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The biosynthetic genes for prenylated phenazines are located at two different chromosomal loci of *Streptomyces cinnamonensis* DSM 1042

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Summary

***Streptomyces cinnamonensis* DSM 1042 produces two types of isoprenoid secondary metabolites: the prenylated naphthalene derivative furanonaphthoquinone I (FNQ I), and isoprenylated phenazines which are termed endophenazines. Previously, a 55 kb gene cluster was identified which contained genes for both FNQ I and endophenazine biosynthesis. However, several genes required for the biosynthesis of these metabolites were not present in this cluster. We now re-screened the cosmid library for genes of the mevalonate pathway and identified a separate genomic locus which contains the previously missing genes. This locus (15 kb) comprised orthologues of four phenazine biosynthesis genes known from *Pseudomonas* strains. Furthermore, the locus contained a putative operon of six genes of the mevalonate pathway, as well as the gene *epzP* which showed sequence similarity to a recently discovered class of prenyltransferases. Inactivation and complementation experiments proved the involvement of *epzP* in the prenylation reaction in endophenazine biosynthesis. This newly identified genomic locus is more than 40 kb distant from the previously identified cluster. The protein EpzP was expressed in *Escherichia coli* in form of a his-tag fusion protein and purified. The enzyme catalysed the prenylation of 5,10-dihydrophenazine-1-carboxylic acid (dihydro-PCA) using dimethylallyl diphosphate (DMAPP) as isoprenoid substrate. K_m values were determined as 108 μM for dihydro-PCA and 25 μM for DMAPP.**

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Introduction

Streptomycetes are prolific producers of secondary metabolites, including polyketides, non-ribosomal peptides and other structural classes. However, isoprenoid secondary metabolites are relatively uncommon in streptomycetes. Isoprenoids identified in *Streptomyces* comprise, e.g. the sesquiterpene geosmin (Jiang *et al.*, 2007), diterpenes like terpentecin (Dairi *et al.*, 2001) and phenalinolactone (Binz *et al.*, 2008), isoprenylated naphthalene derivatives like naphterpin (Shin-ya *et al.*, 1990), prenylated phenazines (Gebhardt *et al.*, 2002) and prenylated indole derivatives (Takahashi *et al.*, 2010), and the aminocoumarin antibiotics novobiocin and clorobiocin (Pojer *et al.*, 2003).

By the discovery of the prenyltransferases of clorobiocin and naphterpin biosynthesis (Pojer *et al.*, 2003; Kuzuyama *et al.*, 2005), it has been revealed that the prenylation reactions in the formation of such isoprenylated aromatic compounds are catalysed by a unique, new class of enzymes. Due to their αββα structural motif, these enzymes have been termed ABBA prenyltransferases (Tello *et al.*, 2008; Heide, 2009).

In our search for new members of the ABBA prenyltransferase class, we investigated *Streptomyces cinnamonensis* DSM 1042 which produces two different types of isoprenylated aromatic secondary metabolites, i.e. furanonaphthoquinone I (FNQ I) and endophenazines (Fig. 1) (Tax *et al.*, 1983; Sedmera *et al.*, 1991). We identified a 55 kb gene cluster which contained genes for both FNQ I and endophenazine biosynthesis (Haagen *et al.*, 2006). Inactivation experiments confirmed the involvement of these genes in the respective pathways. The cluster was found to contain genes for two new members of the ABBA prenyltransferases. One of these, *fnq26*, was shown to encode the prenyltransferase of FNQ I biosynthesis (Haagen *et al.*, 2007). Unexpectedly, the other gene, *fnq28*, was proven not to be involved in FNQ I or endophenazine biosynthesis (Haagen *et al.*, 2006). Therefore, the prenyltransferase of endophenazine biosynthesis in *S. cinnamonensis* remained unknown.

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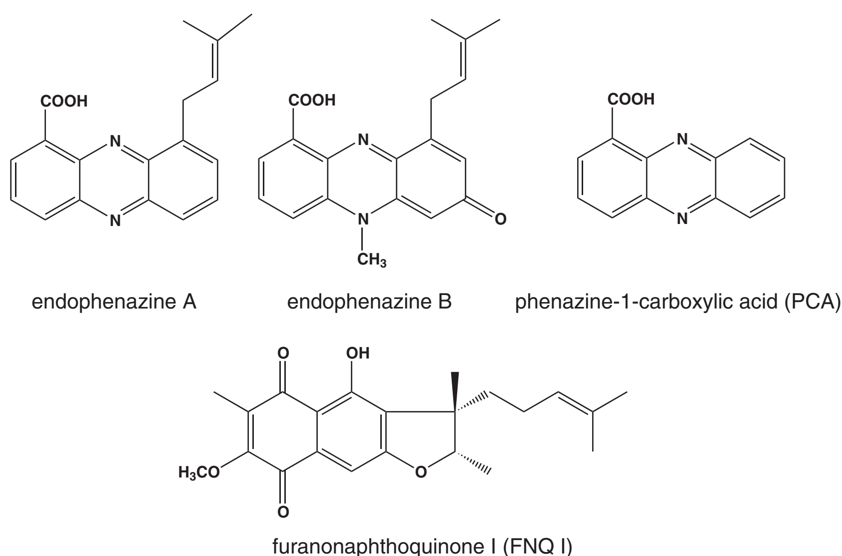

Fig. 1. Secondary metabolites of *S. cinnamonensis* DSM 1042.

In the present study, we identified a genomic locus which contained the gene for the missing prenyltransferase as well as several other genes involved in both endophenazine and FNQ I biosynthesis. The function of the prenyltransferase could be confirmed by inactivation experiments and biochemical investigation. This study provides one of the few examples that the genes of the biosynthesis of a secondary metabolite in a *Streptomyces* strain are not clustered at a single locus of the genome, but distributed to two different loci.

Results

Identification of the putative ABBA prenyltransferase gene epzP *in* S. cinnamonensis

The previously identified prenyltransferases with aromatic substrates showed little or no sequence similarity to each other (Tello *et al.*, 2008; Heide, 2009). Therefore, direct screening of a genomic library of *S. cinnamonensis* for the desired prenyltransferase gene did not appear promising. However, in four *Streptomyces* strains which form prenylated aromatic secondary metabolites the responsible prenyltransferase gene has been identified in the immediate vicinity of a cluster of genes encoding the enzymes of the mevalonate pathway. In *S. cinnamonensis* endophenazines and FNQ I are predominantly formed via the mevalonate pathway (Bringmann *et al.*, 2007). Therefore, the presence of a cluster of mevalonate pathway genes was expected in the genome of *S. cinnamonensis*, and we speculated that the missing prenyltransferase gene may be localized in its vicinity.

Using conserved sequences of mevalonate pathway genes from different streptomycetes, we designed degenerate primers for the hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA reductase and mevalonate diphosphate decarboxylase genes, using the CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) program (Rose *et al.*, 1998; 2003). A cosmid library of *S. cinnamonensis* had been established previously (Haagen *et al.*, 2006) and was screened with the three primer pairs. This led to the identification of cosmid 8-4D which gave PCR products for all three mevalonate pathway genes. This cosmid was subjected to full-length sequencing using a shotgun library of DNA fragments. The cosmid insert comprised 45 167 bp and was deposited in the GenBank database under Accession No. HQ228364. Approximately in the middle of the insert sequence, we found the expected cluster of six mevalonate pathway genes, and directly upstream thereof a gene with obvious sequence similarity to the ABBA prenyltransferase class which was termed *epzP* (Fig. 2).

The exact distance between the previously identified gene cluster (cosmid 3-6H) and the newly identified locus (cosmid 8-4D) in the genome of *S. cinnamonensis* is not known. We screened the cosmid library for cosmids overlapping with cosmid 3-6H, and found all overlapping cosmids not to contain genes of the mevalonate pathway or of phenazine biosynthesis. It can therefore be concluded that those two gene clusters must be more than 40 kb apart.

Sequence analysis of the insert of cosmid 8-4D

The central part of the insert of cosmid 8-4D contained 17 genes apparently related to the biosynthesis of prenylated phenazines. The results of the comparisons of these genes with database entries are listed in Table 1.

The genes upstream of the putative prenyltransferase gene *epzP* show very high similarity to genes found in

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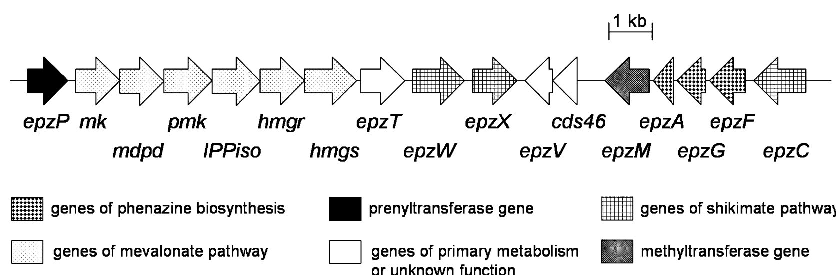

Fig. 2. Gene cluster on cosmid 8-4D of *S. cinnamonensis* DSM 1042, containing phenazine biosynthesis genes, mevalonate pathway genes and the prenyltransferase gene *epzP*. Further genes of endophenazine and furanonaphthoquinone I biosynthesis are contained in another genomic locus (Haagen *et al*., 2006).

many actinomycetes and probably code for primary metabolic enzymes. The gene *epzP* is therefore likely to mark the left border of the identified secondary metabolic gene cluster.

Immediately downstream of *epzP*, the six mevalonate pathway genes are situated. These genes apparently form a single operon, as the first two genes are only separated by 3 bp, while the following genes overlap, suggesting a translational coupling. Highly similar operons have been reported from several other *Streptomyces* strains forming isoprenoid secondary metabolites (Dairi, 2005). It appears likely that these genes are responsible for the formation of dimethylallyl diphosphate and isopentenyl diphosphate as precursors of the isoprenoid moieties of the endophenazines and of FNQ I in *S. cinnamonensis* (Bringmann *et al*., 2007), although no direct experimental proof has been provided.

Approximately 7 kb downstream of the mevalonate pathway genes, and orientated in the opposite direction, is a group of genes with high similarity to genes of phenazine biosynthesis which have previously been examined in *Pseudomonas* strains (Mavrodi *et al*., 2008). These genes include *epzF*, with high similarity to *phzF* which in *Pseudomonas* has been shown to code for an essential enzyme of phenazine biosynthesis, catalysing the isomerization of *trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHHA, Fig. 3) to a highly reactive aminocyclohexenone derivative. A second gene, *epzA*, showed similarity to *phzA* of *Pseudomonas*, involved in the condensation of two aminocyclohexenone moieties to a tricyclic phenazine precursor (Fig. 3) (Ahuja *et al*., 2008). This reaction requires a second, similar protein, PhzB, in *Pseudomonas*. In *S. cinnamonensis*, a *phzB* orthologue, as well as an additional *phzA* orthologue, are found in the previously

Table 1. Deduced functions of genes in the insert of cosmid 8-4D from a genomic library of *S. cinnamonensis* DSM 1042.

Gene	AA	Protein homologue, organism	Accession No.	Identity (%)	Proposed function
epzP	302	5,10-Dihydrophenazine-1-carboxylate-9-dimethylallyltransferase, *S. anulatus* 9663	CAX48655.1	56	Prenyltransferase of endophenazine biosynthesis
mk	345	Mevalonate kinase, *S. anulatus* 9663	CAX48656.1	75	Mevalonate kinase
mdpd	351	Diphosphomevalonate decarboxylase, *Streptomyces* sp. CL190	BAB07791.1	75	Diphosphomevalonate decarboxylase
pmk	371	Phosphomevalonate kinase, *Streptomyces* sp. CL190	BAB07792.1	68	Phosphomevalonate kinase
IPP_{iso}	363	Isopentenyl diphosphate isomerase, *Streptomyces* sp. CL190	Q9KWG2.1	78	IPP isomerase
hmgr	353	3-Hydroxy-3-methylglutaryl CoA reductase, *Streptomyces* sp. CL190	BAB70975.1	91	HMG-CoA reductase
hmgs	391	3-Hydroxy-3-methylglutaryl CoA synthase, *Streptomyces* sp. CL190	BAB07795.1	82	HMG-CoA synthase
epzT	333	3-Oxoacyl-[acyl-carrier-protein] synthase, *S. anulatus* 9663	CAX48662.1	80	Unknown
epzW	425	3-Phosphoshikimate-1-carboxyvinyltransferase, *Streptomyces* sp. Mg1	ZP_04998765.1	64	EPSP synthase
epzX	361	Chorismate synthase, *Streptomyces* sp. e14	ZP_06706902.1	85	Chorismate synthase
epzV	203	PpzV, *S. anulatus* 9663	CAX48664.1	63	Unknown
cds46	176	Tail sheath protein, *Natrialba magadii* ATCC 43099	YP_003478909.1	47	Viral protein
epzM	345	PpzM, *S. anulatus* 9663	CAX48665.1	76	*N*-methyltransferase of endophenazine biosynthesis
epzA	169	EphzA, *S. cinnamonensis* DSM 1042	CAL34112.1	98	Oxidoreductase of phenazine biosynthesis
epzG	213	EphzG, *S. cinnamonensis* DSM 1042	CAL34111.1	93	FMN-dependent oxidase of phenazine biosynthesis
epzF	278	PpzF, *S. anulatus* 9663	CAX48668.1	86	*Trans*-2,3-dihydro-3-hydroxyanthranilate isomerase
epzC	391	EphzC, *S. cinnamonensis* DSM 1042	CAL34108.1	97	DAHP synthase

The sequence of the insert of cosmid 8-4D has been deposited in the GenBank database under Accession No. HQ228364.

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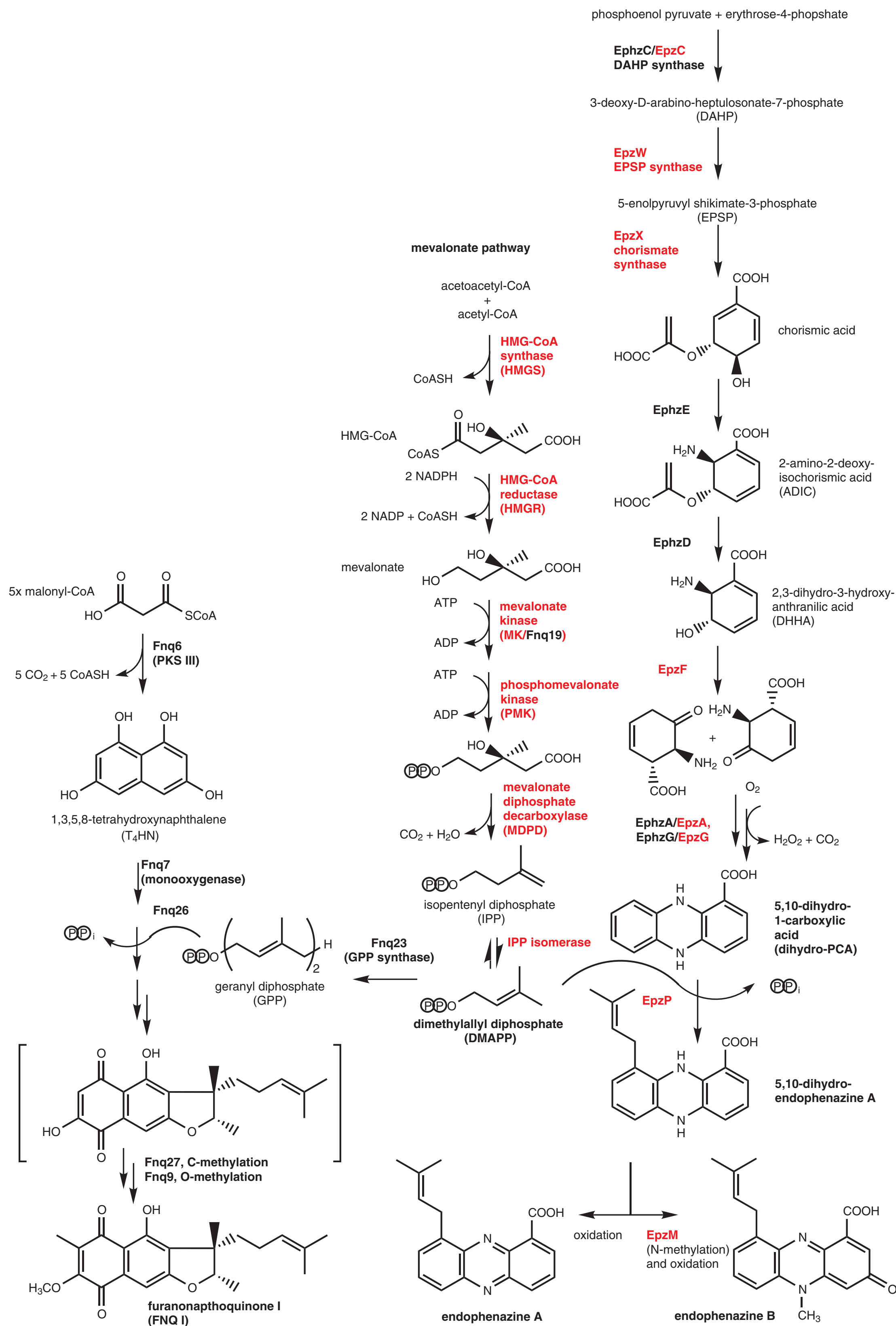

Fig. 3. Biosynthetic pathway to endophenazine A, endophenazine B and furanonaphthoquinone I. The enzymes which are encoded on cosmid 8-4D are marked in red, and those encoded at a previously identified locus (Haagen *et al*., 2006) are marked in black.

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identified locus for endophenazine and FNQ I biosynthesis genes (Haagen *et al*., 2006).

In the biosynthesis of phenazines, the initial tricyclic phenazine precursor is oxidized to 5,10-dihydroxyphenazine-1-carboxylic acid (Fig. 3). *epzG* (similar to *phzG* in *Pseudomonas*) is likely to be involved in this oxidation (Parsons *et al*., 2004). An additional copy of this gene is contained in the previously identified locus (Haagen *et al*., 2006).

Most, but not all phenazine gene clusters identified so far contain a gene (*phzC*) coding for a DAHP synthase (Fig. 3), i.e. the first enzyme of the shikimate pathway (Mavrodi *et al*., 2010). A similar gene, *epzC*, was found in the present gene locus. Additionally, and for the first time in any phenazine biosynthetic gene cluster, we found two further genes with obvious sequence similarity to shikimate pathway genes: *epzW*, coding for a putative 5-enolpyruvyl shikimate-3-phosphate synthase (EPSP), and *epzX*, with similarity to chorismate synthase (Fig. 3). It appears likely that these three genes contribute to the generation of chorismate for phenazine biosynthesis.

Streptomyces cinnamonensis produces the N-methylated compound endophenazine B (Figs 1 and 3). Correspondingly, the identified gene cluster contained the gene *epzM*, with high similarity to a known *N*-methyltransferase of phenazine biosynthesis (Parsons *et al*., 2007). Two further genes, designated as *epzT* and *epzV,* have close orthologues in a recently identified gene cluster for prenylated phenazine biosynthesis (Saleh *et al*., 2009a). It is yet unknown which function, if any, they may have in this pathway.

The genes located upstream of *epzC* could not be unambiguously assigned to either primary or secondary metabolism, and therefore the position of the right border of the identified gene cluster cannot be decided at present.

Inactivation of the prenyltransferase gene epzP

A principal aim of the present study was the identification of the prenyltransferase of endophenazine biosynthesis in *S. cinnamonensis*. The predicted gene product of *epzP* showed sequence similarity to aromatic prenyltransferases of the ABBA family and presented a very likely candidate for the desired gene. In order to confirm the involvement of *epzP* in endophenazine biosynthesis, we carried out an inactivation experiment (Fig. 4A). Using Red/ET-mediated recombination, *epzP* was replaced on cosmid 8-4D by an apramycin resistance gene (Gust *et al*., 2004). The modified cosmid was introduced into *S. cinnamonensis* by conjugation. Integration mutants, resulting from homologous recombination, were selected using their apramycin resistance, and the desired double-cross-over event was confirmed by the absence of the kanamycin resistance, encoded in the cosmid backbone. Two independent mutant strains were generated, and the replacement of *epzP* by the apramycin resistance gene was confirmed by PCR (Fig. 4B).

HPLC-UV and HPLC-ESI-MS analysis of cultures grown in endophenazine production medium confirmed the formation of both endophenazine A (*m/z* 293, $[M+H^+]$) and FNQ I (*m/z* 371, $[M+H^+]$) in the wild-type strain. HPLC-ESI-MS also confirmed the presence of the N-methylated compound endophenazine B (*m/z* 323, $[M+H^+]$) (Fig. 4C). In both Δ*epzP* mutant strains, the production of endophenazine A and B was completely abolished (Fig. 4C), but FNQ I was still produced (wild type 3.4 μmol l^{-1}; Δ*epzP* 6.6 μmol l^{-1}).

To confirm that the abolishment of endophenazine production was indeed due to the inactivation of *epzP*, both mutants were complemented with an intact copy of this gene. For this purpose, *epzP* was amplified by PCR (see *Experimental procedures*) and cloned into the *Escherichia coli–Streptomyces* shuttle vector pUWL-*hyg*^{R}/*ori*T which contains the strong *erm*E* promoter. DNA sequencing confirmed the absence of mutations. The resulting construct pKG08 was introduced into the Δ*epzP* mutants by conjugation, and two independent exconjugants were cultivated in production medium and analysed by HPLC-UV and HPLC-ESI-MS. In both cases, the production of both endophenazine A and endophenazine B was restored (Fig. 4C). The amount of endophenazine A in the complemented mutants, however, reached only 20% of the wild-type strain, possibly due to an inappropriate regulation of the expression of the introduced gene.

These experiments strongly supported the hypothesis that *epzP* codes for the prenyltransferase of phenazine biosynthesis in *S. cinnamonensis*.

Biochemical investigation of EpzP

For the expression and purification of EpzP, its structural gene was again amplified by PCR and cloned into two different expression vectors, pET28a and pHis8. The correct sequence was confirmed for the insert of both constructs and the protein was expressed in *E. coli* as a fusion protein with an N-terminal His_6 or His_8 tag respectively. Both constructs generated equal amounts of protein, and the activity of both fusion proteins were very similar. Ni^{2+} affinity chromatography resulted in a protein of apparent homogeneity. When this protein was incubated with 5,10-dihydrophenazine-1-carboxylic acid (dihydro-PCA) and dimethylallyl diphosphate (DMAPP) as substrates, the enzyme-dependent formation of a single prenylated product was observed (Fig. 5A). The instable prenylated dihydro-PCA was oxidized to the stable endophenazine A using sodium peroxodisulfate, and the identity of the resulting compound to endophenazine A

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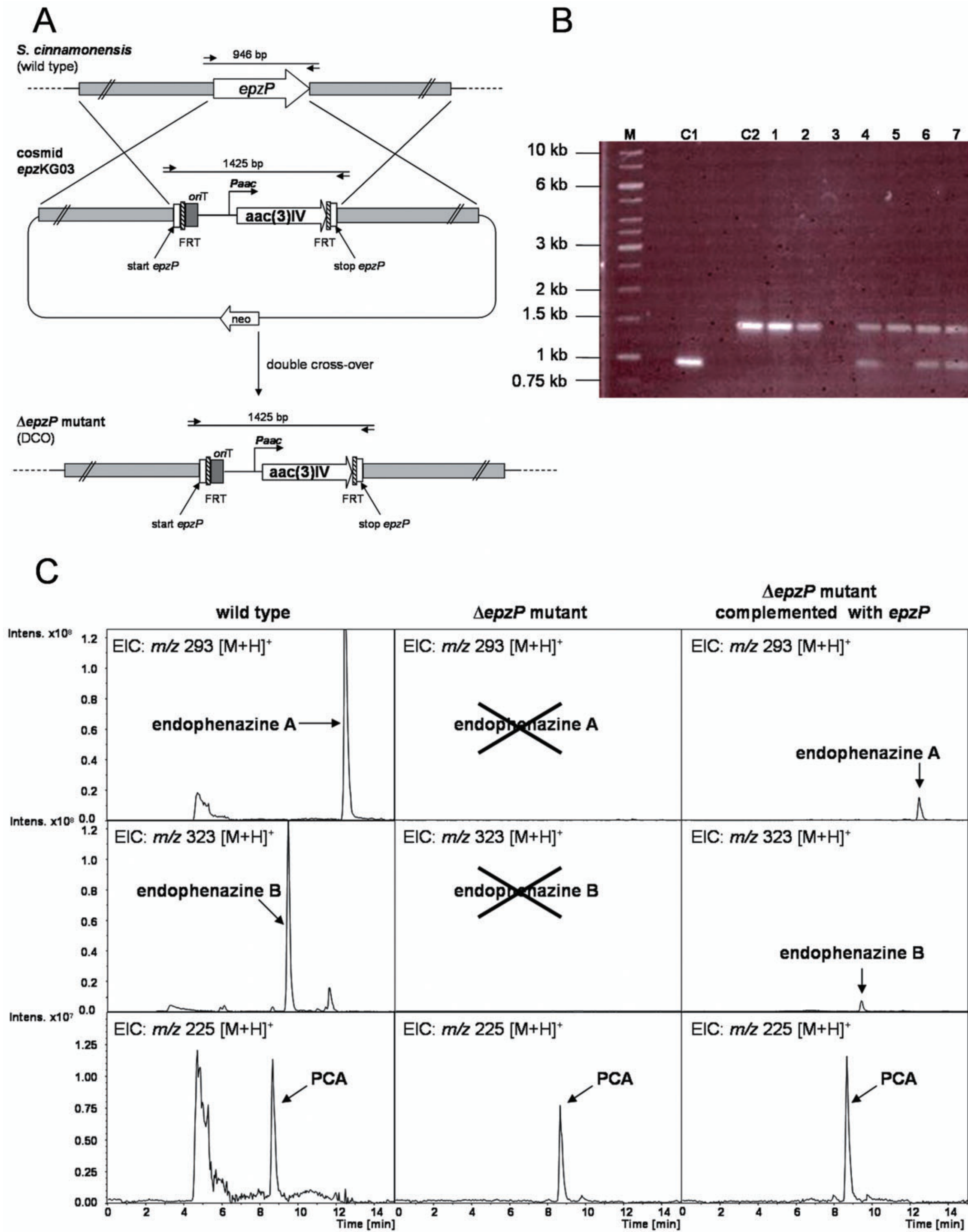

Fig. 4. A. Scheme of the gene inactivation of *epzP*. *aac(3)IV*, apramycin resistance gene; *Paac*, promoter of the apramycin resistance gene; FRT, FLP recognition target; *ori*T, origin of transfer from RK2; *neo*, kanamycin resistance gene. Out of scale.
B. Confirmation of the genotype of single-cross-over mutants (SCO) and double-cross-over mutants (DCO). Lane M: marker, C1: cosmid 8-4D (wild type), C2: cosmid *epz*KG03 (Δ*epzP*). Lanes 4, 6 and 7: SCO mutants. Lanes 1, 2 and 5: DCO mutants.
C. HPLC/MS analysis of the formation of endophenazine A, endophenazine B and phenazine-1-carboxylic acid (PCA) in *S. cinnamonensis* DSM 1042 (wild type), the Δ*epzP* mutant and the Δ*epzP* mutant complemented with intact *epzP*.

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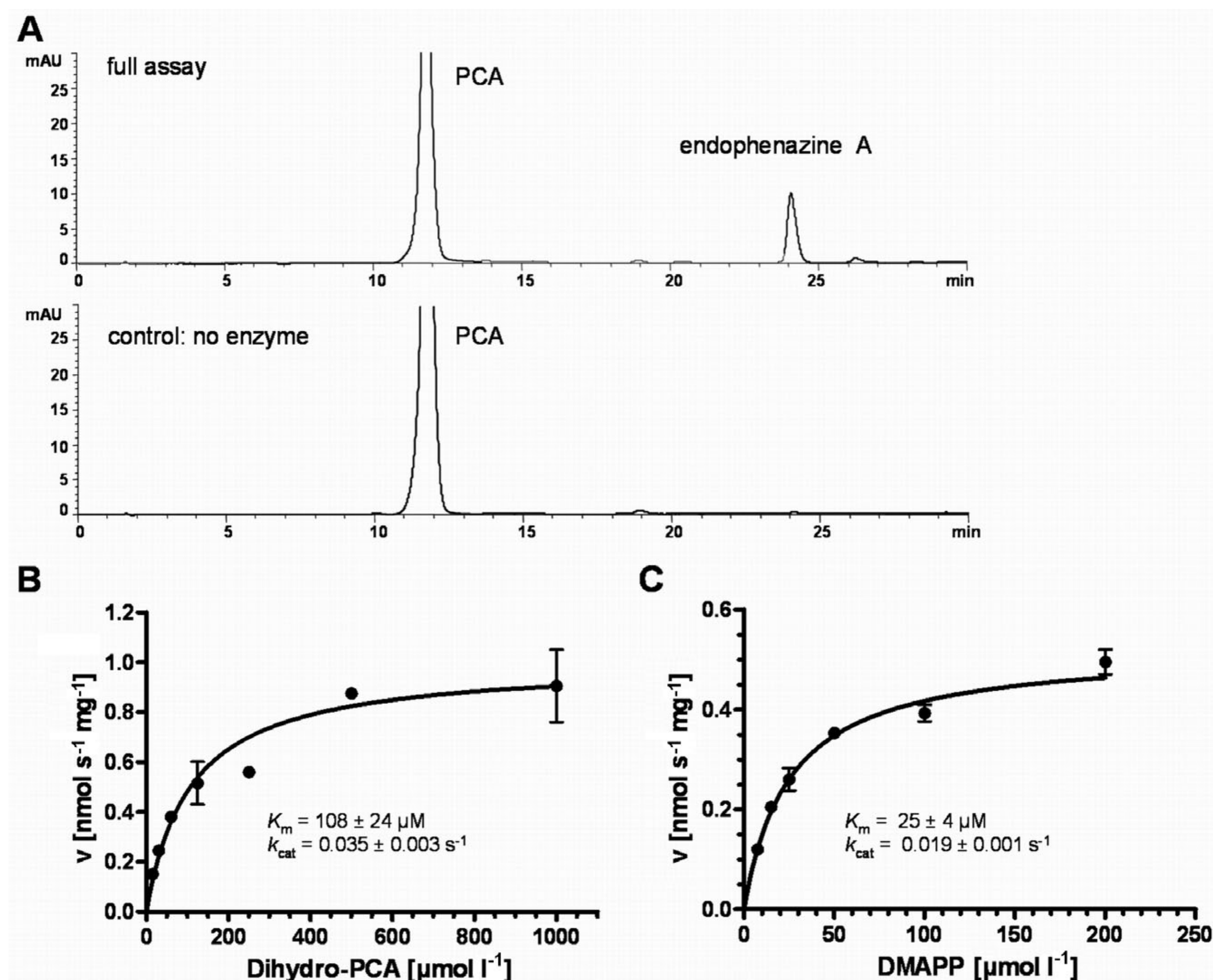

Fig. 5. A. HPLC analysis of prenyltransferase assays with purified EpzP, dihydro-PCA and DMAPP. UV chromatograms were recorded at 365 nm.
B and C. Determination of K_m values of EpzP for dihydro-PCA and DMAPP. In (B), DMAPP was kept constant at 0.4 mM. In (C), dihydro-PCA was kept constant at 0.8 mM. K_m and k_{cat} values were determined by non-linear regression, using GraphPad Prism software (GraphPad Software, La Jolla, CA).

was confirmed by HPLC-UV and HPLC-ESI-MS in comparison with an authentic reference sample. Both compounds gave identical fragmentation patterns in mass spectrometry.

EpzP was specific for dihydro-PCA as aromatic substrate. No reaction product was obtained with PCA. In contrast to the prenyltransferase Fnq26 from the same organism (Haagen *et al.*, 2007), no product was obtained with flaviolin, using either DMAPP or GPP as isoprenoid substrates; also 4-hydroxyphenylpyruvate, substrate of the ABBA prenyltransferases CloQ and NovQ (Pojer *et al.*, 2003), was not accepted by EpzP (Fig. S1).

As is typical for most members of the ABBA prenyltransferase family, the enzymatic activity of EpzP did not depend on the presence of Mg^{2+} or other divalent cations. Addition of $MgCl_2$ up to 10 mM did not increase the activity significantly, and upon addition of EDTA (10 mM) 77% of the activity was retained (Table S1).

Using a constant concentration of DMAPP (0.4 mM) and varying concentrations of dihydro-PCA, the K_m value for the aromatic substrate was determined as 108 ± 24 μM. And correspondingly, using a constant concentration of dihydro-PCA (0.8 mM) and varying concentrations of DMAPP, the K_m value for DMAPP was determined as 25 ± 4 μM (Fig. 5B and C).

Discussion

In a previous study, we identified a cluster of genes for endophenazine and FNQ I biosynthesis in *S. cinnamon-*

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ensis DSM 1042 (Haagen *et al*., 2006). This cluster will hereafter be referred to as locus A. Locus A did not contain a gene for the prenyltransferase which has to be postulated for endophenazine biosynthesis. In the present study, we could identify this missing prenyltransferase gene in a separate genomic locus, together with mevalonate pathway and phenazine biosynthesis genes. This genomic locus will hereafter be called locus B. The presence of genes for enzymes of the mevalonate pathway was expected in *S. cinnamonensis*, as feeding studies in this organism had confirmed that the isoprenoid moieties of endophenazines and FNQ I were derived from mevalonate (Bringmann *et al*., 2007). Likewise, the presence of the putative phenazine-*N*-methyltransferase EpzM was expected, as *S. cinnamonensis* produces the N-methylated compound endophenazine B (Figs 1 and 3), but the two methyltransferase genes present in locus A (*fnq9* and *fnq27*) had been experimentally assigned to methyltransferase reactions in FNQ I biosynthesis (Haagen *et al*., 2006).

Locus A contains a contiguous DNA region comprising orthologues of most genes required for phenazine biosynthesis in *Pseudomonas* (Mavrodi *et al*., 1998), however with the notable exception of a *phzF* orthologue. *phzF* has invariably been found in all functional biosynthetic gene clusters for phenazine biosynthesis identified in *Pseudomonas*, *Burkholderia*, *Pectobacterium* and others (Mavrodi *et al*., 2010). Locus A of *S. cinnamonensis* was the first phenazine biosynthetic gene cluster published from any *Streptomyces* strain, and the absence of *phzF* raised the question whether the essential biosynthetic step catalysed by PhzF in *Pseudomonas* may be accomplished in a different way in *Streptomyces*. However, as we now identified a *phzF* orthologue in locus B of *S. cinnamonensis*, and also in an endophenazine gene cluster from *Streptomyces anulatus* (Saleh *et al*., 2009a), it appears likely that the phenazine biosynthetic pathway in the Gram-positive actinobacteria (including *Streptomyces*) is identical to that in the Gram-negative gamma-proteobacteria (including *Pseudomonas* spp.) and beta-proteobacteria (including *Burkholderia* spp.).

In *S. anulatus*, we found all genes for endophenazine biosynthesis to be clustered in a single genomic locus (Saleh *et al*., 2009a). Likewise, in all previously investigated microbial producer strains of phenazines, all genes required for phenazine biosynthesis were invariably found to be clustered in a single locus (Mavrodi *et al*., 2010). The present study now provides the first example that the genes for phenazine biosynthesis are distributed to two different loci of the genome of a phenazine-producing strain.

Nearly all gene clusters for phenazine biosynthesis contain a paralogue of the first gene of the shikimate pathway, i.e. DAHP synthase (Fig. 3). This gene is usually termed PhzC. However, the present study provides the first example that two further genes of the shikimate pathway are contained in a phenazine gene cluster, i.e. *epzW* and *epzX*, with obvious similarity to EPSP synthase and chorismate synthase (Fig. 3). It appears likely that these genes have a similar role as generally assumed for the *phzC*, i.e. to ensure the supply of chorismate for phenazine biosynthesis, independently from the tightly regulated pathway to the aromatic amino acids.

The family of aromatic prenyltransferases to which EpzP, CloQ, NphB, Fnq26 and Fnq28 belong has originally been found to prenylate only phenolic substrates (Heide, 2009; Saleh *et al*., 2009b). EpzP now provides the second example of an enzyme of this family which prenylates not a phenolic substrate but a dihydrophenazine derivative. The first such example was PpzP (Saleh *et al*., 2009a).

Experimental procedures

Bacterial strains, plasmids and culture conditions

Streptomyces cinnamonensis DSM 1042 was grown in liquid YMG medium or on solid MS medium (Kieser *et al*., 2000) at 30°C. The medium described by Sedmera and colleagues (1991) was used for production of secondary metabolites. *Escherichia coli* XL1 Blue MRF' (Stratagene, Heidelberg, Germany) was used for cloning, and was grown in liquid or on solid (2% agar) Luria–Bertani or SOB medium at 37°C. The REDIRECT technology kit for PCR targeting was obtained from Plant Bioscience Limited (Norwich, UK). The *aac(3)IV/oriT* (apramycin resistance) cassette from pIJ773 (Gust *et al*., 2004) was used. For the selection of the recombinant mutants carbenicillin (50 $\mu g\ ml^{-1}$), apramycin (50 $\mu g\ ml^{-1}$), kanamycin (50 $\mu g\ ml^{-1}$), chloramphenicol (25 $\mu g\ ml^{-1}$) and nalidixic acid (25 $\mu g\ ml^{-1}$) were added to DNA (Kieser *et al*., 2000) and MS medium respectively.

Chemicals and enzymes

Carbenicillin and kanamycin were purchased from GenAxxon BioSciences GmbH, Biberach, Germany; apramycin, nalidixic acid and sodium persulfate ($Na_2S_2O_8$) from Sigma-Aldrich, Steinheim, Germany; chloramphenicol and sodium dithionite ($Na_2S_2O_4$) from Merck, Darmstadt, Germany; and phenazine-1-carboxylic acid from InFarmatik, Hungary. Dimethylallyl diphosphate was synthesized as described by Woodside and colleagues (1993) and endophenazines were isolated according to Sedmera and colleagues (1991), with modification described in *Production and analysis of secondary metabolites*. Restriction enzymes were purchased from New England BioLabs, Ipswich, MA.

Genetic procedures

Standard methods for DNA isolation and manipulation were performed as described by Kieser and colleagues (2000) and Sambrook and Russell (2001). DNA fragments were isolated

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from agarose gels by using the GFX PCR and gel band purification kit (Amersham Biosciences). Chromosomal DNA was isolated by lysozyme treatment and phenol/chloroform extraction as described by Kieser and colleagues (2000).

Screening of the cosmid library

The preparation of the cosmid library has been described previously (Haagen *et al.*, 2006). Screening was performed by PCR with degenerated primers (CODEHOP) (Rose *et al.*, 1998; 2003) for genes of the mevalonate pathway. The primer for the HMG-CoA synthase gene were HMGS_for (5′-GCC AAG TCC GCC GG(A/C/G/T) GT(A/C/G/T) TA(C/T) GT-3′) and HMGS_rev (5′-AGC CGG AAG GGG CC(A/C/G/T) GT(A/C/G/T) GT(C/T) TG-3′); for the HMG-CoA reductase gene CodeHMG_for (5′-GGC CAC CTA CGA GAC CCC (A/C/G/T)(C/T)T (A/C/G/T)TG (A/C/G/T)TG GCC-3′) and CodeHMG_rev (5′-CGC ATC AGC TCG CCG (G/C)(G/T)(A/G) TT(A/C/G/T) GT(C/T) TG-3′); for mevalonate diphosphate decarboxylase gene MDPD_for (5′-GAC CCT GGA CGT CTT CCC (A/C/G/T)AC (A/C/G/T)AC (A/C/G/T)AC-3′) and MDPD_rev (5′-GCG TTC CGC TCG GC(A/G/T) AT(C/T) TC(A/C/G/T) CC-3′).

Inactivation of the gene epzP

The inactivation was carried out with the REDIRECT technology kit for PCR targeting. The cosmid 8-4D was transformed into *E. coli* BW25113 (pIJ790) by electroporation and the bacteria were grown at 30°C. We used the following primers for the amplification of the apramycin resistance cassette (*aac*(*3*)*IV*) from pIJ773: orf16_PT_for_1 (5′-TTC GCC AAA TTC GAT CAT TCG ATC AGT GGA GGA ACC ATG ACT AGT ATT CCG GGG ATC CGT CGA CC-3′) and orf16_PT_rev (5′-CCT TTT GAA TGC CCG CCC CGG CGG GCC GGA GCG TGG TCA TCT AGA TGT AGG CTG GAG CTG CTT C-3′). The resulting PCR product had the size 1436 bp and contained restriction sites (underlined) for XbaI and SpeI. The PCR product was used to replace the gene *epzP* on cosmid 8-4D by Red/ET mediated recombination, resulting in cosmid *epz*KG03. The resulting cosmid was transformed into the non-methylating *E. coli* ET12567 (pUZ8002), and subsequently the non-methylated DNA was introduced by conjugation into *S. cinnamonensis* DSM1042 (wild type). Double-cross-over (DCO) mutants were selected by replica plating (DCO mutants are kanamycin-sensitive and apramycin-resistant) on solid DNA medium with kanamycin or apramycin respectively. DNA was isolated of resulting mutant strains and analysed by PCR with the primers *epz*KG03_for (5′-CAT TCG ATC AGT GGA GGA ACC ATG-3′) and *epz*KG03_rev (5′-GGC GGG CCG GAG CGT GGT CA-3′). Gene replacement mutants showed a single PCR band with the size of 1400 bp, whereas the wild-type gene resulted in a band at 950 bp.

Production and analysis of secondary metabolites

Mutants and wild-type *S. cinnamonensis* strains were precultured for 48 h in liquid YMG medium (50 ml) at 30°C and 180 r.p.m. Fifty millilitres of production medium (Sedmera *et al.*, 1991) was inoculated with 3 ml of the pre-culture in a 300 ml Erlenmeyer flask with spring and baffle and cultivated for 120 h. In case of the Δ*epzP* mutants apramycin (50 μg ml^{-1}) was added to the medium. For isolation of endophenazines and FNQ I, 50 ml of culture was centrifuged at 3500 *g* for 10 min. The supernatant was discarded and the cells were extracted with methanol (10 ml) by vortexing and treatment in an ultrasonic bath for 5 min. The extract was mixed with sodium acetate buffer (10 ml; 1 M, pH 4.0) and extracted with dichloromethane (5 ml). After separation of the organic phase, the solvent was evaporated and the residue was dissolved in methanol (50 μl). Extracts were analysed with HPLC (Agilent 1100 series; Waldbronn, Germany) by using an Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm; Agilent) at a flow rate of 1 ml min^{-1} with a linear gradient from 10% to 100% of solvent B in 30 min (solvent A: 1% formic acid in water; solvent B: 1% formic acid in acetonitrile). Detection was carried out at 252 and 365 nm. Additionally, a UV spectrum from 200 to 400 nm was logged by a photodiode array detector (DAD). The absorbance at 365 nm was used for quantitative analysis, employing authentic samples of PCA and endophenazine as external standards.

Complementation of ΔepzP *mutants*

The Δ*epzP* mutants were complemented with plasmid pKG08, carrying an intact copy of *epzP* in the shuttle vector pUWL-*hyg*^{R}/*ori*T (Zhao *et al.*, 2010). The gene *epzP* was amplified from cosmid 8-4D by PCR with the primers K_orf16_for (5′-AAG CTT ATG TCG GAA AGC GCC GAC-3′) with a HindIII restriction site (underlined) and K_orf16_rev (5′-ACT AGT TCA GCC GTC GGA ACG CAG-3′) with a SpeI restriction site. The PCR product was first cloned into pGEM®-T (Promega Corporation, Madison, WI) to give plasmid pKG03. After restriction with HindIII and SpeI, isolation of the 915 bp fragment, it was ligated into pUWL-*hyg*^{R}/*ori*T (linearized with HindIII and SpeI), resulting in plasmid pKG08. The plasmid was introduced into the non-methylating *E. coli* ET12567 (pUZ8002) and then transferred into Δ*epzP* mutants via conjugation. For cultivation of the complemented Δ*epzP* mutants apramycin (50 μg ml^{-1}) and hygromycin (40 μg ml^{-1}) were added to the production medium. The extraction procedure for the endophenazines and FNQ I has been described above.

LC-ESI-MS and -MS/MS analysis

The extracts were examined with LC-ESI-MS using a Nucleosil 100-C18 column (3 μm, 100 × 2 mm) coupled to an ESI mass spectrometer (LC/MSD Ultra Trap System XCT 6330; Agilent Technology). Analysis was performed at a flow of 0.4 ml min^{-1} with a linear gradient from 10% to 100% of solvent B in 15 min (solvent A: 0.1% formic acid in water; solvent B: 0.06% formic acid in acetonitrile). Detection was carried out at 230, 260, 280, 360 and 435 nm (± 10 nm). Electron spray ionization (positive and negative ionization) in Ultra Scan mode with capillary voltage of 3.5 kV and heated temperature of 350°C was used. LC-MS/MS analysis was carried out in positive ionization mode with the same capillary voltage and temperature. For

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endophenazine A, the mass 293 ± 0.5 Da was selected for fragmentation.

Expression and purification of EpzP

For the construction of the expression plasmids pBB09 (*epzP* in pET28a) and pKG14 (*epzP* in pHis8), *epzP* was amplified with Phusion® DNA Polymerase (Finnzymes, Woburn, MA) using the cosmid 8-4D as template. The following primers were used: for construction of pBB09, orf16_NdeI_F (5′-GGG AAT TCC ATA TGT CGG AAA GCG CCG ACC-3′) and orf16_XhoI_Stop_R (5′-GCC CTC GAG TCA GCC GTC GGA ACG CAG-3′), for construction of pKG14, epz16_EcoRI_F (5′-GTG CCG CGC GAA TTC CAT ATG TCG-3′) and the same reverse primer as above, i.e. orf16_XhoI_Stop_R. The resulting PCR products were cloned into pGEM®-T (Promega). After sequencing (Eurofins MWG Operon, Martinsried, Germany) *epzP* was cloned into pET28a to give pBB09, and into pHis8 to give pKG14. The plasmid pKG14 was transformed into *E. coli* Rosetta 2 (DE3) pLysS (Stratagene) and a pre-culture of 100 ml of liquid LB medium was cultured overnight at 37°C and 200 r.p.m. Thirty-five millilitres of the pre-culture were added to liquid TB medium (1 l) containing kanamycin (50 μg ml^{-1}) and chloramphenicol (25 μg ml^{-1}) and grown at 37°C to an A_{600} of 0.6. The temperature was lowered to 20°C and isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After 20 h of cultivation at 20°C, the cells were harvested by centrifugation for 10 min at 2700 *g* at 4°C. The cells (45 g from 2 l of culture) were resuspended in 110 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 1 M NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 20 mM imidazole, 0.5 mg ml^{-1} lysozyme, 0.5 mM phenylmethylsulfonyl fluoride, PMSF). After stirring at 4°C for 30 min, cells were ruptured with a sonifier (Branson W-250 D, Branson, Danbury, CT) and centrifuged for 45 min at 55 000 *g* at 4°C. The supernatant was purified by nickel affinity chromatography (5 ml HisTrap™ HP column, GE Healthcare). For elution of the protein imidazole buffer [50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 250 mM imidazole] was used. The buffer was changed using PD-10 desalting columns according to the protocol (GE Healthcare Life Sciences), equilibrated with 100 mM Tris pH 7.5, 15% (v/v) glycerol, 2 mM DTT. Ninety-three milligrams of His_8-EpzP could be purified.

Assay for prenyltransferase activity

One hundred microlitres of the reaction mixture contained 100 mM Na-TAPS pH 7.5 (Sigma-Aldrich, Steinheim, Germany), 0.4 mM freshly prepared dihydro-PCA, 1 mM DMAPP and 0.5 μg of EpzP. Dihydro-PCA was prepared by using 90 μl of freshly dissolved 50 mM sodium dithionite and 10 μl of 100 mM PCA (in 1 M Tris-HCl pH 8). After incubation of the assay for 4 min at 30°C, 15 μl of 100 mM sodium persulfate were added to oxidize dihydro-PCA to PCA and dihydro-endophenazine A to endophenazine A. The mixture was extracted with 200 μl of ethylacetate : formic acid (40:1) and after centrifugation 175 μl of the organic phase was evaporated. The residue was dissolved in 100 μl of methanol. Ninety microlitres were investigated by HPLC analysis (Eclipse XDB-C18 column, 4.6 × 150 mm, 5 μm, Agilent 1200 series, Waldbronn, Germany) with the same method and liquid phase as described for the analysis of the secondary metabolites. The analysis by LC-MS was the same as for extracts.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Activity of EpzP with different substrates. EpzP prenylated 9,10-dihydrophenazine-1-carboxylic acid (H2PCA), but not phenazine-1-carboxylic acid (PCA), 4-hydroxyphenylpyruvate (4-HPP) or flaviolin. The prenylation of flaviolin with GPP by Fnq26 results in two structurally different prenylated products (Haagen *et al*., 2007). 4-HPP and its prenylated product show two peaks due to keto-enol tautomerism. Detection in HPLC was carried out at 365 nm for PCA and endophenazine A, 308 nm for 4-HPP and its products, and 306 nm for flaviolin and its products. All assays contained 0.4 mM aromatic substrate and 0.4 mM isoprenoid substrate and were incubated for 30 min at 30°C. EpzP assays were performed with 500 mM NaCl and 100 mM TAPS pH 7.5, the assay with CloQ with 2 mM $MgCl_2$ and 75 mM Tris-HCl pH 7.5 and the assay with Fnq26 with 2 mM $MgCl_2$ and 100 mM TAPS pH 8.5 respectively.

Table S1. Influence of $MgCl_2$ and EDTA on the activity of EpzP. Endophenazine A formation was assayed with 0.4 mM H_2PCA, 1 mM DMAPP, 10 μg of EpzP in 100 mM TAPS pH 7.5 (100 μl, 30 min, 30°C).

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