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The parasporal crystals of *Bacillus pumilus* strain 15.1: a potential virulence factor?

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Diana C. Garcia-Ramon[a], Colin Berry[b], Carmen Tse[b], Alberto Fernández-Fernández[a], Antonio Osuna[a], Susana Vílchez[a, c] #

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[a]Institute of Biotechnology, Campus Fuentenueva, University of Granada, Spain,

[b]Cardiff School of Biosciences, Cardiff University, UK

[c]Department of Biochemistry and Molecular Biology I, Campus Fuentenueva, University of Granada, Spain.

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Running Title: Parasporal crystal in *Bacillus pumilus* 15.1

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#Address correspondence to Susana Vílchez, Department of Biochemistry and Molecular Biology I, Faculty of Science, Campus Fuentenueva, University of Granada, 18071, Granada, Spain. Phone: +34 958240071; Fax: 34 9589947; svt@ugr.es

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Abstract

Bacillus pumilus strain 15.1 was previously found to cause larval mortality in the Med-fly *Ceratitis capitata* and was shown to produce crystals in association with the spore. As parasporal crystals are well-known as invertebrate-active toxins in entomopathogenic bacteria such as *Bacillus thuringiensis* (Cry and Cyt toxins) and *Lysinibacillus sphaericus* (Bin and Cry toxins), the *B. pumilus* crystals were characterised. The crystals were composed of a 45 kDa protein that was identified as an oxalate decarboxylase by peptide mass fingerprinting, N-terminal sequencing and by comparison with the genome sequence of strain 15.1. Synthesis of crystals by a plasmid-cured derivative of strain 15.1 (produced using a novel curing strategy), demonstrated that the oxalate decarboxylase was encoded chromosomally. Crystals spontaneously solubilized when kept at low temperatures and the protein produced was resistant to trypsin treatment. The insoluble crystals produced by *B. pumilus* 15.1 did not show significant toxicity when bioassayed against *C. capitata* larvae, but once the OxdD protein was solubilized, an increase of toxicity was observed. We also demonstrate that the OxdD present in the crystals has oxalate decarboxylate activity as the formation of formate was detected, which suggests a possible mechanism for *B. pumilus* 15.1 activity. To our knowledge, the characterization of the *B. pumilus* crystals as oxalate decarboxylase is the first report of the natural production of parasporal inclusions of an enzyme.

Introduction

The production of spore-associated (parasporal) crystals by several species of bacteria within the genus *Bacillus* and related genera is well known. These proteins are almost always entomopathogenic toxins, active against a wide range of invertebrate targets (Bechtel and Bulla, 1976; 2007) although crystals without known targets (sometimes termed parasporins) are also known. Such parasporins are related in sequence and structure to known

invertebrate-active toxins and it is likely that their natural target merely remains to be discovered (although activity against certain human cancer cells in culture has been reported (Ohba et al., 2009)). The most studied proteinaceous toxins are the Cry and Cyt toxins, produced mainly by *Bacillus thuringiensis* (*Bt*), which are the principal agents responsible for the toxicity of these bacteria toward insects. The insecticidal activity of crystal proteins produced by *Bt* has been extensively used as the basis of many commercial products. The ability to produce parasporal crystals is not restricted to *Bt* as some strains of *Lysinibacillus sphaericus* (Jones et al., 2007), *Clostridium bifermentans* (Barloy et al., 1996), *Paenibacillus popilliae* (Zhang et al., 1997), *Brevibacillus laterosporus* (Smirnova et al., 1996) and *P. lentimorbus* (Yokoyama et al., 2004), also produce parasporal inclusions active against insects. The mechanisms of action proposed for the parasporal crystal toxins generally require solubilization and proteolytic activation of the protoxin form in the midgut of the target invertebrate (Haider et al., 1986; Palma et al., 2014). Serine proteases are important in both solubilization and activation of *Bt* protoxins and, in some insects, changes in the protease profile of their guts have been associated with resistance to *Bt* toxin (Li et al., 2004; Karumbaiah et al., 2007).

Our research group reported a *Bacillus pumilus* strain toxic toward the Mediterranean fruit fly, *Ceratitis capitata* (Molina et al., 2010). Previous assays showed that the toxicity of *B. pumilus* 15.1 can be inactivated either by heat or by proteases, suggesting that the virulence factor produced by this strain could be proteinaceous (Molina, 2010). Since its initial isolation and testing, our strain appears to have decreased in toxicity, even though it continues to produce the parasporal crystals, mainly composed of a 45 kDa protein, that we have previously described (Garcia-Ramon et al., 2016). Despite the loss of toxicity, the crystal protein was still considered as a candidate toxin (possibly interacting with another factor, now lost or under-expressed). In this work we describe the characterization of the crystal

inclusions produced by *B. pumilus* 15.1 as the first example of a parasporal enzyme crystal and we propose a potential mechanism of action for this entomopathogenic strain.

Results

Identification of the crystal protein

The spore-crystal complex of a *B. pumilus* 15.1 culture, sporulated in T3 medium was used to isolate crystals using sucrose density gradient centrifugation (Garcia-Ramon et al., 2016). Crystals formed bands at the interface formed between the solutions of 72% and 79% sucrose (like many Cry toxins (Thomas and Ellar, 1983; Koller et al., 1992; Jones et al., 2007; Swiecicka et al., 2008)). They were also found at the 79% / 84% sucrose interface. The enriched crystal proteins from both bands produced a major band of 45 kDa on SDS-PAGE, as previously observed (Garcia-Ramon et al., 2016) and this was excised for fingerprint analysis using MALDI-TOF MS. Mass spectrometry of the intact protein revealed a mass of 43,799 Da. Comparison of mass peaks obtained from fingerprinting with the recently published *B. pumilus* 15.1 genome (Garcia-Ramon et al., 2015a) and *Bacillus* databases produced matches (40.8% sequence coverage) with OxdD, a putative oxalate decarboxylase encoded in Contig 4 of the *B. pumilus* 15.1 strain genome and an OxdD from *B. pumilus* ATCC 7061. The predicted MW of this *B. pumilus* 15.1 OxdD protein was 43,799.1 Da, corresponding to the molecular weight determined by MS. The N-terminal analysis of this ~45 kDa protein, after treatment with trypsin (see below), rendered the sequence S-E-K-P-D/N-G-I-P. The SEKPNGIP sequence showed 100% identity and 100% sequence coverage with oxalate decarboxylase from *B. pumilus* 15.1 (accession number KLL01117) and other *B. pumilus* strains (KIL13977) from their second amino acid (the initiator methionine was missing as frequently occurs with *in vivo* methionine aminopeptidase activity, particularly when the next residue is small, such as the Ser residue in this case (Xiao el al., 2010).

102 The 45 kDa protein was also subjected to 2D electrophoresis for protein characterization

103 (Figure 1) and two spots at approximately pI 5.5 (spot A) and pI 10 (spot B) were observed.

104 Both spots were analysed by MALDI-TOF MS and identified as oxalate decarboxylase. The

105 theoretical pI of oxalate decarboxylase is 5.22, which corresponds with the pI observed for

106 spot A. The appearance of spot B at pI 10 is unexplained since this does not fit with the

107 theoretical value and, as far as we know, there is no reported oxalate decarboxylase with a pI

108 ≥10 in the literature.

109 Taken together, the data above indicate that the 45 kDa protein is that encoded by the

110 *B. pumilus* strain 15.1 *oxdD* gene and the protein will be described from this point as oxalate

111 decarboxylase. Features of this protein family, including the two Mn^{2+} binding sites, are

112 conserved in the *B. pumilus* protein and we were able to construct a molecular model of the

113 protein based on the known structure of the *B. subtilis* enzyme (PDB accession 5HI0) using the

114 Swiss model program (Schwede et al., 2003) as shown in Figure 2.

115

116 ***The* oxdD *gene***

117 The *oxdD* gene is located on Contig 4 of the draft *B. pumilus* 15.1 genome. Analysis of the

118 region upstream of this gene using the DBTBS database of transcription factors in *B. subtilis*

119 (Sierro et al., 2008) predicts that the gene is preceded by a putative sigma K promoter

120 (GGCCTTTTGTCACCTCACACCATACGATG) beginning 47 nt upstream of the initiator ATG.

121 Regulation by this late mother cell sigma factor would be consistent with previous studies that

122 demonstrated that *B. pumilus* strain 15.1 produces the crystal protein during sporulation when

123 cultured in T3 medium, showing a maximal accumulation after 72 h (Garcia-Ramon et al., 2016).

124 Sigma K is also used in the production of some Cry proteins in *Bt* (reviewed in (Deng et al.,

125 2014)). In addition, beginning 80 nt upstream of the ATG is a putative MntR transcription

126 factor site (GTTTCACCTTATGAAAACG). This site is normally associated with regulation of

127 Mn^{2+} transport with repression of *mntH* at high Mn^{2+} concentrations. The Mn^{2+} ion is the only

128   trace element present in T3 medium with a standard concentration of 5 mg/L of $MnCl_2 \cdot 4H_2O$

129   (25 µM), so we analyzed the accumulation of oxalate decarboxylase at concentrations ranging

130   from 0 to 0.5 g/L (0 to 2.5 mM). The cultures all reached comparable cell densities at the end of

131   the incubation period and the results showed (Figure 3) that oxalate decarboxylase was present

132   at all Mn^{2+} concentrations tested, showing maximal accumulation at 0.5 mg/L and 5 mg/L of

133    $MnCl_2$ (Figure 3, lanes 1 and 2). The variation of oxalate decarboxylase seen in these

134   experiments may be due to variations in expression, possibly mediated via the putative MntR

135   region. Alternatively, the stability of the oxalate decarboxylase could also be involved since the

136   Mn^{2+} binding sites are conserved in the *B. pumilus* 15.1 protein (Figure 2). However, we might

137   expect stabilisation to be greater at higher Mn^{2+} concentrations, which is the opposite to the

138   effect seen in our experiments.

139

140   ***The oxalate decarboxylase protein shows unexpected solubilisation behaviour***

141   When protein crystals are formed, subsequent solubilisation can be expected to occur to

142   release their potential (as occurs with crystal toxins). The crystal toxins of Bt often solubilise

143   at pH values ≥9.0, so solubility of the oxalate decarboxylase crystals was tested under similar

144   conditions. In our standard procedure, crystals from the sucrose gradient, washed with PBS,

145   were resuspended in milliQ water and kept at -20°C. When crystals were used, an aliquot of

146   the thawed crystal suspension was centrifuged, the supernatant was discarded and the pellet

147   resuspended for 1 h at 37°C in 0.1 M sodium phosphate pH 9.0. After this time, samples were

148   centrifuged and soluble and insoluble fractions were analysed by SDS PAGE. Approximately

149   50% of the crystal protein was solubilized at pH 9.0 (results not shown) but total protein

150   content (soluble and insoluble) was considerably lower than expected. Reanalysis of the

151   stored sample revealed that the protein content of the crystals kept at -20°C (pellet fraction)

152   decreased over time, with the crystals of oxalate decarboxylase protein becoming solubilized

153   into the supernatant fraction on low temperature storage. To verify this phenomenon, a fresh

154 crystal preparation was divided into two fractions. One was kept at -20℃ and the second at

155 room temperature (RT). Ten microliter samples were taken over time from each aliquot,

156 centrifuged, pellet and supernatant separated, and analyzed by SDS-PAGE gels. The results

157 presented in Figure 4 showed that when the crystal preparation was kept at RT the oxalate

158 decarboxylase was observed only in the pellet fractions (Figure 4A). In contrast, when the

159 sample was kept at -20℃, the concentration of oxalate decarboxylase in the supernatant

160 fraction increased as the incubation time at -20℃ progressed (Figure 4B). Transmission

161 electron microscopic analysis of crystals revealed that the sample incubated at RT contained

162 parasporal crystals, while the sample incubated at -20℃ (for longer than 24 h) showed almost

163 no crystals at all (data not shown). The protein from the pellet and supernatant fractions

164 obtained after incubation at -20℃ were identified by MALDI-TOF MS and LC-MS/MS (soluble

165 fraction only) to rule out the possibility that other proteins may have been present in the

166 crystals. Once again, MALDI-TOF results identified only oxalate decarboxylase, in the pellet

167 (36% coverage) and in the supernatant (34% coverage). Size exclusion chromatography of

168 the soluble protein indicates that the protein exists in solution in a multimeric form with the

169 protein eluting from the column at a volume, compared to molecular weight standards,

170 consistent with a hexameric assembly (Figure 1S supporting information).

171

172 ***The oxalate decarboxylase protein is resistant to trypsin.***

173 The soluble protein (obtained by incubation of the crystals at -20℃) was digested with a

174 range of proteases to determine whether the protein was susceptible to their action or was

175 (partially) resistant (as would be expected, eg for Cry toxins). Trypsin, chymotrypsin, papain

176 and "Proteinase from *B. subtilis*" were tested at a 10:1 ratio (w/w) protein:enzyme. SDS-PAGE

177 analysis revealed that the oxalate decarboxylase protein was completely digested by papain

178 and "Proteinase from *B. subtilis*" (Figure 5, Panel A, lanes 4 and 5), while trypsin and

179 chymotrypsin gave no visible digestion at this protein:enzyme ratio (Figure 5, Panel A, lanes 2

and 3 respectively). Increasing the quantity of chymotrypsin (1:1 and 1:10 protein:enzyme),
produced increasing degradation of the oxalate decarboxylase (Figure 5, Panel C) but
protein:trypsin ratios of 1:1 to 1:500 still produced no change in the band (Figure 5, Panel B,
lanes 2-6) while this enzyme was able to activate solubilized Cry1Aa13 used as control to
produce the expected 66 kDa product (Figure 5, Panel D).

Investigating the location of the oxdD *gene in* B. pumilus *15.1*

The majority of crystal toxin genes of *Bt* are encoded on extrachromosomal elements and we
decided to investigate the location of the *oxdD* gene. We have recently shown that the
B. pumilus 15.1 strain bears one plasmid of 7,785 bp named pBp15.1S (Contig 38) and one
megaplasmid of unknown size named pBp15.1B (Garcia-Ramon et al., 2015b). The *oxdD* gene
was found in Contig 4 (Accesion number LBDK01000004), a contig of 57,329 bp that encodes
51 predicted proteins. This contig is distinct from the small plasmid pBp15.1S but has a size
that could either represent part of a megaplasmid or a chromosome fragment. In order to
determine if the crystals produced by *B. pumilus* 15.1 strain were encoded by the
chromosome or the megaplasmid we decided to cure the strain of its extrachromosomal
elements.

Obtaining B. pumilus *15.1 variants without extrachromosomal elements*

Different methodologies described in the literature for curing extrachromosomal elements
such as heat and SDS treatment, acridine orange and promethazine treatment (detailed in the
Materials and Methods section) were used without any success (data not shown).
In a previous characterization of the *B. pumilus* 15.1 strain under electron microscopy (Garcia-
Ramon et al., 2016) we observed that the strain showed a particularly thick cell wall. We
hypothesized that the lack of effect of the compounds tested for plasmid curing might be
caused by the difficulty that these compounds might encounter in penetrating the cells to

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206 interfere with plasmid replication. For that reason, we designed a strategy in order to

207 improve the success of compound internalization and hence the success of plasmid curing.

208 The strategy consisted of obtaining spheroplasts from *B. pumilus* 15.1 with the use of

209 lysozyme prior to the treatment with the replication-interfering compounds. We tested our

210 hypothesis with acridine orange and promethazine, two very well known curing compounds.

211 *B. pumilus* 15.1 spheroplasts were obtained from vegetative cells as detailed in the Materials

212 and Methods section and then they were diluted in LB medium containing acridine orange

213 (0.03%) or promethazine (0.12%). As controls, the same amount of vegetative cells, without

214 the lysozyme treatment, were treated under the same conditions in the presence of the

215 replication-interfering compounds. When total DNA was extracted from one colony obtained

216 from each treatment (Figure 6) no extrachromosomal elements were observed in those cells

217 previously treated with lysozyme (Figure 6, lanes 3 and 4). In contrast, those cells not treated

218 with lysozyme (Figure 6, lanes 5 and 6) showed the presence of extrachromosomal elements

219 in their cytoplasm. The use of the spheroplasts instead of the vegetative cells seems to

220 improve the efficiency of acridine and promethazine in curing the strain *B. pumilus* 15.1. The

221 acridine orange strain was selected for further studies and named *B. pumilus* 15.1C (cured

222 from plasmid (pBp15.1S) and megaplasmid (pBp15.1B)). Since, in contrast to the

223 megaplasmid, the smaller pBp15.1S plasmid has been completely characterised and its copy

224 number was found to be 33 (Garcia-Ramon et al., 2015b), we were able to verify its absence

225 by PCR since, using the same methodology: no amplification was obtained from *B. pumilus*

226 strain 15.1C (data not shown). Southern blot analysis using a Dig-labeled probe designed in

227 the *orf*7 of the plasmid pBp15.1S was also carried out. The probe hybridises to the smaller

228 band in the gel, corresponding to the small plasmid and also interacts with the chromosomal

229 band, most likely due to entanglement of the plasmid with chromosomal DNA. No signal (for

230 either band) was observed in the lane corresponding to total DNA from cured *B. pumilus*

231 15.1C (Figure 7 Panel B, lane 2), verifying the absence of plasmid pBp15.1S.

The gene encoding oxalate decarboxylase in* B. pumilus *15.1 has a chromosomal location

The protein profile of the pellet fraction of a 72 h culture of the cured strain *B. pumilus* 15.1C was obtained, analyzed by SDS-PAGE and compared to the *B. pumilus* 15.1 protein profile previously described (Garcia-Ramon et al., 2016). Although the general pattern of proteins was conserved, two main differences were observed: *i*) the accumulation of the 45 kDa oxalate decarboxylase seems to be higher in the cured strain compared to the wild type (Figure 8) and *ii*) an approximately 17 kDa protein was missing in the cured strain compared to the wild-type (Figure 8, lower white arrow).

A MS fingerprinting analysis of the 17 kDa protein treated with trypsin produced two amino-acid sequences (VLPAAGTYTFR and FYAEDTLDIQTRPVVVTPPDPCGC) both showing identity with the product of the *yuaB* gene from *B. pumilus* 15.1 localized in Contig 48 and with the hypothetical protein BPUM_1610 of *B. pumilus* SAFR-032 (accession number ABV62292.1). The coverage of the sequence was around 19%, the predicted molecular weight of the 175 aa protein was 19,297 Da including a predicted signal peptide of 27 aa, the removal of which would yield a 16.3 kDa protein, consistent with the size observed in SDS PAGE gels. This protein shows 67% identity with the *Bacillus subtilis* BslA protein; a protein with an immunoglobulin-like fold that forms a hydrophobic coat on biofilms (Hobley et al., 2013; Bromley et al., 2015).

Taking these results together, we can conclude that the oxalate decarboxylase of *B. pumilus* 15.1 is not encoded by the megaplasmid pBp15.1B, as it is expressed in the cured strain *B. pumilus* 15.1C, and, therefore, the *oxdD* gene is localized in the chromosome. We can also conclude that it is highly probable that the gene encoding the 17 kDa BslA-like protein is present in the megaplamid pBp15.1B as the protein does not express in the cured strain. In order to prove this, two primers based on the gene *yuaB* in the strain 15.1 genome (Garcia-Ramon et al., 2015a) were designed. A 727 bp product was detected only when DNA from the

258   wild type strain was used as template, but not when total DNA from *B. pumilus* 15.1C was used

259   (data not shown). As the *yuaB* gene is not present in the known sequence of pBp15.1S

260   (Garcia-Ramon et al., 2015b) and as the strain contains only one plasmid and one

261   megaplasmid, we must conclude that *yuaB* gene is present in the megaplasmid pBp15.1B. The

262   24,079 bp Contig 48 (LBDK01000048), where the *yuaB* gene is present must therefore, be

263   part of this megaplasmid and contains 27 CDSs, most of them encoding hypothetical proteins.

264   When *B. pumilus* 15.1C was analyzed under transmission electron microscopy no

265   morphological differences were observed compared to *B. pumilus* 15.1 strain (data not

266   shown). The only remarkable difference was that the number of crystals in *B. pumilus* 15.1C

267   cultures was higher than in *B. pumilus* 15.1. A quantification of the number of crystals and

268   spores from different fields of the micrographs obtained, showed that the ratio crystals:spore

269   observed in a culture of *B. pumilus* 15.1C was 0.17:1 compared to the ratio 0.09:1 previously

270   determined for *B. pumilus* 15.1 (Garcia-Ramon et al., 2016). This result seems to indicate that

271   the production of the crystals in the cured strain was higher (almost double) than in the wild

272   type strain, a fact that is in agreement with the observation from SDS-PAGE that the

273   expression of the oxalate decarboxylase protein is higher in the cured strain (Figure 8).

274

275   ***Purified and insoluble crystals produced by* B. pumilus *15.1 are not toxic***

276   The crystal bands from sucrose gradients obtained from the wild type *B. pumilus* 15.1,

277   containing the majority of the oxalate decarboxylase, were tested in bioassays against first-

278   instar larvae of *C. capitata* using deionized water as negative control. As stated above, the

279   activity of strain 15.1 has decreased since initial isolation but it is possible that the purified

280   crystal, assayed at high concentrations might produce an increase in toxicity. When biossayed

281   (Table 1) crystals obtained from *B. pumilus* 15.1 showed a mortality of only 4.2% compared to

282   that obtained in the negative control (6.25% mortality). We then tested the activity of the

283   crystal fractions after being frozen at -20℃ for 4 hours to promote solubilization, performing

284 bioassays with the pellet and supernatant separately. The pellet fraction of *B. pumilus* 15.1

285 caused 6.79% mortality, while supernatant caused 18.8%. In the negative control, where just

286 water was bioassayed, a mortality of 2.08% was recorded. We observed that solubilised

287 crystals were slightly more toxic (3 fold) than the non-solubilised protein, even though a very

288 short period of time for solubilisation was allowed (only 4 h). These results may indicate that

289 oxalate descarboxylase could be involved in toxicity and it needs to be in a soluble form to

290 exert its action.

291

292 ***Oxalate decarboxylase is enzymatically active and produces formate from oxalate.***

293 With the objective of demonstrating if the oxalate decarboxylase produced by *B. pumilus* 15.1

294 as inclusion crystals is enzymatically active, two different enzymatic assays were set up. In the

295 first assay, the oxalate decarboxylase activity assay kit (Sigma Aldrich) was used to assay

296 approximately 1 μg of solubilised crystal protein. The *B. pumilus* protein produced

297 approximately 7 times more formate than the positive control enzyme (7 μl) provided with

298 the kit (9.27 and 1.25 nmol formate respectively). In the second assay, *B. pumilus* 15.1

299 crystals were purified in a sucrose gradient, resuspended in Mili Q water, kept at -20°C for 96

300 h for solubilisation and quantified by the Bradford method. Five or ten micrograms of soluble

301 protein were included in the enzymatic assays using sodium oxalate as a substrate. The

302 activity of the enzyme was evaluated in the presence and absence of Mn^{2+} (as this ion is a

303 cofactor for the enzyme). After stopping the reaction, the production of formate was analysed

304 by ^{1}H-NMR. For quantification purposes, 5 mM methanol was added to each sample as an

305 internal reference just before the ^{1}H-NMR spectra were obtained. The spectra are detailed in

306 Figure 2S as supporting information. Formate production was detected as a singlet at 8.40

307 ppm in all the spectra. After integrating the area of the formate peak and comparing with the

308 area of the methanol signal (3.31 ppm), the concentration of formate was estimated (Table 2).

309 Enzymatic assays containing 10 μg of the enzyme produced twice the amount of formate as

310 those containing 5 μg enzyme. When the enzyme was not included in the assay, formate was

311 not detected (data not shown), ruling out the possibility of spontaneous decomposition of

312 oxalate. Although Mn^{2+} is described to be cofactor for oxalate decarboxylase, the production of

313 formate was significantly reduced (around 50%) when 1 mM of the ion was present in the

314 enzymatic reaction.

315

316 ***Formate has an effect on the development of* C. capitata *larvae.***

317 After demonstrating that oxalate decarboxylase has enzymatic activity, a new set of bioassays

318 was performed in order to test whether the ingestion of formate has any effect on *C. capitata*

319 larvae. For this experiment, 100 mM ammonium formate was included in the larval artificial

320 diet. As a control, 100 mM sodium oxalate was also included in the bioassay. In parallel,

321 solubilised OxdD (5 mg/well) with and without oxalate and a whole culture of *B. pumilus*

322 strain 15.1, with and without oxalate were also assayed in order to determine if the

323 combination of these elements showed any effect on toxicity (Table 3). The presence of

324 oxalate or formate in the diet showed twice the mortality of the water control. However, while

325 no effect on larval size was observed in oxalate bioassays compared to the control, a

326 substantial reduction was noticed when formate was present (larvae did not progress further

327 than first instar), indicating that formate interfered in larval development. When solubilised

328 OxdD (5 mg/well) was included in the diet with or without oxalate, similar mortalities were

329 obtained (around twice that of the water control). No differences in mortality were observed

330 when *B. pumilus* 15.1 strain was assayed either in the presence/absence of oxalate (around

331 three times more mortality than control). These results seem to indicate that the addition of

332 oxalate to the larval diet has no mayor effects on *C. capitata* mortality, either when it was

333 bioassayed alone or together with solubilised crystals/whole *B. pumilus* culture. However,

334 when the formate was present in the diet, larvae were highly undeveloped.

335

Discussion

In this work we have characterised the parasporal crystals of *B. pumilus* strain 15.1 and shown them to consist of a member of the oxalate decarboxylase family of proteins. To our knowledge, this is the first example of a member of an enzyme family found in parasporal crystals.

In order to establish the location of the gene encoding the parasporal crystals of *B. pumilus* 15.1, both plasmids of the strain (Garcia-Ramon et al., 2015b) were removed. The conventional plasmid curing methods, involving culture at high temperature and/or in the presence of replication-interfering chemical compounds, have been applied to many bacteria (Hara et al., 1982; Ward and Ellar, 1983; Mahillon et al., 1988; Sivropoulou et al., 2000). Unfortunately, these techniques are not successful in all strains (Rajini Rani and Mahadevan, 1992; Feng et al., 2013). In fact, using the most conventional treatments (Ward and Ellar, 1983; Mahillon et al., 1988; Ghosh et al., 2000; Molnar et al., 2003) we were not able to isolate a plasmid-free variant of *B. pumilus* 15.1. We assayed sub-inhibitory concentrations of SDS, acridine orange and promethazine combined with high temperature (42°C), but plasmids were not eliminated (data not shown). Based on previous studies, it was proposed that the cell wall/cell membrane could serve as a barrier resulting in inefficient plasmid elimination (Spengler et al., 2003). Hence, the curing strategy developed here was based on obtaining spheroplasts of the cells before the treatment with the replication-interfering compounds. The strategy was highly efficient compared to the conventional methods used for spore-forming bacteria and was faster, as no successive culturing steps were needed. The method described here could represent a useful approach in those strains resilient to plasmid loss using conventional methods, especially in Gram-positive bacteria (we note that *B. pumilus* may be tolerant to higher levels of acridine orange than other species and that this sensitivity should be determined before carrying out this step at an appropriate permissive concentration). Our experiments demonstrated that the *oxdD* gene of *B. pumilus* strain 15.1

362 was located on the chromosome. Although many genes encoding crystals (such as Cry toxins)

363 are encoded by plasmids, there are some encoded in the chromosome (Hu et al., 2008; Wang

364 et al., 2014). The cured *B. pumilus* strain 15.1C, showed a parasporal crystal production

365 approximately double that of the wild type strain. This may indicate that either the small

366 plasmid pBp15.1S or the megaplasmid exerts some kind of direct or indirect regulation on the

367 expression of the *oxdD* gene. Most of the CDSs on these plasmids represent hypothetical

368 proteins but the strain 15.1 genome contig 48, here shown to be part of the megaplasmid in

369 this strain, does appear to encode a YdeB-like putative transcription factor, an HTH-type MerR

370 family transcriptional regulator, a potential RNA binding regulator of transcription that is Hfq-

371 like, and a response regulator protein; although no link between these CDSs and OxdD

372 production has yet been established. The megaplasmid also appears to encode the 17 kDa

373 YuaB protein, which has homologs in *B. subtilis* and a hypothetical protein, BPUM_1610 in

374 *B. pumilus* SAFR-032. In *B. subtilis*, YuaB is a small, secreted protein that is localized at the cell

375 wall, plays a role during biofilm formation (Ostrowski et al., 2011) and is responsible for

376 forming a layer on the surface of the biofilm making it hydrophobic (Kobayashi and Iwano,

377 2012). In contrast to *B. pumilus* 15.1, in *B. subtilis* the *yuaB* gene appears to be encoded

378 chromosomally.

379

380 Oxalate decarboxylase, is a member of the cupin family of proteins, which has enzymatic

381 members but also includes non-enzymatic proteins including seed storage proteins. The

382 *B. pumilus* 15.1 oxalate decarboxylase, along with storage proteins such as canalvalin and

383 phaseolin is a bicupin as it has 2 beta sandwich cupin domains (Tanner et al., 2001)(Figure 2)

384 each one containing one manganese binding site (Anand et al., 2002). The seed proteins are

385 known to show proteinase resistance, as seen for the protein described here. The protein

386 from *B. pumilus* crystals appears to form a hexameric complex, consistent with the oxalate

387   decarboxylase from *B. subtilis* that in solution (Svedružić et al., 2007) and in X-ray

388   crystallographic analysis (Anand et al., 2002) also forms hexamers.

389   Oxalate decarboxylase (EC 4.1.1.2) catalyzes the conversion of oxalate to formate and carbon

390   dioxide. The first bacterial oxalate decarboxylase was identified in *B. subtilis* (OxdC, formerly

391   known as YvrK) as a cytosolic enzyme (Tanner and Bornemann, 2000). Subsequently, a

392   second hypothetical protein (YoaN) from *B. subtilis* exhibited oxalate decarboxylase activity

393   and was named OxdD (Tanner et al., 2001), which was found to be present in the interior

394   layer of the spore coat (Costa et al., 2004). In *B. subtilis*, OxdC and OxdD are spore-associated

395   proteins (Kuwana et al., 2002) and the recombinant proteins overexpressed in *E. coli* are

396   soluble showing oxalate decarboxylase activity only when expressed in the presence of

397   manganese salts (Tanner et al., 2001). We have demonstrated that the accumulation of the

398   *B. pumilus* 15.1 oxalate decarboxylase is dependent on the Mn^{2+} concentration in the medium,

399   consistent with putative promoter elements identified upstream of the gene.

400   The oxalate decarboxylase crystals were found to solubilize at low temperature (-20°C), a

401   phenomenon that has not previously been described for a crystal protein. This is interesting

402   in light of the fact that toxicity of the original *B. pumilus* 15.1 strain was dependent on the

403   incubation of the whole culture at low temperature for at least 4 days (Molina et al., 2010). In

404   addition, oxalate decarboxylase parasporal crystals purified from *B. pumilus* 15.1 were not

405   significantly toxic in diet contamination assays against *C. capitata* larvae but a slight increase

406   of toxicity (2-3 times) was observed when solubilized protein was used (Table 1 and Table 3).

407   Although the oxalate decarboxylase protein is not able to induce the mortality of *C. capitata*

408   larvae by itself, we cannot rule out the possibility that this protein may play some role in this

409   process as other virulence factors could be necessary for full toxicity. There are few reports in

410   the literature of oxalate decarboxylase in relation to virulence. The substrate for this enzyme

411   (oxalic acid or oxalate) is associated with several plant pathogenic fungi from the genus

412   *Sclerotinia* (Bateman and Beer, 1965; Kritzman et al., 1977; Magro et al., 1984). Although the

16

exact mechanism of oxalic acid as a virulence factor is not completely understood, its ability to chelate calcium ions, or to change pH, favoring some cellulolytic enzymes (Lumsden, 1979) or to act as a plant defense inhibitor (Mayer and Harel, 1979; Ferrar and Walker, 1993) seems to help the fungi to invade host plants. Pseudomonad-like bacterial strains synthesising oxalate degrading enzymes (Dickman and Mitra, 1992) are reported to prevent *Sclerotinia sclerotiorum* infections in plants by removing the fugal virulence factor oxalate. Oxalate decarboxylase has been used in biological control of fungal plant diseases (Kesarwani et al., 2000; Dias et al., 2006) making transgenic plants resistant to fungal pathogens.

The fact that the oxalate decarboxylase is overexpressed in *B. pumilus* 15.1 suggests an important role for the bacterium. We have demonstrated that oxalate decarboxylase present in *B. pumilus* 15.1 crystals shows enzymatic activity when solubilised, as formate production was detected in *in vitro* enzymatic assays. The action of oxalate decarboxylase on its only described substrate, oxalate (Brenda database (Schomburg, 2015)), could produce a significant amount of formate when *B. pumilus* 15.1 is bioassayed and this could explain the toxicity of the strain toward *C. capitata* larvae. Formate is well known for being a compound toxic for insects and other arthropods and higher organisms (Elzen et al., 2004; Chaskopoulou et al., 2009; Underwood and Currie, 2009; Chen et al., 2012; Chen et al., 2013) and we have shown it to have a particularly detrimental effect on *C. capitata* larvae development. The origin of the oxalate substrate for the enzyme to produce formate in the environment is not known. The production of oxalate in bacteria is not a very frequent characteristic but in a few cases its production has been related with virulence. This has been demonstrated for *Burkholderia glumae*, a plant pathogen that causes seedling and grain rot via the production of oxalate (Li et al., 1999). Although we cannot state definitively whether strain 15.1 is able to produce oxalate, the genome data for this strain (Garcia-Ramon et al., 2015a) does not appear to exhibit genes encoding ascorbate 2,3 dioxygenase (which can produce oxalate from L-ascorbate), (S)-hydroxyl acid dehydrogenase (which can produce oxalate from glyoxalate) or

17

oxalate CoA transferase and glyoxylate dehydrogenase (which together can produce oxalate from glyoxylate via oxalylCoA). The genome does, however, encode a putative oxalate:formate symporter in the MSF family, which is present in other *B. pumilus* genomes and is conserved in other bacilli but is found in few species outside this genus, so we could speculate that the strain could utilise oxalate from the medium and use oxalate decarboxylase to produce formate as a virulence factor. However, our data showed that an external supply of oxalate in the larval diet seems not to have any effect on toxicity. Clearly many questions still remain unanswered in the mode of action of *B. pumilus* strain 15.1 but this work represents a step forward in the understanding of this bacterium in relation to putative novel virulence factors that may be used by entomopathogenic bacteria. Characterization of the kinetics of the enzyme and further investigations of its relationship with toxicity will be undertaken in further studies.

Experimental Procedures

Bacterial strain and growth conditions

The bacterial strain used in this study was *Bacillus pumilus* 15.1 (Molina et al., 2010). Luria-Bertani (LB) medium was routinely used for growing bacteria. When sporulation was required, T3 medium (Travers et al., 1987) was used and incubation was at 30℃ for 72 h at 240 rpm. Modified T3 medium was also used with different concentrations of $MnCl_2$ (ranging from 0 to 0.5 g/L).

Protein expression profile determination under different conditions

B. pumilus 15.1 was grown in 3 mL of LB at 30℃ and 240 rpm overnight and used to inoculate 50 mL of T3 medium for growth under the conditions described above for 72 h. Samples (1 mL) were centrifuged for 1 min at 16,000 x g. Pellets were resuspended in 50 μL of PBS,

465   analyzed by SDS-PAGE and stained with Coomassie brilliant blue, according to standard

466   procedures. Precision Plus Protein™ Standards (Bio-rad) molecular weight marker was used

467   in all SDS-PAGE gels.

468

469   ***Discontinuous sucrose gradient***

470   To isolate the parasporal crystals, sporulated cultures (72 h of incubation) grown in T3

471   medium were subjected to the procedure described by Garcia-Ramon *et al.* (2016) for

472   discontinuous sucrose gradient separation.

473

474   ***Protein analysis by 2D gel electrophoresis***

475   Analyses by 2-dimensional (2D) gel electrophoresis were carried out according to the

476   manufacturer's recommendations (Biorad). Briefly, 15 µL of each protein sample were mixed

477   with 115 µL of re-hydratation solution (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT and

478   0.2% ampholytes) and loaded onto IPG strips (Ready Strip™ IPG Strips 11 cm, pH 3-10, Bio-

479   Rad). The strips were re-hydrated at 20°C for 16 h (passive rehydration) in a Protean® IEF

480   Cell (Bio-Rad). Isoelectric focusing (IEF) was carried out using the following four-step

481   program: (*i*) 250 V for 1 h in a linear mode; (*ii*) 4,000 V for 2 h in a linear mode; (*iii*) 4,000 V

482   until 18,000 Vh in a rapid mode; 500 V until 50 µA per strip in a rapid mode. After IEF, strips

483   were equilibrated for 10 min in equilibration buffer I (6 M urea, 0.375 M Tris-HCl pH 8.8, 2%

484   SDS (wt/vol), 20% glycerol (vol/vol)) containing 130 mM DTT, followed by an incubation in

485   equilibration buffer II, containing 135 mM iodoacetamide instead of DTT, for 10 min. Proteins

486   were then separated by their molecular weight by placing the strip on the top of a 12% SDS-

487   PAGE in a vertical electrophoretic unit (Bio-Rad). Electrophoresis was performed at 120 V for

488   60 min. Two dimensional gels were stained with Coomassie blue.

489

Solubilization of crystals and protease treatment

491 Fractions from a discontinuous sucrose gradient containing most of the crystals produced by

492 *B. pumilus* 15.1 were kept frozen at -20℃ until use. To determine protease stability of the 45

493 kDa protein, the sample was thawed on ice and centrifuged at 13,000 rpm for 3 min and the

494 supernatant was collected in a fresh tube. Protein concentration was determined in the

495 supernatant using Bradford's reagent (Sigma), following the manufacturer's

496 recommendations and using bovine serum albumin BSA (Sigma) as a standard. Supernatant

497 fractions were incubated with four different proteolytic enzymes: trypsin, chymotrypsin,

498 papain and "Proteinase from *Bacillus subtilis*" (cat No. 96887) from Sigma. Buffers and

499 incubation temperatures for each enzyme were chosen according the instructions provided

500 by the supplier. The standard ratio used for protease treatment was 10:1 (w/w)

501 (protein:protease), although other ratios were tested. Samples were incubated for 1 h and a

502 BSA control was carried out in parallel to verify protease activity. A sample without proteases

503 was also incubated under the same conditions as a negative control. For comparative

504 purposes, the solubilized Cry1Aa13 (expressed in *Escherichia coli* from plasmid pCP10 (Pigott,

505 2006) was also digested at the same protein:trypsin ratios (between 1:1 to 1:500,

506 protein:trypsin). All the digested proteins were analyzed by SDS-PAGE.

507

508 ***Transmission electron microscopy***

509 Fresh aliquots from the sucrose gradient fractions were pelleted and washed following the

510 methodology previously described (Garcia-Ramon et al., 2016) and sent to the "Biological

511 Sample Preparation Laboratory" at the Scientific Instrumentation Center of the University of

512 Granada (CIC-UGR) for processing. Samples were observed under a Transmission Electronic

513 Microscope (LIBRA 120 PLUS from Carl Zeiss SMT) in the Microscopy Service of the CIC-UGR.

514 Ten images of 12.6 μm in size were used to determine the crystal:spore ratio.

515

Plasmid curing procedures

Three procedures reported in the literature were tested for the curing of the extrachromosomal elements present in the strain *B. pumilus* 15.1. In the first place, the methods described by Ward and Ellar (1983) and Mahillon et al. (1988), based on culturing the strain at high temperature were used with slight modifications. *B. pumilus* strain 15.1 was grown in 3 mL LB for 24 h at 42°C and 240 rpm. Successive dilutions of the culture (1:100) into fresh medium were made after 12 h of incubation during a total period of 72 h. The second method tested was performed as described above, with the difference that LB medium was supplemented with 0.002% SDS (Sivropoulou et al., 2000). In the third procedure, the *B. pumilus* 15.1 strain was grown in LB supplemented with 0.03% acridine orange or 0.12% promethazine for 24 h, either at 30°C or at 42°C. Bacterial cultures were transferred (1:100 dilution) into fresh LB medium supplemented with the interfering compounds every 12 h for 5 days.

Cells derived from these procedures were plated on LB medium and incubated for 12-24 h at 30°C. Randomly selected colonies were used for total DNA extraction using the methodology described by Reyes-Ramirez and Ibarra (2008). Total DNA was analyzed by electrophoresis in a 0.8% (wt/vol) agarose gel with SYBR Green from Invitrogen.

In addition to the standard methods, above, we also developed a novel curing strategy. For this, *B. pumilus* 15.1 was cultured in 5 mL of LB medium to an optical density at 600 nm of 0.9 to 1.1. One millilitre of the culture was pelleted at 16,000 x g for 1 min. The pellet was resuspended in 1 mL PBS containing 2% (wt/vol) lysozyme and 20% (wt/vol) sucrose, and was incubated at 37°C for 90 min. In this period of time, more than 90% spheroplast formation was achieved as monitored under the microscope. The spheroplast suspension was diluted 1:100 in LB medium supplemented with 0.03% acridine orange or 0.12% promethazine and cultured at 30°C and 240 rpm for 48 h until growth was observed. Serial dilutions were plated on LB plates and incubated at 30°C overnight.

542

Plasmid copy number determination

Plasmid copy number was determined by quantitative real time PCR as previously described (Garcia-Ramon et al., 2015b). Briefly, total DNA was used to amplify the *smc* gene that is present in a single copy on the chromosome with smc_F and smc_R primers, and orf7_F and orf7_R primers were used to amplify a unique region in the pBp15.1S plasmid.

548

Southern blot analysis

Total DNA was electrophoresed on a 0.8% (wt/vol) agarose gel and stained with ethidium bromide and transferred to a nylon membrane. The PCR product (855 ng) amplified with orf7_F and orf7_R primers (Garcia-Ramon et al., 2015b) and cleaned with QIAquick® PCR Purification kit (Qiagen) were used as a probe for the pBp15.1S plasmid. DNA labelling, transfer and fixation to the membrane, hybridization and immunological detection were performed with a DIG DNA Labeling and Detection Kit (Roche No. 11093657910) following the instructions provided by the supplier.

557

Mass spectrometric analysis of protein samples

Bands or spots identified for analysis from the 1D or 2D SDS-PAGE gels were individually excised and sent to "Centro de Investigación Principe Felipe", Valencia-Spain, for the peptide identification by Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI TOF-MS). Digestion products were analyzed by MALDI MS (4700 Proteomics analyser of the Applied Biosystems). Searches of the *B. pumilus* 15.1 genome (Garcia-Ramon et al., 2015a) and public databases were performed using MASCOT search engine (Matrix-Science, London, UK). The services from "SCSIE University of Valencia Proteomics Unit" and "CBMSO Protein Chemistry Facility" that belong to the ProteoRed Proteomics Platform were also used. At the SCSIE University of Valencia Proteomics Unit a

568   MALDI-TOF MS/MS analysis (5800 MALDI TOFTOF ABSciex) was performed. The MS and

569   MS/MS information was analyzed by MASCOT via the Protein Pilot (ABSciex). Database

570   search was performed on NCBInr.

571   At the CBMSO Protein Chemistry Facility (Madrid) a Liquid chromatography tandem mass

572   spectrometry (LC-MS/MS) analysis (Orbitrap-LTQ-Velos-Pro) was performed and the search

573   was made on UniProt-*Bacillus* and UniProt-*Bacillus pumilus* databases, using Proteome

574   Discoverer 1.4 software.

575

576   ***N-terminal amino acid sequencing***

577   The solubilized and trypsinized protein of 45 kDa was separated in a 12% acrylamide SDS

578   PAGE gel with Tris Tricine running buffer. Separated proteins were blotted onto PVDF

579   membrane using a semi-dry transfer blotter. N-terminal sequencing was performed by

580   Abingdon Health Laboratory Services, Birmingham, UK.

581   The sequence obtained was compared with protein sequences from the genome of *B. pumilus*

582   15.1 (GenBank LBDK00000000.1) (Garcia-Ramon et al., 2015a).

583

584   ***Size exclusion chromatography***

585   Soluble oxalate decarboxylase protein from *B. pumilus* strain 15.1 was analysed by size

586   exclusion chromatography using a HiLoad 16/600 Superdex 200 prepacked column (GE

587   Healthcare) in 50 mM sodium phosphate (pH 5.0), 300 mM NaCl using an AKTAPure 25

588   system (GE Healthcare). The molecular weight of oxalate decarboxylase in solution was

589   determined by reference to a calibration curve obtained on the same column with gel

590   filtration standards (BioRad).

591

Primer design and PCR amplification of the hypothetical protein YuaB

593 To PCR amplify the *yuaB* gene, the primers YuabF (5'

594 AAAAAGATCTAACCAAATGCGCTATTCCCC 3') and YuabR (5'

595 AAGAATTCCTTTGTCAACAATCTGAAGCGC 3') were designed based on the sequence from

596 *B. pumilus* 15.1 (Garcia-Ramon et al., 2015a). Total DNA from the wild type and the cured

597 strain were used under the following PCR conditions: 95°C for 5 min, followed by 30 cycles of

598 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and then a final extension at 72°C for 5 min.

599 Amplification was checked by electrophoresis on a 1% (wt/vol) agarose gel.

600

601 **C. capitata *larval bioassays***

602 Bioassays with *B. pumilus* strain 15.1 were performed as described previously (Molina et al

603 2010). When ammonium formate or sodium oxalate were bioassayed, solid powder from

604 these compounds was dissolved in the diet to a final concentration of 100 mM. The

605 insecticidal activity of insoluble parasporal inclusion suspensions obtained from *B. pumilus*

606 15.1 was tested at a cell density approximately 40 times greater than the original culture

607 following Molina *et al.* (2010) with some modifications. When solubilised, oxalate

608 decarboxylase was assayed at 10 μg/mL of diet (5 μg/well). Briefly, 100 μL of the samples

609 were dispensed into each well and mixed with 500 μL of artificial diet. One larva of *C. capitata*

610 was placed in each well. The bioassays were performed in 48-well sterile Cellstar microplates

611 (Greiner Bio-one) at 25°C. Deionized water was used as negative control. All bioassays were

612 performed at least twice using different cultures or crystal samples obtained from separate

613 cultures and gradients. In all bioassays mortality was recorded 10 days after the beginning of

614 the bioassay.

615 **Enzymatic assays**

616 The activity of oxalate decarboxylase was evaluated by the production of formate using two

617 methods. In the first, the oxalate decarboxylase activity assay kit (Sigma Aldrich) was used

618 according to the manufacturer's instructions. Results were compared to a range of

619 concentrations of formate and with the activity of an oxalate carboxylase positive control

620 (both provided in the kit). The second assay detected formate production by nmr. Briefly,

621 300 μl of sodium phosphate buffer (100 mM, pH 5.0) was mixed with 200 μl of sodium oxalate

622 (300 mM, pH 5.0) in a final volume of 600 μl containing 0, 5 or 10 μg of oxalate decarboxylase

623 enzyme (previously purified by sucrose gradient and solubilized in Milli Q water at low

624 temperature as described above). When indicated, 1 mM $MnCl_2$ was included in the assay. The

625 mixture was incubated for 2 h at 37°C and the reaction was stopped with 1 mL of sodium

626 phosphate buffer (150 mM, pH 9.5). Then, methanol (reagent grade, Sharlau) was added to

627 each sample to a final concentration of 5 mM as an internal reference for ^{1}H-NMR analysis.

628 Samples were analysed in a Varian Direct Drive Spectrometer of 500 MHz at the Centro de

629 Instrumentación Científica of the University of Granada. Spectra were obtained under fully

630 relaxed conditions and the water signal was suppressed. The area of each peak was integrated

631 using MestReNova 9.0 software taking the methanol signal as an internal reference.

632

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Table 1: Mortality results obtained after 10 days in *C. capitata* larvae bioassays using insoluble and soluble crystals obtained from *Bp* 15.1 after sucrose gradient purification and incubation at -20ºC for solubilisation. The increase in toxicity compared to the negative control was also calculated.

Bioassay	% Mortality	Fold increase
H₂0 (-ve control)	6.25 ± 2	1
Untreated crystals from *Bp*15.1	4.2 ± 1	0.6
Solubilized crystals from *Bp*15.1	18.8 ± 3	3.0
Pellet remaining after solubilisation	6.79 ± 2	1.1

Table 2: Integral value of peaks at 8.40 ppm (corresponding to formate) and estimated formate concentration using methanol as internal reference. Formate production was evaluated in the presence (1 mM) and the absence of Mn^{2+} ions and with different amounts of oxalate decarboxylase enzyme.

µg of enzyme	- Mn^{2+}		+ Mn^{2+}	
	Integral value [a]	Formate [b] concentration (mM)	Integral value [a]	Formate [b] concentration (mM)
0	0	0	0	0
5	0.19 ± 0.00	0.31 ± 0.00	0.11 ± 0.00	0.18 ± 0.01
10	0.42 ± 0.01	0.60 ± 0.02	0.18 ± 0.00	0.29 ± 0.00

[a] Mean of the integral values obtained in two different enzymatic assays.
[b] Estimated formate concentration using 5 mM methanol as internal reference.

Table 3: Mortality results obtained after 10 days in *C. capitata* larvae bioassays using different chemicals (oxalate and formate), *B. pumilus* 15.1, and soluble oxalate decarboxylase. The increase in toxicity compared to the negative control was also calculated.

Bioassay	% Mortality	Fold increase
H₂0 (-ve control)	14.35	1
Formate (100 mM)	27.35 ± 3[a]	1.8
Oxalate (100 mM)	29.84 ± 2	2.0
Oxalate (100 mM) + Soluble OxdD[b]	28.3 ± 24	1.9
Soluble OxdD[b]	27.19 ± 6	1.8
*Bp*15.1	41.67 ± 10	2.8
*Bp*15.1+oxalate (100 mM)	44.79 ± 25	3.0

[a] The body size of larvae found in this bioassay was similar to first instar larvae.
[b] The amount of soluble oxalate decarboxylase (OxdD) was 5 µg/well (10 µg/mL of diet)

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Figure 1. Two-dimensional electrophoresis of a fraction obtained from the sucrose gradient of a *B. pumilus* 15.1 culture. The pH (p*I*) range is shown horizontally and molecular weight (kDa) is shown vertically. The p*I* ranged from 3 to 10. Arrow A shows p*I* 5.5; Arrow B shows p*I* ≥ 10.

Figure 2. OxdD model. The model of *B. pumilus* 15.1 OxdD was produced using Swiss model. The two conserved Mn^{2+} binding sites (H96, H98, E102 and H274, H276, E281) are coloured red and shown with sticks and dots. The symmetry of the molecule with its two cupin domains (left and right) can be seen clearly.

Figure 3. Protein profile of the pellet fractions of *B. pumilus* 15.1 cultures grown on T3 medium in the presence of different concentrations of $MnCl_2$. The standard conditions for $MnCl_2$ were 5 mg/L (lane 2). Lane 0 shows a pellet fraction of a culture without $MnCl_2$, lane 1 with 0.5 mg/L $MnCl_2$, lane 3 with 50 mg/L $MnCl_2$, and lane 4 with 0.5 g/L $MnCl_2$. Lane M shows a molecular weight marker (Precision Plus Bio-rad) in kDa. The arrow shows the oxalate decarboxylase protein.

Figure 4. SDS-PAGE analysis of the pellet and supernatant fraction of oxalate decarboxylase crystals obtained from a fresh sucrose gradient and kept at room temperature RT (panel A) or low temperature (Panel B). The incubation at -20°C solubilized the 45 kDa oxalate decarboxylase over time while during incubation at RT the protein remained in the insoluble fraction. Lanes S represent the supernatant fractions and lanes P represent the pellet fractions of the samples. The arrows indicate the oxalate decarboxylase protein. Lanes M show the Precision Plus Bio-rad molecular weight marker in kDa.

Figure 5. SDS-PAGE analysis of the oxalate decarboxylase and the Cry1Aa13 digested with different proteases.
Panel A shows the oxalate decarboxylase digested with trypsin (lane 2), chymotrypsin (lane 3), papain (lane 4) and "proteinase from *B. subtilis*" (lane 5). Panel B and C shows digestions of the oxalate decarboxylase with trypsin (Panel B) and chymotrypsin (Panel C) at protein:protease ratios 1:1 (lanes 2), 1:10 (lanes 3), 1:50 (lanes 4), 1:100 (lanes 5) and 1:500 (lanes 6). Panel D shows the digestion of Cry1Aa13 at the same protein:protease ratios as Panel B. As control, lanes 1 show the soluble proteins with no protease treatment. Lanes M show the molecular mass marker (Precision Plus Bio-rad) in kDa.

Figure 6. DNA electrophoresis in 0.8% agarose gel of total DNA extracted from several *B. pumilus* 15.1 variants. Wild-type strain is shown in lanes 1 and 2. Variants obtained with the prior formation of spheroplasts are shown in lanes 3 (treated with acridine orange) and 4 (treated with promethazine). Lanes 5 and 6 show two variants treated with acridine orange and promethazine respectively without lysozyme treatment. M: Molecular weight marker (HyperLadder I from Bioline) in base pairs. White arrows indicate the megaplasmid (pBp15.1B) and the plasmid (pBp15.1S) respectively, and black arrow indicates the chromosomal DNA.

Figure 7. DNA electrophoresis (Panel A) and Southern blot (Panel B) of total DNA from *B. pumilus* 15.1 wild type (lanes 1) and *B. pumilus* 15.1C (lanes 2). Electrophoresis was performed in a 1% agarose gel and stained with ethidium bromide. Sothern blot was performed with a DIG labeled probe designed in the *orf*7 of the plasmid pBp15.1S (Garcia-Ramon et al., 2015b). M: Molecular weight marker (HyperLadder I from Bioline) in base pairs. The white arrows indicate the megaplasmid, the chromosome and the plasmid from top to bottom, respectively.

Figure 8. SDS-PAGE analysis of the pellets from *B. pumilus* 15.1 and *B. pumilus* 15.1C cultures.
White arrows show the oxalate decarboxylase protein at 45 kDa in the wild type (lane 1) which is more intense in the cured strain (lane 2) and the 17 kDa protein present only in the wild type strain. Lane M shows the molecular weight marker (Precision Plus Bio-rad) in kDa.

Suplementary Figures

Figure 1S: Multimeric form of *B. pumilus* 15.1 Oxalate decarboxylase determined by size-exclusion chromatography. The column was calibrated with the gel filtration standards from Bio-Rad (grey circles) vitamin B12 (1.3 kDa) (1), myoglobin (17 kDa) (2), ovalbumin (44 kDa) (3), γ-globulin (158 kDa) (4) and thyroglobulin (670 kDa) (5). Red circles represent the theoretical elution volume for the OxdD monomer (a), hexamer (b) and heptamer (c). The blue circle represents the experimental elution volume obtained for *B. pumilus* 15.1 OxdD.

Figure 2S: Representative spectra obtained in the H-NMR analysis. The spectra were obtained from oxalate decarboxylase enzymatic reactions using 5 ☐g (panels A and C) and 10 ☐g of enzyme (panels B and D). Reactions were performed in the absence (panels A and B) and in the presence (panels C and D) of 1 mM of Mn^{2+}. Integration values for formate (8.404 ppm) were calculated using methanol (3.3 ppm) as an internal reference.