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Low-Molecular-Weight Metabolites Secreted by *Paenibacillus larvae* as Potential Virulence Factors of American Foulbrood

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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 median lethal times. Furthermore, we 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* subsp. *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.

Honeybees are the most important economic 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 than the other three genotypes (2, 5). The different lethal times (times to 100% mortality of bees [LT_{100}]) are relevant because infection symptoms or deaths may be detected by nurse bees 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 honeybee disease that only affects the larval stages of the honeybee (2, 12), with 24- to 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 killing the larvae (13). After the larvae die, their tissue decays while the infected larval body changes to a brownish and viscous mass (ropy stage). It next dries to a hard scale, which tightly adheres 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 given that 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 lar-

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vae 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 the host's hemocoel (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.

MATERIALS AND 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 an 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% rH 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 from 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, Braine-l'Alleud, Belgium) was selected as the larval and the postdefecation rearing container. One larva was placed into each well onto 100 μl of the larva diet. During the first 5 days, larvae were transferred every 24 h to a fresh well with a new spore-free diet. From day 6 until defecation, larvae were grafted every 48 h to reduce handling stress. The infectious larval diet was fed only during the first 24 h 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 larva diet consisting of 50% (wt/wt) royal jelly (Imkerei Ullmann, Erlensee, Germany), 6% (wt/vol) glucose (Sigma-Aldrich, Deutschland), and 6% (wt/vol) 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 (approximately 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 feces. The larvae were maintained in an incubator (type 6120; Heraeus Instruments) at 35°C and 96% rH. The experiments were terminated at day 15, because most bees have reached the prepupal stage by then. Clinical symptoms of AFB should have been diagnosed before this point, such that only a 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, color 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 were genotype ERIC II or AB (33, 34). NRLL B-41635 was obtained from the United States Department of Agriculture (Agricultural Research Service [ARS]). The genome of strain *P. larvae* subsp. *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 larva 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; described above) and subsequently mixed with 50% (vol/vol) 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 a 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% (vol/vol) 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 a diet containing only MYPGP or J medium.

The Amberlite XAD16 extract of the most virulent strain, DSM 16116, was fractionated further using solid-phase extraction, leading to five fractions (0.1% trifluoroacetic acid [TFA], 10% acetonitrile [ACN], 50% ACN, 99.9% ACN, and flowthrough) 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, MD) 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 (all descriptions are in wt/vol) 0.5% tryptone, 1.5% yeast extract (both from Becton, Dickinson, and Company, Sparks, MD), 0.3% K_2HPO_4, and 0.5% glucose, which was separately added after sterilization (both from Carl Roth GmbH, Karlsruhe, Germany). Alternatively, strains were kept in 75 ml MYPGP medium (36, 37) containing 1.5% yeast extract, 0.3% K_2PO_4, 0.2% glucose, and 0.1% sodium pyruvate, as well as 1.0% Mueller-Hinton broth (CM0405; Oxoid). In the case of those cultures, which would later be used for infection of honeybee larvae, 0.01% (wt/vol) mangan sulfate 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 described 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 a 72-h total cultivation time. Two culture volumes of analytical reagent-grade methanol (Thermo Scientific, Loughborough, United Kingdom) 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 4,000 rpm in a Megafuge 10R (Heraeus Sepatech, Hanau, Germany). To remove residual CFU, the supernatant was filtered through a polytetrafluoroethylene (PTFE) filter (pore size, 0.45 μm; diameter, 22 mm; Phenomenex, Aschaffenburg, Germany). The sterility of the filtrate was tested by streaking 300 μl of the filtrate on J medium agar plates containing 1.5% (wt/vol) agar. The fil-

tered 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 (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 was tested. For this purpose, the larval diet was prepared with the above-mentioned culture supernatants. Briefly, the sugars which were used for the sugar solution part of the larva 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 C18-E reversed-phase solid-phase extraction (RP-SPE) cartridges (20-g bed mass; Phenomenex). The extract was dissolved in 0.1% TFA and loaded onto a C18E-SPE cartridge. 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 a Bruker AmaZon X mass spectrometer and an Acquity UPLC BEH C_{18} 1.7-μm RP column (Waters) using a gradient of acetonitrile-0.1% formic acid in H_2O ranging from 5 to 95% in 22 min at a flow rate of 0.6 ml min^{-1}. For MALDI analysis, extracts were diluted in 70% ACN with 0.1% TFA. All samples were mixed 1:2 with 1 μl of 20 mM 4-chloro-α-cyanocinnamic acid (ClCCA) (40, 41) in 70% ACN, spotted onto a polished stainless steel target, and air dried. MALDI-MS and MALDI tandem mass spectrometry (MALDI-MS^2) analyses were 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, 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* subsp. *larvae* B-3650 and *Paenibacillus larvae* subsp. *larvae* BRL-23001, the contigs of both genome-sequencing projects were concatenated with spacers of 25 N (unspecified nucleotides) to obtain two individual linear DNA strands. The fully closed genome of *P. larvae* subsp. *larvae* DSM 25430, recently made 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 bp was allowed as the minimal ORF size. To identify possible functions of the encoded proteins, the protein BLAST was used to identify homologous proteins as well as conserved motifs. For the identification of nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) domain architectures, the NRPS-PKS analysis website was used (44).

Statistics. The mean times of death were estimated with the Kaplan-Meier test. The survival rates of control and treatment groups were compared using a log-rank 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.

FIG 1 Honeybee larvae 9 days after eclosion. After defecation, the larvae were transferred to pupation wells lined with paper tissues (right).

RESULTS

Artificial rearing. In order to assess larva survival during *in vitro* rearing, we monitored survival daily (up to day 7) for three replicates per experimental condition. 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 median survival time (MST) of bees was evaluated until day 15 twice in control groups with medium (for assay 1, MST = 10.79 days, n = 24 larvae and 8 plates; for assay 2, MST = 11.92 days, n = 36 larvae and 12 plates) and once with methanol (MST = 11.79 days, n = 72 larvae and 24 plates) (Table 1). In each assay, these groups did not differ from the control group without medium or methanol (degrees of freedom [df] = 1; $P > 0.05$ by Mantel-Cox test) (assay 1, MST = 10.17 days, n = 78 larvae and 21 plates; assay 2, MST = 11.5 days, n = 183 larvae and 43 plates; Kaplan-Meier test) (Table 1). Similarly, the median survival times between the medium and the methanol control groups in the same assay did not differ (df = 1; $P > 0.05$ by Mantel-Cox test) (Table 1). This indicates that 1% methanol is not toxic for the larvae and that the medium either contains no compounds harmful to the larvae or their concentrations are 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 honeybee larvae were infected by ingestion of spore-contaminated food. Compared to the control group, which was fed 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 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 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 the Kaplan-Meier test did not differ significantly (df = 1; $P > 0.05$ by Mantel-Cox test)

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

Treatment[c]	MST[a]	SE	Significant difference[b]
Infection (assay 1)			
DSM 16116 (*n* = 45) (3 plates)	9.47	0.55	A
DSM 16115 (*n* = 63) (6 plates)	10.11	0.50	AB
DSM 17237 (*n* = 14) (2 plates)	10.58	1.19	AB
NRLL B-41635 (*n* = 63) (6 plates)	10.11	0.45	AB
Medium control (*n* = 24) (8 plates)	10.79	0.92	B
Control (*n* = 78) (21 plates)	10.17	0.43	AB
Supernatant (assay 2)			
Amberlite XAD16-extracted supernatant (*n* = 21) (3 plates)	8.10	0.52	A
Supernatant (*n* = 20) (3 plates)	5.60	0.42	B
Control (*n* = 183) (43 plates)	11.50	0.25	C
Amberlite XAD16 extract (assay 2)			
DSM 16116 (*n* = 34) (3 plates)	3.82	0.10	A
DSM 16115 (*n* = 36) (3 plates)	4.11	0.14	A
DSM 17237 (*n* = 36) (3 plates)	5.61	0.20	B
NRLL B-41635 (*n* = 81) (6 plates)	12.23	0.39	C
Methanol control (*n* = 72) (24 plates)	11.79	0.42	DC
Medium control (*n* = 36) (12 plates)	11.92	0.59	DC
Control (*n* = 183) (43 plates)	11.50	0.25	D
Fractions (3.5 mg/ml) (assay 2)			
VT1 (*n* = 21) (3 plates)	7.76	0.44	A
10% ACN (*n* = 25) (3 plates)	6.28	0.38	B
30% ACN (*n* = 27) (3 plates)	7.15	0.50	AB
50% ACN (*n* = 27) (3 plates)	3.81	0.25	C
99.9% ACN (*n* = 27) (3 plates)	3.07	0.05	D
0.1 % TFA (*n* = 27) (3 plates)	4.704	0.139	E
Flowthrough (*n* = 21) (3 plates)	13.05	0.81	F
Control (*n* = 183) (43 plates)	11.50	0.25	F

[a] Median survival time in days, estimated with the Kaplan-Meier test to day 15.
[b] If two treatments have different letters, their MST are significantly different from each other ($P < 0.05$ by log-rank test and Mantel-Cox test).
[c] *n*, number of honeybee larvae.

(Table 1; also see Fig. S1 in the supplemental material). The survival time of honeybee larvae after infection with *P. larvae* ERIC I strain NRLL B-41625 was similar to that of the control group. In 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 compared to the controls (MST were estimated with Kaplan-Meier test and compared to each other with the Mantel-Cox test; df = 1; $P < 0.001$) (Table 1 and Fig. 2). The mortality of larvae fed with Amberlite XAD16 extract from strain NRLLB-41635 (MST = 12.2 days by Kaplan-Meier test) did not differ from the control (MST = 11.5 days by Kaplan-Meier test; df = 1; $P > 0.05$ by Mantel-Cox test) (Table 1). Nevertheless, these larvae showed the symptoms of AFB (ropy and scale stages).

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 larva diet. Adding XAD16-extracted culture supernatant or nonextracted supernatant to the diet reduced the survival time of larvae compared to that of the control larvae. Furthermore, the nonextracted supernatant showed a significantly higher toxicity toward the honeybee larvae than the extracted supernatant (df = 1; $P > 0.001$ by Mantel-Cox test) (Table 1 and Fig. 3). This indicates that XAD16 extraction 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% trifluoroacetic acid, 10% ACN, 50% ACN, 99.9% ACN, and flowthrough) that were also analyzed for their toxicity. All fractions were fed at

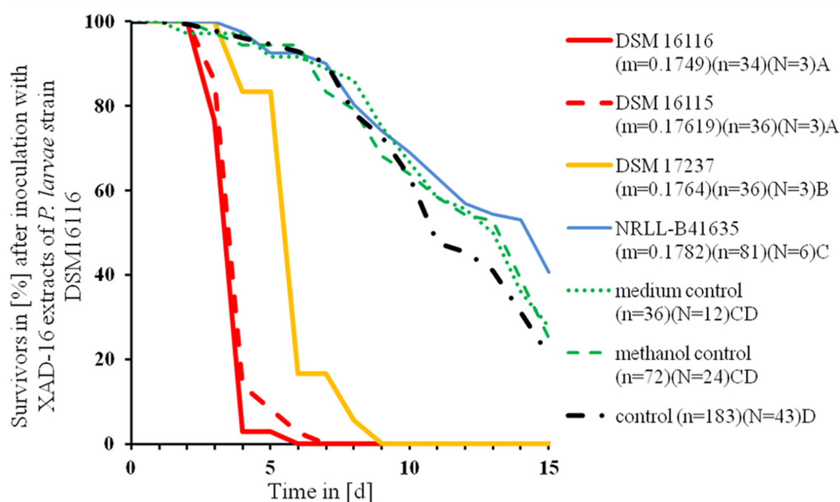

FIG 2 Larval survival after inoculation with Amberlite XAD16 extracts (3.5 mg/ml) from different strains of *P. larvae*. Strains are compared to each other and to control groups by mean time of death (MST) estimated with the Kaplan-Meier test. Statistically different MSTs are indicated by different letters; treatments sharing the same letter do not differ. $P < 0.01$ by log-rank test and Mantel-Cox test. m = original amount of extract (g), n = number of honeybee larvae, N = number of plates used.

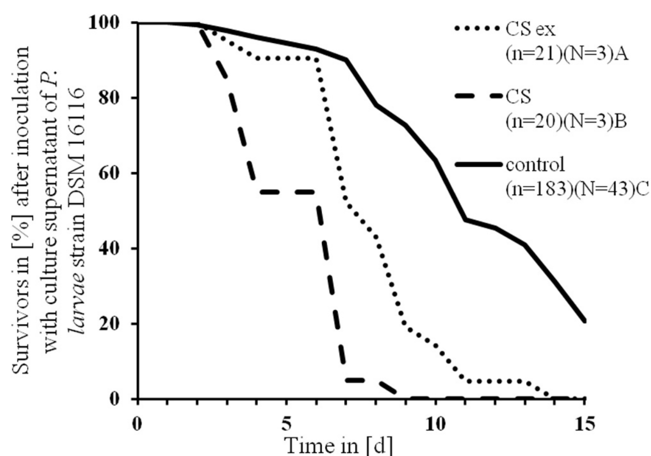

FIG 3 Larval survival after inoculation with spore-free culture supernatant of *P. larvae* DSM 16116 before (CS) and after Amberlite XAD16 extraction (CS ex). CS is compared to CS ex, where low-molecular-mass molecules are absent, and to a control group by mean time of death (MST) estimated with the Kaplan-Meier test. The different letters indicate significant differences between the MST. $P < 0.001$ by log-rank test and Mantel-Cox test. n = number of honeybee larvae, N = number of plates used.

the identical concentration of 3.5 mg/ml, except for 99.9% ACN with 0.003 mg/ml larval diet, because only small amounts could be extracted.

All fractions were toxic except for the flowthrough ($df = 1$; $P > 0.001$ by Mantel-Cox test) (Table 1). Larvae fed with VT1, the full extract of DSM 16116, died with an MST of 7.76 days; that of the control was 11.5 days.

Larvae fed with the flowthrough fraction had an MST of 13.05 days and no negative effects on larval development. The MST of the 100% ACN fraction was only 3.07 days, the 50% ACN fraction had an MST of 6.95 days, and the 10% ACN fraction had an MST of only 5 days. It was conspicuous that larvae of the 10% ACN fraction were clearly smaller than the control larvae. The MST of the 30% ACN fraction did not differ from that of the VT1 fraction, while all other fractions differed from each other, from the control, and from the VT1 fraction (MST were estimated with Kaplan-Meier test and compared to each other with the Mantel-Cox test; $df = 1$; $P < 0.001$) (Table 1 and 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 (see Fig. S2 in the supplemental material). Additionally, the raw culture supernatants (without Amberlite XAD16 extraction) were analyzed by MALDI-TOF mass spectrometry (see 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 (see Fig. S4).

***In silico* analyses of the *P. larvae* genomes for bioactive natural product biosynthesis genes.** Since no genomic information of the *Paenibacillus* strains cultivated in this study were available, the genomes of *P. larvae* subsp. *larvae* B-3650, *P. larvae* subsp. *larvae* BRL-23001 (35), and *P. larvae* subsp. *larvae* DSM 25430 were analyzed. An antiSMASH analysis of all genomes was followed by a manual inspection of all identified putative secondary

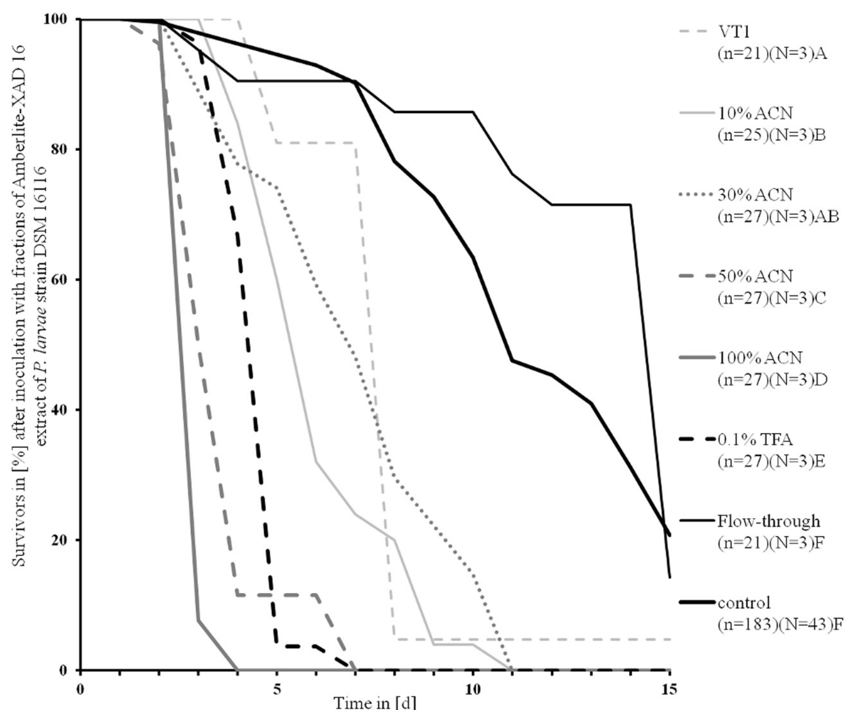

FIG 4 Larval survival after inoculation with fractions (3.5 mg/ml) of Amberlite XAD16 extracts of *Paenibacillus larvae* DSM 16116. Fractions are compared to each other and to the control group by mean time of death (MST) estimated with the Kaplan-Meier test. The different letters indicate significant differences between the MST. $P < 0.001$ by log-rank test and Mantel-Cox test. n = number of honeybee larvae, N = number of plates used.

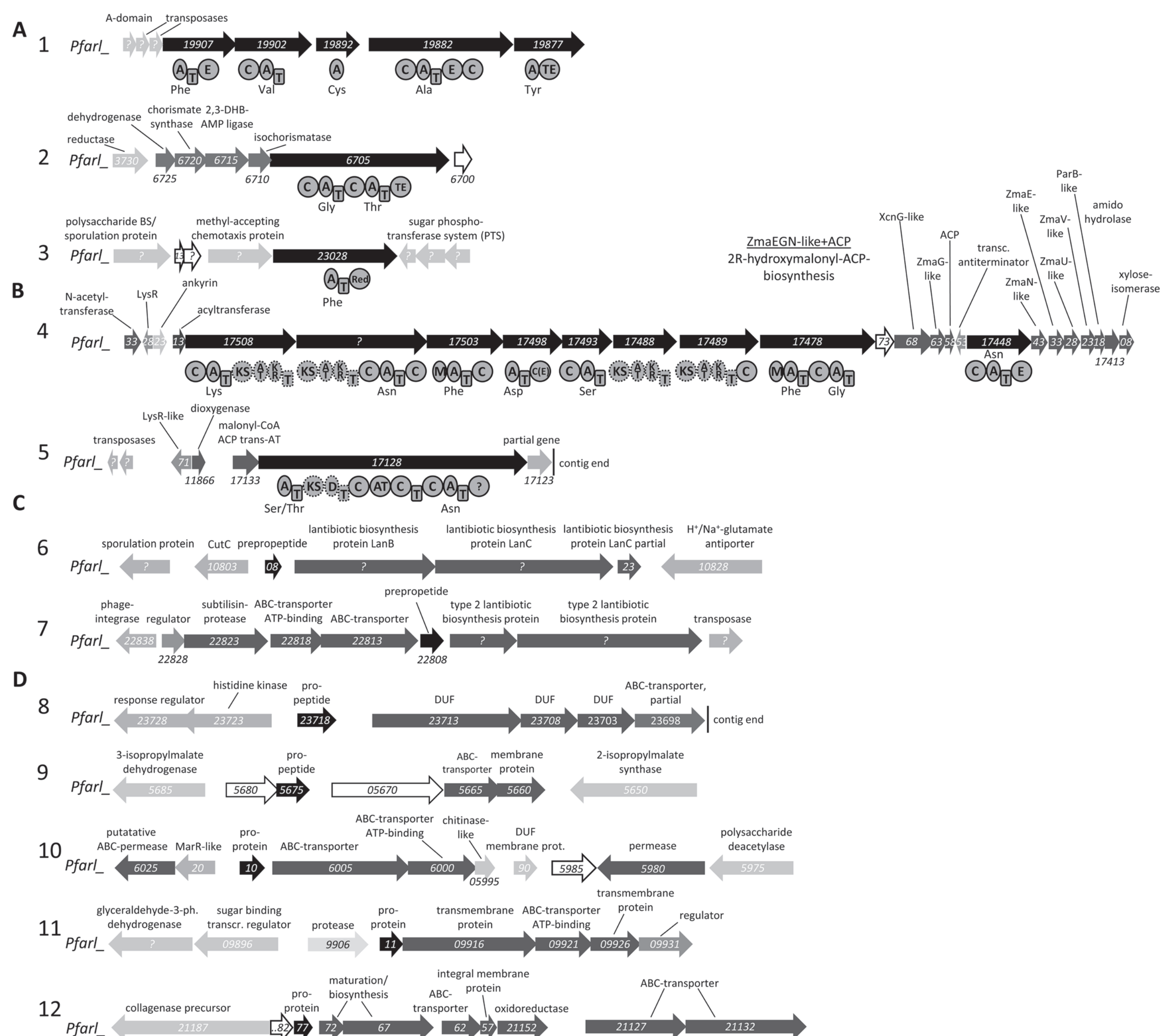

FIG 5 Putative secondary metabolite gene clusters identified in the genome of *Paenibacillus larvae* subsp. *larvae* B-3650. (A) NRPS cluster; (B) NRPS-PKS hybrid clusters; (C) lantibiotic biosynthesis cluster; and (D) bacteriocin biosynthesis cluster. Black arrows represent genes encoding NRPS or NRPS-PKS hybrids or lantibiotic or bacteriocin propeptides. Dark gray arrows represent genes putatively involved in the biosynthesis and transport of the corresponding secondary metabolite. Gray, light gray, and white arrows represent genes encoding putative regulatory proteins, proteins probably not involved in secondary metabolite biosynthesis, and hypothetical proteins, respectively. Trans-AT, *trans*-acting acyltransferase; BS, biosynthesis; AT, aminotransferase; CoA, coenzyme A; ACP, acyl carrier protein; prot., protein; transcr., transcription; 3-ph., 3-phosphate.

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 (see Fig. S5 in the supplemental material). However, several similar gene clusters have been identified in these genomes (numbered identically in Fig. 5 and in Fig. 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; also see Fig. S5). Gene cluster 2 resembles the paenibactin biosynthesis gene cluster responsible for the biosynthesis of the siderophore paenibactin from *Paenibacillus eglii* B69 (Fig. 5; also see Fig. S5) (22). Cluster 3 encodes a three-domain nonribosomal peptide synthetase (NRPS) with an adenylation (A), thiolation (T), and terminal reduction (Red) domain (Fig. 5; also see 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 an NRPS-PKS hybrid with all structural features of the prodrug activation mechanism previously identified in the biosynthesis of xenocoumacin, zwittermicin, and colibactin (39, 50–52). Additionally, a putatively incomplete NRPS-PKS hybrid gene cluster (cluster 5 in Fig. 5) responsible

for the biosynthesis of bacillomycin/mycosubtilin-like lipopeptides was identified in strain B-3650. The NRPS-PKS hybrid protein Pfarl_17128 (Fig. 5B) exhibited a highly similar domain architecture and 69% sequence identity to the mycosubtilin NRPS in *Bacillus atrophaeus* 1942 (subunit A) as well as 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 on the contig. In strain DSM 25430, an additional PKS-NRPS hybrid that is encoded by cluster 5 (see Fig. S5) also shows some similarity to the mycosubtilin biosynthesis enzymes. Two and one biosynthesis gene clusters involved in the biosynthesis of lantibiotics and five and two clusters involved in the biosynthesis of bacteriocins were identified in the genomes of strains B-3650 and DSM 25430, respectively (Fig. 5; also see Fig. S5). The alignment of both lantibiotic prepropoteins (gene clusters 6 and 7) (see Fig. S6) against other database-deposited lantibiotic prepropotein structures showed only weak sequence similarity to other lantibiotic prepropoteins, except for the conservation of 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, which was assigned to genotype ERIC II (E. Genersch, personal communication). Accordingly, the incubation times of ERIC II and ERIC IV strains are shorter than those of ERIC I strains (6). The LT_{100} values of ERIC II and VI were 6 to 7 days, whereas that of ERIC I was approximately 12 days. As DSM 17237, with an unknown ERIC subtype, behaves similarly to DSM 16116 and DSM 16115, it might also represent an ERIC II subtype. Interestingly, NRLL B-41635 (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 (see Fig. S1 in the supplemental material) of the same strain might be the result of the direct application of these putative virulence factors in the food source. In the case of spores, *P. larvae* needs to germinate in the midgut and then produces the respective toxic compounds during the infection process, which might delay the toxicity. We have also shown that not all toxic compounds bind to the XAD resin, which implies 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 masses of up to 100 kDa would be too large to bind to the XAD resin, which is described as binding mainly low-molecular-weight compounds, such as antibiotics and other secondary metabolites, but also hydrophobic compounds of up to 40 kDa.

Unfortunately, we were not able to isolate a pure toxic compound, but we could show active fractions that can be purified in the future using established *in vitro* honeybee larva-rearing methods (Fig. 1). Mass spectrometry revealed the presence of several different compounds in the *P. larvae* crude extracts (see Fig. S2 in the supplemental material) as well as in the bioactive fractions (see Fig. S4). Interestingly, the analysis of the different *P. larvae* supernatants revealed the presence of highly similar or identical compounds in these strains (see 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 because the toxic substance(s) is not visible based on mass(es) overlapping those of other compounds. When *P. larvae* extracts were analyzed by MALDI-MS (see Fig. S2 in the supplemental material), 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.

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 12 identified gene clusters are biosynthesis gene clusters encoding NRPS, hybrids of 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 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 *Escherichia coli* strains (51).

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

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