Unprecedented Diversity of Catalytic Domains in the First Four Modules of the Putative Pederin Polyketide Synthase

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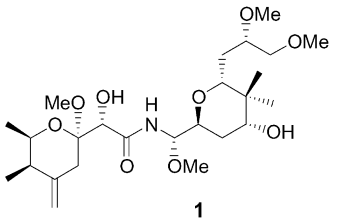
Polyketides of the pederin group are highly potent antitumor compounds found in terrestrial beetles and marine sponges. Pederin is used by beetles of the genera Paederus *and* Paederidus *as a chemical defense. We have recently identified a group of putative pederin biosynthesis genes and localized them to the genome of an as yet unculturable* Pseudomonas *sp. symbiont, the likely true pederin producer. However, this polyketide synthase cluster lacks several genes expected for pederin production. Here we report an additional polyketide synthase encoded on a separate region of the symbiont genome. It contains at least three novel catalytic domains that are predicted to be involved in pederin chain initiation and the formation of an unusual exomethylene bond. The region is bordered by mobility pseudogenes; this suggests that gene transposition led to the disjointed cluster organization. With this work, all putative pederin genes have been identified. Their heterologous expression in a culturable bacterium will provide important insights into how sustainable sources of invertebrate-derived drug candidates can be created.*

Introduction

It has been proposed for many years that natural product symbiosis, that is, the biosynthesis of secondary metabolites by symbiotic organisms, plays an important role in the chemistry of invertebrates.[1–4] Such suspected symbiont metabolites include many promising drug candidates isolated from marine animals. The existence of producing bacteria could be exploited to overcome the supply problem that currently hampers animal-derived drug development and production. However, our current general inability to cultivate such symbionts represents a serious technical challenge in pinpointing the true producer. So far, the most compelling evidence for symbiotic producers exists for the complex polyketide pederin (**1**), a highly potent



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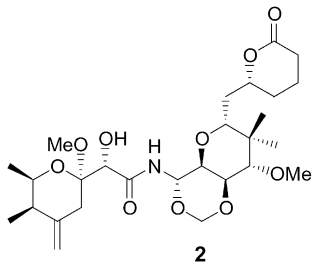
anticancer agent. It is found in apparently all species of the rove beetle genera *Paederus* and *Paederidus*,[5] whose females use pederin to chemically defend their offspring against predators.[6] Remarkably, closely related compounds have also been isolated from several sponges during marine antitumor and antiviral screening programs, for example, theopederin C (**2**) and onnamide A (**3**) from *Theonella swinhoei*.[5] Recently we have reported the *ped* biosynthesis gene cluster encoding a hybrid type I



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polyketide synthase (PKS) – nonribosomal peptide synthetase system, whose architecture perfectly matches a large portion of the pederin (**1**) structure.[7] Although these genes were isolated from the total DNA of *Paederus fuscipes* beetles, they belong to the genome of an as yet unculturable symbiotic bacterium with a very close relationship to *Pseudomonas aeruginosa*.[7, 8]

Bacterial type I PKSs are giant, modular enzymes harboring a multitude of catalytic domains.[9] Each module typically consists of at least a ketoacyl synthase (KS), an acyltransferase (AT), and

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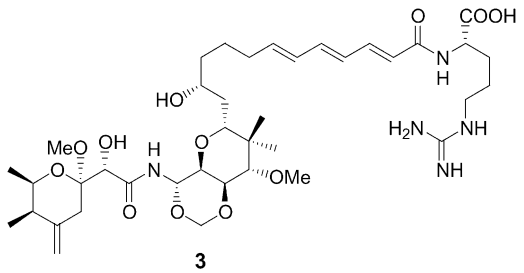

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an acyl carrier protein (ACP) domain, and attaches one polyketide building block, usually a small malonyl-CoA derivative, to the growing polyketide chain. During each of these elongation steps, various optional additional domains present on the module can introduce further structural modifications. The order and domain architecture of modules on the PKS enzymes usually strictly correspond to the order and structure of each polyketide building block, a phenomenon known as the colinearity rule. This rule can be used to predict whether an isolated PKS gene cluster encodes the biosynthesis of a specific metabolite. It also permits a rational design of novel polyketides by genetic modification of the domain architecture.[10, 11]

An analysis of the domain organization of the *ped* system revealed a number of surprises. The AT domains, usually found within each module, were instead encoded by separate genes.[7] Although this was the first reported example of monofunctional ATs encoded by type I PKS clusters, a number of similar systems have been discovered recently; this suggests that such enzymes might be more widespread among bacteria.[12–14] In addition, several partial gene clusters have been published, whose modules lack ATs and that probably also belong to this group.[15–17] Work on the leinamycin biosynthesis gene cluster revealed that these ATs acylate each module *in trans*,[12] and we have shown that such "*trans*-AT" PKSs are phylogenetically distinct from regular "*cis*-AT" systems.[18] The second deviation from the general PKS rules was that the genes encoding the first elongation steps were not found within the *ped* cluster. Unclustered genes of a bacterial PKS pathway are very rare: to the best of our knowledge, the ansamitocin system is the only other published example of a disconnected PKS.[19] To isolate the remaining pederin genes from the bacterial consortium of *P. fuscipes*, we used a phylogeny-based PCR strategy that specifically targets *trans*-AT PKS systems.[18] This approach led to the isolation of additional PKS gene fragments from metagenomic DNA of beetle bacteria. In this paper we present evidence that one of these fragments belongs to the missing PKS catalyzing the first three rounds of elongation.

Results and Discussion

Screening of a bacterial cosmid library for additional *trans*-AT PKS genes

We have previously reported the PCR amplification from metagenomic bacterial DNA of three PKS gene fragments (PS1 – PS3) that do not belong to the already sequenced *ped* cluster.[18] PCR products covering the KS domain of *trans*-AT PKSs can be distinguished from those of *cis*-AT systems by phylogenetic analysis.[18] Such an analysis placed the amplified product designated as PS3 close to the *cis*-AT, and PS1 and PS2 into the *trans*-AT group. Since the missing part of the *ped* PKS should belong to the latter group, we intended to isolate the gene(s) belonging to these PCR products. We therefore screened 2000 clones of a cosmid library prepared from DNA of *P. fuscipes* bacteria with probes derived from PS1 and PS2. Unexpectedly, restriction mapping of the positive clones revealed that the two PCR products did not originate from the same genomic region and apparently belonged to different secondary metabolites. Partial sequencing of both regions identified the PS2 system as a mixed peptide synthetase – PKS unrelated to the pederin (**1**) structure (data not shown), while the domain arrangement of the PS1 PKS was in very good agreement with the proposed architecture. The cosmid pPS9D2 containing the entire PS1 region, as judged from sequencing of insert ends, was therefore selected for further analysis.

Sequence analysis of the PS1 region

A map of the completely sequenced insert of cosmid pPS9D2 is shown in Figure 1, and the deduced functions of the identified open reading frames (ORFs) are listed in Table 1. As with the previously sequenced *ped* region, several genes with remarkable homology to *Ps. aeruginosa* ORFs were found in proximity to the PKS; this indicates that the two systems are located on the same genome. These homologues comprise a cluster involved in the uptake of branched-chain amino acids, a putative heat-shock gene, and a probable hemin receptor gene. In addition, the cosmid harbors various pseudogenes, including 11 decayed IS-like elements (IS = insert sequence). Framed by two of these ISs is a small gene cluster highly similar to PKS systems. Its three genes, designated *pedI*, *pedJ*, and *pedK*, do not contain frameshifts or stop codons and therefore seem to be intact.

An inspection of the domain arrangement of PedI revealed several unprecedented features among type I PKSs. Nevertheless, its unusual architecture clearly mirrors the structure of the pederin (**1**) starter moiety. The deduced product of *pedI* resembles a type I PKS consisting of four modules. By phylogenetic analysis of a PCR product covering a KS domain of this gene, we had previously predicted that the PKS lacks AT domains. In accordance with this, no homology to such domains was found within PedI. The first module lacks a KS domain and should therefore catalyze chain initiation. However, instead of a conventional AT, the module harbors an unusual N-terminal domain of about 240 aa similar to proteins of the GCN5-related *N*-acetyltransferase (GNAT) family[20] and sequence patterns diagnostic for this group (InterPro: IPR000182). The [RQ]XXGX[GA] motif involved in binding of the acetyl-CoA substrate[20] was identified at aa 135 – 140 (QQYNLG). GNATs catalyze the transfer of an acetyl group from acetyl-CoA to various heteroatoms,[20] which suggests that this domain primes the loading module with acetate. GNAT domains as part of PKSs have not been reported before.[9, 21] All published AT-less PKS systems are loaded with a starter other than acetate. In these cases, the absence of a GNAT domain, which usually accepts only

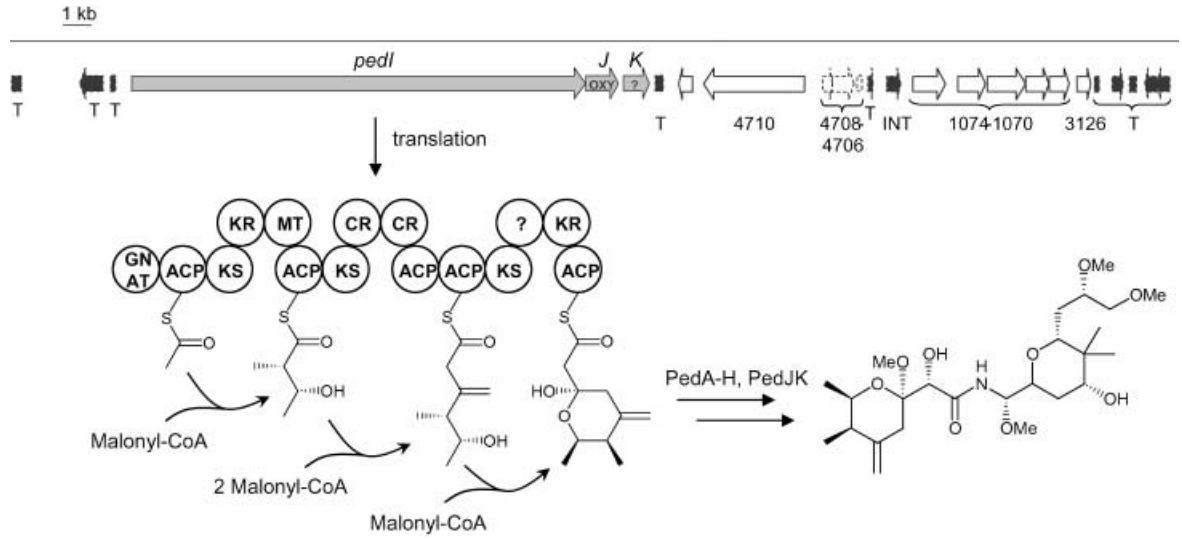


Figure 1. *Map of the genomic region belonging to the PCR product PS1 and proposed biosynthesis of pederin (**1**). Dotted boxes and arrows indicate degraded ORFs. Pseudogenes in black are involved in horizontal gene transfer.* Ped *genes are marked in gray. GNAT, GCN5-related* N*-acetyltransferase domain; ACP, acyl carrier protein domain; KS, ketosynthase domain; KR, ketoreductase domain; MT, methyltransferase domain; CR, crotonase domain; OXY, oxygenase; T, transposase; INT, phage integrase. Numbered genes are highly similar to genes from* Pseudomonas aeruginosa. *The numbers refer to the PA number of the closest homologue. PedA – PedH are encoded on a different part of the symbiont genome.*[7]

acetyl-CoA as substrate, is therefore not surprising. The sequencing of additional AT-less PKSs will reveal if the PedI GNAT domain is an exception or a common feature to acetate-primed members of this group.

In the second module, signature motifs of a KS, a ketoreductase (KR), a methyltransferase (MT), and an ACP domain were found, indicating that the corresponding polyketide extension unit bears a methyl group in an α-position to a hydroxy function. The KR domain contains a characteristic Asp residue at position 1270, which has recently been shown to be conserved in domains generating alcohols with D stereochemistry[22] (B-type alcohols, as defined in ref. [23]). This precisely corresponds to the first extension unit of the pederin molecule, which bears a B-type hydroxyl function adjacent to a methyl group. Cardani et al. attempted to elucidate the biosynthesis of **1** by feeding radioactively labeled precursors to *P. fuscipes*.[24] They reported the presence of 40% activity of administered labeled propionate in the *N*-acyl moiety constituting the western half of pederin (**1**). This seemingly suggests that the first extension unit is derived from methylmalonyl-CoA instead of malonyl-CoA and SAM-dependent methylation by an MT domain. However, propionate is catabolized in many bacteria[25] and insects[26] to acetate by several different routes and can then enter polyketide biosynthesis. Since the radioactive label was located on the propionate C2 position, it would be retained after chain shortening and be incorporated into malonyl-CoA-derived moieties. The fact that all known methyl groups generated by AT-less PKSs are attached by MT domains[12, 13, 15] is consistent with this hypothesis.

The second extension unit bears a rare exomethylene group at its C1 position. Carbon atoms that branch off such positions are known from a number of other complex polyketides, for example, myxovirescin (=TA),[27] mupirocin,[13] and leinamycin.[12] The PKS clusters of these compounds encode 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase homologues that were proposed to catalyze the addition of acetyl- or malonyl-CoA to a keto group, followed by decarboxylation, dehydration, and/or reduction steps. We had initially expected a similar enzyme in the biosynthesis of pederin (**1**), but such a homologue is not among the genes on pPS9D2 and could neither be detected in the symbiont DNA by hybridization with a probe derived from the HMG-CoA synthase homologue *mupH* nor by PCR with degenerate primers based on conserved motifs (data not shown). Instead, two unprecedented domains were found in the third module of PedI. In addition to a KS and an ACP domain, two domains were identified that show homology to enzymes of the crotonase (CR) superfamily. These enzymes catalyze a wide range of diverse reactions all involving a stabilized CoA thioester enolate intermediate.[28] The CR domain adjacent to the KS exhibits relatively low homology to known enzymes (42% similarity to the next homologue). A PSI-BLAST search revealed that it is related to naphthoate synthases.[29] The second CR domain is highly similar to discrete enzymes found in *trans*-AT systems, such as PksI (66%) from *Bacillus subtilis*[15] and MupK from *Pseudomonas fluorescens*.[13] These enzymes are annotated as putative enoyl-CoA hydratases, but their exact functions are presently unknown. If the colinearity rules apply, this module could catalyze the attachment of the exomethylene group, possibly by action of the two unusual CR domains. Indeed, members of the CR superfamily perform reactions very similar to those proposed for exomethylene group formation (Scheme 1). Among the enzymes acting on C–C bonds, naphthoate synthase from menaquinone metabolism catalyzes a Claisen condensation,[29] and hydroxycinnamate lyase from *Ps. fluorescens* is responsible for a retro-aldol reaction.[30] Decarboxylation and dehydration steps are known from the *E. coli* methylmalonyl-CoA decarboxylase and crotonase,[28] respectively. Moreover, homologues of the second CR domain are located directly adjacent to HMG-CoA synthase-like genes in the *mup*

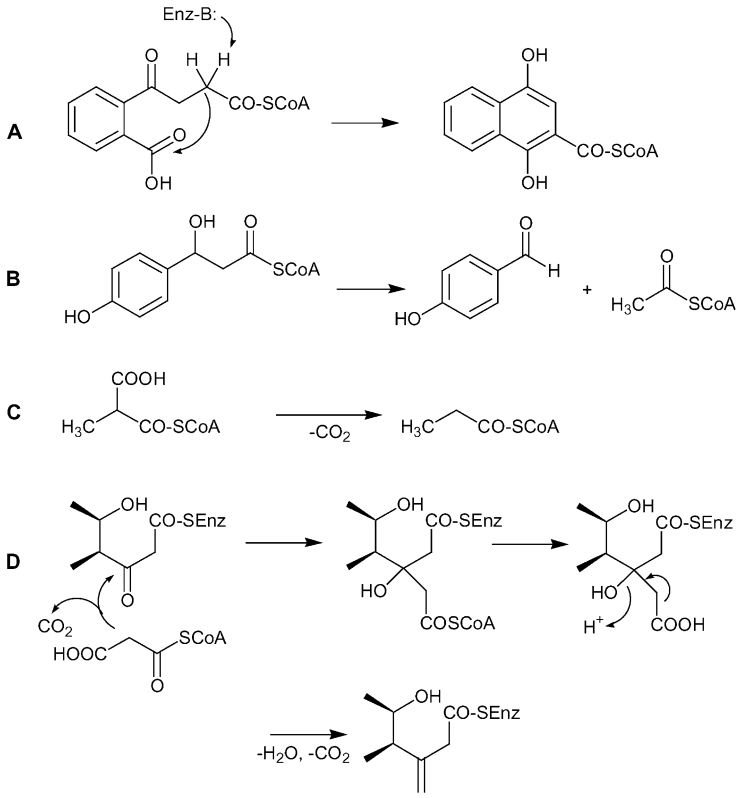
Table 1. *Genes and pseudogenes identified in the PS1 region.*

Gene	Putative function	Sequence similarity (protein, origin)	Similarity/ identity [%]	Amino acids	Accession number
	(PilL)[a]	PilL2, *Ps. aeruginosa*	63/56		AY273869
	(transposase)	XAC2604, *X. axonopodis* pv. *citri*	ca. 55/45		AE011901
	(transposase)	SCO5297, *Streptomyces coelicolor*	58/41		AL079356
pedI	PKS			4882	
pedI domains:	GNAT, GCN5-related histone acetyltransferase family	BT0804, *Bacteroides thetaiotaomicron*	46/23		AE016929
	ACP	NdaD, *Nodularia spumigena*	46/26		AY210783
	KS	PksP, *Bacillus subtilis*	59/43		Z99113
	KR	PedF, symbiont of *P. fuscipes*	54/36		AY059471
	MT	PksR, *B. subtilis*	74/56		Z99113
	ACP	PksM, *B. subtilis*	70/46		Z99113
	KS	PedF, symbiont of *P. fuscipes*	75/58		AY059471
	CR, crotonase superfamily	PksI, *B. subtilis*	42/24		Z99112
	CR, crotonase superfamily	PksI, *B. subtilis*	66/50		Z99112
	ACP	PksL, *B. subtilis*	51/34		Z14098
	ACP	PksL, *B. subtilis*	43/25		Z14098
	KS	PksL, *B. subtilis*	61/45		Z14098
	unknown				
	KR	PksL, *B. subtilis*	56/34		Z14098
	ACP	MmpD, *Pseudomonas fluorescens*	49/26		AF318063
pedJ	monooxygenase luciferase family	MupA, *Ps. fluorescens*	75/60	370	AF318063
pedK	unknown			318	
	(transposase)	Rv1150, *Mycobacterium tuberculosis*	51/31		NP 215666
	hypothetical protein	PP4615, *Ps. putida*	82/68	156	AE016791
phuH	probable outer membrane hemin receptor	PhuH, PA4710, *Ps. aeruginosa*	75/68	763	AE004885
	(hypothetical protein)	PA4708, *Ps. aeruginosa*	82/74		AE004884
	(ABC transporter permease)	PhuU, PA4707, *Ps. aeruginosa*	83/79		AE004884
	(ABC transporter ATP-binding component)	PhuV, PA4706, *Ps. aeruginosa*	75/71		AF055999
	(transposase)	IS2000 TnpA, *Ps. aeruginosa*	62/58		AF133699
	(integrase)	ECS1090, *Azotobacter* sp. FA8	57/46		AJ496234
braC	Leu/Ile/Val/Thr/Ala-binding protein	BraC, PA1074, *Ps. aeruginosa*	92/86	372	AE004539
braD	branched-chain amino acid transporter permease protein	BraD, PA1073, *Ps. aeruginosa*	97/94	307	AE004539
brae	branched-chain amino acid transporter permease protein	BraE, PA1072, *Ps. aeruginosa*	94/89	417	AE004539
braF	branched-chain amino acid transporter ATP-binding protein	BraF, PA1071, *Ps. aeruginosa*	95/91	255	AE004539
braG	branched-chain amino acid transporter ATP-binding protein	BraG, PA1070, *Ps. aeruginosa*	96/91	233	AE004539
ibpA	heat-shock protein	IbpA, PA3126, *Ps. aeruginosa*	94/89	149	AE004736
	(transposase)	OB1837, *Oceanobacillus iheyensis*	60/40		AP004599
	(transposase)	TnpA, *Enterococcus faecium*	70/50		AF403298
	(transposase)	OB1838, *O. iheyensis*	57/37		AP004599
	(transposase)	ISPs1 ORFB, *Ps. syringae* pv. tomato	87/77		AE016871
	(transposase)	ISPs1 ORFA, *Ps. syringae* pv. tomato	85/78		AE016871

[a] Entries in parentheses belong to pseudogenes.

and *pksX* clusters, suggesting that these genes participate in methyl(ene) group formation. The first CR domain without close homologues might substitute the HMG-CoA synthase, catalyzing the aldol addition of acetyl- or malonyl-CoA (Scheme 1). The CR related to PksI and MupK could then be involved in the further processing of the intermediate and perform the final decarboxylation and/or dehydration steps. If the two CR domains are indeed sufficient for exomethylene group formation, this module could represent a valuable tool in engineered biosynthesis that might dramatically increase the possible structural range of recombinant polyketides. However, at present it cannot be excluded that PedK or additional enzymes encoded elsewhere on the symbiont genome participate in this reaction.

Module 4 represents the last module of this PKS and contains a KS, a KR, and an ACP domain. The presence of this module was unexpected, since the corresponding polyketide extension step was initially assigned to the first module of PedF. However, the N-terminal KS domain of PedF lacks the conserved His residue necessary for the decarboxylation of the extender unit.[31] This module should therefore be unable to perform a polyketide elongation, which in fact necessitates an additional module on PedI. The same feature is known from the *mup* system, in which a seemingly superfluous module lacking a decarboxylation motif is located at the N-terminus of MmpA.[13] Thomas and co-workers proposed that such modules could have a pseudo-loading function and be involved in the proper channeling of polyketide chains between two PKS proteins.[13] Alignment of the last PedI module with other PKSs (data not shown) revealed that the KS and KR domains are separated by an unusually large region of approximately 250 aa without convincing homology to known sequences or patterns. Further functional studies are needed to determine whether it represents a novel domain, perhaps catalyzing the stereospecific intramolecular acetalization that likely takes place at this point in the biosynthesis of pederin (**1**),



Scheme 1. *Reactions catalyzed by members of the crotonase family and hypothetical pathway leading to the formation of the pederin exomethylene group. A) napthoate synthase (MenB); B) 4-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) from* Ps. fluorescens *AN103 (last step of a two-step reaction); C) methylmalonyl-CoA decarboxylase (MMCD) from* E. coli*; D) proposed pathway catalyzed by the PedI CRs. Acetyl-CoA could be used instead of malonyl-CoA in this route.*

or whether it is only an unusually long spacer region between the KS and the KR. The KR domain present in this module has no structural counterpart in pederin (**1**). It could be an inactive evolutionary remnant, similar to many other known PKS domains with no apparent function.[9] Alternatively, it could aid in the acetalization by polarizing the carbonyl bond.

PedJ, encoded by a gene downstream of *pedI*, is highly similar to several oxidoreductases of the bacterial luciferase type and contains the characteristic Pfam pattern (PF00296). The two closest database matches are MupA with unknown function[13] and MtaG from the myxothiazol pathway.[32] The latter enzyme was proposed by Müller and coworkers to catalyze the α-hydroxylation of an amino acid residue. Mupirocin contains no amino acid but features an α-hydroxylated polyketide extender position. PedJ could therefore be responsible for the α-hydroxylation of either the glycine residue or the third extension unit. Adjacent to *pedJ*, the ORF *pedK* encoding a putative protein of 318 aa was identified. However, homology and pattern analysis provided no insight into the possible function of this gene.

In contrast to all other known PKS gene clusters, no domain or gene was identified on the cosmid that would be involved in release of the assembled chain from the PKS or in the transfer of acyl-CoA units to the ACP domains. Therefore, this PKS evidently needs additional genes to be functional. These features strongly suggest that PedIJK and the previously identified *ped* genes complement each other and jointly encode pederin (**1**) biosynthesis. The current unculturability of the *Paederus* spp. symbiont prohibits standard experiments to verify the function of *ped* genes, such as knockout. Present studies in our laboratory therefore aim at expressing the *ped* genes in a heterologous host.

Although the *ped* system is to the best of our knowledge only the second example of PKS genes distributed among two genomic regions,[19] the numerous IS elements located on both ends of the two *ped* regions readily explain the disjointed structure. Their presence indicates that the entire PKS cluster was originally acquired as a genomic island by horizontal gene transfer. By a second IS-mediated rearrangement process, the *pedIJK* region could then have transposed to a different genome position. Ultimately, the region would have been stabilized by decay of all adjacent mobility genes. In free-living bacteria, frequent horizontal gene transfer selects for the clustering of genes from secondary metabolism.[33] In contrast, gene exchange would be a rare event in the *Paederus* spp. symbiont, which has lived in a shielded environment for millions of years. These conditions could have resulted in the observed gene scattering.

The pederin system represents an ideal model to understand the factors governing natural product symbioses and how sustainable sources of drug candidates from invertebrates can be created. In this work, the availability of all gene candidates necessary for pederin (**1**) biosynthesis now sets the stage for the production of pederin-type antitumor compounds in a culturable bacterium. In addition, the small repertoire of known PKS domains has been expanded by at least three new members. Their further study could significantly improve the versatility of engineered biosynthesis and our understanding of polyketide assembly.

Experimental Section

Isolation of bacteria from *Paederus fuscipes*: One hundred *P. fuscipes* beetles collected at Aydın, Turkey were ground in liquid nitrogen and resuspended in LB medium (5 mL). The mixture was kept for 10 min on ice to let beetle residues settle. The supernatant was centrifuged at 100 g for 10 min to sediment eukaryotic cells. The supernatant was then passed through a 40-μm nylon filter (Millipore), and the bacteria were pelleted by centrifugation at 5000 g for 10 min. The purity of the bacterial fraction was checked by DAPI staining and microscopic inspection.

Preparation and screening of a *P. fuscipes* symbiont genomic library: Genomic DNA from *P. fuscipes* bacteria was prepared by standard SDS lysis and used for the construction of a cosmid library in the pWEB vector (Epicentre) according to the manufacturer's instructions. Two thousand clones were deposited in 96-well plate format and transferred to Hybond N^+ membranes. Membranes were screened by using a fluorescein-labeled probe prepared from a 1.5-

kb fragment of the *ped* cluster and the ECL Direct Labeling and Detection kit (Amersham Biosciences) according to the manufacturer's protocol, except that Church buffer was used during prehybridization and hybridization. The probes were prepared from PCR fragments corresponding to the KS domain as described elsewhere.[7, 18] Hybridization was performed at 60 °C, followed by low-stringency washes for 2 × 7 min at 25 °C and 2 × 15 min at 60 °C.

Sequence analysis of positive cosmids: Inserts of positive cosmids were end-sequenced to gain information about the location of PKS genes. The cosmid pPS9D2 with genes unrelated to PKSs at its termini was chosen for complete sequencing. After shearing of the DNA by using a Standard Nebulizer (Octurno), the fragment ends were repaired with T4 DNA polymerase and Klenow fragment. Fragments of 1 – 1.5 kb were isolated by agarose electrophoresis, cloned into the pUC18 vector, and end-sequenced by using the BigDye Terminator Ready Mix (Applied Biosystems) and an ABI 3700 sequencer (Applied Biosystems). Sequence data were assembled by using GAP4 software[34] and analyzed by using the BLASTX, PSI-BLAST, FramePlot, and InterProScan algorithms.

The nucleotide sequence has been deposited at GenBank under accession number AY426537.

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