic cells**, contributing
87 to bacterial defense against host responses [30].

88

89 Epidemiological landscape of *B. cereus*

90 Although *B. cereus* infections have been documented worldwide, the body of literature
91 reporting these infections mainly relates to foodborne outbreaks of gastroenteritis in
92 isolated countries [4, 17, 31]. The limited global epidemiological data on *B. cereus*
93 infections is attributed to: (a) the mild and short-duration of symptoms and self-limiting
94 nature of most *B. cereus* infections, which means that individuals generally do not
95 seek medical attention; (b) lack of laboratory testing to confirm whether *B. cereus* is
96 attributed to patient symptoms and/or disease; and (c) *B. cereus* infections are not
97 notifiable to health authorities in most countries, leading to undocumented cases
98 and/or transmission in the community [4, 17, 32]. These factors together lead to
99 underreporting and underestimation of the actual incidence of this pathogen in
100 humans. Epidemiological surveillance is further complicated by a lack of testing for
101 other pathogens which can induce symptoms similar to *B. cereus* [32, 33]. For
102 example, food poisoning caused by infection with the Gram-positive bacteria
103 *Staphylococcus aureus* and *Clostridium perfringens* leads to **emesis** and diarrhea.
104 These infections are largely indistinguishable from *B. cereus* infection [32, 33], which

105 further confounds the validity of epidemiological reporting of *B. cereus* in the context
106 of foodborne outbreaks.
107
108 Despite the lack of accurate global estimates, several consistent trends have emerged
109 amongst foodborne outbreaks associated with *B. cereus* infection. Epidemiological
110 data have shown that rice, pasta, pastry and noodles are associated with emesis,
111 whereas vegetables, meat products and milk products are associated with diarrhea
112 [17, 31, 32, 34]. Furthermore, the diarrheal-type disease has been reported more
113 frequently in Bulgaria, Finland, Hungary and Norway, whereas the emetic-type
114 disease in Japan and the United Kingdom [17]. These trends might reflect the eating
115 habits of individual countries and suggest that certain food groups act as a vehicle for
116 strains of *B. cereus* harboring a specific subset of virulence factors that are more likely
117 to cause either emetic or diarrheal symptoms. Additional epidemiological analyses of
118 *B. cereus* isolates, including profiling of their genetic markers and virulence factors, in
119 different outbreak settings, would provide insights into the distribution and
120 transmission of this pathogen. Further, research efforts focusing on improved
121 biosecurity, food safety and methods of detection would further prevent future
122 outbreaks and minimize the health and economic burden of *B. cereus* infection.
123
124 **Virulence factors of *B. cereus***
125 *B. cereus* produces a collection of virulence factors including pore-forming toxins,
126 **cereulide**, **hemolysins**, enterotoxins, proteases and **phospholipases** (**Fig. 3, Key
127 Figure**). These virulence factors are discussed in detail below:
128
129 *Pore-forming toxins*

130 *B. cereus* secretes two three-component pore-forming toxins called hemolysin BL
131 (HBL) and non-hemolytic enterotoxin (NHE), and a single-component toxin called
132 cytotoxin K (CytK, also known as hemolysin IV). These toxins are produced and
133 secreted primarily during the exponential phase of bacterial growth and are regulated
134 by the master transcriptional regulator PlcR [35].
135
136 HBL is composed of subunits B, L_1 and L_2 [36], whereas NHE is composed of subunits
137 A, B and C [37]. More recent studies have demonstrated that all three subunits of HBL
138 and NHE assemble in a linear and specific order to form a pore on mammalian
139 membranes; HBL subunits bind in the order of B, L_1 and L_2, whereas NHE subunits
140 bind in the order of C, B and A (**Fig. 4**) [38-42]. Furthermore, HBL and NHE pores
141 have been visualized in lipid bilayers of liposomes using cryo-transmission electron
142 microscopy [39, 40]. Subunits of HBL and NHE have also been found to form
143 complexes in solution [43, 44], which could be attributed to the highly hydrophobic
144 nature of these proteins or experimental conditions that induced conformational
145 changes enabling interactions between hydrophobic proteins [44]. In addition, the
146 different techniques used to generate recombinant toxins in these studies may have
147 affected protein folding and exposure of otherwise hidden binding domains [38, 44-
148 46].
149
150 During an infection, the anaerobic or microaerobic environment of the intestine
151 promotes the production and secretion of HBL and NHE [47-49]. These enterotoxins
152 cause damage to epithelial cells by forming pores leading to microvilli injury, osmotic
153 lysis of intestinal epithelial cells and subsequent diarrhea [24]. Indeed, genomic
154 analysis of clinical, food and environmental strains of *B. cereus* has revealed that HBL,

155 NHE and CytK are highly prevalent, with HBL being present in 40-92% of the isolates,
156 NHE in 95-98% of the isolates, and CytK in 50-80% of the isolates [32, 50, 51]. The
157 high prevalence of these pore-forming toxins suggests that the probability of a strain
158 carrying at least one of these toxins is very high and that any of these toxin-bearing
159 strains would be capable of causing disease.
160
161 *Cereulide*
162 Consumption of food products contaminated with the emetic toxin, cereulide, leads to
163 emesis in humans. Cereulide, a cyclic **dodecadepsipeptide,** is similar to a potassium
164 **ionophore**. It is resistant to heat, proteolysis and acidic environments [52]. Cereulide
165 is encoded by a non-ribosomal peptide synthetase gene cluster called *ces,* located on
166 a **mega plasmid** called pCER270. This mega plasmid is related to the pXO1 plasmid
167 of *B. anthracis* [53]. It is hypothesized that cereulide biosynthesis follows a canonical
168 non-ribosomal peptide assembly process [54] similar to other well-characterized cyclic
169 antimicrobial peptides, including gramicidin S [55] and surfactin [56]. However, a more
170 recent non-canonical mechanism of non-ribosomal peptide assembly for cereulide
171 biosynthesis has been proposed [57]. In contrast to the canonical pathway where
172 single monomers are building blocks of **tetradepsipeptide** assembly, the non-
173 canonical pathway utilizes dipeptides as building blocks of tetradepsipeptide assembly
174 [57]. This observation indicates that further structural and functional studies are
175 required to elucidate the complex biosynthesis pathway of cereulide.
176
177 Transcription of the cereulide toxin is governed by the nutrient-responsive
178 transcriptional regulator CodY, the sporulation transcription factor Spo0A, and the
179 sporulation-vegetative, transitional-state transcription factor ArbB [11, 54, 58-60]. The

180 extrinsic environment, and the intrinsic nutritional and developmental cell status
181 collectively dictate CodY- and Spo0A-regulated transcription of cereulide [11, 54, 59,
182 60]. Furthermore, a putative hydrolase CesH encoded by the *cesH* gene present in
183 the *ces* gene cluster was found to be a transcriptional repressor of cereulide [59, 61].
184
185 Cereulide is secreted predominantly during the stationary phase of bacterial growth. It
186 is found in food products, including rice, pasta, milk and dairy products [17, 62].
187 Following ingestion, cereulide is absorbed in the intestine and distributed throughout
188 the body where it can cross the **blood-brain-barrier** or accumulate in the liver,
189 kidneys, fat, and muscle tissue [63]. The emetic effect of cereulide is thought to be
190 dependent on its interaction with the **serotonin 5-HT3** receptors expressed in the
191 stomach and small intestine, inducing gut-to-brain signaling via the **vagus nerve** [64]
192 (**Fig. 3**). In the house musk shrew, the chemical antagonist of 5-HT3 receptors called
193 ondansetron hydrochloride, or surgical severing of the vagus nerve, inhibited
194 cereulide-induced emesis [64]. These findings suggest an involvement of 5-HT3
195 receptors in cereulide-induced emesis, however, whether cereulide directly interacts
196 with 5-HT3 receptors at vagal sensory endings or that it indirectly stimulates secretion
197 of serotonin to activate 5-HT3 receptors is not known [64].
198
199 *Hemolysins and enterotoxins*
200 Hemolysis is a key feature of many strains of *B. cereus*. Indeed, HBL, NHE and CytK
201 induce hemolysis in erythrocytes owing to their pore-forming ability, however, *B.*
202 *cereus* also expresses hemolysin I, hemolysin II and hemolysin III (**Fig. 3**). Hemolysin
203 I (also known as **cereolysin O**) is a cholesterol-dependent **cytolysin** similar to
204 perfringolysin O of *Clostridium perfringens*, whereas hemolysin II and hemolysin III are

205 cholesterol-independent cytolysins [51]. Hemolysin II induces **apoptosis** in mouse
206 macrophages and human monocytes via activation of the apoptotic caspase, caspase-
207 3 [65]. The biochemical properties, structure and mechanism of pore formation of
208 these hemolysins have remained unclear.

209

210 Other enterotoxins of *B. cereus* include the lesser characterized enterotoxin T and
211 enterotoxin FM [66, 67] (**Fig. 3**). Both enterotoxins have been found to be non-
212 cytotoxic to mammalian cells [68] and enterotoxin FM is thought to function as a cell-
213 wall peptidase rather than a functional enterotoxin [69]. The exact role of these toxins
214 in bacterial growth and virulence is unknown and requires further investigation. Given
215 that the production of toxins requires substantial cost to the bacteria in terms of energy
216 and resources, these toxins are probably important at some stage in the lifecycle of *B.*
217 *cereus*. It is intriguing to speculate that these enterotoxins might be secreted to defend
218 against other competing bacteria or single-cell predatory protozoa in the environment
219 [70, 71]. Indeed, an example from another *Bacillus* species is *Bacillus subtilis*, which
220 secretes the tRNase toxin WapA used to extract nutrients from and kill its target prey,
221 *Bacillus megaterium* [72, 73].

222

223 *Metalloproteases*

224 In addition to secretion of hemolysins and enterotoxins, spores of *B. cereus* express
225 two **metalloproteases** called InhA1 and NprA, used for escaping immune surveillance
226 and promote germination leading to establishment of infection in a host [74-76] (**Fig.**
227 **3**). Genomic analysis has revealed that InhA1 and NprA share 90% nucleotide
228 similarity with proteases found in *B. anthracis* and *B. thuringiensis* [74-76]. In *B.*
229 *anthracis,* homologues of InhA1 and NprA are thought to degrade host protease

230 inhibitors, extracellular matrix proteins, and epithelial barrier proteins [75, 77]. It is
231 possible that InhA1 and NprA of *B. cereus* may have similar functions.
232
233 *Phospholipases*
234 An important class of virulence factors of *B. cereus* used to establish an infection in
235 the host is mammalian membrane-damaging phospholipases. Specifically, *B. cereus*
236 produces a **sphingomyelinase (SMase)**, a **phosphatidylinositol-specific**
237 **phospholipase C (PI-PLC)**, and a phosphatidylcholine-specific phospholipase C [78]
238 (**Fig. 3**).
239
240 Previous studies have shown that phospholipases can facilitate hemolysis [79, 80].
241 With the exception of SMase, the role of phospholipases in the pathogenesis of *B.*
242 *cereus* has remained largely unclear [81]. The presence of SMase is associated with
243 increased severity of *B. cereus* infection in mice, leading to lethality which can be
244 abrogated following pharmacological inhibition of SMase [82, 83]. The biochemical
245 properties and pathogenicity of SMase in *B. cereus* infection are discussed further in
246 another review [84]. PI-PLC of *B. cereus* has been shown to complex with
247 Glucosaminyl(α1→6)-d-myo-inositol and facilitates cleavage of membrane-bound
248 GPI-anchored proteins [85-87]. In addition, two synthetic chiral compounds called R-
249 7ABO and S-7ABO were found to be novel inhibitors of *B. cereus* phosphatidylcholine-
250 specific phospholipase C [88], and therefore, these compounds could be further tested
251 in animal models of *B. cereus* infection to validate their potential use as antibacterial
252 therapeutics.
253
254 **Membrane-bound receptors of HBL in mammalian cells**

255 HBL can form pores in the plasma membrane and exert cytotoxicity on mammalian
256 immune and non-immune cells. Whether HBL binds to a specific surface receptor to
257 mediate pore formation was unknown until now. In most cases, HBL subunits bind in
258 the order of B, L_1 and L_2 [38, 39, 41]. A previous study has shown that the B subunit
259 lacking its hydrophobic transmembrane region exhibited stronger binding to the cell
260 membrane of primary mouse macrophages than the wildtype B subunit, suggesting
261 that an unknown structural domain/s of HBL-B can mediate binding to the membrane
262 and that this domain/s might be concealed by the hydrophobic region [40]. Deletion of
263 this hydrophobic region in HBL-B might have stereochemically uncovered a putative
264 binding site to the putative receptor, resulting in a reduction in steric hindrance and
265 stronger binding [40].
266
267 Using a genome-wide CRISPR-Cas9 screen in RAW276.4 mouse macrophages, two
268 mammalian surface receptors called LPS-induced TNF-α factor (LITAF, also known
269 as small integral membrane protein of lysosome/late endosome, or SIMPLE) and the
270 LITAF-like protein called Cell Death Inducing P53 Target 1 (CDIP1) were identified as
271 surface receptors of HBL [89] (**Fig. 3**). This study demonstrated that primary mouse
272 macrophages or Chinese hamster ovary (CHO) epithelial cells lacking the gene
273 encoding LITAF were resistant to HBL-induced cytotoxicity [89]. Further, mice lacking
274 LITAF survived a lethal challenge of purified HBL [89]. Analysis of truncated variants
275 of LITAF showed that the C-terminal residues of this receptor are critical in mediating
276 HBL binding [89]. In the mouse melanoma cell line B16F10, deletion of CDIP1 in
277 addition to LITAF was required to provide resistance to HBL-mediated cytotoxicity [89].
278 These results indicate that LITAF functions as the primary receptor of HBL, whereas,

279 in certain cell types, CDIP1 serves as an independent and alternative receptor to
280 LITAF [89].
281
282 How individual subunits of HBL interact with either of the two receptors and what
283 signaling cascades are initiated following their engagement are intriguing questions
284 for future research. In addition, what might be the physiological advantage of encoding
285 a receptor for HBL which would render a host susceptible to HBL-induced lethality?
286 Earlier studies had shown that LITAF is expressed in multiple cell types, tissues and
287 organs in humans, such as peripheral blood leukocytes, lymph nodes, and the spleen
288 [90]. In human and mouse macrophages, **Toll-like receptors (TLRs)** can induce the
289 expression of LITAF, which serves as a transcription factor mediating the expression
290 of pro-inflammatory cytokines, such as TNF and IL-6 [90, 91]. Further, mutations in
291 LITAF, potentially causing impaired endosomal protein trafficking and degradation, are
292 associated with an inherited motor and sensory neuropathy known as Charcot-Marie-
293 Tooth disease [92]. Therefore, LITAF functions as a trigger of inflammation and
294 guardian of fundamental cellular processes. Given its widespread expression and
295 critical functionality in the cellular physiology of humans, it is intriguing to speculate
296 that *B. cereus* would strategically use HBL to target LITAF to trigger cell death
297 responses in the mammalian host.
298
299 **Novel membrane-bound receptor/s of NHE**
300 Identification of LITAF and CDIP1 opens up exciting opportunities to search for other
301 surface receptors of *B. cereus* toxins which are important for pathogenesis. Indeed,
302 isolates of *B. cereus* lacking HBL can still cause disease in humans [13, 93, 94]. These
303 strains probably express NHE and other virulence factors capable of contributing to

304 the infection [13, 93, 94]. In the context of NHE, the three NHE subunits bind, in most
305 cases, in the order of C, B and A. Several studies have shown that the C subunit NHE
306 lacking its transmembrane region is unable to bind and form pores in the plasma
307 membrane or induce cell death [40, 46]. This observation highlights potential structural
308 differences between the apical binding subunit of HBL and the apical binding subunit
309 of NHE, and further indicates that NHE likely binds to a different cell-surface receptor
310 to that of HBL [40, 46]. It will be interesting to identify the surface receptors of NHE
311 and other *B. cereus* toxins.
312

313 **Immune response to *B. cereus* infection**

314 Innate immunity mounts the first line of defense against infectious diseases. The
315 innate immune system recognizes pathogens and danger signals via germline-
316 encoded **pattern-recognition receptors (PRRs)**. These PRRs include, but are not
317 limited to, cytosolic **inflammasome** sensor proteins and surface and endosomal TLRs
318 [95, 96]. PRRs are expressed in immune cells and non-immune cells, including
319 macrophages, dendritic cells, neutrophils, epithelial cells, endothelial cells, and NK
320 cells [97]. Previous studies have shown that macrophages, dendritic cells and
321 neutrophils recognize both vegetative cells and the spore of *B. cereus*, triggering an
322 immune response [98-100].
323

324 **Inflammasome responses**

325 The inflammasome is a cytosolic protein complex comprising of a sensor protein, the
326 adaptor protein called apoptosis-associated speck-like protein containing a caspase
327 recruitment and activation domain (also known as ASC or PYCARD), and the cysteine
328 protease caspase-1 [101, 102]. The inflammasome sensor proteins belong to either

329 the nucleotide-binding oligomerization domain-like receptors, absent in melanoma 2
330 (AIM2)-like receptors, or the tripartite motif family [103, 104]. To date, NLRP1, **NLRP3**,
331 NAIP-NLRC4, NLRP6, NLRP9b, Caspase-11, AIM2 and Pyrin are known to form
332 inflammasome complexes in response to a wide range of pathogens and danger
333 signals [95, 96]. Activation of the inflammasome results in proteolytic cleavage and
334 secretion of pro-inflammatory cytokines IL-1β and IL-18, and proteolytic cleavage of
335 the pore-forming protein **gasdermin D**, which induces an inflammatory form of cell
336 death known as pyroptosis [105, 106].
337
338 In mouse macrophages and human **monocytes**, *B. cereus* infection activates the
339 inflammasome very rapidly, generally within 3 h [39, 40]. Both HBL and NHE of *B.*
340 *cereus* are sensed by the inflammasome sensor NLRP3, owing to the efflux of
341 potassium through plasma membrane pores formed by either toxin [39, 40] (**Fig. 4**).
342 Assembly of the NLRP3 inflammasome triggers the secretion of IL-1β and IL-18 and
343 induction of gasdermin D-dependent pyroptosis [39, 40].
344
345 The ability of HBL to induce overt inflammation via the NLRP3 inflammasome also
346 results in lethality in mice [39]. Mice lacking NLRP3 were less susceptible to *B. cereus*
347 infection compared with wildtype mice when an intraperitoneal route was used to
348 deliver the bacteria [39]. This finding suggests that NLRP3 is detrimental in response
349 to *B. cereus* infection. This is not entirely unexpected because intraperitoneal
350 administration of the bacteria results in systemic dissemination, and the lack of NLRP3
351 would prevent overt inflammation and lethality in mice. Indeed, administration of a
352 small molecule inhibitor of NLRP3 called MCC950 to mice infected with *B. cereus* can
353 inhibit inflammation, and that this is sufficient to rescue mice from lethality [39, 40].

354 The use of MCC950 or similar inflammasome blockers might be beneficial for those
355 suffering from systemic infection of *B. cereus* and other NLRP3-activating pathogens.
356 In contrast to systemic infection, it is likely that NLRP3 would be protective in response
357 to a localized infection. This speculation would require experimental validation, such
358 as in an orogastric model of infection in mice, whereby *B. cereus* can establish a more
359 localized infection site in the gastrointestinal tract, and that NLRP3 would be able to
360 mount a more localized immune response to the infection.
361
362 Although both toxins can activate the NLRP3 inflammasome, HBL induces a quicker
363 activation than does NHE [40] (**Fig. 4**). The smaller-sized membrane pores assembled
364 by HBL might lead to rapid potassium efflux and inflammasome activation, whereas
365 the larger-sized membrane pores assembled by NHE might lead to delayed potassium
366 efflux and inflammasome activation [107, 108]. It is also possible that, in mouse
367 macrophages, the expression of HBL receptors, LITAF and CIDP1, is higher than the
368 expression of NHE receptors, leading to differential kinetics in the activation of NLRP3
369 in response to the two toxins [89]. Nevertheless, the observation that a single cytosolic
370 inflammasome sensor is used to sense HBL and NHE suggests that this is perhaps a
371 host strategy to exploit the functional conservation and similarity between HBL and
372 NHE. It would be interesting to interrogate how *B. cereus* strains deficient in both HBL
373 and NHE are detected by inflammasome sensors and other cytosolic PRRs.
374
375 **TLRs**
376 The innate immune response triggered by TLRs has been predominantly
377 characterized in mouse and rabbit models of endophthalmitis, a severe intraocular
378 infection mostly associated with post-traumatic injury and vision loss [109]. Earlier

379 studies have shown that TLR2 mediated **polymorphonuclear leukocytes (PMN)**
380 infiltration in the eyes in response to *B. cereus*-induced endophthalmitis in mice [110].
381 The lack of TLR2 did not affect intraocular growth of *B. cereus,* but reduced the
382 secretion of TNF, IL-6 and IFN-γ in the infected eyes [110] (**Fig. 3**). In addition, mice
383 lacking TLR2 exhibited delayed loss of retinal function. However, the inability of TLR2-
384 deficiency to abolish inflammatory cytokine production and provide full protection
385 against loss of retinal function suggests contributions from other innate immune
386 receptors [110].
387
388 Subsequent studies revealed that mice lacking either one of the two adaptor proteins
389 of TLRs, called Myeloid differentiation primary response protein MyD88, or TIR
390 domain-containing adapter molecule 1 (also known as TICAM-1 or TRIF) exhibited
391 reduced intraocular inflammation following *B. cereus* infection [111]. TLR4 signals
392 through MyD88 and TRIF, and thus, an involvement of TLR4 might explain the
393 previous observation that mice lacking TLR2 displayed residual intraocular
394 inflammation following *B. cereus* infection [110, 111]. Indeed, mice lacking TLR4
395 exhibited reduced influx of PMN, retinal damage and intraocular inflammation in
396 response to *B. cereus* infection [111]. However, this result was unexpected because
397 *B. cereus* does not encode LPS, a ligand of TLR4 found in Gram-negative bacteria.
398 The component/s of *B. cereus* that activate TLR2 are likely to be teichoic and
399 lipoteichoic acid or lipoproteins (**Fig. 3**), whereas an unknown virulence factor of *B.*
400 *cereus* might trigger TLR4 signaling. Previous studies have shown that cholesterol-
401 dependent cytolysins, anthrolysin of *B. anthracis* [112] and **pneumolysin** of another
402 Gram-positive bacterium *Streptococcus pneumoniae* [113], can induce TLR4-

403 dependent apoptosis in mouse macrophages. The cholesterol-dependent cytolysin of
404 *B. cereus,* cereolysin, could therefore be a putative ligand of TLR4.
405
406 **CONCLUDING REMARKS**
407 Since the discovery of *B. cereus*, microbiological investigations have largely focused
408 on the biology of HBL, NHE, and cereulide, leaving the role of many other toxins and
409 enzymes undefined. In light of this knowledge gap, key questions concerning the
410 molecular mechanisms in which *B. cereus* virulence factors lead to disease have
411 remained unanswered (see Outstanding Questions).
412
413 Several mammalian receptors of *B. cereus* have now been identified. Identification of
414 NLRP3 as a mammalian cytosolic sensor of HBL and NHE links *B. cereus*, a toxin-
415 producing "extracellular" bacterium, to an innate immune inflammasome pathway.
416 This toxin-inflammasome axis is a critical determinant of inflammation, cell death and
417 disease outcome in response to infection. Additional virulence factors of *B. cereus* are
418 likely to activate the inflammasome pathway; a search for these novel activators
419 represents an exciting area of future research. The subsequent identification of LITAF
420 and CDIP1 as mammalian surface receptors of HBL further shed light on the
421 mechanisms of toxin-receptor interactions. Future studies will focus on understanding
422 the structure-function of these mammalian surface receptors and how their
423 interactions with HBL subunits leads to formation of a multi-component membrane
424 pore. For example, do specific subunits of HBL interact with these receptors? How do
425 these receptors induce a conformational change in subunit B of HBL which leads to
426 recruitment of the other HBL subunits? These structural insights, potentially gained
427 from a crystal structure of HBL in concert with LITAF and CDIP1, might reveal critical

428 sites on the toxins and/or receptors that can be targeted by small molecule inhibitors.
429 This information could inspire a new research avenue for the development of therapies
430 aimed at blocking toxin-receptor-mediated pathology.
431
432 Continuing application of CRISPR-based screening techniques will identify new host
433 receptors and components mediating toxin binding, intracellular signaling, and cell
434 death in response to *B. cereus* infection. This technological advance will be key to
435 characterizing the role of many poorly described virulence factors in the pathogenesis
436 of *B. cereus* infection, such as enterotoxins, phospholipases and metalloproteases.
437
438 Another area of research that is underdeveloped is the role of host genetics in *B.*
439 *cereus* infection. Since LITAF, CDIP1, NLRP3 and 5-HT3 all contribute to the
440 immunopathology and pathogenesis of *B. cereus* infection, it will be interesting to
441 explore whether mutations in these components confer natural resistance to *B. cereus*
442 infection and/or specific virulence factors. Identification of protective mutations, such
443 as mutations in host receptors that would abolish their toxin-binding function, could
444 provide an additional explanation for epidemiological evidence showing a
445 predominance of emetic disease or diarrheal disease observed in foodborne
446 outbreaks. The use of whole exome sequencing on patients from *B. cereus* outbreaks
447 which display a predominance for either emesis or diarrhea may provide clues as to
448 whether such natural resistance exists. Overall, future studies exploring the role of
449 host genetics in *B. cereus* infection will provide a more holistic appreciation of *B.*
450 *cereus* pathogenesis and complement the existing microbiological- and
451 immunological-focused studies in the field.
452

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CONFLICTS OF INTEREST

None to declare.

GLOSSARY

- **Anthrax:** A potentially deadly disease which results in inflammation of the skin, lungs and gastrointestinal tract.

- **Apoptosis:** A programmed cell death pathway induced by apoptotic caspases.

- **Bacteremia:** Bacterial infection of the bloodstream.

- **Blood-brain-barrier:** A complex network of cells within the brain's microvasculature protecting the central nervous system against circulating toxins or pathogens.

- **Cereulide:** An emetic toxin produced by *B. cereus.*

- **Cytolysin:** A toxin that causes cell death.

- **Dodecadepsipeptide:** A oligomeric form of depsipeptide (bicyclic peptide) composed of twelve monomers.

- **Emesis:** The act of vomiting.

- **Endophthalmitis:** An infection of the tissue and/or fluid within the eyeball.

- **Facultative anaerobe:** A microorganism capable of aerobic and anaerobic respiration.

- **Gasdermin D:** A pore-forming protein mediating inflammasome-induced cell death known as pyroptosis.

- **Gas-gangrene:** An infection caused by toxin-producing bacteria characterized by accumulation of gas within the dead tissue.

- **Hemolysins:** Virulence factors of bacteria that lyse red blood cells.

- **Inflammasome:** An innate immune signaling complex assembled within the cytoplasm.

- **Ionophore:** A compound that facilitates ion transport across a cell membrane.

- **Mega plasmid:** A large plasmid >100 kb, found as an extrachromosomal genetic element in bacteria.

- **Mesophilic:** A class of microorganisms that grow well at temperatures between 15 to 45 ^{o}C.

- **Metalloproteases:** Proteases requiring metal ions for catalytic activity.

- **Monocytes:** A type of mononuclear white blood cell.

514
515 • **NLRP3 (Nucleotide-Binding Oligomerization Domain-like Receptor 3):** An
516 inflammasome sensor that responds to a diverse range of stimuli, such as
517 pathogens or endogenous danger signals.
518
519 • **Pattern-recognition receptors (PRRs):** Receptors of the innate immune system
520 which recognize pathogens or endogenous danger signals.
521
522 • **Pellicle:** A type of bacterial biofilm that forms at the liquid-air interphase (i.e.
523 floating).
524
525 • **Peritrichous flagella:** Flagella projecting from all surfaces of a bacterium.
526
527 • **Phospholipase:** An enzyme that hydrolyses lipids into fatty acids.
528
529 • **Phosphatidylinositol-specific phospholipase C:** An enzyme responsible for
530 hydrolyzing lipid phosphatidylinositol and phosphatidylinositol-glycan found on
531 eukaryotic membranes.
532
533 • **Planktonic cells:** Individual bacteria not attached to one another or a surface.
534
535 • **Pneumolysin:** A toxin which has pore-forming activity and is a key virulence factor
536 of several Gram-positive bacteria.
537
538 • **Polymorphonuclear leukocytes (PMNs):** White blood cells with multi-lobed
539 nucleus and cytoplasmic granules.
540
541 • **Psychrotrophic:** An extremophile class of organism capable of growth at less than
542 7 ^{o}C.
543
544 • **Serotonin:** An important chemical that acts as a neurotransmitter and a hormone
545 within the body.
546
547 • **S-layer:** A protein layer of the cell envelope within some species of archaea and
548 bacteria.
549
550 • **Sphingomyelinase:** An enzyme responsible for the degradation of sphingomyelin
551 (a eukaryotic membrane sphingolipid).
552
553 • **Superoxide dismutase (SOD):** An enzyme capable of catalyzing the dismutation
554 of superoxide radicals to oxygen and hydrogen peroxide.
555
556 • **Tetradepsipeptide:** A oligomeric form of depsipeptide (bicyclic peptide)
557 composed of four monomers.
558
559 • **Toll-like receptors (TLRs):** a group of membrane-bound innate immune receptors
560 that recognize pathogens and their products to initiate immune responses.
561

562 563 564 565 566 567

- **Vagus nerve:** One of the 12 cranial nerves that provides autonomic innervation to organs and systems within the body.

- **5-HT3 receptor:** A receptor within the ligand-gated ion channel superfamily.

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842

843 **FIGURE LEGENDS**

844

845 **Figure 1. Environmental reservoirs, routes of transmission, and clinical**
846 **manifestations associated with *B. cereus* infection.**
847 *B. cereus* can be transmitted to humans through consumption of undercooked or
848 contaminated food via the oral-gastric route. Food contamination caused by *B. cereus*
849 can occur during any stages of food harvesting, processing, storage, preparation and
850 consumption. Nosocomial transmission (also known as hospital-acquired
851 transmission) has been reported to occur due to the formation of *B. cereus* biofilms on
852 items such as catheters and bedsheets. These transmission routes account for the
853 majority of gastrointestinal and/or extra-intestinal manifestations in the hospital setting.

854

855 **Figure 2. Macroscopic and microscopic morphologies of *Bacillus cereus*.** (A)
856 Colony morphology of *B. cereus* grown on a blood agar plate under aerobic conditions
857 at 30°C. *B. cereus* colonies are large in size, and have a rough surface and irregular
858 edges, often surrounding by a zone of clearing indicating hemolysis. (B) Gram staining
859 reveals that *B. cereus* is a Gram-positive bacterium. (C) Transmission electron
860 microscopy and negative staining shows peritrichous flagella protruding from *B.*
861 *cereus*. Scale bar, 1 μm. (D) Scanning electron microscopy shows that *B. cereus* is a
862 rod-shaped bacterium. Scale bar, 2 μm. (D) Transmission electron microscopy reveals
863 the ultrastructure of *B. cereus*. Scale bar, 0.5 μm.

864

865 **Figure 3. The virulence factors of *Bacillus cereus***

866 The *B. cereus* cell wall comprises an inner lipid bilayer membrane, a peptidoglycan

867 layer, and an outermost crystalline surface glycoprotein layer (S-layer). Interspersed
868 within the cell wall are teichoic and lipoteichoic acid molecules. *B. cereus* infection
869 may involve TLR2 and/or TLR4 activation, however the bacterial ligands for either
870 receptor have remained unknown (indicated by a question mark). *B. cereus* contains
871 several enzymes involved in virulence, including the metalloproteases InhA1 and
872 NprA, sphinogomyelinase (SMase) and several phospholipases (PL's). Collectively,
873 these enzymes are thought to degrade proteins and lipids within and/or associated
874 with the host cell membrane to establish an infection. Several virulence factors of *B.*
875 *cereus* are responsible for causing diarrheal symptoms following infection and include
876 the tripartite toxins hemolysin BL (HBL) and non-hemolytic enterotoxin (NHE) as well
877 as cytotoxin K (CytK, also known as hemolysin IV). HBL-induced pore formation
878 occurs after binding to the surface cell receptors LITAF (also known as small integral
879 membrane protein of lysosome/late endosome, or SIMPLE) and the LITAF-like protein
880 called Cell Death Inducing P53 Target 1 (CDIP1). Furthermore, HBL- and NHE-
881 induced pore formation drives activation of the NLRP3 inflammasome. HBL and NHE
882 also possess additional hemolytic activity, along with CytK, cereolysin, (CLO, also
883 known as hemolysins I), hemolysin II and hemolysin III. The emetic toxin, cereulide, is
884 encoded on the plasmid pCER270 and is thought to induce emesis through either a
885 direct or indirect interaction (indicated by a question mark) with 5-HT3 receptors of the
886 stomach and intestine that drive gut-to-brain signaling. Several strains of *B. cereus*
887 have acquired the ability to produce an Anthrax-like toxin, encoded by the plasmids
888 putatively assigned pBCXO1 and pBC218. This toxin is thought to drive a severe
889 anthrax-like infection. Enterotoxin T (ET) and Enterotoxin FM (EFM) are also thought
890 to play an important role in virulence, although their roles as enterotoxins and/or
891 enzymes have remained unclear.

892

893 **Figure 4. HBL and NHE induce an immune response via the NLRP3**
894 **inflammasome**

895 *B. cereus* secretes two tripartite pore forming toxins called HBL and NHE, whose
896 expression is controlled by the master transcriptional regulator PlcR. HBL assembles
897 on the mammalian cell membrane in the linear order of B, L_1 and L_2, whereas, NHE
898 assembles in the order of C, B, and A. Assembly of the three components for either
899 toxin on the host cell membrane leads to pore formation. HBL- and NHE-induced pore
900 formation facilitates potassium (K^+) efflux, which is subsequently sensed by the
901 cytosolic innate immune sensor NLRP3. *B. cereus* strains carrying HBL potentiate
902 rapid activation of NLRP3 compared to *B. cereus* strains carrying NHE but lacking
903 HBL. NLRP3 recruits ASC and caspase-1 to form an inflammasome complex.
904 Activation of the NLRP3 inflammasome results in auto-proteolytic processing of
905 caspase-1, which then cleaves pro-interleukin (IL)-1β and -18, and the pro-pyroptotic
906 factor gasdermin D (GSDMD) into their active forms. The active N-terminal fragment
907 of GSDMD forms pores in the cell membrane that results in a type of cell death called
908 pyroptosis. The GSDMD pores also facilitate the release of bioactive IL-1β and IL-18,
909 which promote inflammation in the host.

HIGHLIGHTS

- *Bacillus cereus* is an important human pathogen and new findings have expanded our understanding of how this bacterium causes disease.
- *Bacillus cereus* Hemolysin BL (HBL) and Non-Hemolytic Enterotoxin (NHE) induce membrane pore formation, leading to the activation of the NLRP3 inflammasome, systemic inflammation, and death.
- Lipopolysaccharide Induced TNF Factor (LITAF) and Cell Death Inducing P53 Target 1 (CDIP1) are bona fide mammalian surface receptors of HBL.
- These newly identified toxin receptors and the NLRP3 inflammasome represent unique targets for potential future therapies against severe *Bacillus cereus* infections.

OUTSTANDING QUESTIONS BOX

- **Do the enterotoxins NHE and CytK have a surface receptor?**
  LITAF and CDIP1 have emerged as newly discovered surface receptors for HBL, however it remains unknown whether NHE and CytK also require a receptor to exert their activity. The identification of receptors of these toxins, if any, will be fundamental to understanding the mechanisms in which these enterotoxins cause disease.

- **Does enterotoxin pore-formation always require a receptor?**
  Studies have shown that HBL can induce pore formation in synthetic liposomes in the absence of cellular proteins (i.e. receptors). In contrast, new evidence indicates that the mammalian receptors LITAF and, in some cases, CDIP1, are required for membrane binding by HBL. This conflicting evidence suggests that perhaps HBL does not always require a receptor to mediate pore formation and has receptor-dependent and receptor-independent modes of activity.

- **What is the mechanism in which cereulide mediates activation of 5-HT3 receptors?**
  The emetic toxin cereulide is believed to induce activation of 5-HT3 receptors in the stomach and intestine. However, it is unknown whether this toxin interacts directly or indirectly with these receptors. Further pharmacological and structural analysis will be essential to appreciate the mechanism in which cereulide induces emesis.

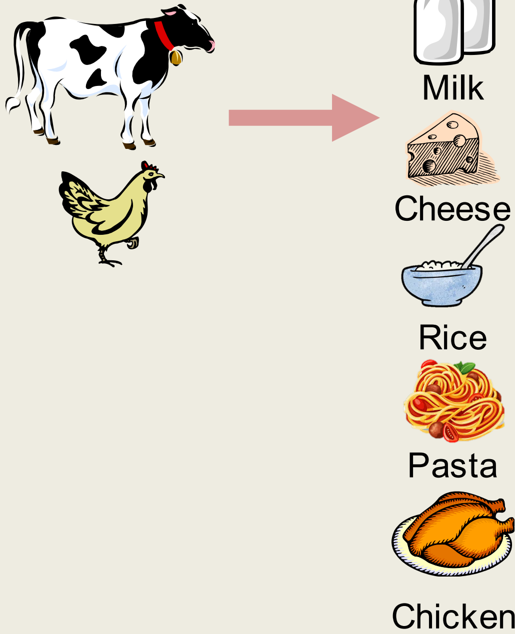
- **Are there other *B. cereus* virulence factors that activate the inflammasome?**
  The enterotoxins HBL and NHE activate the NLRP3 inflammasome. An important question is whether any other virulence factors of *B. cereus* can activate the inflammasome and if inflammasome activation by this pathogen is restricted to NLRP3.

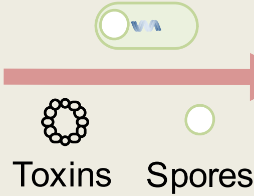
- **What is the mechanism in which *B. cereus* causes anthrax-like disease?**
  It is hypothesized that, in some cases of *B. cereus* infection, the anthrax-like toxin is responsible for anthrax-like disease. However, strains lacking the plasmid encoding this toxin have also been reported in anthrax-like disease, and thus, the actual virulence factor/s causing this disease have remained unknown.

Environmental reservoir and route of transmission

Clinical manifestation

Consumption of contaminated food

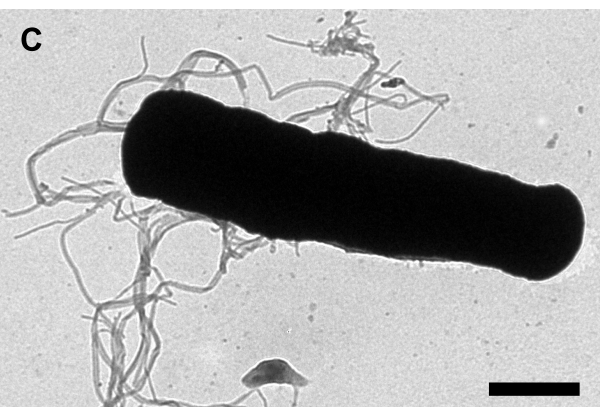


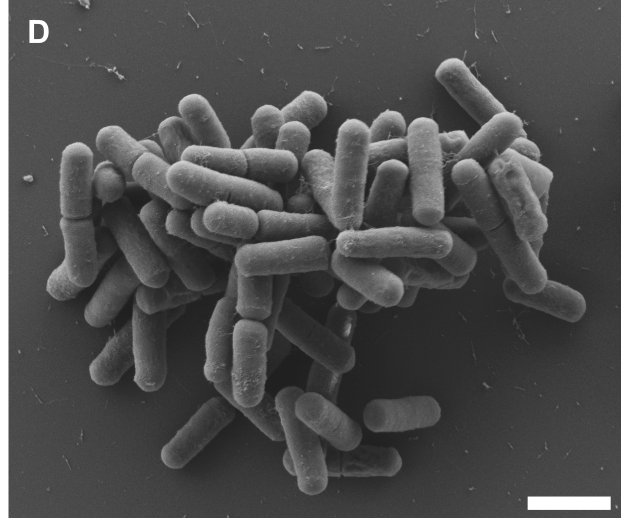


Nosocomial infection

- Brain abscess
- Meningitis
- Endophthalmitis
- Emesis
- Fulminant bacteraemia
- Respiratory tract infection
- Endocarditis
- Hepatitis
- Bone infection
- Gastroenteritis
- Gas gangrene-like infection

Figure 1





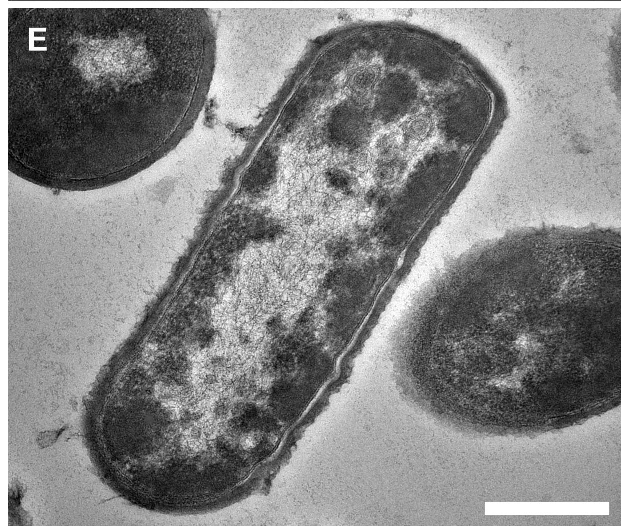


Figure 2

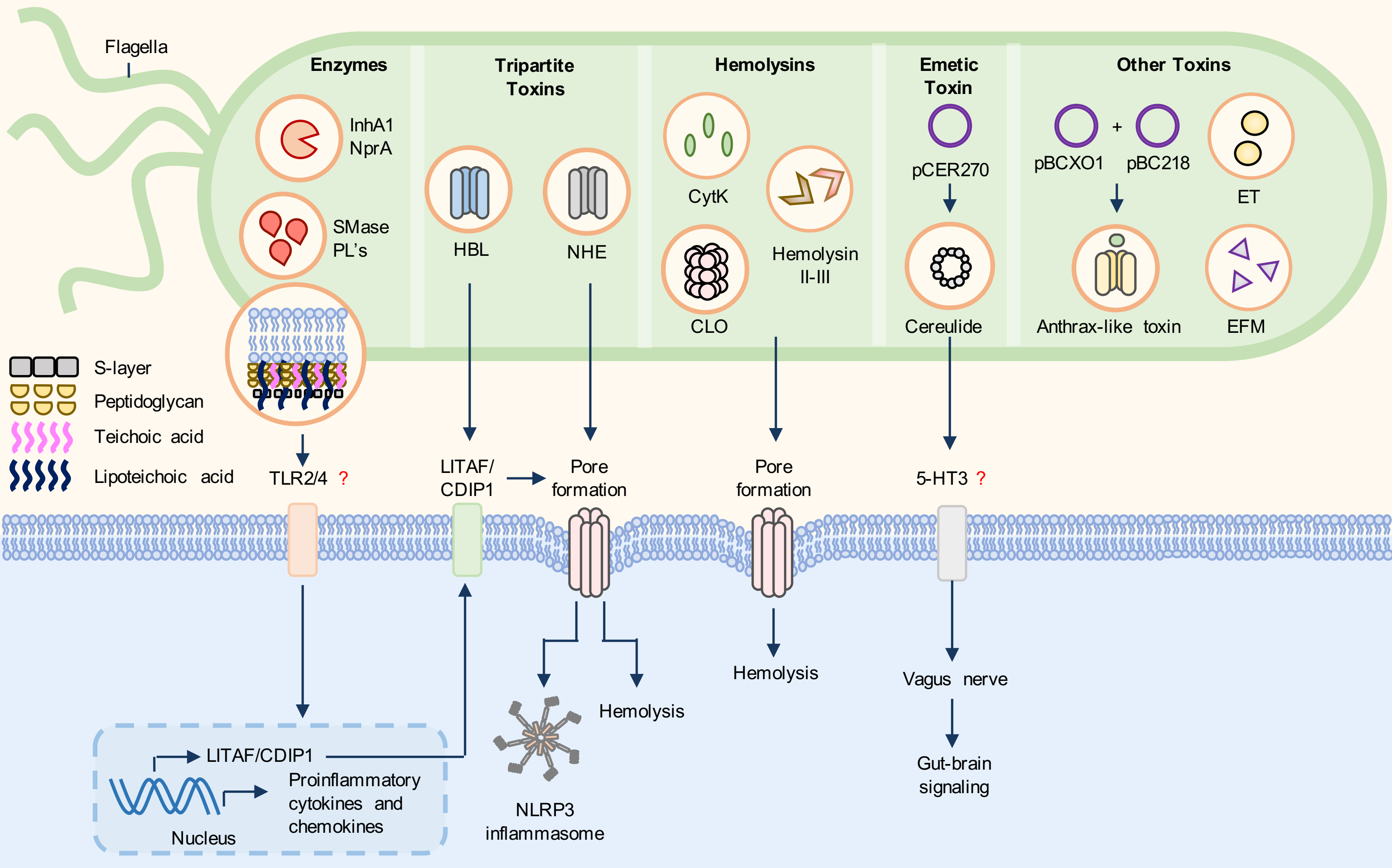


Figure 3

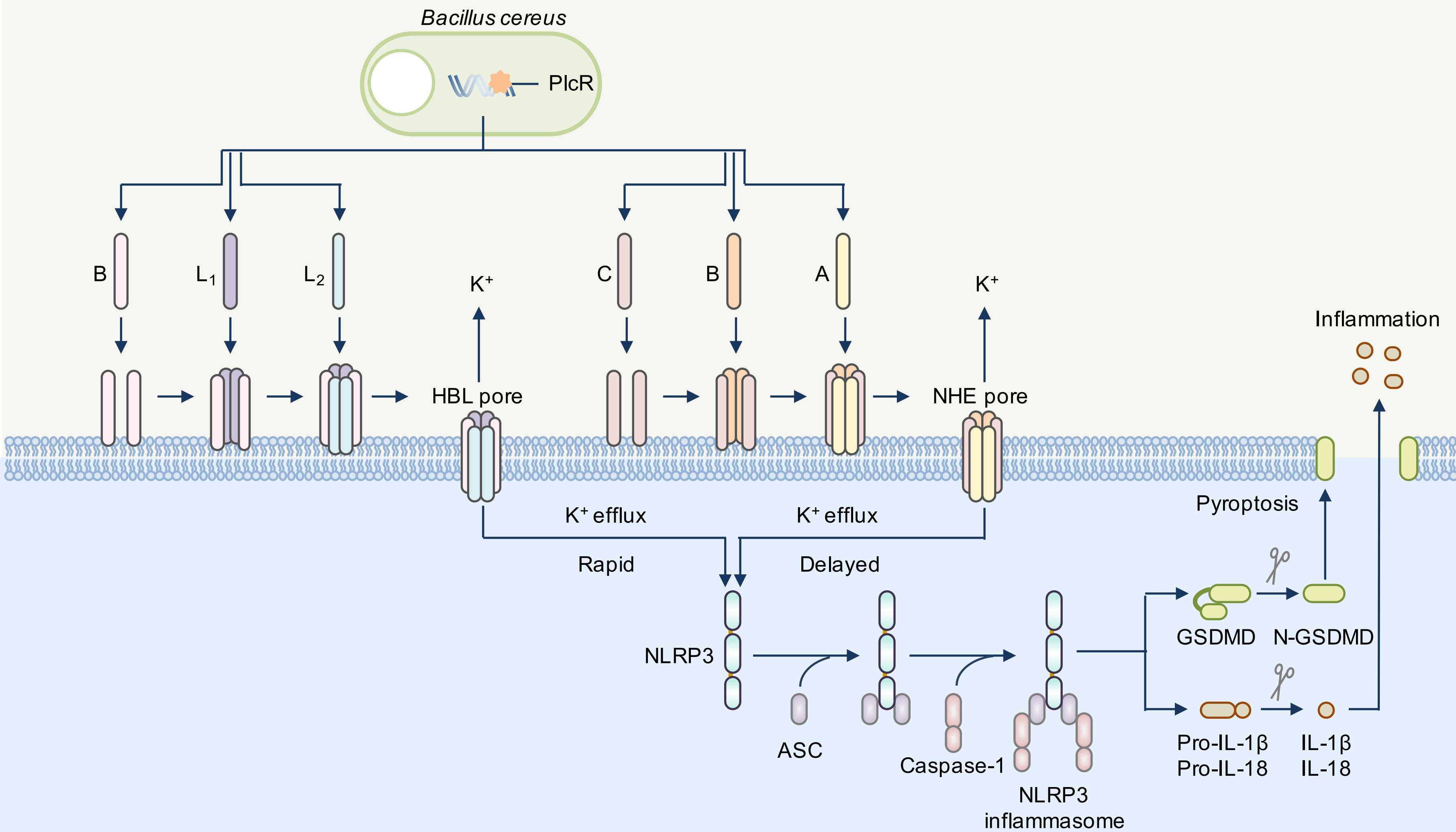


Figure 4