Molecular basis of antibiotic self-resistance in a bee larvae pathogen

Tam Dang1, Bernhard Loll2, Sebastian Müller1, Ranko Skobalj1, Julia Ebeling3, Timur Bulatov1, Sebastian Gensel1, Josefine Göbel3, Markus C. Wahl2,4, Elke Genersch3,5, Andi Mainz1 & Roderich D. Süßmuth1

*Paenibacillus larvae*, the causative agent of the devastating honey-bee disease American Foulbrood, produces the cationic polyketide-peptide hybrid paenilamicin that displays antibacterial and antifungal activity. Its biosynthetic gene cluster contains a gene coding for the N-acetyltransferase PamZ. We show that PamZ acts as self-resistance factor in *Paenibacillus larvae* by deactivation of paenilamicin. Using tandem mass spectrometry, nuclear magnetic resonance spectroscopy and synthetic diastereomers, we identified the N-terminal amino group of the agmatinamic acid as the N-acetylation site. These findings highlight the pharmacophore region of paenilamicin, which we very recently identified as a ribosome inhibitor. Here, we further determined the crystal structure of PamZ:acetyl-CoA complex at 1.34 Å resolution. An unusual tandem-domain architecture provides a well-defined substrate-binding groove decorated with negatively-charged residues to specifically attract the cationic paenilamicin. Our results will help to understand the mode of action of paenilamicin and its role in pathogenicity of *Paenibacillus larvae* to fight American Foulbrood.
Pollination of wild and cultivated flowering plants is an indispensable ecosystem service, which is mainly provided by pollinating insects. Among the insect pollinators, managed honey bee colonies play a particularly important role in agriculture, where they are widely used as commercial pollinators and contribute to 35% of the production volume of global food crops. In order to secure human food supply, it is therefore important to ensure the health of honey bees, which is continuously threatened by the overuse of insecticides such as neonicotinoids in agriculture and also by various viral, bacterial, and fungal pathogens as well as metazoan parasites.

The Gram-positive, facultative anaerobic, spore-forming bacterium, *Paenibacillus larvae* (*P. larvae*), is the causative agent of the epizootic American Foulbrood (AFB) of honey bees. AFB is the most serious bacterial disease of honey bees and is classified as notifiable disease in most countries because it is highly contagious and lethal to entire colonies. Furthermore, most authorities consider the killing of diseased colonies and burning of the hive material the only workable control measure resulting in considerable economic losses in apiculture. AFB is a fatal intestinal infection of the honey bee brood initiated in first instar larvae by ingestion of spore-contaminated food. The distribution of the spores, the infectious form of *P. larvae*, within a colony and between colonies, also within apiary and between apiaries, consequently leads to honey bee colony losses. The use of enterobacterial repetitive intergenic consensus (ERIC) sequence primers has revealed four well-described genotypes ERIC I to ERIC IV for *P. larvae* which differ in virulence on the larval and colony level as well as in pathogenesis strategies employed to kill the host. The existence of another ERIC genotype, ERIC V, has recently been proposed. From contemporary outbreaks of AFB all over the world, only *P. larvae* ERIC I and ERIC II can be isolated, suggesting that the hypervirulent genotypes ERIC III to ERIC V did not become established in the honey bee population.

In our quest to find sustainable control measures against this most serious bacterial disease of honey bees, we started to unravel AFB pathogenesis by analyzing the interaction between *P. larvae* and honey bee larvae on a molecular level. We identified several virulence factors of *P. larvae* ERIC I and ERIC II and showed that two AB toxins, a chitin-degrading enzyme, and also an S-layer protein have a pivotal role in the virulence of this pathogen and that *P. larvae* also produces various secondary metabolites. Bacterial secondary metabolites, with polyketides and (non-)ribosomal peptides as important representatives, provide highly valuable lead structures, among them antibiotics with novel modes of action for drug development to fight various infectious diseases. Secondary metabolites can also act as virulence-like factors, functioning as signal molecules in gene regulation of defense or growth mechanisms. The search for secondary metabolites produced by *P. larvae* led to the structural elucidation of paenilamicin that shows cytotoxic, antibacterial and antifungal activities. It is currently assumed that paenilamicin is produced as a defense molecule against microbial competitors, since only *P. larvae* can usually be isolated as a pure culture from the cadavers of AFB-killed larvae, suggesting that other saprophytic competitors are absent in the degradation process of the larval cadavers to the characteristic ropy mass.

We recently substantiated this view by showing that paenilamicin was active against the bee-associated saprophyte *P. alvei* in *P. larvae*-infected larvae.

Paenilamicin is a linear, cationic aminopolyol peptide antibiotic and is synthesized via an unusual nonribosomal peptide synthetase-polyketide synthase (NRPS-PKS) hybrid assembly line that exhibits several fascinating biosynthetic features. It contains unusual structural motifs such as galantinamic acid (Glm), agmatinamic acid (Aga), N-methylidiaminopropionic acid (mDap), galantinic acid (Gla) and a 4,3-spermidine (Spd) at the C-terminus (Fig. 1). *P. larvae* produces a mixture of paenilamicin variants A1, A2, B1, and B2. They only differ in two positions of the paenilamicin backbone: at the N-terminus and in the center between mDap1 and Gla. Either a lysine (series A) or an arginine (series B) is activated by the adenylation domain of NRPS1 and NRPS4 (pamD), respectively (Fig. 1).

The *pam* gene cluster harbors a gene encoding the putative acetyl-CoA-dependent N-acetyltransferase PamZ, which belongs to the Gcn5-related N-acetyltransferase (GNAT) superfamily. One prominent member of this superfamily is the bacterial

![Fig. 1 Biosynthetic gene cluster and structure of paenilamicin variants.](https://example.com/fig1.png)
aminoglycoside N-acetyltransferase (AAC) that plays an important role in antibiotic resistances, particularly in clinical and environmental settings. Aminoglycoside antibiotics have been widely used in the treatment of bacterial infections but they rapidly lose activity against multi-resistant bacteria due to adaptation and the development of resistance. By contrast, self-resistance is an innate, non-adaptation-based mechanism for the protection against self-produced antimicrobial agents. Since self-produced antimicrobial agents could also harm the bacterial host, self-resistance is critical for survival and territorial competition.

Our results demonstrate the deactivation of paenilamicins by the regio- and stereoselective self-resistance protein PamZ including its high-resolution crystal structure that shows how its tandem-domain arrangement may organize substrate binding. Together with a parallel study, in which we report on the total synthesis and the biological evaluation of paenilaminic, we have here unambiguously identified the N-terminal building block of paenilaminics as an essential switch for target binding, biological activity and self-resistance.

**Results and discussion**

Regio- and stereoselective N-acetylation of paenilaminic by PamZ. To confirm our hypothesis that PamZ (NCBI WP_023484187) is an acetyl-CoA-dependent N-acetyltransferase that targets paenilaminics, we monitored PamZ-mediated antibacterial effects in vitro by agar diffusion assays against *Bacillus megaterium* (*B. megaterium*) as indicator strain as well as by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. To this end, the pamZ gene was amplified from the wild-type (WT) *P. larvae* ERIC II strain, inserted into the commercial pET28a(+) vector, and transformed into *E. coli* BL21-Gold(DE3) for heterologous expression. PamZ was then purified (Supplementary Fig. 1) and used for the assays including four native paenilaminic variants as substrates and acetyl-CoA as co-substrate. The paenilaminic variants were purified from *P. larvae* ERIC I and ERIC II, which preferably produce the paenilaminic mixtures A2/B2 and A1/B1, respectively (Fig. 1 and Supplementary Fig. 2). In addition, we also tested synthetic paenilaminic B2 (PamB2_3).

The agar diffusion assays clearly showed that paenilaminics incubated with PamZ and acetyl-CoA were not able to inhibit the growth of *B. megaterium*, whereas antibacterial activity was observed in the absence of acetyl-CoA and/or PamZ (Fig. 2). This loss of biological activity correlated with the conversion of paenilaminics to the corresponding N-acetylpaenilaminics as observed by HPLC-ESI-MS. ESI mass spectra revealed that the mass-to-charge ratios of natural and synthetic paenilaminics exhibited a characteristic mass shift of 42 Da indicative of the addition of an acetyl group (Supplementary Figs. 3–7).

Paenilaminic contains several primary and secondary amino groups that are potential candidates for N-acetylation. To determine the site of acetylation, we monitored PamZ-mediated effects in fingerprint tandem MS and NMR spectra of paenilaminic before and after treatment with PamZ/acetyl-CoA. Besides the mass shift of 42 Da for the acetylation, characteristic MS2 fragmentation patterns originated from the difference between Glm and Aga residues in series A and B (+28 Da) as well as the difference between Lys and Orn residues in series 1 and 2 (+14 Da). MS2 fragmentation mainly resulted in fragment ions *b*<sub>4</sub>*y*<sub>4</sub> and *y*<sub>6</sub> of each paenilaminic and N-acetylpaenilaminic variant acquired by collision-induced dissociation (Supplementary Table 1). Fragment ion *b*<sub>4</sub> varied depending on the paenilaminic series showing mass shifts of 14 Da and 28 Da. Importantly, we observed a mass shift of 42 Da only for fragment ion *b*<sub>4</sub>*y*<sub>4</sub> indicating acetylation in the N-terminal half of paenilaminic. By contrast, the fragment ions *y*<sub>6</sub> did not exhibit any mass shifts of 42 Da between paenilaminics and N-acetylpaenilaminics.

Thus, we excluded acetylation in the C-terminal half of paenilaminic (Supplementary Figs. 8–18). In addition, we detected and isolated small amounts of N-acetylpaenilaminic A1, B1, and B2 from supernatants of *P. larvae* ERIC I and ERIC II (Supplementary Fig. 19), and compared them with our products formed in vitro. The MS2 fragmentation analysis confirmed that the mono-acetylation in the N-terminal half of paenilaminic also occurred in vivo (Supplementary Figs. 20–22). The MS2 experiments did not reveal whether the N-terminal amino group of Aga/Glm or its side chain (amino/guanidino group) was acetylated.

To ultimately identify the functional group that is modified by PamZ, we acquired a 1H-13C hetero-nuclear single-quantum coherence (HSQC) NMR spectra of paenilaminic B2 before and after incubation with PamZ/acetyl-CoA. Although both spectra were mostly superimposable, severe chemical shift perturbations (CSPs) were observed for a minor fraction of cross-peaks (Fig. 3a). Mapping CSPs onto the structure of paenilaminic B2 revealed a well-defined region comprising the N-terminal half, with the strongest effect being located at position 6 of Aga (Fig. 3b and Supplementary Table 2). N-acetylpaenilaminic B2 also showed an additional cross-peak compared to paenilaminic B2, which we tentatively assigned to the methyl moiety of the newly attached acetyl group (Fig. 3a).

Our data unequivocally demonstrated that PamZ mono-N-acetylates the N-terminal amino group at Aga-6 position of paenilaminic and thereby abolishes its antibacterial activity. Ultimately, this result is further supported by two synthetic diastereomers of paenilaminic B2 with L- instead of the native d-configuration at Aga-6 (PamB2_1 and PamB2_2), that were both antibacterially less active and that were not modified by PamZ (Fig. 4 and Supplementary Fig. 23).

The structure of PamZacetyl-CoA binary complex. A BLAST search indicated that PamZ belongs to the GNAT superfamily with a sequence identity of 31% to the N-acetyltransferase, ZmaR, whose structure has not yet been determined and which confers resistance against the aminopolyol peptide antibiotic, zwetitermicin A, in *Bacillus cereus* UW85 (Supplementary Fig. 24). We solved the crystal structure of PamZ in complex with acetyl-CoA at a resolution of 1.34 Å by using the uncharacterized N-acetyltransferase from *Streptococcus suis* 89/1591 (PDB 3G3S) for molecular replacement (Supplementary Table 3). The electron density was of excellent quality, allowed the modeling of the entire polypeptide chain, and unambiguously revealed the bound acetyl-CoA (Supplementary Fig. 25). PamZ comprises an N-terminal domain (NTD, residues 1–128, secondary structure elements indicated by primes) and a C-terminal domain (CTD,
residues 140–275), which both adopt the characteristic GNAT fold (Fig. 5a). The two tandem-GNAT domains, that may have originated from a gene duplication event, share low sequence identity (<20%) and are connected by an α-helical linker (α-bridge, residues 129–139). The overall fold of each domain is very similar to that of bacterial aminoglycoside N-acetyltransferases (AACs), as pairwise structural alignments with several AACs (PDB1BO4, 1M4I, 1S3Z) gave root-mean-square deviations (RMSDs) of 2.9–4.2 Å for both the NTD and CTD (Supplementary Fig. 26). A structural superposition of the NTD and CTD of PamZ yielded an RMSD of 4.2 Å for 75 pairs of Cα atoms (Supplementary Fig. 27).

However, a comparison with the typical GNAT fold revealed several unique features in PamZ. Instead of two N-terminal α-helices, α1 and α2, both domains of PamZ contain three short helical segments, α0–α1–α2 (α0–α1–α2), which pack onto one face of the central antiparallel β-sheet, β2–β3–β4 (β2–β3–β4), whereas helix α3 (α3) buries its other side. A kink in the backbone conformation of strand β3, involving residues T199 and C200, causes a strong right twist and thus a distortion of the antiparallel β3–β4 arrangement, which led us to discriminate these strands as β3a/β3b and β4a/β4b (Fig. 5a). The central β-sheet is extended by strand β5 in the NTD, whereas the CTD shows the characteristic β-bulge of GNAT enzymes — a V-shaped cavity between strands β4b and β5 accommodates the pantetheine segment of CoA (Fig. 6a). Furthermore, the well-conserved pyrophosphate-binding loop (P-loop) of the GNAT family (R/Q-X-X-G-X-A/G)26 is only present in the CTD of PamZ (Q-N-K-G-L-A) between strand β4b and helix α3 (Fig. 6a), whereas the NTD is missing this signature motif. Accordingly, there is only one acetyl-CoA molecule canonically bound in the PamZ structure, namely to the CTD.

Hence, we concluded that the NTD is incompetent in binding acetyl-CoA and rather plays a structural role, in particular for substrate binding (see below). Notably, many GNAT enzymes exist as homodimers in solution with various arrangements of the monomer-monomer interface32. Likewise, AACs have often been crystallographically observed in a homodimeric state, although their quaternary structure in solution may vary35. PamZ exists as a monomer, both in solution and in the crystal (Supplementary...
Fig. 4 Substrate specificity and stereoselectivity of PamZ. The natural product (PamB2), synthetic paenilamicin B2 (PamB2_3) and synthetic diastereomers of paenilamicin B2 (PamB2_2, PamB2_1) were incubated with PamZ and acetyl-CoA in vitro and tested in an agar diffusion assay against Bacillus megaterium (insets). From the chemical structure of paenilamincins, only the agmatinamic and galantionic acid are depicted to emphasize the changes in stereoconfiguration highlighted in purple and circles. Each single reaction was verified by HPLC-ESI-MS. Dashed lines indicate the mass shift of 42 Da (4 × 10.5 Da) due to N-acetylation.

Fig. 28). However, the tandem-GNAT domain constellation of PamZ achieves an intramolecular domain-domain interface that resembles that of some GNAT homodimers. There are several GNAT enzymes that utilize domain swapping of strand $\beta_6$ to stabilize their homodimeric structure. Interestingly, a major interface in PamZ is achieved by domain swapping of strand $\beta_6$ ($\beta_6^*$), which inserts between strands $\beta_5$ and $\beta_6$ ($\beta_5$ and $\beta_6$) of the opposing domain and thus forms an extended, antiparallel, and strongly-twisted $\beta$-sheet throughout the enzyme (Fig. 5b). This $\beta$-sheet is only interrupted by the $\beta$-bulge in the CTD accommodating the cofactor and allowing the amide groups of its pantetheine portion to form pseudo-$\beta$-sheet hydrogen bonds to strand $\beta_4b$ (Fig. 6a). A very similar tandem arrangement of a pseudo-GNAT NTD and a canonical GNAT CTD can be found in the template protein (PDB 3G3S). Another example is the structure of mycothiol synthase MshD from Mycobacterium tuberculosis, which is also organized as a tandem repeat of two GNAT domains with a catalytically inactive NTD.

PamZ appears to utilize its NTD to form a well-defined substrate pocket with strands $\beta_5$ and $\beta_6^*$ representing its floor. A second interface between the NTD and CTD is accomplished through tight packing of helix $\alpha_2^*$ onto the small $\beta_3b$-$\beta_4a$ sheet. Further interactions involve helix $\alpha_2$ of the CTD and the loops between $\alpha_2^*$ and $\beta_2^*$ as well as $\beta_3$ and $\beta_4$ of the NTD. These inter-domain contacts fully cover the central groove that is normally found at the interface of homodimeric structures of GNAT enzymes and restrict substrate entry to the opening that is also used by the cofactor. This remaining cleft between the two domains of PamZ is decorated with several acidic residues (e.g. E89, E116, E118, D120, D162, D170, D215, E216, E217, E218, E272, E274, and the C-terminus) and thus deploys a large negatively charged surface to attract its polycationic substrate (Fig. 5c). A corridor that lies aside and beyond the acetyl group of the cofactor is approximately 7–8 Å deep and 8–9 Å wide with respect to the thioester carbonyl atom. Although we did not obtain crystals of a ternary PamZ-acetyl-CoA-paenilamicin complex, the position of acetyl-CoA, the well-defined shape of the neighboring pocket and our knowledge about the substrate’s N-terminal acetylation site allows us to predict that the Glm/Aga side chain of paenilamicin very likely penetrates into this pocket. Acidic residues D25 (loop between $\alpha_1^*$ and $\alpha_2^*$), E122 ($\beta_6^*$), and E208 ($\beta_4a$) are well-positioned within the pocket to accommodate and stabilize the guanidine group of Aga, as well as to tolerate the $\text{NH}_2$ amine of Glm. Other residues that shape the substrate pocket include T58/T59 (loop between $\beta_3$ and $\beta_4$), T98 ($\beta_5$) and Y124 ($\beta_6^*$) of the NTD as well as C200/Y201 ($\beta_5b$) and S245/F247 ($\beta_5$) of the CTD (Fig. 6c). This shows that both domains most likely contribute to substrate recognition. Moreover, the structure of PamZ explains its regioselectivity: if PamZ was to modify e.g. the terminal amino group of spermidine in paenilamicin, the enzyme would not require such a deep substrate-binding pocket. The architecture of the central groove between the NTD and CTD has evolved to optimally accommodate the N-terminal Glm/Aga building block of paenilamicin, whilst terminal amines such as those of spermidine, ornithine and lysine side chains would not occupy this binding pocket, as they would experience significantly less binding stabilization.

Such accommodation of Glm/Aga in the substrate pocket would position the N-terminal amino group of Aga-6 close to the thioester carbonyl of the cofactor. An active site aspartate or glutamate residue commonly acts as a general base to trigger the $\text{N}$-acetylation reaction by deprotonation of the amine followed by a nucleophilic attack at the carbonyl of the thioester. In PamZ, the side chains of E122 ($\beta_6^*$) as well as E208 ($\beta_4a$) exhibit an interatomic distance of ~7 Å to the carbonyl atom of acetyl-CoA and thus might be in close proximity to the N-terminal amino group of Aga-6 (Fig. 6c). Residue S245 ($\beta_5$) is sandwiched between E122 and E208, and may mediate deprotonation and/or proton shuttling. Furthermore, we cannot exclude the involvement of water molecules during proton transfer. An oxyanion...
hole as described for myristoyl-CoA transferase is not present in PamZ, but the amide proton of V211 ($\beta^{4b}$) facilitates hydrogen bonding to the carbonyl oxygen of the thioester, which would increase the electrophilicity of the carbonyl carbon and stabilize the tetrahedral transition state after nucleophilic attack.

Self-resistance mechanism of *P. larvae*. The deactivation of paenilamicin through formation of N-acetylpaenilamicin by the action of PamZ (Supplementary Figs. 3–7) implicates that the enzyme may confer self-resistance to the producer strain *P. larvae*. To test this hypothesis, we exposed the deletion mutant *P. larvae ΔpamZ* to a mixture of paenilamicin A1/B1 in an agar diffusion assay. The mixture, which was purified from *P. larvae* ERIC II, inhibited bacterial growth of the deletion mutant ΔpamZ, but not that of the WT strain (Fig. 7a).

This result demonstrated that *P. larvae* requires the resistance gene, *pamZ*, to protect itself from the deleterious effects of its own antibacterial agent, paenilamicin. For further experimental

---

**Fig. 5 X-ray crystal structure of Gcn5-related N-acetyltransferase PamZ.** (a) Structural topological view of PamZ with its characteristic tandem-GNAT fold. The protein structure is divided into an N-terminal (NTD) and a C-terminal (CTD) domain. Color coding of protein regions follows that of other bacterial GNATs, such as aminoglycoside N-acetyltransferases (AACs). (b) Cartoon representations of PamZ from two perspectives. The first perspective (left) follows the color code as in panel a. Acetyl-CoA (AcCoA) bound to the P-loop and $\beta$-bulge of the CTD is depicted as sticks. The $\beta$-bulge is formed by strands $\beta^{4b}$ and $\beta^{5}$. The tandem-GNAT domains are highlighted (right) in blue (NTD) and purple (CTD). Secondary structure elements are labeled according to the protein topology. (c) Identical view as in panel b with the electrostatic potential mapped on the surface of PamZ, illustrating positive (blue) and negative (red) charges. The acetyl group attached to CoA (sticks) points into the active site highlighted by an asterisk. GNAT is an abbreviation of Gcn5-related N-acetyltransferase (Gcn5: general control non-repressed protein 5).
support, we analyzed supernatants and cell pellets of *P. larvae* WT and ΔpamZ for paenilamicins and *N*-acetylpaenilamicins. In cell lysates of *P. larvae* WT, we exclusively found *N*-acetylpaenilamicin, whereas for the deletion mutant ΔpamZ only unmodified paenilamicin (Fig. 7b) was detected. From paenilamicin isolates of the WT strain, primarily paenilamicin and only small amounts of *N*-acetylpaenilamicin were found in the supernatant by HPLC-ESI-MS (Supplementary Fig. 2). These findings demonstrate that the self-resistance factor PamZ enables *P. larvae* WT to acetylate and thus inactivate intracellular paenilamicin. *N*-acetylation functions as an efficient self-protection mechanism by scavenging paenilamicin that reenters the cells of *P. larvae*. However, this mechanism may not apply to intracellular paenilamicin after its release from the NRPS-PKS assembly line. Instead, it seems very likely that an inactive precursor, i.e. a prodrug, of paenilamicin is produced to mask the strong antibacterial activity before cellular export. Along these lines, the biosynthetic gene cluster of paenilamicin harbors the *pamJ* gene, which shows significant sequence similarity to a cyclic-peptide export ABC transporter with *d*-asparagine-specific peptidase activity that has been reported to be involved in a prodrug resistance mechanism in nonribosomal peptide synthesis. The peptidase recognizes and cleaves an *N*-acyl-*d*-asparagine unit of the prodrug. Accordingly, *P. larvae* must have developed a dual self-resistance mechanism against paenilamicin both potentially addressing the N-terminal Glm/Aga residue, specifically the N-terminal amino group at Aga-6 position, as modification site. Not only *P. larvae*, but also other bacteria belonging to the Firmicutes refer to a dual self-resistance mechanism associated with NRPS-PKS-derived compounds like amicoumacin, zwittermicin, and edeine (Supplementary Fig. 29). In a very recent study, paenilamicin B2 showed an inhibitory effect (IC₅₀ of ~0.3 µM) on the *E. coli* ribosome in vitro, whereas the non-native diastereomer PamB₂₂ was approximately tenfold and the *N*-acetylpaenilamicin B2 approximately 100-fold less active. The modifications of the N-terminal amino group at Aga-6 thus point to the importance of the N-terminal Glm/Aga residue as a major pharmacophore mediating recognition at the molecular target.

The insights into the pharmacophore region of paenilamicins and the structure of PamZ including its substrate-binding pocket may lead to the development of inhibitors against the self-resistance factor to weaken the bee larvae pathogen. This study lays an appreciable foundation to understand the self-resistance

**Fig. 6 Active site of PamZ.** a Motifs A (β₄-α₃) and B (β₅-α₄) located in the C-terminal domain (CTD) interact with co-substrate acetyl-CoA. b Close-up view of the active site displaying the negatively charged groove (color code as in Fig. 5c). c Highlighted amino acid residues with hydrogen-donating and -accepting groups form the groove and are well-positioned to potentially interact with the substrate paenilamicin.

**Fig. 7 Self-resistance of *P. larvae* against paenilamicin.** a Deactivation of a paenilamicin mixture A₁/B₁ (left) was tested by an agar diffusion assay against *P. larvae* WT (top) and *P. larvae* ΔpamZ (bottom). The negative control (right) contained water only. b HPLC-ESI-MS spectra of cell lysates of *P. larvae* WT (top) and *P. larvae* ΔpamZ (bottom) are depicted. Relevant peaks for paenilamicin (A₁/B₁) and *N*-acetylpaenilamicin (Ac-A₁/Ac-B₁) species are labeled with corresponding m/z ratios (z = 4). WT - wild-type.
mechanisms of *P. larvae* and to screen for potential small molecule inhibitors that target the active site of PanZ and compete with the naturally occurring paenilamcin. Consequently, the inhibitors would disable the self-resistance of *P. larvae* to paenilamcin resulting in the suicide of the bacterial pathogen for the benefit of the honey bee larvae. Such an anti-virulence strategy has the advantage that it circumvents the direct selection pressure on *P. larvae* to produce resistant strains because it only disarms the bacterium instead of interfering in essential bacterial functions. Therefore, it is not expected that inhibiting PamZ will readily lead to the development of resistant strains, as has already happened with the classical antibiotic treatment of AFB. Assuming that the potential candidate has been confirmed in lab-scale applications, it could be applied in honey bee colonies by preventingly impregnating the brood comb wax foundations with the substance so that it is dissolved in the brood food and then ingested by the larvae during early larval stages when *P. larvae* poses the most serious threat. Certainly, finding a small molecule inhibitor for PanZ and optimizing its application will be further investigated in future studies. In summary, these results expand our knowledge of the molecular strategies exploited by *P. larvae* to survive in its ecological niche — knowledge that is needed to combat this pathogen and secure the health of bee colonies worldwide.

**Methods**

**Bacterial strains and culture conditions.** The field strain *Paenibacillus larvae* (P. larvae) 04-309 (DSM 25430) and the deletion mutant 04-309 AapamZ were cultivated as follows: bacteria were grown on Columbia sheep blood agar (CSA, Thermo Fisher Scientific Oxoid, Schwerte, Germany) medium plates at 37 °C for 2-3 days. A preculture of 2 mL Mueller-Hinton-yeast-physphate-glucose-pyruvate (MYPG) broth was inoculated with single colonies and grown overnight. A 50 mL culture of MYPGP broth was inoculated with the preculture to reach an optical density measured at 600 nm (OD600) of 0.001. This main culture was incubated at 30 °C for 72 h with gentle shaking (80 rpm). Cultures were centrifuged at 3200g, 4 °C for 30 min, and supernatants were stored at −20 °C until further use.

*Escherichia coli* (E. coli) BL21-Gold(DE3) cells were cultivated in Luria-Bertani (LB) medium at 37 °C and 180 rpm. The medium was supplemented with kanamycin (50 µg mL−1) as an antibiotic based on the selection marker of the plasmid after transformation. Indicator strains like *B. megaterium* used for the agar diffusion assay were cultivated in LB medium at 37 °C and 180 rpm.

**Deletion mutant generation.** The generation of the pamZ deletion mutant was realized through a well-established protocol for *P. larvae* using the TargeTron Gene Knockout System (Sigma-Aldrich, Germany) based on group II intron insertion as previously described. The *pamZ* gene of *P. larvae* (DSM 25430) was disrupted with the analysis tool antiSMASH 5.0, cloned into vector pET28a(+) introducing an N-terminal histidine-tag and a TEV site. Reactions were performed in the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles (105 s per cycle) at 98 °C for 30 s, at 61 °C for 30 s, and at 72 °C for 45 s, followed by a final extension step at 72 °C for 10 min. The PCR analysis was performed with the GenDoc System v0.2.14 (Intas Imaging Instruments GmbH). The amplicons were purified and digested with Nhel and Xhol, and transformed into *E. coli* BL21-Gold(DE3).

**Heterologous expression and protein purification.** Terrific broth (TB) medium was inoculated with an overnight culture of pET28a pamZ-transformed in *E. coli* BL21-Gold(DE3) cells to reach an OD600 of 0.1 for the purification of PamZ. The culture was incubated at 37 °C and 180 rpm until OD600 of 0.8-1.0. Expression was induced by addition of 0.2 mM (Ec) isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were further incubated at 160 rpm, 18 °C for 20 h. Cells were harvested at 5000g, 4 °C for 30 min (Beckman Coulter, Avanti J-26 XP with rotor JLA 8.1), and the pellet was resuspended in lysis buffer (500 mM sodium chloride, 50 mM TRIS/HCL pH 8.0, 20 mM imidazole). Then, 10 mM magnesium chloride, 5 µg mL−1 DNase, 0.25 mg mL−1 lysozyme, and 0.2 M benzamidin were added to the solution. The cell disruption was performed by the cell homogenizer at 15 000 psi (Constan Systems Ltd, United Kingdom). The cell lysate was centrifuged at 50 000g, 4 °C for 30 min (Beckman Coulter, Avanti J-26 XP with rotor JA 25.50). The supernatant was loaded onto a His-Trap column using an AKTA protein purification system (AKTA Purifier 10, GE Healthcare). The chromatography was run with a two-step gradient started with 100% starting buffer (500 mM sodium chloride, 50 mM TRIS/HCL pH 8.0, 20 mM imidazole) and switched to 50% elution buffer (500 mM sodium chloride, 50 mM TRIS/HCL pH 8.0, 200 mM imidazole) within 20 CV to elute the His-tagged PamZ. A His-Trap crude FF column (GE Healthcare) was used for this purification. Fractions of interest were collected and combined to increase protein concentration. Subsequently, TEV protease (1 mg per 10 mg of protein) was added to the concentrated protein solution and incubated at 4 °C for 16 h. The N-terminal, TEV-cleavable His-tag was separated from the untagged PamZ by a second nickel affinity chromatography. Size-exclusion chromatography was performed with a HiLoad 16/60 Superdex 75 pg column (GE Healthcare) to remove residual imidazole from the protein sample with buffer solution (150 mM sodium chloride, 20 mM TRIS/HCL pH 8.0). The flow rate was set to 1 mL min−1. The chromophore was recorded with the需要用 v5.20 (Biopest) and the scanner system ViewPit 900 based on Epson scanner technology.

**Analytical size-exclusion chromatography**. Mixture A and B were used as standards. Mixture A contained aprotinin (3 mg mL−1), carbonic anhydrase (3 mg mL−1), conalbumin (3 mg mL−1), and mixture B ribonuclease (3 mg mL−1), ovalbumin (4 mg mL−1). The chromatograms of mixture A and B were acquired as references to determine the elution volume of PamZ. Untagged PamZ (1.25 mg mL−1) was prepared to obtain the best-fitted chromatogram. The size-exclusion chromatography was run with the AKTA protein purification system (AKTA-purifier 10, GE Healthcare), equipped with Superdex 75 10/300 GL and run with buffer solution (150 mM sodium chloride, 20 mM TRIS/HCL pH 8.0). The flow rate was set to 0.5 mL min−1. The chromatograms were recorded with Unicorn v5.20 (GE Healthcare).

**Protein crystallization, structure determination, and refinement.** For crystalization experiments, PamZ was concentrated to 71 mg mL−1. Crystallization was performed in a sitting drop vapor diffusion setup at 293 K. The reservoir solution was composed of 40% (w/v) PEG 3350, 50 mM ammonium sulfate, and 100 mM sodium acetate at pH 4.6. Prior to flash cooling, the crystals were cryo-protected in a 20% (v/v) glycerol solution with a 10% (v/v) glycerol protection solution. Data were collected at beamline 14.2 at BESSY. Diffraction data were processed with XDS (Supplementary Table 3). The structure was solved by molecular replacement with PHASER 2.8.152 using the N-terminal domain of the PDB 3G3S. Since the C-terminal domain could not be readily placed the model was completed by Arp/ wArp 8.053. The structure was re

---

**NATURE COMMUNICATIONS** | https://doi.org/10.1038/s41467-022-29829-w | www.nature.com/naturecommunications
Compound isolation from the supernatant. In all, 1 L of frozen supernatants of *P. larvae* ATCC 9545 or DSM 25430 cultures were thawed and then incubated with Amberlite XAD16 adsorption beads (1 g of beads per 10 mL culture filtrate, Sigma, St. Louis, MO, USA) and stirred for 16 h at room temperature. Then, the flow through was separated from the beads and a three-step gradient elution applied using 1 L of 10% (v/v) methanol followed by 1 L each of 90% (v/v) methanol plus 0.1% formic acid (LC) to finally obtain paenilamicin (and also N-acetylpaenilamicin). (N-acetyl)paenilamin-containing fractions were concentrated and purified subsequently by using a Grace HPLC column (GROM-SI 125 ODS-5 ST, 10 µm, 250 × 20 mm) coupled to an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) with a MWD UV detector. The separation was accomplished by a linear gradient elution using water plus 0.1% (v/v) formic acid as solvent A and acetonitrile plus 0.1% (v/v) formic acid as solvent B. The gradient started from 3% (v/v) to 15% (v/v) solvent B for 8 min, followed by 100% (v/v) solvent B for 2 min, and finished with an isocratic gradient of 100% (v/v) solvent B for 3 min. The flow rate was set to 20 mL min\(^{-1}\). The column was equilibrated with 5% (v/v) solvent B for 3 min. The flow rate was set to 0.5 mL min\(^{-1}\). Other parameters were set as follows: positive mode, gas temperature to 300 °C, drying gas to 8 L min\(^{-1}\), nebulizer to 35 psi, sheath gas temperature to 350 °C, sheath gas flow to 11 L min\(^{-1}\), capillary voltage to 3500 V, fragmentor to 330 V, sminner to 65 V, acquired rate to 1 spec s\(^{-1}\). The MS data derived from the Q-TOF were acquired with MassHunter LC/MS Data Acquisition B.06.01 (Agilent Technologies). The Q-TOF was attached to an Agilent 1200 Infinity HPLC system and equipped with an HPLC column (Poroshell 120, EC-C8, 2.7 µm, 2.1 × 50 mm, Agilent Technologies, Waldbronn, Germany). The HPLC was started with a linear gradient from 5% (v/v) to 100% solvent B for 10 min using water plus 0.1% (v/v) formic acid as solvent A and acetonitrile plus 0.1% (v/v) formic acid as solvent B, followed by an isocratic gradient of 100% (v/v) for 1 min. The column was equilibrated with 5% (v/v) solvent B for 2 min. The flow rate was set to 0.5 mL min\(^{-1}\). The MS data derived from the Q-TOF were acquired with MassHunter LC/MS Data Acquisition B.06.01 (Agilent Technologies). The Q-TOF was attached to an Agilent 1200 Infinity HPLC system and equipped with an HPLC column (Poroshell 120, EC-C8, 2.7 µm, 2.1 × 50 mm, Agilent Technologies, Waldbronn, Germany). The HPLC was run with a linear gradient using water plus 0.1% (v/v) formic acid as solvent A and acetonitrile plus 0.1% (v/v) formic acid as solvent B from 5% (v/v) to 100% (v/v) solvent B for 6 min, followed by an isocratic gradient of 100% (v/v) solvent B for 2 min. The column was equilibrated with 5% (v/v) solvent B for 2 min. The flow rate was set to 0.5 mL min\(^{-1}\). The ES source parameters were set as follows: product ion spectra were recorded in data-dependent acquisition (DDA) mode with a mass range from m/z 180 to m/z 2000 (MS1: FTMS, normal, 60000, full, positive. MS2: FTMS, normal, 30000). The parameter for the DDA mode was set as follows: dynamic exclusion (repeat count: 3, repeat duration: 30 s, exclusion size list: 50, exclusion duration: 180 s), current scan event (minimum signal threshold: 10,000, activation (type: CID, default charge state: 2, isolation width: m/z 2.0, normalised collision energy: 35), activation time: 30 ms), data-dependent acquisition (DDA). The LTQ-Orbitrap XL was acquired with XCalibur 2.2, and displayed with MassHunter Qualitative Analysis B.07.00 (Agilent Technologies). An LTQ-Orbitrap XL hybrid ion trap-orbitrap (Thermo Fisher Scientific GmbH, Bremen, Germany) was used to verify in vitro activation assays and to generate tandem mass spectra of paenilamicin and N-acetylpaenilaminic in data-dependent acquisition (DDA) mode with a mass range from m/z 180 to m/z 2000 (MS1: FTMS, normal, 60000, full, positive. MS2: FTMS, normal, 30000). The LTQ-Orbitrap XL was acquired with XCalibur 2.2, and displayed with MassHunter Qualitative Analysis B.07.00 (Agilent Technologies). An LTQ-Orbitrap XL hybrid ion trap-orbitrap (Thermo Fisher Scientific GmbH, Bremen, Germany) was used to verify in vitro activation assays and to generate tandem mass spectra of paenilamicin and N-acetylpaenilaminic in data-dependent acquisition (DDA) mode with a mass range from m/z 180 to m/z 2000 (MS1: FTMS, normal, 60000, full, positive. MS2: FTMS, normal, 30000). The LTQ-Orbitrap XL was acquired with XCalibur 2.2, and displayed with MassHunter Qualitative Analysis B.07.00 (Agilent Technologies). An LTQ-Orbitrap XL hybrid ion trap-orbitrap (Thermo Fisher Scientific GmbH, Bremen, Germany) was used to verify in vitro activation assays and to generate tandem mass spectra of paenilamicin and N-acetylpaenilaminic in data-dependent acquisition (DDA) mode with a mass range from m/z 180 to m/z 2000 (MS1: FTMS, normal, 60000, full, positive. MS2: FTMS, normal, 30000). The LTQ-Orbitrap XL was acquired with XCalibur 2.2, and displayed with MassHunter Qualitative Analysis B.07.00 (Agilent Technologies). An LTQ-Orbitrap XL hybrid ion trap-orbitrap (Thermo Fisher Scientific GmbH, Bremen, Germany) was used to verify in vitro activation assays and to generate tandem mass spectra of paenilamicin and N-acetylpaenilaminic in data-dependent acquisition (DDA) mode with a mass range from m/z 180 to m/z 2000 (MS1: FTMS, normal, 60000, full, positive. MS2: FTMS, normal, 30000). The LTQ-Orbitrap XL was acquired with XCalibur 2.2, and displayed with MassHunter Qualitative Analysis B.07.00 (Agilent Technologies). An LTQ-Orbitrap XL hybrid ion trap-orbitrap (Thermo Fisher Scientific GmbH, Bremen, Germany) was used to verify in vitro activation assays and to generate tandem mass spectra of paenilamicin and N-acetylpaenilaminic in data-dependent acquisition (DDA) mode with a mass range from m/z 180 to m/z 2000 (MS1: FTMS, normal, 60000, full, positive. MS2: FTMS, normal, 30000).
Chemical synthesis. The total synthesis of paenilamicin B2 (PamB2_3) and its diastereomers (PamB2_2, PamB2_1) have been recently described12.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support this study are available from the corresponding author upon reasonable request. The crystallographic data generated in this study have been deposited in the Protein Data Bank under accession code 7B3A. Diffraction images have been deposited at www.proteindiffraction.org. The MS data generated in this study have been deposited in Mass Spectrometry Interactive Virtual Environment (MassIVE) under project identifier M5V088695 (https://doi.org/10.25345/C5M68T). Source data are provided with this paper.

Received: 11 November 2021; Accepted: 30 March 2022; Published online: 29 April 2022

References
1. Klein, A. M. et al. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. B Biol. Sci. 274, 303–313 (2007).
2. Henry, M. et al. A common pesticide decreases foraging success and survival in honey bees. Science 336, 348–350 (2012).
3. Cormann, R. S. et al. Pathogen webs in collapsing honey bee colonies. Environ. Microbiol. Rep. 26, 185–230 (2013).
4. Wyatt, M. A. et al. Staphylococcus aureus nonribosomal peptide secondary metabolites regulate virulence. Science 329, 294–296 (2010).
5. Beceiro, A., Tomás, M. & Bou, G. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? Clin. Microbiol. Rev. 26, 862–890 (2013).
6. Morrissey, B. J. et al. Biogeography of Paenibacillus larvae spores between honey bee (Apis mellifera) colonies through robbing. PLoS ONE 9, e104562 (2014).
7. García-González, E. et al. Pathogen webs in collapsing honey bee colonies. Environ. Microbiol. Rep. 26, 185–230 (2013).
8. García-González, E. & Genersch, E. Honey bee larval peritrophic matrix degradation during infection with Paenibacillus larvae, the aetiological agent of American foulbrood. Int. J. Med. Microbiol. 303, 515–522 (2008).
9. García-González, E. & Genersch, E. Identification and characterization of two novel toxins expressed by the lethal honey bee pathogen Paenibacillus larvae, the causative agent of American foulbrood. Environ. Microbiol. 15, 2951–2965 (2013).
10. Garcia-González, E. & Genersch, E. Honey bee larval peritrophic matrix degradation during infection with Paenibacillus larvae, the aetiological agent of American foulbrood of honey bees, is a key step in pathogenesis. Environ. Microbiol. 15, 2894–2901 (2013).
11. García-González, E. et al. Paenibacillus larvae chitin-degrading protein PlCBP49 is a key virulence factor in American foulbrood of honey bees. PLoS Pathog. 10, e1004284 (2014).
12. García-González, E. et al. Paenibacillus larvae chitin-degrading protein PlCBP49 is a key virulence factor in American foulbrood of honey bees. PLoS Pathog. 10, e1004284 (2014).
13. García-González, E. & Genersch, E. Proteome analysis of Paenibacillus larvae reveals the existence of a putative S-layer protein. Environ. Microbiol. Rep. 4, 194–202 (2012).
14. Müller, S. et al. Paenilamicin: structure and biosynthesis of a hybrid nonribosomal peptide/polyketide antibiotic from the bee pathogen Paenibacillus larvae. Angew. Chem. Int. Ed. 53, 10821–10825 (2014).
15. García-González, E. et al. Biogeography of Paenibacillus larvae spores between honey bee (Apis mellifera) colonies through robbing. PLoS ONE 9, e104562 (2014).
16. Fünfhaus, A. & Genersch, E. Proteome analysis of Paenibacillus larvae spores between honey bee (Apis mellifera) colonies through robbing. Environ. Microbiol. 15, 2894–2901 (2013).
17. Müller, S. et al. Paenilamicin: structure and biosynthesis of a hybrid nonribosomal peptide/polyketide antibiotic from the bee pathogen Paenibacillus larvae. Angew. Chem. Int. Ed. 53, 10821–10825 (2014).
18. García-González, E. et al. Pathogen webs in collapsing honey bee colonies. Environ. Microbiol. Rep. 26, 185–230 (2013).
19. Wyatt, M. A. et al. Staphylococcus aureus nonribosomal peptide secondary metabolites regulate virulence. Science 329, 294–296 (2010).
20. García-González, E. et al. Virulence factors which correlate with epidemiological prevalences of Paenibacillus larvae. Appl. Environ. Microbiol. 81, 4555–4566 (2015).
21. Dang, T. & Süssmuth, R. D. Bioactive peptide natural products as lead structures for medicinal use. Acc. Chem. Res. 50, 1566–1576 (2017).
well as by the DFG, grant numbers GE1365/1-1, GE1365/1-2, and GE1365/2-1 to E.G. Brandenburg, Sachsen-Anhalt, Thüringen, Sachsen and the Senate of Berlin, Germany, as funded by the Ministries responsible for Agriculture of the German Federal States of

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) with SU 239/21-1 (project no. 279410221) for funding the project. This research was also funded by the Ministries responsible for Agriculture of the German Federal States of Brandenburg, Sachsen-Anhalt, Thüringen, Sachsen and the Senate of Berlin, Germany, as well as by the DFG, grant numbers GE1365/1-1, GE1365/1-2, and GE1365/2-1 to E.G. T.B. is grateful for the support of the DFG with RTG 2473 (Bioactive Peptides, project no. 39293329). We are grateful to Claudia Alings, Freie Universität Berlin, for help with crystallization. We acknowledge access to beamlines of the BESSY II storage ring (Berlin, Germany) via the Joint Berlin MX-Laboratory sponsored by Helmholtz-Zentrum Berlin für Materialien und Energie, Freie Universität Berlin, Humboldt-Universität zu Berlin, Max-Delbrück-Centrum, Leibniz-Institut für Molekulare Pharmakologie, and Charité-Universitätsmedizin Berlin.

Author contributions

T.D., S.M., A.M., and R.D.S. designed the experiments. T.D., S.M., and R.S. purified paenilamicins and PamZ and also conducted the in vitro activation assays. T.D. set up the tandem-MS experiments and analyzed the MS data. R.L. performed the crystallization and elucidated the protein structure of PamZ. J.E. generated the deletion mutant P. larvum ΔpamZ and performed the in vivo activation assay of paenilamin against wild-type and deletion mutant. T.B. and S.G. synthesized paenilamin B2 and the two diastereomers. J.G. cultivated P. larvum wild-type and deletion mutant ΔpamZ and prepared the corresponding supernatants. A.M. acquired and analyzed the NMR data. T.D., M.C.W., E.G., A.M., and R.D.S. wrote the manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-29829-w.

Correspondence and requests for materials should be addressed to Roderich D. Süssmuth.

Peer review information Nature Communications thanks Alessandro Caputo, Jurgen Rohr, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and you intend use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022