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

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Running Title: Parasporal crystal in *Bacillus pumilus* 15.1

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Keywords: *entomopathogenic bacteria, crystal inclusions,* Bacillus pumilus, *plasmid curing*, *oxalate decarboxylase*

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

51 invertebrate-active toxins and it is likely that their natural target merely remains to be
52 discovered (although activity against certain human cancer cells in culture has been reported
53 (Ohba et al., 2009)). The most studied proteinaceous toxins are the Cry and Cyt toxins,
54 produced mainly by *Bacillus thuringiensis* (*Bt*), which are the principal agents responsible for
55 the toxicity of these bacteria toward insects. The insecticidal activity of crystal proteins
56 produced by *Bt* has been extensively used as the basis of many commercial products. The
57 ability to produce parasporal crystals is not restricted to *Bt* as some strains of *Lysinibacillus*
58 *sphaericus* (Jones et al., 2007), *Clostridium bifermentans* (Barloy et al., 1996), *Paenibacillus*
59 *popilliae* (Zhang et al., 1997), *Brevibacillus laterosporus* (Smirnova et al., 1996) and *P.*
60 *lentimorbus* (Yokoyama et al., 2004), also produce parasporal inclusions active against insects.
61 The mechanisms of action proposed for the parasporal crystal toxins generally require
62 solubilization and proteolytic activation of the protoxin form in the midgut of the target
63 invertebrate (Haider et al., 1986; Palma et al., 2014). Serine proteases are important in both
64 solubilization and activation of *Bt* protoxins and, in some insects, changes in the protease
65 profile of their guts have been associated with resistance to *Bt* toxin (Li et al., 2004;
66 Karumbaiah et al., 2007).
67 Our research group reported a *Bacillus pumilus* strain toxic toward the Mediterranean fruit
68 fly, *Ceratitis capitata* (Molina et al., 2010). Previous assays showed that the toxicity of
69 *B. pumilus* 15.1 can be inactivated either by heat or by proteases, suggesting that the virulence
70 factor produced by this strain could be proteinaceous (Molina, 2010). Since its initial isolation
71 and testing, our strain appears to have decreased in toxicity, even though it continues to
72 produce the parasporal crystals, mainly composed of a 45 kDa protein, that we have
73 previously described (Garcia-Ramon et al., 2016). Despite the loss of toxicity, the crystal
74 protein was still considered as a candidate toxin (possibly interacting with another factor,
75 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).

The 45 kDa protein was also subjected to 2D electrophoresis for protein characterization (Figure 1) and two spots at approximately p*I* 5.5 (spot A) and p*I* 10 (spot B) were observed. Both spots were analysed by MALDI-TOF MS and identified as oxalate decarboxylase. The theoretical p*I* of oxalate decarboxylase is 5.22, which corresponds with the p*I* observed for spot A. The appearance of spot B at p*I* 10 is unexplained since this does not fit with the theoretical value and, as far as we know, there is no reported oxalate decarboxylase with a p*I* ≥10 in the literature.

Taken together, the data above indicate that the 45 kDa protein is that encoded by the *B. pumilus* strain 15.1 *oxdD* gene and the protein will be described from this point as oxalate decarboxylase. Features of this protein family, including the two Mn^{2+} binding sites, are conserved in the *B. pumilus* protein and we were able to construct a molecular model of the protein based on the known structure of the *B. subtilis* enzyme (PDB accession 5HI0) using the Swiss model program (Schwede et al., 2003) as shown in Figure 2.

The oxdD *gene*

The *oxdD* gene is located on Contig 4 of the draft *B. pumilus* 15.1 genome. Analysis of the region upstream of this gene using the DBTBS database of transcription factors in *B. subtilis* (Sierro et al., 2008) predicts that the gene is preceded by a putative sigma K promoter (GGCCTTTTGTCACCTCACACCATACGATG) beginning 47 nt upstream of the initiator ATG. Regulation by this late mother cell sigma factor would be consistent with previous studies that demonstrated that *B. pumilus* strain 15.1 produces the crystal protein during sporulation when cultured in T3 medium, showing a maximal accumulation after 72 h (Garcia-Ramon et al., 2016). Sigma K is also used in the production of some Cry proteins in *Bt* (reviewed in (Deng et al., 2014)). In addition, beginning 80 nt upstream of the ATG is a putative MntR transcription factor site (GTTTCACCTTATGAAAACG). This site is normally associated with regulation of Mn^{2+} transport with repression of *mntH* at high Mn^{2+} concentrations. The Mn^{2+} ion is the only

trace element present in T3 medium with a standard concentration of 5 mg/L of $MnCl_2 \bullet 4H_2O$ (25 μM), so we analyzed the accumulation of oxalate decarboxylase at concentrations ranging from 0 to 0.5 g/L (0 to 2.5 mM). The cultures all reached comparable cell densities at the end of the incubation period and the results showed (Figure 3) that oxalate decarboxylase was present at all Mn^{2+} concentrations tested, showing maximal accumulation at 0.5 mg/L and 5 mg/L of $MnCl_2$ (Figure 3, lanes 1 and 2). The variation of oxalate decarboxylase seen in these experiments may be due to variations in expression, possibly mediated via the putative MntR region. Alternatively, the stability of the oxalate decarboxylase could also be involved since the Mn^{2+} binding sites are conserved in the *B. pumilus* 15.1 protein (Figure 2). However, we might expect stabilisation to be greater at higher Mn^{2+} concentrations, which is the opposite to the effect seen in our experiments.

The oxalate decarboxylase protein shows unexpected solubilisation behaviour

When protein crystals are formed, subsequent solubilisation can be expected to occur to release their potential (as occurs with crystal toxins). The crystal toxins of Bt often solubilise at pH values ≥9.0, so solubility of the oxalate decarboxylase crystals was tested under similar conditions. In our standard procedure, crystals from the sucrose gradient, washed with PBS, were resuspended in milliQ water and kept at -20˚C. When crystals were used, an aliquot of the thawed crystal suspension was centrifuged, the supernatant was discarded and the pellet resuspended for 1 h at 37˚C in 0.1 M sodium phosphate pH 9.0. After this time, samples were centrifuged and soluble and insoluble fractions were analysed by SDS PAGE. Approximately 50% of the crystal protein was solubilized at pH 9.0 (results not shown) but total protein content (soluble and insoluble) was considerably lower than expected. Reanalysis of the stored sample revealed that the protein content of the crystals kept at -20˚C (pellet fraction) decreased over time, with the crystals of oxalate decarboxylase protein becoming solubilized into the supernatant fraction on low temperature storage. To verify this phenomenon, a fresh

crystal preparation was divided into two fractions. One was kept at -20˚C and the second at room temperature (RT). Ten microliter samples were taken over time from each aliquot, centrifuged, pellet and supernatant separated, and analyzed by SDS-PAGE gels. The results presented in Figure 4 showed that when the crystal preparation was kept at RT the oxalate decarboxylase was observed only in the pellet fractions (Figure 4A). In contrast, when the sample was kept at -20˚C, the concentration of oxalate decarboxylase in the supernatant fraction increased as the incubation time at -20˚C progressed (Figure 4B). Transmission electron microscopic analysis of crystals revealed that the sample incubated at RT contained parasporal crystals, while the sample incubated at -20˚C (for longer than 24 h) showed almost no crystals at all (data not shown). The protein from the pellet and supernatant fractions obtained after incubation at -20˚C were identified by MALDI-TOF MS and LC-MS/MS (soluble fraction only) to rule out the possibility that other proteins may have been present in the crystals. Once again, MALDI-TOF results identified only oxalate decarboxylase, in the pellet (36% coverage) and in the supernatant (34% coverage). Size exclusion chromatography of the soluble protein indicates that the protein exists in solution in a multimeric form with the protein eluting from the column at a volume, compared to molecular weight standards, consistent with a hexameric assembly (Figure 1S supporting information).

The oxalate decarboxylase protein is resistant to trypsin.

The soluble protein (obtained by incubation of the crystals at -20˚C) was digested with a range of proteases to determine whether the protein was susceptible to their action or was (partially) resistant (as would be expected, eg for Cry toxins). Trypsin, chymotrypsin, papain and "Proteinase from *B. subtilis*" were tested at a 10:1 ratio (w/w) protein:enzyme. SDS-PAGE analysis revealed that the oxalate decarboxylase protein was completely digested by papain and "Proteinase from *B. subtilis*" (Figure 5, Panel A, lanes 4 and 5), while trypsin and 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

interfere with plasmid replication. For that reason, we designed a strategy in order to improve the success of compound internalization and hence the success of plasmid curing. The strategy consisted of obtaining spheroplasts from *B. pumilus* 15.1 with the use of lysozyme prior to the treatment with the replication-interfering compounds. We tested our hypothesis with acridine orange and promethazine, two very well known curing compounds. *B. pumilus* 15.1 spheroplasts were obtained from vegetative cells as detailed in the Materials and Methods section and then they were diluted in LB medium containing acridine orange (0.03%) or promethazine (0.12%). As controls, the same amount of vegetative cells, without the lysozyme treatment, were treated under the same conditions in the presence of the replication-interfering compounds. When total DNA was extracted from one colony obtained from each treatment (Figure 6) no extrachromosomal elements were observed in those cells previously treated with lysozyme (Figure 6, lanes 3 and 4). In contrast, those cells not treated with lysozyme (Figure 6, lanes 5 and 6) showed the presence of extrachromosomal elements in their cytoplasm. The use of the spheroplasts instead of the vegetative cells seems to improve the efficiency of acridine and promethazine in curing the strain *B. pumilus* 15.1. The acridine orange strain was selected for further studies and named *B. pumilus* 15.1C (cured from plasmid (pBp15.1S) and megaplasmid (pBp15.1B)). Since, in contrast to the megaplasmid, the smaller pBp15.1S plasmid has been completely characterised and its copy number was found to be 33 (Garcia-Ramon et al., 2015b), we were able to verify its absence by PCR since, using the same methodology: no amplification was obtained from *B. pumilus* strain 15.1C (data not shown). Southern blot analysis using a Dig-labeled probe designed in the *orf*7 of the plasmid pBp15.1S was also carried out. The probe hybridises to the smaller band in the gel, corresponding to the small plasmid and also interacts with the chromosomal band, most likely due to entanglement of the plasmid with chromosomal DNA. No signal (for either band) was observed in the lane corresponding to total DNA from cured *B. pumilus* 15.1C (Figure 7 Panel B, lane 2), verifying the absence of plasmid pBp15.1S.

232

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

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

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

251 Taking these results together, we can conclude that the oxalate decarboxylase of *B. pumilus*
252 15.1 is not encoded by the megaplasmid pBp15.1B, as it is expressed in the cured strain
253 *B. pumilus* 15.1C, and, therefore, the *oxdD* gene is localized in the chromosome. We can also
254 conclude that it is highly probable that the gene encoding the 17 kDa BslA-like protein is
255 present in the megaplamid pBp15.1B as the protein does not express in the cured strain. In
256 order to prove this, two primers based on the gene *yuaB* in the strain 15.1 genome (Garcia-
257 Ramon et al., 2015a) were designed. A 727 bp product was detected only when DNA from the

wild type strain was used as template, but not when total DNA from *B. pumilus* 15.1C was used (data not shown). As the *yuaB* gene is not present in the known sequence of pBp15.1S (Garcia-Ramon et al., 2015b) and as the strain contains only one plasmid and one megaplasmid, we must conclude that *yuaB* gene is present in the megaplasmid pBp15.1B. The 24,079 bp Contig 48 (LBDK01000048), where the *yuaB* gene is present must therefore, be part of this megaplasmid and contains 27 CDSs, most of them encoding hypothetical proteins.

When *B. pumilus* 15.1C was analyzed under transmission electron microscopy no morphological differences were observed compared to *B. pumilus* 15.1 strain (data not shown). The only remarkable difference was that the number of crystals in *B. pumilus* 15.1C cultures was higher than in *B. pumilus* 15.1. A quantification of the number of crystals and spores from different fields of the micrographs obtained, showed that the ratio crystals:spore observed in a culture of *B. pumilus* 15.1C was 0.17:1 compared to the ratio 0.09:1 previously determined for *B. pumilus* 15.1 (Garcia-Ramon et al., 2016). This result seems to indicate that the production of the crystals in the cured strain was higher (almost double) than in the wild type strain, a fact that is in agreement with the observation from SDS-PAGE that the expression of the oxalate decarboxylase protein is higher in the cured strain (Figure 8).

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

The crystal bands from sucrose gradients obtained from the wild type *B. pumilus* 15.1, containing the majority of the oxalate decarboxylase, were tested in bioassays against first-instar larvae of *C. capitata* using deionized water as negative control. As stated above, the activity of strain 15.1 has decreased since initial isolation but it is possible that the purified crystal, assayed at high concentrations might produce an increase in toxicity. When biossayed (Table 1) crystals obtained from *B. pumilus* 15.1 showed a mortality of only 4.2% compared to that obtained in the negative control (6.25% mortality). We then tested the activity of the crystal fractions after being frozen at -20˚C for 4 hours to promote solubilization, performing

bioassays with the pellet and supernatant separately. The pellet fraction of *B. pumilus* 15.1 caused 6.79% mortality, while supernatant caused 18.8%. In the negative control, where just water was bioassayed, a mortality of 2.08% was recorded. We observed that solubilised crystals were slightly more toxic (3 fold) than the non-solubilised protein, even though a very short period of time for solubilisation was allowed (only 4 h). These results may indicate that oxalate descarboxylase could be involved in toxicity and it needs to be in a soluble form to exert its action.

Oxalate decarboxylase is enzymatically active and produces formate from oxalate.

With the objective of demonstrating if the oxalate decarboxylase produced by *B. pumilus* 15.1 as inclusion crystals is enzymatically active, two different enzymatic assays were set up. In the first assay, the oxalate decarboxylase activity assay kit (Sigma Aldrich) was used to assay approximately 1 μg of solubilised crystal protein. The *B. pumilus* protein produced approximately 7 times more formate than the positive control enzyme (7 μl) provided with the kit (9.27 and 1.25 nmol formate respectively). In the second assay, *B. pumilus* 15.1 crystals were purified in a sucrose gradient, resuspended in Mili Q water, kept at -20˚C for 96 h for solubilisation and quantified by the Bradford method. Five or ten micrograms of soluble protein were included in the enzymatic assays using sodium oxalate as a substrate. The activity of the enzyme was evaluated in the presence and absence of Mn^{2+} (as this ion is a cofactor for the enzyme). After stopping the reaction, the production of formate was analysed by ^{1}H-NMR. For quantification purposes, 5 mM methanol was added to each sample as an internal reference just before the ^{1}H-NMR spectra were obtained. The spectra are detailed in Figure 2S as supporting information. Formate production was detected as a singlet at 8.40 ppm in all the spectra. After integrating the area of the formate peak and comparing with the area of the methanol signal (3.31 ppm), the concentration of formate was estimated (Table 2). Enzymatic assays containing 10 μg of the enzyme produced twice the amount of formate as

those containing 5 μg enzyme. When the enzyme was not included in the assay, formate was not detected (data not shown), ruling out the possibility of spontaneous decomposition of oxalate. Although Mn^{2+} is described to be cofactor for oxalate decarboxylase, the production of formate was significantly reduced (around 50%) when 1 mM of the ion was present in the enzymatic reaction.

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

After demonstrating that oxalate decarboxylase has enzymatic activity, a new set of bioassays was performed in order to test whether the ingestion of formate has any effect on *C. capitata* larvae. For this experiment, 100 mM ammonium formate was included in the larval artificial diet. As a control, 100 mM sodium oxalate was also included in the bioassay. In parallel, solubilised OxdD (5 mg/well) with and without oxalate and a whole culture of *B. pumilus* strain 15.1, with and without oxalate were also assayed in order to determine if the combination of these elements showed any effect on toxicity (Table 3). The presence of oxalate or formate in the diet showed twice the mortality of the water control. However, while no effect on larval size was observed in oxalate bioassays compared to the control, a substantial reduction was noticed when formate was present (larvae did not progress further than first instar), indicating that formate interfered in larval development. When solubilised OxdD (5 mg/well) was included in the diet with or without oxalate, similar mortalities were obtained (around twice that of the water control). No differences in mortality were observed when *B. pumilus* 15.1 strain was assayed either in the presence/absence of oxalate (around three times more mortality than control). These results seem to indicate that the addition of oxalate to the larval diet has no mayor effects on *C. capitata* mortality, either when it was bioassayed alone or together with solubilised crystals/whole *B. pumilus* culture. However, when the formate was present in the diet, larvae were highly undeveloped.

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

413 exact mechanism of oxalic acid as a virulence factor is not completely understood, its ability to
414 chelate calcium ions, or to change pH, favoring some cellulolytic enzymes (Lumsden, 1979) or
415 to act as a plant defense inhibitor (Mayer and Harel, 1979; Ferrar and Walker, 1993) seems to
416 help the fungi to invade host plants. Pseudomonad-like bacterial strains synthesising oxalate
417 degrading enzymes (Dickman and Mitra, 1992) are reported to prevent *Sclerotinia*
418 *sclerotiorum* infections in plants by removing the fugal virulence factor oxalate. Oxalate
419 decarboxylase has been used in biological control of fungal plant diseases (Kesarwani et al.,
420 2000; Dias et al., 2006) making transgenic plants resistant to fungal pathogens.
421 The fact that the oxalate decarboxylase is overexpressed in *B. pumilus* 15.1 suggests an
422 important role for the bacterium. We have demonstrated that oxalate decarboxylase present
423 in *B. pumilus* 15.1 crystals shows enzymatic activity when solubilised, as formate production
424 was detected in *in vitro* enzymatic assays. The action of oxalate decarboxylase on its only
425 described substrate, oxalate (Brenda database (Schomburg, 2015)), could produce a
426 significant amount of formate when *B. pumilus* 15.1 is bioassayed and this could explain the
427 toxicity of the strain toward *C. capitata* larvae. Formate is well known for being a compound
428 toxic for insects and other arthropods and higher organisms (Elzen et al., 2004; Chaskopoulou
429 et al., 2009; Underwood and Currie, 2009; Chen et al., 2012; Chen et al., 2013) and we have
430 shown it to have a particularly detrimental effect on *C. capitata* larvae development. The
431 origin of the oxalate substrate for the enzyme to produce formate in the environment is not
432 known. The production of oxalate in bacteria is not a very frequent characteristic but in a few
433 cases its production has been related with virulence. This has been demonstrated for
434 *Burkholderia glumae*, a plant pathogen that causes seedling and grain rot via the production of
435 oxalate (Li et al., 1999). Although we cannot state definitively whether strain 15.1 is able to
436 produce oxalate, the genome data for this strain (Garcia-Ramon et al., 2015a) does not appear
437 to exhibit genes encoding ascorbate 2,3 dioxygenase (which can produce oxalate from L-
438 ascorbate), (S)-hydroxyl acid dehydrogenase (which can produce oxalate from glyoxalate) or

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˚C 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˚C 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,

analyzed by SDS-PAGE and stained with Coomassie brilliant blue, according to standard procedures. Precision Plus Protein™ Standards (Bio-rad) molecular weight marker was used in all SDS-PAGE gels.

Discontinuous sucrose gradient

To isolate the parasporal crystals, sporulated cultures (72 h of incubation) grown in T3 medium were subjected to the procedure described by Garcia-Ramon *et al.* (2016) for discontinuous sucrose gradient separation.

Protein analysis by 2D gel electrophoresis

Analyses by 2-dimensional (2D) gel electrophoresis were carried out according to the manufacturer's recommendations (Biorad). Briefly, 15 µL of each protein sample were mixed with 115 µL of re-hydratation solution (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT and 0.2% ampholytes) and loaded onto IPG strips (Ready Strip™ IPG Strips 11 cm, pH 3-10, Bio-Rad). The strips were re-hydrated at 20˚C for 16 h (passive rehydration) in a Protean® IEF Cell (Bio-Rad). Isoelectric focusing (IEF) was carried out using the following four-step 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 until 18,000 Vh in a rapid mode; 500 V until 50 µA per strip in a rapid mode. After IEF, strips were equilibrated for 10 min in equilibration buffer I (6 M urea, 0.375 M Tris-HCl pH 8.8, 2% SDS (wt/vol), 20% glycerol (vol/vol)) containing 130 mM DTT, followed by an incubation in equilibration buffer II, containing 135 mM iodoacetamide instead of DTT, for 10 min. Proteins were then separated by their molecular weight by placing the strip on the top of a 12% SDS-PAGE in a vertical electrophoretic unit (Bio-Rad). Electrophoresis was performed at 120 V for 60 min. Two dimensional gels were stained with Coomassie blue.

490 ***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˚C 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

516 ***Plasmid curing procedures***

517 Three procedures reported in the literature were tested for the curing of the
518 extrachromosomal elements present in the strain *B. pumilus* 15.1. In the first place, the
519 methods described by Ward and Ellar (1983) and Mahillon et al. (1988), based on culturing
520 the strain at high temperature were used with slight modifications. *B. pumilus* strain 15.1 was
521 grown in 3 mL LB for 24 h at 42˚C and 240 rpm. Successive dilutions of the culture (1:100)
522 into fresh medium were made after 12 h of incubation during a total period of 72 h. The
523 second method tested was performed as described above, with the difference that LB medium
524 was supplemented with 0.002% SDS (Sivropoulou et al., 2000). In the third procedure, the
525 *B. pumilus* 15.1 strain was grown in LB supplemented with 0.03% acridine orange or 0.12%
526 promethazine for 24 h, either at 30˚C or at 42˚C. Bacterial cultures were transferred (1:100
527 dilution) into fresh LB medium supplemented with the interfering compounds every 12 h for
528 5 days.

529 Cells derived from these procedures were plated on LB medium and incubated for 12-24 h at
530 30˚C. Randomly selected colonies were used for total DNA extraction using the methodology
531 described by Reyes-Ramirez and Ibarra (2008). Total DNA was analyzed by electrophoresis
532 in a 0.8% (wt/vol) agarose gel with SYBR Green from Invitrogen.

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

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.

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.

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

MALDI-TOF MS/MS analysis (5800 MALDI TOFTOF ABSciex) was performed. The MS and MS/MS information was analyzed by MASCOT via the Protein Pilot (ABSciex). Database search was performed on NCBInr.

At the CBMSO Protein Chemistry Facility (Madrid) a Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis (Orbitrap-LTQ-Velos-Pro) was performed and the search was made on UniProt-*Bacillus* and UniProt-*Bacillus pumilus* databases, using Proteome Discoverer 1.4 software.

N-terminal amino acid sequencing

The solubilized and trypsinized protein of 45 kDa was separated in a 12% acrylamide SDS PAGE gel with Tris Tricine running buffer. Separated proteins were blotted onto PVDF membrane using a semi-dry transfer blotter. N-terminal sequencing was performed by Abingdon Health Laboratory Services, Birmingham, UK.

The sequence obtained was compared with protein sequences from the genome of *B. pumilus* 15.1 (GenBank LBDK00000000.1) (Garcia-Ramon et al., 2015a).

Size exclusion chromatography

Soluble oxalate decarboxylase protein from *B. pumilus* strain 15.1 was analysed by size exclusion chromatography using a HiLoad 16/600 Superdex 200 prepacked column (GE Healthcare) in 50 mM sodium phosphate (pH 5.0), 300 mM NaCl using an AKTAPure 25 system (GE Healthcare). The molecular weight of oxalate decarboxylase in solution was determined by reference to a calibration curve obtained on the same column with gel filtration standards (BioRad).

592 ***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
633 **Acknowledgements**
634 We are very grateful to Dr. Manuel Martínez Bueno and Dr. Rubén Cebrian, from the
635 University of Granada, for their help with the Southern-blot technique. We also thank the
636 Scientific Instrumentation Center of the University of Granada for the service and support of
637 the microscopy and ^{1}H-NMR service. Also, thanks to the "Centro de Investigación Principe
638 Felipe" Valencia – Spain, "SCSIE University of Valencia Proteomics Unit", especially to Oreto
639 Antúnez Temporal; and "CBMSO Protein Chemistry Facility" for the service and support on
640 the MALDI-TOF analyses. We also thank to Dr. Barba from Vall d'Hebron Hospital and Dr.
641 Álvarez de Cienfuegos from University of Granada for their help in ^{1}H-NMR analysis. SEC
642 analysis was undertaken in the Cardiff School of Biosciences Protein Technology Research

643 Hub. This work was partially supported by the MEC project CGL2008-02011 and project AGR-
644 6409 from the Junta de Andalucía Research Council. All author declare any conflict of interest.

645

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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
H_20 (-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.

	- Mn^{2+}		**+ Mn^{2+}**	
µg of enzyme	**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
H_20 (-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)

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.