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Survey of *Brevibacillus laterosporus* insecticidal protein genes and virulence factors

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15 **Abstract**

16 The pathogenic action of the bacterium *Brevibacillus laterosporus* against invertebrates
17 involves a toxin-mediated mechanism. Several studies, conducted with specific strains against
18 diverse targets, suggested the implication of different toxins. Recent genome sequencing and
19 annotation of some insecticidal strains revealed several putative virulence factors highly
20 conserved in this species. After determining the pathogenicity of strain UNISS 18 against
21 different Lepidopteran and Dipteran larvae, in this study we have investigated the actual
22 expression of genes encoding for enzymes (i.e., chitinases, proteases), toxins, and other
23 virulence factors, either *in vitro* and *in vivo* at the transcriptional level. Selected genes encode
24 for two chitinases, a collagenase-like protease, a GlcNAc-binding protein, two protective
25 antigen proteins, a bacillolysin, a thermophilic serine proteinase, two spore surface proteins,
26 an insecticidal toxin homologous to Cry75Aa. All target genes were well expressed in pure
27 bacterial cultures with significant differences between bacterial growth phases. Their
28 expression level was generally enhanced in the bacterial population developing in the insect
29 body cavity, compared with pure culture. The expression of certain genes increased
30 substantially over time after insect inoculation. These results support a complex mechanism
31 of action leveraging a variety of available virulence factors, and can also explain the ability of
32 this bacterial species to act against diverse invertebrate targets.

33

34 *Keywords:* bioinsecticide; entomopathogenic bacteria; pathogenesis; toxins; gene expression.

35

36 **1. Introduction**

37 The growing demand for food quality and safety, the importance of environment protection
38 and the modern legislative frameworks foster the use of environmentally friendly pesticidal
39 products to protect crops, animal and human from pests and parasites (Villaverde et al., 2016).
40 Accordingly, the interest in the discovery and development of new active substances,
41 including bio-based compounds produced by microorganisms is significantly increasing
42 (Lacey et al., 2015). Among these, a main role is played by insecticidal toxins of
43 entomopathogenic bacteria, like the crystal (Cry) proteins synthesized by a variety of *Bacillus*
44 *thuringiensis* Berliner strains during sporulation that have considerable scientific and
45 industrial importance (Bravo et al., 2011). These toxins normally act in the gut of their host,
46 after being ingested, through specific interaction with insect epithelial membrane receptors,
47 followed by a sequence of events leading to cell membrane disruption and gut paralysis
48 (Adang et al., 2014). Different protein toxins and virulence factors are instead associated with
49 the pathogenicity of other bacterial species acting against insects, whose mode of action has
50 in many cases not been clarified (Ruiu, 2015).

51 *Brevibacillus laterosporus* Laubach, is another entomopathogenic bacterium, featuring the
52 production of a canoe-shaped parasporal body (CSPB) attached to one side of the spore and,
53 in the case of some strains, the production of cytoplasmic inclusions containing insecticidal
54 crystal proteins (Zubasheva et al., 2010). Accordingly, a mechanism of action involving the
55 alteration of the insect gut epithelial barrier has been observed (Ruiu. et al., 2012; Ferreira et
56 al., 2016). While the implication of crystal proteins in the insecticidal action has been
57 reported, a significant pathogenicity is also associated with *B. laterosporus* strains lacking
58 parasporal crystals (Ruiu et al., 2007), which supports the involvement of other active
59 substances. Among these, highly conserved putative virulence factors have been identified on
60 the spore surface (Marche et al., 2017). However, bioassays with these proteins were not able

61 to reproduce the full bacterial toxicity, thus suggesting that additional factors are involved in
62 the insecticidal action.
63 In order to increase our understanding of *B. laterosporus* entomopathogenicity, investigations
64 were conducted on a selection of putative virulence factors that we have identified following
65 the whole genome sequencing and annotation of the insecticidal strain UNISS 18 (Camiolo et
66 al., 2017), characterized by the lack of parasporal crystals (Ruiu et al., 2007). For this purpose
67 the actual expression of genes encoding for enzymes (i.e., chitinases, proteases) and toxins,
68 have been studied either *in vitro* and *in vivo* at the transcriptional level, thus proving their
69 activation during the pathogenic process. Because most of these genes are shared among
70 known *B. laterosporus* genomes, this work provides useful data and information for future
71 studies on different strains of this insect pathogenic species.

72

73 **2. Materials and methods**

74 *2.1. Bacterial strain and growth conditions*

75 The entomopathogenic strain *B. laterosporus* UNISS 18 (= NCIMB 41419) was used in this
76 study. Bacterial cultures were routinely conducted in conical flasks containing Luria–Bertani
77 (LB) broth at 30 °C in an orbital incubator shaking at 180 rpm. Synchronized cultures were
78 obtained as described in Ruiu et al. (2007), using a sequence of LB pre-cultures at the
79 exponential phase. Bacterial growth was checked by phase microscopy and cell cultures were
80 harvested at different growth phases (vegetative, early stationary, sporulation). For this
81 purpose cell fractions were centrifuged at 5000 rpm for 20 min and used fresh in bioassays or
82 stored at -80° for analysis. Living cells used in bioassays were centrifuged at room
83 temperature and resuspended in PBS buffer, while cells and sporangia used for analysis were
84 harvested at 4°C. Living cell counts were routinely performed through plating serial dilutions

85 of bacterial suspensions on LB agar, in order to determine the number of colony forming units
86 (CFU).

87

88 2.2. Insect bioassays

89 Larvae of different insect species were used in preliminary bioassays to test bacterial
90 pathogenicity by injection. These included the wax moth *Galleria mellonella* L. (Lepidoptera:
91 Pyralidae), the gypsy moth *Lymantria dispar* L. (Lepidoptera: Erebidae), the lackey moth
92 *Malacosoma neustria* L. (Lepidoptera: Lasiocampidae), the house fly *Musca domestica* L.
93 (Diptera: Muscidae), and the blow fly *Lucilia caesar* L. (Diptera: Calliphoridae). Mass reared
94 *G. mellonella* and *L. caesar* larvae were provided by Microvita (Bologna, Italy). Larvae of *L.*
95 *dispar* and *M. neustria* were collected in a cork oak forest in central Sardinia (Italy). Larvae
96 of *M. domestica* were provided by the Insect Rearing facilities of the Department of
97 Agriculture of the University of Sassari (Italy).

98 In order to determine pathogenicity, bacterial suspensions (2 µl) were injected into each
99 surface sterilized larva in between the ventral intersegmental region using a Hamilton syringe.
100 Larvae of each insect species were injected a higher (1000 CFU/larva) and a lower (100
101 CFU/larva) dose of vegetative cells of *B. laterosporus*. Control larvae were injected 2 µl PBS
102 solution. Treated and control larvae were maintained in groups of 10 inside plastic plates on
103 filter paper inside a growth chamber at 25° C and 50% R.H. Insects were inspected every 12 h
104 for two days, and the number of dead larvae was recorded after each time interval.

105 Experiments were conducted with a four replicated design and were repeated three times with
106 different batches of insects and bacterial preparations.

107 Further injection experiments following the same design were conducted on *L. caesar* larvae
108 that were subjected to RT qPCR analyses. Haemolymph samples from pools of these larvae (1
109 pool = 10 larvae) were analyzed to determine the number of *B. laterosporus* colony forming

110 units (CFU) at different time intervals after injection. These experiments involved three
111 independent biological replicates, and each analysis was performed with three technical
112 replicates.

113

114 2.3. *Gene selection and sequencing*

115 A selection of putative *B. laterosporus* genes associated with pathogenicity and virulence
116 against insects, was identified on the genome of strain UNISS 18, as a result of preliminary
117 genome sequencing, assembly and annotation (Camiolo et al., 2017).

118 Gene selection has taken into account previous reports on the insect pathogenicity of various
119 *B. laterosporus* strains (Ruiu, 2013; Marche et al., 2017) and other entomopathogenic bacteria
120 (Castagnola and Stock, 2014). Selected genes encode for chitinases, spore surface proteins,
121 insecticidal proteins, metalloproteinases and other enzymes.

122 While the whole genome of *B. laterosporus* UNISS 18 is deposited at DDBJ/ENA/GenBank
123 under the accession no. MBFH00000000, the complete sequences of target genes have been
124 deposited in the GenBank database under the accession numbers provided in Table 1.

125 The NCBI Basic Local Alignment Search Tool (BLAST) was used for quick sequence
126 alignments between different strains.

127

128 2.4. *RT qPCR*

129 The expression of selected *B. laterosporus* genes in correspondence of different growth
130 phases was determined by analyzing the level of mRNA transcripts on pure cell suspensions
131 collected at vegetative (12 h), early stationary (24 h), and sporulation (36 h) phases. Similarly,
132 expression of the same genes was determined at the transcriptional level on haemolymph
133 samples from pools of live *L. caesar* larvae collected at different time intervals (2,6,12 h)
134 after injection with 1000 CFU per larva.

135 Total RNA was extracted from cultured bacterial suspensions and from haemolymph pools
136 employing TRIzol® Reagent (Life Technologies) according to manufacturer's protocol
137 (Chomczynski and Sacchi, 1987). RNA was routinely quantified and purity checked using a
138 NanoDrop ND-1000 Spectrophotometer (Thermo Scientific), before being treated with RQ1
139 RNase-Free DNase (Promega). An aliquot (1 µg) was then used to synthesize first-strand
140 cDNA employing Random Hexamer Primers (Life Technologies), SuperScript® II Reverse
141 Transcriptase (Life Technologies) and RNaseOUT™ Recombinant Ribonuclease Inhibitor
142 (Life Technologies) according to the manufacturers' instructions. Power SYBR® Green PCR
143 Master Mix (Life Technologies) was used to run quantitative PCR reactions on an Applied
144 Biosystems 7900HT Fast Real-Time PCR System according to manufacturer's instructions,
145 with the following cycle conditions: denaturation at 95 °C for 10 min, followed by 40 cycles
146 of 95 °C for 15 s, annealing at 57-60 °C for 1 min, and extension at 60 °C for 1 min.
147 Forward and reverse primers listed in Table 1 were designed on target gene sequences using
148 Primer3web (version 4.0.0) (Untergasser et al., 2012). Their PCR efficiency was preliminarily
149 tested by standard curve and dissociation curve analyses (Pfaffl, 2001). The relative
150 abundance of qPCR transcripts was determined as suggested by Livak and Schmittgen (2001)
151 using 16S rRNA gene as *B. laterosporus* internal control gene in the *in-vitro* experiments, and
152 both bacterial 16S rRNA and insect actin as reference genes in the *L. caesar* experiments, for
153 PCR normalization (Marche et al., 2017; Mura and Ruiu, 2017). All experiments involved at
154 least three independent biological replicates, and each analysis was performed with three
155 technical replicates.

156

157 2.5. Statistical analysis

158 Statistical analyses were performed with SAS software (version 9.1) with significance level
159 set at $\alpha = 0.05$ (SAS Institute Inc., 2004).

160 Overtime mortality data of each insect species were analyzed using repeated measures
161 ANOVA (PROC MIXED), and means were separated using LSMEANS comparison (adjust =
162 Tukey).

163 Linear regression analyses were used for analyzing the relationship between time and
164 bacterial growth (CFU) into growth media or insect haemolymph.

165 The relative expression of the target genes was analyzed using the comparative $2^{-\Delta\Delta C_t}$ method
166 (Livak and Schmittgen, 2001). Fold changes in gene expression in different experiments were
167 subjected to two-ways ANOVA (factors: gene and time), followed by multiple comparison of
168 means (adjust = Tukey).

169

170 **3. Results**

171 *3.1. Pathogenicity of Brevibacillus laterosporus against different targets*

172 The pathogenicity of *B. laterosporus* UNISS 18 against larvae of different insect species was
173 assessed by injecting vegetative cells into their body and determining the mortality level after
174 different time intervals. As shown in Table 2, a significant pathogenicity with 100% mortality
175 achieved within 48 h was observed on all treated species compared with the control (*G.*
176 *mellonella*: $F_{2,33} = 2821.31$, $P < 0.0001$; *L. dispar*: $F_{2,33} = 2525.36$, $P < 0.0001$; *M. neustria*
177 $F_{2,33} = 2935.36$, $P < 0.0001$; *M. domestica*: $F_{2,33} = 1377.94$, $P < 0.0001$; *L. caesar*: $F_{2,33} =$
178 2184.43 , $P < 0.0001$).

179 A more rapid time to death was generally observed on Lepidopteran compared with Dipteran
180 species, and a faster pathogenic action was detected at higher dose (*G. mellonella*: $F_{2,66} =$
181 1276.75 , $P < 0.0001$; *L. dispar*: $F_{2,66} = 869.56$, $P < 0.0001$; *M. neustria*: $F_{2,66} = 508.12$, $P <$
182 0.0001 ; *M. domestica*: $F_{2,66} = 1139.55$, $P < 0.0001$; *L. caesar*: $F_{2,66} = 924.68$, $P < 0.0001$).

183

184 *3.2. Time course bacterial growth in the insect body*

185 The over time bacterial growth in the haemocoel of *L. caesar* larvae injected with two
186 different doses of vegetative cells (100 and 1000 CFU) was determined by counting the
187 number of CFU at different time intervals after injection and until insect death. The *B.*
188 *laterosporus* population was found to grow fast in the insect body and the amount of living
189 bacteria (CFU) was positively correlated with time after injection of either a lower (adjusted
190 $R^2 = 0.7630$, $F = 191.0$, $P < 0.0001$) or a higher (adjusted $R^2 = 0.8855$, $F = 457.5$, $P <$
191 0.0001) dose (Fig. 1).

192

193 *3.3. In-vitro expression of genes related to bacterial pathogenicity and virulence*

194 The relative expression (fold change) of target genes at different *B. laterosporus* stages of
195 growth is shown in Fig. 2. All target genes were well expressed in pure culture and the
196 relative level of expression was significantly affected by the gene ($F_{10,264} = 10.99$, $P < 0.0001$)
197 and the bacterial growth phase ($F_{2,264} = 11.99$, $P < 0.0001$). A significant interaction gene x
198 time was also observed ($F_{20,264} = 5.71$, $P < 0.0001$). Significantly higher expression was
199 detected for collagenase-like protease (*prtC*), insecticidal toxin (*mtx*), bacillolysin (*bll8*) and
200 spore surface protein (*cpbA*) genes (exponential vegetative phase: $F_{10,88} = 42.13$, $P < 0.0001$;
201 early stationary phase: $F_{10,88} = 7.73$, $P < 0.0001$; sporulation phase: $F_{10,88} = 6.80$, $P < 0.0001$).
202 Collagenase-like protease (*prtC*) and insecticidal toxin (*mtx*) genes achieved the highest
203 expression level during the vegetative phase, while bacillolysin (*bll8*) gene was highly
204 expressed during both vegetative and sporulation phases. The expression of spore surface
205 protein A (*cpbA*) gene increased gradually over time achieving a maximum at sporulation.

206

207 *3.4. In-vivo time-course expression of genes related to bacterial pathogenicity and virulence*

208 The relative expression (fold change) of different target genes in blow fly larvae, at
209 progressive time intervals (2, 6 and 12 h) after injection of *B. laterosporus* vegetative cells, is
210 shown in Fig. 3.

211 The relative level of expression in the insect haemolymph was significantly affected by the
212 gene ($F_{10,264} = 28.12$, $P < 0.0001$) and time after inoculum injection ($F_{2,264} = 34.25$, $P <$
213 0.0001). The interaction gene x time was also significant ($F_{20,264} = 27.59$, $P < 0.0001$).
214 For all genes a significant increase in their expression level was achieved at 12 h after
215 injection (*chiA*: $F_{2,24} = 23.25$, $P < 0.0001$; *pal*: $F_{2,24} = 7.35$; $P = 0.0032$; *gpb*: $F_{2,24} = 6.27$; $P =$
216 0.0064 ; *chiD*: $F_{2,24} = 18.28$; $P < 0.0001$; *prtC*: $F_{2,24} = 13.64$; $P < 0.0001$; *mtx*: $F_{2,24} = 14.52$; $P <$
217 0.0001 ; *pa2*: $F_{2,24} = 21.87$; $P < 0.0001$; *bll8*: $F_{2,24} = 15.75$; $P < 0.0001$; *tsp*: $F_{2,24} = 43.56$; $P <$
218 0.0001 ; *cpbA*: $F_{2,24} = 28.22$; $P < 0.0001$; *cpbB*: $F_{2,24} = 11.76$; $P = 0.0003$). A mixture of
219 bacterial growth phases, including vegetative cells and early stage sporangia were observed in
220 the haemolymph of these insect samples by phase microscopy. A higher expression level was
221 detected for spore surface protein A (*cpbA*) gene, followed by bacillolysin (*bll8*), chitinase A
222 (*chiA*), chitodextrinase (*chiD*), collagenase-like protease (*prtC*), and insecticidal toxin (*mtx*),
223 that were significantly more expressed than other target genes (Fig. 3).

224

225 **4. Discussion**

226 The pathogenic properties of *B. laterosporus* against invertebrates, including insects,
227 nematodes and mollusks, are documented by a growing scientific literature (Ruiu, 2013).
228 From the very first observations, significant differences between strains of this bacterial
229 species emerged, thus supporting the implication of diverse and specific virulence factors
230 against different hosts.

231 Our preliminary bioassays revealed the pathogenicity of *B. laterosporus* UNISS 18 against a
232 variety of insect targets in different orders (Lepidoptera and Diptera), which is in line with the

233 ability of this species to act against diverse invertebrate targets (Ruiu, 2013; Marche et al.,
234 2016). A rapid growth of the bacterial population inoculated into the insect haemocoel was
235 also observed, highlighting the ability of this insect pathogen to rapidly overcome the host
236 defense mechanisms leading to lethal septicemia. However, when the bacterium is ingested by
237 the host, it needs to cross the intestinal barrier by opening a breach in the peritrophic matrix
238 and disrupting the intestinal wall, before being able to spread in the body cavity. This
239 complex process, could be achieved by the combined or sequential action of different
240 bacterial toxins and virulence factors, among which a main role is likely to be played by
241 chitinases and proteases dissolving chitin and glycoprotein physical barriers (Castagnola and
242 Stock, 2014), and by pore-forming toxins, such as the well known crystal (Cry) proteins,
243 causing damage to the epithelial cells (Jurat-Fuentes and Crickmore, 2017.). Such destructive
244 action in the midgut of insect ingesting *B. laterosporus* has already been documented (Ruiu et
245 al., 2012; Ferreira et al., 2016), while the role of specific virulence factors at different stages
246 of the pathogenic process has not been clarified. Several studies employing different bacterial
247 strains against diverse targets, showed that most of the insecticidal action is associated with
248 bacterial preparations harvested at the stationary and sporulation growth phases. Accordingly,
249 as a result of the first studies on the interaction between *B. laterosporus* and insects, a higher
250 insecticidal activity was observed after the administration of late vegetative cells or sporangia
251 (Favret and Yousten, 1985; Oliveira et al., 2004). The production of insecticidal crystal
252 proteins during sporulation was thereafter reported, thus supporting the implication of
253 parasporal bodies in the insecticidal action of some strains (Zubasheva et al., 2010). The
254 increase in virulence of bacterial preparations harvested at successive time intervals during
255 growth in artificial media was confirmed in time-course experiments employing synchronised
256 cultures of *B. laterosporus* strain UNISS 18 to investigate the involvement of spore surface
257 proteins in the insecticidal action against the house fly (Marche et al., 2017). More recently,

258 novel Cry proteins exhibiting inhibitory activity against coleoptera and lepidoptera have been
259 identified in some *B. laterosporus* strains (Bowen et al., 2017). Nevertheless, other insect
260 toxins like the insecticidal proteins produced during the vegetative phase (VIP) by certain
261 strains were found to act against Coleoptera (Warren, 1997).

262 Data deriving from recent genome sequencing and annotation of different *B. laterosporus*
263 strains (Djukic et al., 2011; Camiolo et al., 2017) suggested that different strains of these
264 species share a variety of putative toxins, enzymes and virulence factors. However, such
265 genomic studies did not provide information on the actual expression of such bacterial
266 virulence genes. Our study showed that selected genes were well expressed by strain UNISS
267 18 during bacterial growth in pure culture and during pathogenesis. These target genes encode
268 for two chitinases (ChiA, and ChiD), a collagenase-like protease (PrtC) and a GlcNAc-
269 binding protein (Gbp), that can participate in the degradation of chitin (Prasanna et al., 2013);
270 two protective antigen proteins (PA1 and PA2) possibly implied in the translocation of other
271 toxins and virulence factors into the cell (Barth et al., 2004); a bacillolysins (B118) that is
272 common in many *Bacillus* species (Rabideau et al., 2015); a thermophilic serine proteinase
273 (Tsp) that can be associated with a broad substrate specificity (Harrison and Bonning, 2010),
274 two spore surface proteins (CpbA and CpbB), whose involvement in insect pathogenesis has
275 previously been reported (Marche et al., 2017); an insecticidal toxin (Mtx) showing high
276 homology (99%) with the recently discovered Cry75Aa protein acting against Lepidoptera
277 and Coleoptera (Bowen et al., 2017). Consistently, the implication of entomopathogenic
278 bacteria protein fractions, including a mixture of metalloproteases, peptidases, and other
279 enzymes, in the insecticidal action against diptera has already been reported (Ruiu et al.,
280 2015).

281 In pure culture, the bacillolysins (*B118*) gene was the most expressed during the exponential
282 vegetative phase and was also the most expressed, together with the spore surface protein

283 (*cpbA*) gene, during sporulation. The latter, being related to the production of the typical
284 canoe-shaped parasporal body (CSPB), showed a gradual over time increase in the expression
285 level, achieving a maximum at sporulation (Marche et al., 2017). The insecticidal toxin (*mtx*)
286 gene, was well expressed during all bacterial growth phases, showing a higher level during
287 the vegetative phase. The expression level of collagenase-like protease *prtC* gene was always
288 above most target genes with no differences among bacterial stages of growth. Taken
289 together, these results suggest that a different expression level of these genes during different
290 bacterial stages of growth may explain the different level of toxicity associated with each
291 bacterial phase (Ruiu et al., 2007). With a view of developing a bioinsecticide for field
292 applications (Ruiu et al., 2011 and 2014), knowledge on the synthesis activation of toxins and
293 virulence factors during bacterial growth in batch culture is an important information for
294 appropriate manufacturing procedures (Jackson, 2016).

295 All target genes were generally more expressed by the bacterial population developing in the
296 insect body compared with pure cultures, and the expression of each gene increased
297 substantially over time after insect inoculation. The spore surface protein (*cpbA*) gene was the
298 most expressed, followed by chitinase (*chiA*, and *chiD*), collagenase-like protease (*prtC*),
299 bacillolysin (*Bll8*), and insecticidal toxin (*mtx*) genes. Such expression enhancement is an
300 indication of a stimulatory effect within the insect body environment where the bacterium can
301 exploit its arsenal of toxins and virulence factors for a rapid growth. In the hypothesis in
302 which the bacterium entered the host orally, the same enzymes and toxins could support its
303 passage through the intestinal barriers to reach the nutrient-rich haemocoel (Mura and Ruiu,
304 2017). According to this scenario, we can imagine a complex mechanism of action leveraging
305 a variety of available virulence factors. This diversity, support the pathogenic potential of this
306 bacterial species against different invertebrates. Further studies will clarify the specific role of
307 each bacterial virulence factor during pathogenesis.

308

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312

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396 **Table 1** - *Brevibacillus laterosporus* protein genes used for qPCR analysis

| Protein product | Gene ID | Accession Number ^a | Primer sequence | | Annealing temperature (°C) |
|---------------------------------------|-------------|-------------------------------|-------------------------------|-------------------------------|----------------------------|
| | | | Sense 5' - 3' | Antisense 5' - 3' | |
| Chitinase A | <i>chiA</i> | MG725829 | 5'-CGAATACAAACAGGAGCCTAA-3' | 5'-GCATCCCAAACAGAAGTGAG-3' | 57 |
| Chitodextrinase | <i>chiD</i> | MG725830 | 5' -AGTCAGATTGGTGATCGTAGC-3' | 5'-GCGTTTGCTCTACTAATCCCAC-3' | 57 |
| Collagenase-like protease PrtC | <i>prtC</i> | MG725831 | 5'-ATCAAACAAGAGTGGATCGAC-3' | 5'-ATTCTACCTTCGAGCCCAC-3' | 57 |
| GlcNAc-binding protein A | <i>gbp</i> | MG725832 | 5' -TGGGGAGATTACAGGAGCA-3' | 5'-CCATCGTTAAATGTAGCAATGAG-3' | 57 |
| Protective antigen domain protein PA1 | <i>pa1</i> | MG725833 | 5'-GCTGTATTGATTTGATAGGTTCC-3' | 5'-CTAATTCACCGAAGAAGGATG-3' | 57 |
| Protective antigen domain protein PA2 | <i>pa2</i> | MG725834 | 5'-ATCACTCCTCTGCTACGCA-3' | 5'-CTGCTAGATTCATTCTCTGGTTG-3' | 57 |
| Bacillolysin BL18 | <i>bl18</i> | MG725835 | 5'-GTACAAGGCGAGGTAGAGAAT-3' | 5'-GTCCAAAGAAGGAGGTTACAT-3' | 60 |
| Thermophilic serine proteinase | <i>tsp</i> | MG725836 | 5'-ATAATGAATCCCGACCTGGT-3' | 5'-TCGATCAGGATGAGAATCTAG-3' | 60 |
| Spore surface protein A | <i>cpbA</i> | KY124461.1 | 5'-GCTTCACACGATCAGCAACC-3' | 5'TGTAGGCGGGCAGCTAAAAA3' | 60 |
| Spore surface protein B | <i>cpbB</i> | KY124462.1 | 5'-TCACCAAGACACAAAGCCCT -3' | 5'-GGGCTTTGTGTCTTGGTGAG-3' | 60 |
| Insecticidal toxin MTX | <i>mtx</i> | MG725837 | 5'-ATGGTAGCAGTGGTTTTGACT-3' | 5'-CATTAGAAGGAAGTCCAACCG-3' | 57 |

417 ^a Complete gene sequences were deposited in the GenBank database.

418

419

420

Table 2 - In vivo bacterial pathogenicity

| Species | Dose ^a (CFU/larva) | Mortality (%) after injection (mean \pm SE) ^b | | |
|----------------------------|----------------------------------|--|------------------|-------|
| | | 12 h | 24 h | 48 h |
| <i>Galleria mellonella</i> | 0 | 0 a | 0 a | 0 a |
| | 100 | 11.7 \pm 2.0 b | 68.3 \pm 2.1 d | 100 e |
| | 1000 | 42.5 \pm 2.5 c | 100 e | 100 e |
| <i>Lymantria dispar</i> | 0 | 0 a | 0 a | 0 a |
| | 100 | 15.0 \pm 1.9 b | 50.0 \pm 2.1 d | 100 f |
| | 1000 | 43.3 \pm 1.9 c | 85.8 \pm 2.3 e | 100 f |
| <i>Malacosoma neustria</i> | 0 | 0 a | 0 a | 0 a |
| | 100 | 22.5 \pm 2.2 b | 59.2 \pm 2.3 d | 100 f |
| | 1000 | 47.5 \pm 2.2 c | 88.3 \pm 2.7 e | 100 f |
| <i>Musca domestica</i> | 0 | 0 a | 0 a | 0 a |
| | 100 | 5.0 \pm 1.6 b | 37.5 \pm 1.8 d | 100 f |
| | 1000 | 18.3 \pm 2.7 c | 67.5 \pm 2.8 e | 100 f |
| <i>Lucilia caesar</i> | 0 | 0 a | 0 a | 0 a |
| | 100 | 6.6 \pm 2.5 a | 39.2 \pm 2.6 b | 100 d |
| | 1000 | 9.2 \pm 2.3 a | 65.0 \pm 2.3 c | 100 d |

^a Each larva was injected 2 μ l suspension of exponential vegetative cells.

^b For each species, different letters indicate significantly different means (ANOVA Mixed Proc., Tukey adjusted $P < 0.05$).

Figure captions

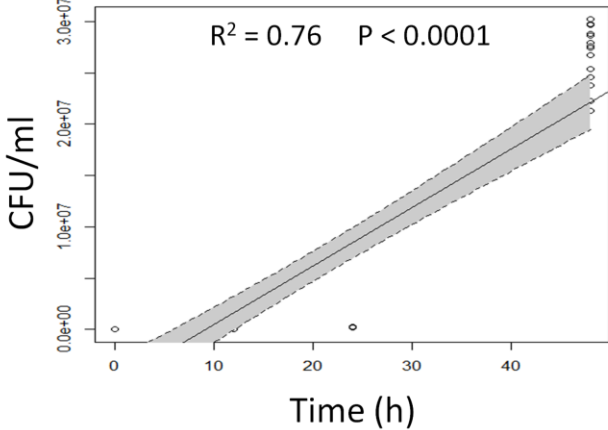
Fig. 1 - Linear regression plots with 95% confidence intervals (shaded areas) showing the predicted relationship between *B. laterosporus* population abundance in the insect haemocoel determined as colony forming units (CFU) and time after injection of a lower (100 CFU) (a) or a higher (1000 CFU) (b) dose of vegetative cells.

Fig. 2 - In-vitro expression of genes related to pathogenicity and virulence in correspondence of different bacterial growth phases. Fold change was obtained using *16S rRNA* transcript abundance at each growth phase for qPCR data normalization. Different letters above bars indicate significantly different means (2-ways ANOVA, Tukey adjusted $P < 0.05$).

Fig. 3 - In-vivo expression of genes related to bacterial pathogenicity and virulence at different time intervals after vegetative cells injection. Fold change was obtained using bacterial *16S rRNA* and insect *actin* transcript abundance at each time interval for qPCR data normalization. Different letters above bars indicate significantly different means (2-ways ANOVA, Tukey adjusted $P < 0.05$).

Fig. 1

a) Lower injection dose



b) Higher injection dose

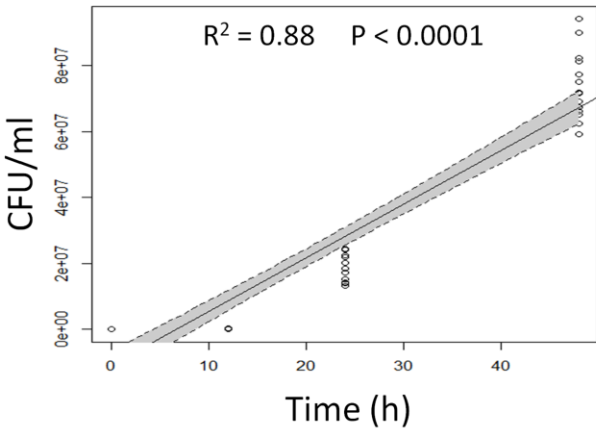


Fig. 2

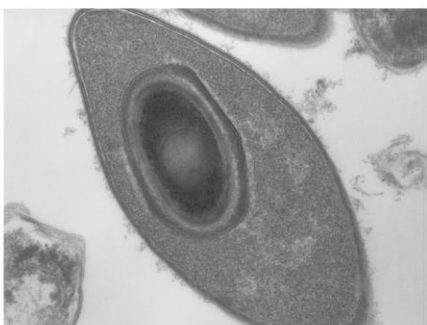
a) Bacterial growth phase



Young vegetative cell



Old vegetative cell



Early-stage sporangium

b) Relative gene expression

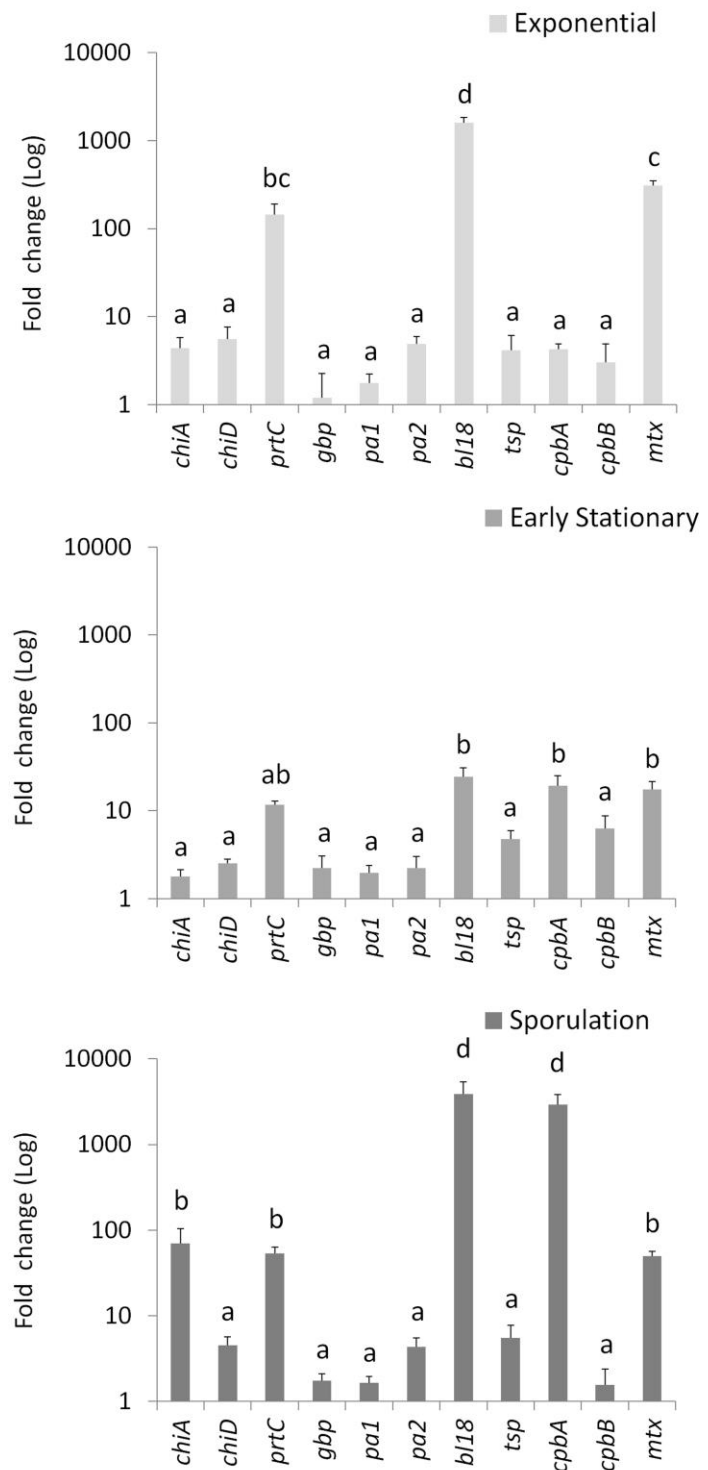


Fig. 3

