Low-molecular weight metabolites secreted by Paenibacillus larvae as potential virulence factors of American Foulbrood

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Abstract

The spore forming bacterium *Paenibacillus larvae* causes a severe and highly infective bee disease, American Foulbrood (AFB). Despite the large economic losses induced by AFB, the virulence factors produced by *P. larvae* are as yet unknown. To identify such virulence factors, we experimentally infected young, susceptible larvae of the honeybee, *Apis mellifera carnica*, with different *P. larvae* isolates. Honeybee larvae were reared *in vitro* in 24-well plates in the laboratory after isolation from the brood comb. We identified genotype-specific differences in the etiopathology of AFB between the tested isolates of *P. larvae*, which were revealed by differences in the LT100. We furthermore confirmed that extracts of *P. larvae* cultures contain low molecular weight compounds, which are toxic to honeybee larvae. Our data indicate that *P. larvae* secretes metabolites into the medium with a potent honeybee toxic activity pointing to a novel pathogenic factor(s) of *P. larvae*. Genome mining of *P. larvae* ssp. *larvae* BRL-230010 led to the identification of several biosynthesis gene clusters putatively involved in natural product biosynthesis highlighting the potential of *P. larvae* to produce such compounds.

Introduction

Honeybees are the most important economical and ecological pollinators of wild plants and insect-pollinated cultivated crops. About 35% of the global crop-based food production benefits from animal-mediated pollination with bees being the primary pollinators for most of the crops requiring animal pollination (1). One factor that contributes to their high pollinator efficiency is the high population densities in bee hives. On the other hand, this high density makes bees especially vulnerable to various pathogens, such as viruses, mites and bacteria. Among them, *Paenibacillus larvae* is the most virulent pathogenic bacterium. It is a spore-forming, Gram-positive, rod shaped, and obligatory pathogenic bacterium, that has to kill its host to form novel spores, which can then be transmitted further. It forms extremely tenacious
spores that remain infectious for over half a century (2, 3). The bacterium has a highly pathogenic reproduction rate producing a huge number of spores within each infected larva (4). Strains of *P. larvae* can be subdivided into four different genotypes that differ in their virulence (5, 6). While larvae infected with *P. larvae* genotypes ERIC II to ERIC IV were killed within 6 to 7 days, it took ERIC I around 12 to 14 days to kill all infected individuals. Therefore, genotype ERIC I was considered to be less virulent to bee larvae as compared to the other three genotypes (2, 5). The different lethal times (LT$_{100}$) are relevant because infection symptoms or deaths may be detected by nurse bee more frequently. Removing infected bees earlier may be beneficial for the whole colony.

*P. larvae* is the only known causative pathogen of American Foulbrood, (AFB) (5, 7, 8). The disease has spread worldwide (9, 10) and is causing a significant decrease in honeybee populations and significant drops of honey, pollen, propolis, royal jelly and beeswax production (11). AFB is a highly deleterious bacterial honey bee disease, that only affects the larval stages of the honeybee (2, 12) with 24-48 h old larvae being most susceptible to *P. larvae* (2). Adult worker bees become contaminated while removing dead larvae, which probably is an important infection pathway to spread the infectious spores in the colony. The infection begins when spore-contaminated food is swallowed by the larvae. After germination in the larval midgut, vegetative cells proliferate and translocate into the hemocoel, probably by penetrating the midgut epithelium, and consecutively spread within the hemolymph causing septicemia and ultimately kill the larvae (13). After the larvae died their tissue decays while the infected larval body changes to a brownish and viscous mass (ropy stage). Next it dries down to a hard scale, which is tightly adhering to the lower cell wall and contains millions of spores (4, 12-15). Although it was commonly assumed that the honeybee, *Apis mellifera*, is the only host species, *P. larvae* may cause bacteremic infections in humans, when the spores are unintentionally self-injected, e.g., via honey-prepared methadone by drug users with a suppressed immune system (16).
Despite the wealth of studies about AFB and *P. larvae*, relatively little is known about the chemicals causing larval toxicity. This is even more astonishing since several bioactive compounds have been described from other *Paenibacillus* species (17-24). Other entomopathogenic bacteria (25, 26) produce and secrete many different pathogenic compounds, for example proteins, antibiotics or polypeptides. This has also been observed for *P. larvae*, which produced a broad spectrum of antibacterial compounds, probably suppressing potential competitors in the honeybee larvae cadaver (27). Moreover, it secretes proteases, which may act as virulence factors during the infection process (28).

The AB toxins Plx1 and Plx2 are among the virulence factors of *P. larvae* and have been identified in the genome of *P. larvae* ERIC I but not in the genome of ERIC II (29). They show considerable homology to MTX1 from *Lysinibacillus sphaericus* (30). MTX1 was described as a toxin that induces rounding up of host cells (31). This effect is needed during the *P. larvae* infection pathway while breaching the midgut epithelium to invade into the host’s haemocoel (13).

The aim of this study was to determine whether *P. larvae* secretes low molecular weight compounds which are toxic to honeybee larvae reared *in vitro* under controlled experimental conditions without social brood care interference. Thus, an *in vitro* rearing of honeybee larvae had to be established and different strains of *P. larvae* were analyzed using the bee larvae as a bioassay as well as by analytical chemistry methods. Additionally, the available genomes of two strains of *P. larvae* were analyzed regarding gene clusters, which might be involved in the biosynthesis of these virulence factors.

**Methods**
Honeybees

All experiments were carried out on larvae from *Apis mellifera carnica*. Larvae derived from the progeny of a single wild-mated queen were maintained in a AFB symptom-free apiary at the Institut für Bienenkunde Oberursel, Germany. The experiments were performed between July and August 2010.

Artificial rearing

Larvae were reared in 24-well tissue plates at 35°C and 95 % R.H. according to published methods (6, 32). First instar larvae (ca. 12 h, larval age was estimated by size), which were grafted from worker brood cells were used throughout the experiments. The larvae were carefully removed out of their cells using a grafting tool (Schweizer Umlarvlöffel, Carl Fritz Imkertechnik GmbH & Co. KG, Mellrichstadt, Germany). A 24-well tissue culture plate (3.4 ml/well Orange Scientific, B-1420 Braine-l'Alleud, Belgium) was selected as the larval and the post-defecation rearing container. One larva was placed into each well onto 100µL of the larvae diet. During the first 5 days larvae were transferred every 24 h to a fresh well with new spore-free diet. From day 6 until defecation larvae were grafted every 48h to reduce handling stress. The infectious larval diet was fed only during the first 24h after grafting. During extract- and supernatant-tests, larvae were fed from day one until they defecated or died. Larvae were fed daily ad libitum (100 µl) with an excess of a liquid larvae diet consisting of 50% (w/w) royal jelly (Imkerei Ullmann, Erlensee, Germany), 6% (w/v) glucose (Sigma Aldrich, Deutschland), 6% (w/v) fructose (Carl Roth GmbH, Karlsruhe, Deutschland) in sterile deionized water. The transfer of the growing larvae to a new well with fresh diet took place on a heating plate adjusted to about 34°C and dead larvae were removed. For the transfer of larger larvae a rounded sterile metal spatula was used. After defecation (approx. at day 9) the larvae were transferred to pupation wells lined with paper tissues. Prior to the transfer into pupation
wells, larvae were placed and rolled briefly on absorbent paper to remove adhering larval diet and faeces. The larvae were maintained in an incubator (Heraeus Instruments Typ t 6120) at 35°C and 96 % R.H. The experiments were terminated at day 15 because most bees have reached the prepupal stage by then and clinical symptoms of AFB should have been diagnosed before such that only little additional mortality is predicted.

Vital parameter estimation

Each day the larvae from control and infected groups were examined under a stereo microscope. The presence of larval exuvia, signs of respiration and injury, disease symptoms, colour change, infestation of fungal infection or other abnormalities were monitored and dead larvae were removed. The number of dead larvae on each plate was determined and surviving larvae were transferred to fresh food.

Bacterial strains

The *P. larvae* strains DSM 16116, DSM 16115 and DSM 17237 used in this study were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). They represent German field isolates of the year 2003 and they were genotype ERIC II or AB, respectively (33, 34). NRLL B-41635 was obtained from the United States Department of Agriculture (Agricultural Research Service; ARS). The genome of strain *P. larvae* ssp. *larvae* BRL-230010 was sequenced recently (35) and was used for the identification of biosynthesis gene clusters. NRLL B-41635 was cultivated in MYPGP media for showing negative effects on larvae development as described previously (36, 37). All other strains were grown in J medium (38).
Exposure bioassay

Cultures containing spores of all four tested *P. larvae* strains were diluted 1:25 in sugar solution (6% fructose, 6% glucose, see above) and subsequently mixed with 50% (v/v) royal jelly. This resulted in a 1:50 dilution of the *P. larvae* cultures in the larval diet. The spore-contaminated larval diet was prepared freshly immediately before each experiment and fed to larvae only once at day during the *in vitro* breeding experiment. Spore suspensions were stored at 4°C before they were used for experiments.

Due to their low water solubility Amberlite® XAD16 extracts were dissolved first in methanol (final methanol concentration in the diet <1%). This solution was mixed with larval diet by dissolving them first in the sugar solution and mixed with 50% (v/v) royal jelly. To control for toxic effects of methanol on developing honeybee larvae, a 1% methanol control was run in parallel. As a further control larvae were fed with a diet containing only MYPGP or J medium.

The Amberlite® XAD16 extract of the most virulent strain DSM 16116 was furthermore fractionated using solid phase extraction leading to five fractions (0.1% trifluoracetic acid, 10% acetonitrile (ACN), 50% ACN, 100% ACN, and Flow-trough) in addition to the full Amberlite® XAD16 extract (VT1) from *P. larvae* strain DSM 16116.

Cultivation of *P. larvae* and extraction of secreted metabolites

All tested *Paenibacillus larvae* strains were kept on Difco columbia blood agar base (Becton, Dickinson and company, Sparks, USA), containing 5% defibrinated sheep blood, or J medium agar plates and single colonies were used for the inoculation of liquid cultures. For infection of larvae with different isolates of *P. larvae*, bacterial strains were cultivated for a maximum of 5 days at 34°C in 75 ml of J medium (38), containing 0.5% tryptone, 1.5% yeast extract (both Becton, Dickinson and company, Sparks, NJ), 0.3% K2HPO4 and 0.5% glucose, which was separately added after sterilization, (both Carl Roth GmbH, Karlsruhe, Germany;...
all descriptions in w/v). Alternatively, strains were kept in 75 ml MYPGP-Medium (36, 37), containing 1.5 % yeast extract, 0.3 % K$_2$PO$_4$, 0.2 % glucose and 0.1 % sodium pyruvate as well as 1.0 % Mueller-Hinton-Broth (Oxoid CM0405). In case of those cultures, which would later on be used for infection of honeybee larvae, 0.01 % (w/v) mangansulfate was added to the cultivation media in order to maximize the sporulation activity, since infection of *A. melifera* larvae is caused by germinated *P. larvae* spores.

For metabolite extraction and successive toxicity tests, *P. larvae* strains were cultivated at 34°C for 48 h under permanent shaking at 120 rpm in 500 ml baffled flasks and for an additional cultivation period of 24 h under identical conditions as above with 2 % Amberlite® XAD16 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) added to the culture medium. Cultures were harvested by removing the Amberlite® XAD16 beads after 72 h total cultivation time. Two culture volumes of analytical reagent grade methanol (Thermo Scientific, Loughborough, UK) were added to the loaded Amberlite® XAD16 to extract resin bound metabolites. After 1 h under permanent shaking, the extract was decanted and centrifuged for 20 min at 4000 rpm in a megafuge 10R (Heraeus sepatech, Hanau, Germany). To remove residual colony forming units, the supernatant was filtered through a polytetrafluoroethylene (PTFE)-filter (pore size: 0.45 µm, diameter: 22 mm; phenomenex, Aschaffenburg, Germany). Sterility of the filtrate was tested by streaking out 300 µl of the filtrate on J medium agar plates containing 1.5 % (w/v) agar. The filtered extract was concentrated by means of a rotary evaporator (Heidolph Instruments GmbH & Co. KG, Schwalbach, Germany) and dried in 4 ml glass vials using a vacuum centrifuge (eppendorf concentrator 5301, Eppendorf, Hamburg, Germany). For feeding of Amberlite® XAD16 extracts to larvae the XAD16 extracts were dissolved first in methanol due to their low water solubility such that the final methanol concentration in the larval diet was less than 1%.

To evaluate the efficiency of the metabolite extraction from liquid *P. larvae* cultures by means of Amberlite® XAD16, the toxicity of sterile filtered *P. larvae* culture supernatants
and identically treated but Amberlite® XAD16 extracted culture supernatants were tested. For this purpose, the larvae diet was prepared with the before mentioned culture supernatants. Briefly, the sugars which were used for the sugar solution part of the larvae diet were diluted in sterile culture supernatants resembling the volume of water that would have been used instead.

XAD extract from DSM 16116 was fractionated with Strata C18E reversed phase solid phase extraction (RP-SPE)-cartridges (Phenomenex) (20 g bed mass). The extract was dissolved in 0.1 % TFA and loaded onto a C18E-SPE-catridge. After washing with 200 ml 0.1 % TFA, compounds were successively eluted with 200 ml of 10, 30, 50, and 99.9 % ACN (0.1 % TFA each). Obtained fractions were dried using a rotary evaporator and stored at –20 °C.

**Analyses of culture extracts**

The Amberlite® XAD16 extracts of culture supernatants as well as the fractions obtained from C18E-SPE were analyzed by means of liquid chromatography electrospray ionization mass spectrometry LC-MS as well as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Briefly, extracts were analyzed as described previously (39), using a Dionex Ultimate 3000 system coupled to an Bruker AmaZon X mass spectrometer and an Acquity UPLC BEH C18 1.7 μm RP column (Waters) using an acetonitrile/0.1 % formic acid in H2O gradient ranging from 5-95 % in 22 min at a flow rate of 0.6 mL min⁻¹.

For MALDI-analysis, extracts were diluted in 70 % acetonitrile (ACN) with 0.1 % trifluoracetic acid (TFA). All samples were mixed 1:2 with 1 μl of a 20 mM 4-chloro-α-cyanocinnamic acid (ClCCA) (40, 41) in 70 % ACN and spotted onto a polished stainless steel target and air-dried. MALDI-MS and MALDI-MS² analysis was performed with a MALDI LTQ Orbitrap XL (Thermo Fisher Scientific, Inc., Waltham, MA) equipped with a nitrogen laser at 337 nm. For MALDI-MS of culture supernatants, samples were prepared as a 1:5 dilution in 70 % ACN/0.1 % TFA and mixed 1:1 with the above mentioned matrix.
solution and measured on a Voyager-DE STR mass spectrometer (Applied Biosystems, Darmstadt, Germany).

**Genome analysis**

For identification of possible secondary metabolite biosynthesis gene clusters in the available genomes of *Paenibacillus larvae* ssp. *larvae* B-3650 and *Paenibacillus larvae* ssp. *larvae* BRL-23001, the contigs of both genome sequencing projects were concatenated with spacers of 25 N to obtain two individual linear DNA strands. The fully closed genome of *P. larvae* ssp. *larvae* DSM 25430 recently accessible at the NCBI website was also analyzed. An antiSMASH (42, 43) analysis of all DNA-sequences was performed and the putative biosynthesis gene clusters were consecutively analyzed manually to dismiss possible artificial clusters that resulted from concatenation or false annotation. Briefly, for B-3650 and BRL-23001 the Geneious software (version 5.6.2) was used to predict possible open reading frames (ORF) in the identified genomic loci. The start codons ATG, GTG, and CTG were considered and to allow the identification of antibiotic and bacteriocin structural genes, 90 base pairs were allowed as minimal ORF-size. To identify possible functions of the encoded proteins, the protein BLAST was used to identify homologous proteins as well as conserved motives. For the identification of NRPS- and PKS-domain architectures, the NRPS/PKS analysis website was used (44).

**Statistics**

The mean times of death were estimated with the Kaplan-Meyer-Test. The survival rates of control and treatment groups were compared using a logrank test (Mantel Cox Test). All statistical tests were performed using SPSS Statistics version 20.0 (IBM, Chicago IL) running on a PC under Windows XP; Service Pack 3.
Results

Artificial Rearing

In order to assess larvae survival during *in vitro* rearing we monitored survival daily (up to day 7) for three replicates per experimental conditions. Results from the control group demonstrate successful *in vitro* rearing with >80% survival of larva up to day 7 (Fig. 1). Control larvae (without treatment) were reared on all plates for all experiments. The middle survival time (MST) of bees was evaluated until day 15 twice in control groups with medium (1. assay: MST=10.79 days, n=24 larvae, N=8 plates; 2. assay: MST=11.92 days, n=36 larvae, N=12 plates, Tab. 1) and once with methanol (MST=11.79 days, n=72 larvae, N=24 plates, Tab. 1). In each assay these groups did not differ from the control group without medium or methanol (Mantel Cox Test, df 1, p>0.05, see Tab.1) (1. assay: MST=10.17 days, n=78 larvae, N=21 plates; 2. assay: MST=11.5 days, n=183 larvae, N=43 plates, Kaplan-Meyer-Test, see Tab. 1). Similarly, the middle survival times between the medium and the methanol control groups in same assay did not differ (Mantel Cox Test, df 1, p>0.05, see Tab.1). This indicates that 1% methanol is not toxic for the larvae and that medium either contains no compounds harmful to the larvae or with their concentrations being too low to impair development (Table 1). Therefore, for all further experiments, the control groups did not contain methanol.

Infections with *P. larvae* spores

Artificially reared honey bee larvae were infected by ingestion of spore-contaminated food. Compared to the control group, which was fed with a diet without *P. larvae* spores, all tested strains affect larval development, because they showed the typical clinical symptoms (45). After the larvae were identified as dead, they were separated from the others and were inspected daily. Their color changed to brown and got darker every day. The dead larvae finally dissolved into a viscous mass sticking to the walls of the storage vessel, as it has been described for the ropy stage and consecutively dried down to a hard scale. Although all tested
strains showed the typical clinical symptoms, the different *P. larvae* genotypes (ERIC I and ERIC II) tend to vary in virulence even if the MST estimated with Kaplan Meyer test did not differ significantly (Mantel Cox Test, df 1, p>0.05, Table 1; Supplementary Figure S1). The survival time of honeybee larvae after infection with *P. larvae* ERIC I strain NRLL B-41625 was similar to the control group. By contrast, all three tested ERIC II strains were more virulent as described previously (6).

*P. larvae* toxicity induced by small molecules

When the Amberlite® XAD16-extracts were tested for toxicity, larvae fed with Amberlite® XAD16-extracts from DSM 16116, DSM 16115 and DSM 17237 cultures had increased mortalities as compared to the controls (MST estimated with Kaplan-Meyer-Test, compared to each other with Mantel Cox Test, df 1, p<0.001; Table 1, Fig. 2). Mortality of larvae fed with Amberlite® XAD16-extract from strain NRLLB-1635 (Kaplan-Meyer-Test, MST= 12.2 d) did not differ from control (Kaplan-Meyer-test, MST= 11.5 d; Mantel Cox Test, df 1, p>0.5, Table 1). Nevertheless these larvae showed the symptoms of AFB (ropy and scale stage).

In order to address whether low molecular weight compounds secreted by *P. larvae* are bound to the XAD16 resin, the supernatant of the most virulent strain DSM 16116 was analyzed for toxicity with and without previous XAD16-extraction. For this, DSM 16116 was grown with or without the adsorber resin Amberlite® XAD16, that binds typical low molecular weight natural products like peptides or polyketides (39, 46, 47), and the cell free culture supernatant was consecutively used to prepare the larvae diet. Adding XAD16-extracted culture supernatant or non-extracted supernatant to the diet reduced the survival time of larvae as compared to the control larvae. Furthermore, the non-extracted supernatant showed a significantly higher toxicity towards the honeybee larvae as compared to the extracted
supernatant (Mantel Cox Test, df 1; p<0.001; Table 1, Fig. 3). This indicates that XAD16-

evacuation removes virulence factors secreted by P. larvae from the supernatant.

Subsequent solid phase extraction of the Amberlite® XAD16-extracts from DSM 16116 led
to five fractions (0.1 % trifluoracetic acid, 10% acetonitrile (ACN), 50 % ACN, 100 % ACN,
and Flow-through) that were also analyzed for their toxicity. All fractions were fed at the
identical concentration of 3.5 mg/ml of except for 100% ACN with 0.003 mg/ml larval diet,
because only small amounts could be extracted.

All fractions were toxic except for the flow-through (Mantel Cox Test, df 1; p<0.001; Table
1). Larvae fed with VT1, the full extract of DSM 16116, died with a middle survival time of
7.76 d different to control with 11.5 d.

Larvae fed with the fraction flow-through had a MST of 13.05 days and no negative effects on
larval development. While MST of fraction 100% ACN were only 3.07 days followed by
fraction 50% ACN with a MST of 6.95 days and 10% ACN with a MST of only 5 days. It was
conspicuous that larvae of fraction 10% ACN were clearly smaller as compared to the control
larvae. The MST of fraction 30 % ACN did not differ to VT1 while all other fractions differed
to each other, to the control and to VT1 (MST estimated with Kaplan-Meyer-Test, compared
to each other with Mantel Cox Test, df1, p<0.001; Table 1, Fig. 4).

Mass spectrometry of prepurified P. larvae extracts

Amberlite® XAD16-extracts from all tested P. larvae strains (DSM 16116, DSM 16115,
DSM 17237, and NRLL B-41635) were analyzed by positive ion mode MALDI-orbitrap mass
spectrometry (Fig. S2). Additionally, the raw culture supernatants (without Amberlite®
XAD16-extraction) were analyzed by MALDI-TOF mass spectrometry (Fig. S3). In both
cases, no qualitative differences were observed, which could explain the significantly
different activities in the toxicity assays. MALDI-MS analysis of the fractions from DSM
16116 showed the presence of different compounds in the different fraction allowing their isolation in future experiments (Fig. S4).

**In silico analyses of the P. larvae genomes for bioactive natural products biosynthesis genes**

Since no genomic information of the *Paenibacillus*-strains cultivated in this study were available, the genome of *P. larvae* ssp. *larvae* B-3650, *P. larvae* ssp. *larvae* BRL-23001 (35) and *P. larvae* ssp. *larvae* DSM 25430 were analyzed. An antiSMASH analysis of all genomes was followed by a manual inspection of all identified putative secondary metabolite gene clusters. The genomes of B-3650 and BRL-23001 showed an identical set of natural product biosynthesis gene clusters (Fig. 5) differing from the genome of DSM 25430 (Fig. S5). However, several similar gene clusters have been identified in all genomes that were numbered identical in Figures 5 and S5 for simplicity. In all genomes gene clusters responsible for the production of nonribosomally made peptides (NRP), NRP-polyketide (PK) hybrids, lantibiotics, as well as bacteriocins were identified (Fig. 5 and S5). Gene cluster 2 resemble the paenibactin biosynthesis gene cluster responsible for the biosynthesis of the siderophore paenibactin from *Paenibacillus eglii* B69 (Fig. 5 and Fig. S5) (22). Cluster 3 encodes a three-domain nonribosomal peptide synthetase (NRPS) with adenylation (A), thiolation (T) and terminal reduction (Red) domain (Fig. 5 and Fig. S5). Similar NRPS have been shown to be involved in the biosynthesis of piperazines or terphenylquinones from fungi (48, 49). The largest gene cluster (cluster 4) encodes a NRPS-PKS hybrid with all structural features of the prodrug activation mechanism previously identified in the biosynthesis of xenocoumacin, zwitermicin and colibactin (39, 50-52). Additionally, a putatively incomplete NRPS-PKS-hybrid gene cluster (cluster 5 in Fig. 5) responsible for the biosynthesis of a bacillomycin/mycosubtilin-like lipopeptides was identified in strain B-3650. The NRPS-PKS hybrid protein Pfarl_17128 (Figure 5B.5) exhibited a highly similar domain architecture and a...
69 % sequence identity with the mycosubtilin NRPS in *Bacillus atrophaeus* 1942 (subunit A) as well as a 68 % sequence identity with BmyA from the bacillomycin biosynthesis machinery in *Bacillus amyloliquefaciens* FZB42 (53, 54). However, no further genes associated with the biosynthesis of a mycosubtilin- or bacillomycin-like compound could be identified due to the end of the contig. In strain DSM 25430 an additional PKS/NRPS hybrid is encoded by cluster 5 (Fig. S5) also showing some similarity to the mycosubtilin biosynthesis enzymes. Two and one biosynthesis gene clusters involved in the biosynthesis of lantibiotics, and five and two involved in the biosynthesis of bacteriocins were identified in the different genomes, respectively (Fig. 5 and S5). The alignment of both lantibiotic preproproteins (gene clusters 6 and 7, Figure S6) against other database-deposited lantibiotic preproprotein-structures showed only weak sequence similarity to other lantibiotic preproproteins, except for the conservation one C-terminal cysteine residue (55).

**Discussion**

We have shown that low molecular weight compounds produced by *P. larvae* are toxic to honeybee larvae. Whereas all strains analyzed showed a similar toxicity profile when spore suspensions were added to the food source of the larvae, extracts from different strains of *P. larvae* differed significantly in their toxicity, pointing to either different virulence factors or different amounts thereof. Our study indicates that DSM 16116 is the most virulent strain. Similarly virulent is DSM 16115 that was assigned to genotype ERIC II (personal communication E. Genersch). Accordingly, the incubation times of ERIC-II and ERIC IV strains are shorter as compared to ERIC I strains (6). The LT$_{100}$ values of ERIC- II und VI were 6-7 d, whereas those of ERIC I-was approx. 12 d. As DSM 17237 with an unknown ERIC subtype behaves similar to DSM16116 and DSM 16115, it might also represent an ERIC II subtype. Interestingly, NRLL B-41625 (ERIC I) differs from the other strains (all ERIC II) as its toxic effects appear later, which might be due to its genotype difference.
The fact that Amberlite® XAD16 extracts (Fig. 2) seemed to be more toxic than spores (Fig. S1) of the same strain might be the result of the direct application of these putative virulence factors in the food source. In case of spores, *P. larvae* needs to germinate in the midgut and then produce the respective toxic compounds during the infection process, which might therefore delay the toxicity. We have also shown that not all toxic compounds bind to the XAD resin, which might imply the presence of different toxic compounds. Indeed, several other protein virulence factors have previously been described from *P. larvae* including metalloproteases, protein toxins and others (29, 56-58). However, these protein virulence factors with molecular weights of up to 100 kDa would be too large to bind to the XAD resin, which is described to bind molecules with a molecular weight of less than 4 kDa.

Unfortunately, we were not able to isolate a pure toxic compound but could show active fractions that can be purified in the future using the established *in vitro* honeybee larvae rearing (Fig. 1). Mass spectrometry revealed the presence of several different compounds in the *P. larvae* crude extracts (Fig. S2) as well as in the bioactive fractions (Fig. S4). Interestingly, the analysis of the different *P. larvae* supernatants revealed the presence of highly similar or identical compounds in these strains (Fig. S3), indicating a conserved class of compounds among the different strains.

Additionally, active compounds are not necessarily visible in the acquired mass spectra because the acquired mass spectra do not cover the compound’s mass or due to the fact that the toxic substance(s) are not visible based on mass-overlapping(s) with other compounds.

When *P. larvae* extracts were analyzed by MALDI-MS (Fig. S2), no masses corresponding to already known secondary metabolites from *Paenibacillus* were identified. Additionally, application of the recently introduced peptidogenomics approach (59) was not reasonable, since no genome sequence of the analyzed strains was available. A manual survey for masses of known natural products from *Paenibacillus* strains in the acquired mass spectra did not result in the identification of the respective mass signals.
In order to address the potential of *P. larvae* to produce low molecular weight toxic compounds, the available genome of *P. larvae* BRL-230010 was analyzed for the presence of biosynthesis gene clusters of different natural product classes. Among the identified 12 gene clusters are biosynthesis gene clusters encoding nonribosomal peptide synthetases (NRPS), hybrids of polyketide synthases (PKS) and NRPS, as well as lantibiotics and bacteriocins, all of which could be responsible for the observed toxic effect. The *m/z* ratios observed in the different extracts are clearly in the range one would expect for the products of the identified gene clusters. Especially interesting is the hybrid PKS/NRPS gene cluster 4 encoding a compound that is most likely activated via proteolytic cleavage as it was shown for the potent antibiotic xenocoumacin from the entomopathogenic bacterium *Xenorhabdus nematophila* (39) and the still unknown compound colibactin, a potent virulence factor of pathogenic *E. coli* strains (51).

Future work must focus on the identification of the compounds responsible for the observed toxicity and also to correlate these compounds with their respective gene clusters. The latter will allow to study also the regulation of the biosynthesis especially in the larval context and thus might shed more light on the molecular mechanism leading to AFB.

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**Table 1.** Survival of artificially reared larvae fed with different treatments are compared to each other and to control groups by time of death after inoculation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MST</th>
<th>Standard error</th>
<th>significant differences</th>
</tr>
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<tbody>
<tr>
<td>DSM 16116 (n=45)(N=3)</td>
<td>9.47</td>
<td>0.55</td>
<td>A</td>
</tr>
<tr>
<td>DSM 16115 (n=63)(N=6)</td>
<td>10.11</td>
<td>0.50</td>
<td>AB</td>
</tr>
<tr>
<td>DSM 17237 (n=14)(N=2)</td>
<td>10.58</td>
<td>1.19</td>
<td>AB</td>
</tr>
<tr>
<td>NRLL B-41635 (n=63)(N=6)</td>
<td>10.11</td>
<td>0.45</td>
<td>AB</td>
</tr>
<tr>
<td>medium control (n=24)(N=8)</td>
<td>10.79</td>
<td>0.92</td>
<td>B</td>
</tr>
<tr>
<td>control (n=78)(N=21)</td>
<td>10.17</td>
<td>0.43</td>
<td>AB</td>
</tr>
<tr>
<td>Infection (Assay1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant (Assay2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amberlite® XAD16 extracted supernatant (n=21)(N=3)</td>
<td>8.10</td>
<td>0.52</td>
<td>A</td>
</tr>
<tr>
<td>supernatant (n=20)(N=3)</td>
<td>5.60</td>
<td>0.42</td>
<td>B</td>
</tr>
<tr>
<td>control (n=183)(N=43)</td>
<td>11.50</td>
<td>0.25</td>
<td>C</td>
</tr>
<tr>
<td>Amberlite® XAD16 extract (Assay2)</td>
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<td></td>
</tr>
<tr>
<td>DSM 16116 (n=34)(N=3)</td>
<td>3.82</td>
<td>0.10</td>
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</tr>
<tr>
<td>DSM 16115 (n=36)(N=3)</td>
<td>4.11</td>
<td>0.14</td>
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</tr>
<tr>
<td>DSM 17237 (n=36)(N=3)</td>
<td>5.61</td>
<td>0.20</td>
<td>B</td>
</tr>
<tr>
<td>NRLL B-41635 (n=81)(N=6)</td>
<td>12.23</td>
<td>0.39</td>
<td>C</td>
</tr>
<tr>
<td>methanol control (n=72)(N=24)</td>
<td>11.79</td>
<td>0.42</td>
<td>DC</td>
</tr>
<tr>
<td>medium control (n=36)(N=12)</td>
<td>11.92</td>
<td>0.59</td>
<td>DC</td>
</tr>
<tr>
<td>control (n=183)(N=43)</td>
<td>11.50</td>
<td>0.25</td>
<td>D</td>
</tr>
<tr>
<td>Fractions (3.5 mg/ml) (Assay2)</td>
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<td></td>
</tr>
<tr>
<td>VT1 (n=21)(N=3)</td>
<td>7.76</td>
<td>0.44</td>
<td>A</td>
</tr>
<tr>
<td>10% ACN (n=25)(N=3)</td>
<td>6.28</td>
<td>0.38</td>
<td>B</td>
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<td>30% ACN (n=27)(N=3)</td>
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<tr>
<td>50% ACN (n=27)(N=3)</td>
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<td>C</td>
</tr>
<tr>
<td>100% ACN (n=27)(N=3)</td>
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<td>0.05</td>
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<tr>
<td>0.1 % TFA (n=27)(N=3)</td>
<td>4.704</td>
<td>0.139</td>
<td>E</td>
</tr>
<tr>
<td>Flow-through (n=21)(N=3)</td>
<td>13.05</td>
<td>0.81</td>
<td>F</td>
</tr>
<tr>
<td>Control (n=183)(N=43)</td>
<td>11.50</td>
<td>0.25</td>
<td>F</td>
</tr>
</tbody>
</table>

**MST:** middle survival time in days, estimated with Kaplan Meyer Test to day 15. If two treatments have different letters (ABCDEF) their MST is significantly different to each other (tested with logrank test, Mantel Cox Test; p<0.05).
Fig. 1. Honey bee larvae 9 days after eclosion. After defecation the larvae were transferred to pupation wells lined with paper tissues (right).
Fig. 2. Larval survival after inoculation with Amberlite® XAD16-extracts (3.5 mg/ml) from different strains of *P. larvae*. Strains are compared to each other and to control groups by mean time of death (MST) estimated with Kaplan-Meyer-Test. Statistically different MSTs are indicated by different letters; treatments sharing the same letter do not differ. Tested with logrank test, Mantel-Cox Test; p<0.01.)
Fig. 3. Larval survival after inoculation with spore-free culture supernatant of *P. larvae* DSM 16116 before (CS) and after Amberlite® XAD16-extraction (CS ex). CS is compared to CS ex, where low molecular molecules are absent, and to a control group by mean time of death (MST) estimated with Kaplan-Meyer-Test. The different letters ABC indicate significant differences between the MST; compared to each other with logrank test, Mantel Cox Test; p<0.001).
Fig. 4. Larval survival after inoculation with fractions (3.5 mg/ml) of Amberlite® XAD16-extracts of *Paenibacillus larvae* DSM 16116. Fractions are compared to each other and to control group by mean time of death (MST) estimated with Kaplan-Meyer-Test. The different letters indicate significant differences between the MST; compared to each other with logrank test, Mantel Cox Test; p<0.001).
Fig. 5. Putative secondary metabolite gene clusters identified in the genome of *Paenibacillus larvae* subsp. *larvae* B-3650. NRPS-cluster (A), NRPS/PKS-hybrid clusters (B), lantibiotic biosynthesis cluster (C), and bacteriocin biosynthesis cluster (D). Black arrows represent genes encoding NRPS or NRPS/PKS hybrids or lantibiotic- or bacteriocin-propeptides. Dark grey arrows represent genes putatively involved in the biosynthesis and transport of the corresponding secondary metabolite. Grey, light grey and white arrows represent genes encoding putative regulatory proteins, proteins probably not involved in the secondary metabolite biosynthesis, and hypothetical proteins, respectively.