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Structure, Biosynthesis, and Occurrence of Bacterial Pyrrolizidine Alkaloids

*Olivia Schimming, Victoria L. Challinor, Nicholas J. Tobias, Hélène Adihou, Peter Grün, Laura Pöschel, Christian Richter, Harald Schwalbe, and Helge B. Bode**

Abstract: *Pyrrolizidine alkaloids (PAs) are widespread plant natural products with potent toxicity and bioactivity. Herein, the identification of bacterial PAs from entomopathogenic bacteria using differential analysis by 2D NMR spectroscopy (DANS) and mass spectrometry is described. Their biosynthesis was elucidated to involve a non-ribosomal peptide synthetase. The occurrence of these biosynthesis gene clusters in Gram-negative and Gram-positive bacteria indicates an important biological function in bacteria.*

Pyrrolizidine alkaloids (PAs) are widespread natural products in plants that are most likely produced as a chemical defense mechanism against herbivores.[1,2] More than 600 derivatives are known from over 6000 different plant species, and they are probably responsible for half of the loss of domestic livestock and countless reports on acute and chronic human food poisoning owing to their high toxicity.[1]

Despite the infamous "success" of these PA toxins, only a few PA derivatives that originate from non-plant organisms have been described, namely the clazamycins,[3,4] jenamidines,[5,6] bohemamine,[7] and NP25302,[8] which have all been isolated from *Streptomyces*. Whereas PA biosynthesis in plants includes homospermidine cyclization,[9–11] nothing is known about bacterial PA biosynthesis.

Herein, we report the identification of PA derivatives from entomopathogenic bacteria using heterologous expression followed by differential analysis by 2D NMR spectroscopy (DANS)[12] and mass spectrometry, structure confirmation by chemical synthesis, identification of the biosynthesis including a novel monooxygenase, and the occurrence of similar biosynthetic gene clusters in several bacteria.

In the context of our continuous efforts to assign functions to natural products from entomopathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus* in the complex life cycles of these bacteria, their nematode host, and their insect prey, we have identified an unknown biosynthetic gene cluster in *Xenorhabdus stockiae* (Figure 1). The cluster encodes a bimodular non-ribosomal peptide synthetase (NRPS; PxaA) and a monooxygenase (PxaB) followed by additional genes. Yeast homologous recombination cloning of the respective gene cluster from *X. stockiae* DSM 17904 and expression in *E. coli* allowed the application of DANS.[12,13] High-resolution COSY and HSQC spectra were acquired with identical parameters for the extracts of *E. coli* strains carrying either the *pxaAB* cluster or the empty plasmid, which were grown in only 10 mL of LB medium (LB = lysogeny broth) in the presence of Amberlite XAD-16 adsorbent resin. Comparison of the COSY spectra revealed two spin systems that were present in the *pxaAB*-carrying *E. coli* system but absent in the control strain, whereas a comparison of the corresponding multiplicity-edited HSQC spectra enabled the assignment of the associated carbon atoms (Figure 2 A and B). The first spin system encompassed resonances at $\delta_H = 3.91$ (br t, $J = 8.3$ Hz, CH, $\delta_C = 70.4$ ppm; Supporting Information, Figure S17), 2.17 and 1.51 (m, CH_2, $\delta_C = 27.5$ ppm), 2.13 (m, CH_2, $\delta_C = 28.9$ ppm), and 3.42 and 3.18 ppm (m, CH_2, $\delta_C = 49.0$ ppm), whereas the second included signals at $\delta_H = 2.46$ (t, $J = 7.5$ Hz, CH_2, $\delta_C = 37.5$ ppm) and 1.66 ppm (m, CH_2, $\delta_C = 25.7$ ppm), overlapping methylene resonances at $\delta_H = 1.36$–1.39 ppm ($2 \times CH_2$, $\delta_C = 32.4$ and 23.5 ppm), and a methyl signal at $\delta_H = 0.93$ ppm (t, $J = 6.9$ Hz, CH_3, $\delta_C = 14.3$ ppm). Furthermore, the ^{1}H NMR spectrum of the *pxaAB* strain displayed a new singlet at $\delta_H = 5.68$ ppm, which correlated with a signal at $\delta_C = 93.6$ ppm in the HSQC spectrum (Figure S16). Therefore, in combination with the bioinformatic analysis of PxaAB suggesting the incorporation of Ser and Pro by the two adenylation (A) domains, the information available through DANS suggested a pyrrolizidine core and a straight-chain acyl moiety.

Subsequent isolation of the compound identified by DANS allowed for the full elucidation of its structure by NMR spectroscopy (Figure 2 C, Table S2) as compound **1**, which was named pyrrolizixenamide A. This structure was also supported by HR-MS experiments and the sum formula derived thereof (Table S3) as well as isotope labeling experiments (Figure S1). HPLC/MS analysis of the *pxaAB E. coli* extract (Figure S2) led to the identification of two minor derivatives, namely pyrrolizixenamides B and C (**2** and **3**),

[*] O. Schimming,[+] Dr. V. L. Challinor,[+] Dr. N. J. Tobias, Dr. H. Adihou, P. Grün, L. Pöschel, Prof. Dr. H. B. Bode
Merck Stiftungsprofessur für Molekulare Biotechnologie
Fachbereich Biowissenschaften
Goethe Universität Frankfurt
Max-von-Laue-Strasse 9, 60438 Frankfurt am Main (Germany)
E-mail: h.bode@bio.uni-frankfurt.de
Homepage: http://www.bio.uni-frankfurt.de/48050101
Dr. C. Richter, Prof. Dr. H. Schwalbe
Institut für Organische und Chemische Biologie
Zentrum für Biomolekulare Magnetische Resonanz
Goethe Universität Frankfurt
Max-von-Laue-Strasse 7, 60438 Frankfurt am Main (Germany)
Prof. Dr. H. B. Bode
Buchmann Institute for Molecular Life Sciences (BMLS)
Goethe Universität Frankfurt
Max-von-Laue-Strasse 15, 60438 Frankfurt am Main (Germany)
[+] These authors contributed equally to this work.

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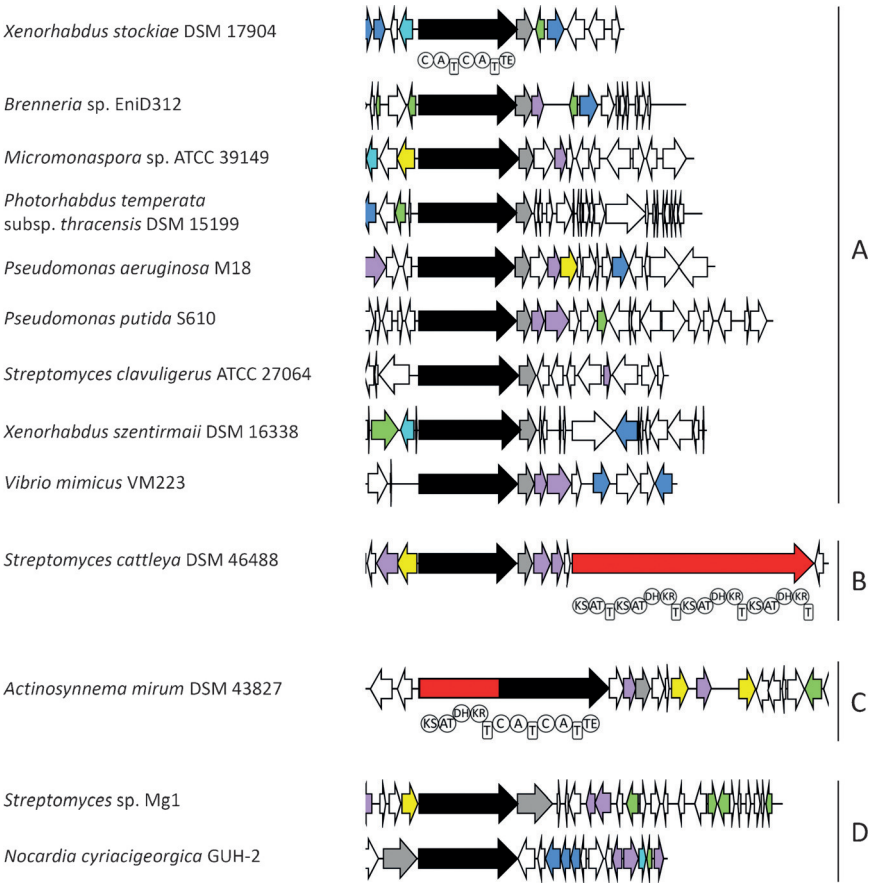
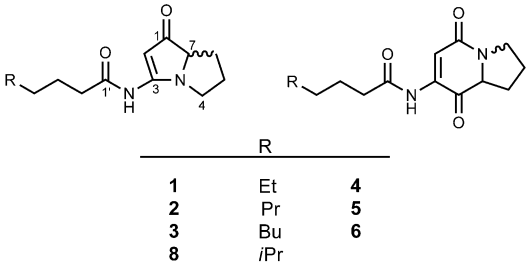


Figure 1. Pyrrolizidine alkaloid biosynthesis gene clusters identified in different bacteria subclassified into types A–D. All NRPS enzymes have the same domain architecture and A domain specificities for Ser and Pro, respectively. NRPS black, monooxygenase gray, PKS red, cytochrome P450 yellow, oxidoreductase purple, transporter blue, transcription factor green, methyltransferase turquoise, other genes white.

with similar MS-MS fragmentation patterns to **1**, that differ only in the length of the acyl side chain (Figure S3), which was also supported by HR-ESI-MS analysis (Table S3) and isotope labeling experiments (Figure S4). From large-scale cultivation, **2** and **3** could be isolated in sufficient amounts for NMR-based structure elucidation, which confirmed their structures (Figure S5, Table S2).

These compounds were shown to be racemic as deduced from their chemical synthesis (Figure S6)[14,15] followed by enantioselective HPLC analysis (Figure S7), as described for the closely related jenamidines.[6,15]

Coexpression of the downstream genes with *pxaAB* showed no difference in PA production (Figure S1). However, expression of *pxaA* alone resulted in the production of compounds **4** to **6**, the structures of which were solved by MS experiments (Figure S3 and Table S2).



	R	
1	Et	**4**
2	Pr	**5**
3	Bu	**6**
8	*i*Pr	

An intermediate that is structurally similar to compounds **4** to **6** has been proposed for the biosynthesis of the lipocyclocarbamate (LCC) brabantamide A (SB-253514),[16,17] which was isolated from two different *Pseudomonas* species, and the PxaAB and BraBC enzymes involved in LCC biosynthesis show 40% similarity. In LCC biosynthesis, it was suggested that this intermediate is due to the catalytic activity of an unusual thioesterase domain that catalyzes the dehydration of the serine moiety followed by the release of **4** (Figure 3B). Interestingly, a serine-derived dehydroalanine has also been proposed as an intermediate of β-lactam formation in nocardicin NRPS-based biosynthesis.[18] In PA or LCC biosynthesis, the flavin-dependent monooxygenase, PxaB or BraC, might catalyze a Baeyer–Villiger oxidation leading to ring expansion, which is followed by different steps that are all catalyzed by PxaB or its analogue: Hydrolysis of the seven-membered intermediate **7** followed by decarboxylation and ring contraction yields the PA skeleton as in *X. stockiae* (Figure 3), whereas an allylic 1,3-transposition of **7** results in the [5,5]bicyclic structure of LCC as in *Pseudomonas*. Further PxaB-catalyzed oxidation of the C7 carbon atom might result in the formation of hydroxylated jenamidines and pyrrolizixenamides, which were detected as trace compounds in *E. coli* that expressed *pxaAB* (Figure S8).

To determine whether this branching point in the biosynthesis of the PA and LCC compounds is a result of the different monooxygenases, *pxaA* was coexpressed with *braC* (Figure S1). Surprisingly, the same compounds as observed for *pxaAB* expression were present, indicating that additional players or substrate features might be required for LCC formation.[19] However, very recently, the biosynthesis of the legonmycins from *Streptomyces* sp. MA37 has been described.[20] For the biosynthesis of these bacterial PA compounds, the PxaB-like monooxygenase LgnC has been characterized in vitro and catalyzes the same carbamate formation and ring contraction also observed in *X. stockiae*.

As PAs are widespread in plants, the presence of PxaAB homologues in the bacterial kingdom was investigated. Using the PxaA and PxaB protein sequences from *X. stockiae*, we identified all genomes containing orthologues of both proteins. The construction of a phylogenetic tree using the protein sequences from PxaB led to the identification of a distinct clade of bacteria related to PxaB (totaling >90

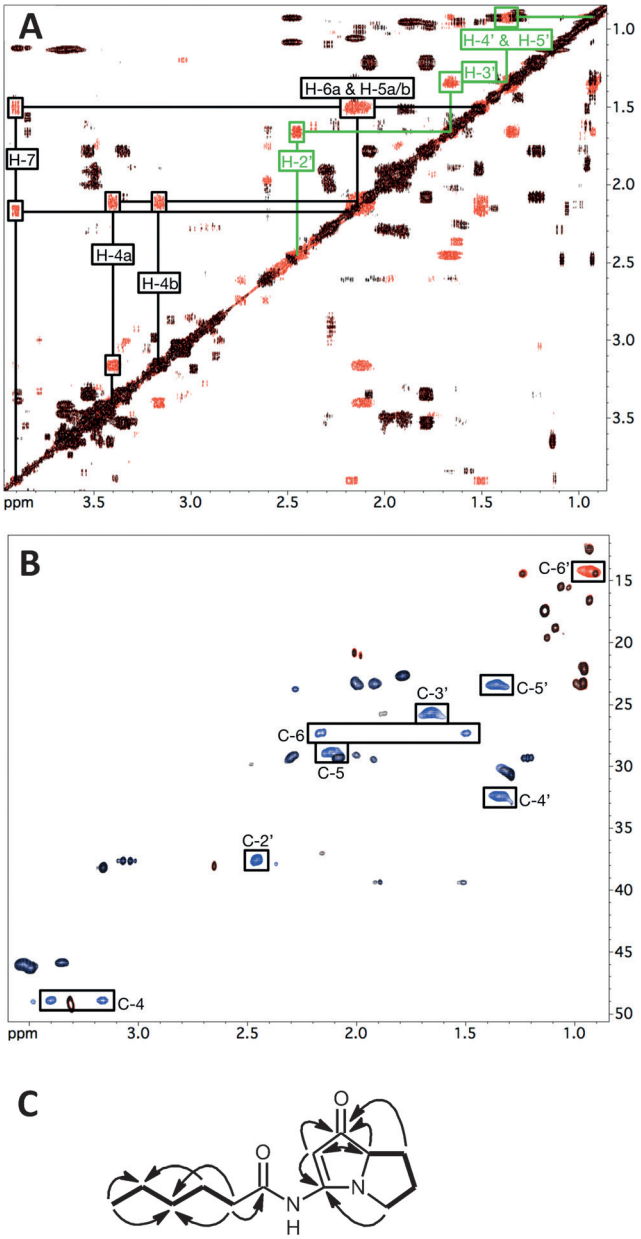


Figure 2. A) Expansion of the DANS overlay of COSY spectra (CD_3OD, 600 MHz) acquired from culture extracts of *E. coli* strains (grown in 10 mL of LB) carrying the *pxaAB* gene cluster (red) or an empty plasmid (black), showing identified spin systems. B) Expansion of the DANS overlay of multiplicity-edited HSQC spectra acquired for culture extracts of *E. coli* strains carrying the *pxaAB* gene cluster (CH_3/CH red, CH_2 blue, CD_3OD, 600 MHz) or an empty plasmid (black, CD_3OD, 600 MHz). C) Structure of **1** deduced from COSY (bold) and HMBC correlations (arrows).

strains from 23 diverse bacterial species) and was indicative of the presence of the entire PxaAB cluster. Among this collection were Gram-positive (*Nocardia*, *Streptomyces*, *Micromonospora*) and Gram-negative bacteria (*Pseudomonas*, *Vibrio*, *Xenorhabdus*, *Photorhabdus*; Figure 1, Figure S9, Table S4), suggesting the broad occurrence of such bacterial PA or LCC compounds. Interestingly, different gene cluster types were identified for PA or LCC biosynthesis, with the *X. stockiae* type being the most widespread (Figure 1 A). In *Streptomyces cattleya*, the PxaAB enzymes are encoded close to a type I polyketide synthase (PKS), which might generate the acyl moiety (Figure S10). In *Actinosynnema mirum*, however, PxaA is part of a PKS–NRPS hybrid that might result in the formation of the unsaturated jenamidine derivatives (Figure S11). In *Streptomyces* Mg1 and *N. cyriacigeorgica*, PxaB is fused to an NRPS-like condensation domain that is most similar to standard elongation domains (Figure S12), and in legonmycin biosynthesis, the PxaA homologue is split into two enzymes.[20]

No PA gene cluster has been linked to the production of such compounds in any of the identified strains except for MA37[20] despite the fact that some of them have been well studied, such as *P. aeruginosa* or *S. clavuligerus*. Therefore, a promoter exchange approach[21] was used to activate the PA gene cluster in the genetically accessible *X. szentirmaii*, which resulted in the production of pyrrolizixenamide D (**8**; Figure S13), differing from **1** in the presence of a branched acyl moiety (Figure S5).

Antibacterial and antitumor activities have been described for some bacterial PAs,[5,8,22] and brabantamide A, a highly potent phospholipase A_2 (PLA_2) inhibitor,[23,24] has been further developed into the first-in-class PLA_2 inhibitor darapladib.[25] It could be speculated that similar targets for bacterial PAs may modulate the immune response of symbiotic hosts (such as nematodes for *Xenorhabdus*) leading to stable symbiosis. Alternatively, these compounds might suppress the immune response during an infection process. *P. aeruginosa* causes severe infections in humans and animals and is one of the most important clinically acquired multiresistant pathogens,[26] whereas *Vibrio mimicus* causes severe gastroenteritis.[27] The presence of *pxaAB* homologues in these bacteria highlights the importance of a better understanding of the structure, biosynthesis, and function of bacterial PA and LCC derivatives, especially in pathogenic bacteria.

In summary, we have identified pyrrolizidine alkaloid derivatives in *Xenorhabdus* bacteria, elucidated their biosynthesis, and revealed their occurrence in Gram-negative and Gram-positive bacteria. Moreover, we have shown that two-dimensional NMR spectroscopy is a powerful method for the identification of natural products even from small-scale cultures that is ideally suited to the comparison of mutant versus wild-type or heterologously expressed gene clusters.

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Keywords: Baeyer–Villiger oxidation · biosynthesis · natural products · non-ribosomal peptide synthetases · pyrrolizidine alkaloids

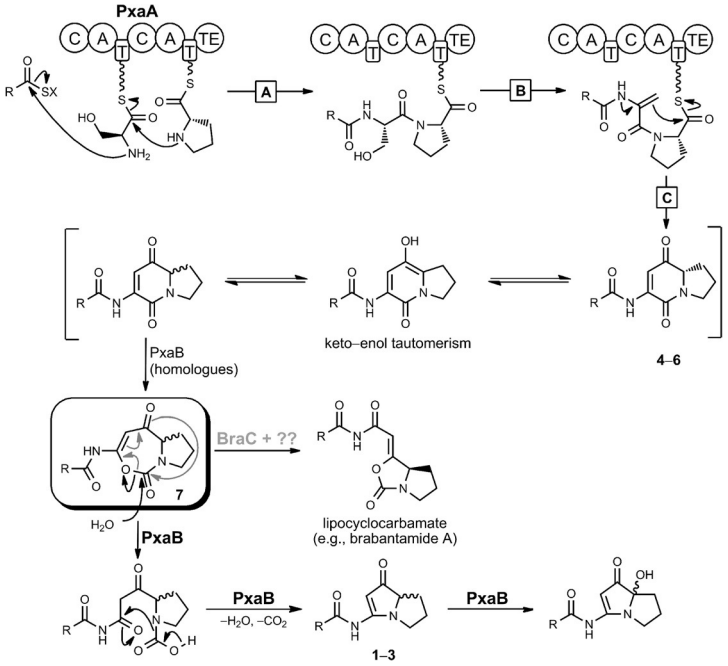

Figure 3. Proposed biosynthesis of bacterial PA and LCC compounds. Standard NRPS biochemical pathways (A) might be followed by TE-catalyzed dehydration and cyclization (B) prior to the enzymatic cascade catalyzed by PxaB.

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