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CONCISE ARTICLE

Activation of a silent phenazine biosynthetic gene cluster reveals a novel natural product and a new resistance mechanism against phenazines†‡

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The activation of silent biosynthetic gene clusters is a principal challenge for genome mining strategies in drug discovery. In the present study, a phenazine biosynthetic gene cluster was discovered in the Gram-positive bacterium *Streptomyces tendae* Tü1028. This gene cluster remained silent under a multitude of cultivation conditions, both in the genuine producer strain and in a heterologous expression strain. However, introduction of a constitutive promoter upstream of the phenazine biosynthesis genes led to the production of phenazine-1-carboxylic acid (PCA) and of a new derivative thereof, *i.e.* a conjugate of PCA and L-glutamine. The linkage of PCA to L-glutamine by amide bond formation was catalyzed by enzymes of the heterologous expression host *Streptomyces coelicolor* M512. PCA showed a strong antibiotic effect, but PCA-Gln did not. Glutamination of PCA therefore appears to represent a resistance mechanism against the antibiotic PCA, which can be produced in significant quantities in soil by *Pseudomonas* strains. The gene cluster also contained genes for all enzymes of the mevalonate pathway and for an aromatic prenyltransferase, thereby resembling gene clusters for prenylated phenazines. However, purification and biochemical investigation of the prenyltransferase proved that it does not prenylate phenazines but hydroxynaphthalene substrates, showing very similar properties as NphB of naphterpin biosynthesis (Kuzuyma *et al.*, *Nature*, 2005, **435**, 983–987.).

Introduction

Secondary metabolites of actinomycetes and fungi, and their chemical derivatives, are the primary source of antibacterial agents for medical use.[1] Between 1981 and 2006, 68% of newly introduced antibacterial agents and 54% of anticancer agents were natural products or semisynthetic derivatives thereof.[2,3] The recently initiated large scale microbial genome sequencing projects have triggered a revolution in the genetics and biochemistry of natural product biosynthesis.[4] They have shown that a single strain, *e.g.* of the actinomycetes, has the genetic capacity to produce 10–30 different secondary metabolites, but usually only 2–3 of them are currently known. This indicates that around 90% of secondary metabolic gene clusters of previously investigated strains are still "cryptic", *i.e.* their products are unknown. The investigation of these "cryptic" gene clusters is likely to allow the discovery of many so far unknown natural products and therefore represents a novel avenue to drug discovery.[2] However, many of these clusters are silent, *i.e.* they are not expressed under the currently used culture conditions. The development of methods for the activation of such silent clusters is one of the most important prerequisites for the successful use of genome mining strategies for drug discovery.[5] So far, there are only few examples for the successful activation of silent gene clusters by genetic manipulation. Activation was achieved by either the overexpression of a positive regulator gene[6,7] or the inactivation of a negative regulatory gene.[8–10] However, the regulation of the biosynthesis of secondary metabolites is complex, encompassing different types of transcriptional regulators, environmental factors and an intricate cascade of intracellular signals to achieve the final regulatory effect. In many cases, it may not be possible to achieve the activation of an entire cluster by simple overexpression or deletion of a certain regulatory gene, and also it may be difficult to identify the genes which are most important for the regulation. Therefore, additional strategies which can be used for the activation of silent gene clusters need to be developed.

We encountered the need for such an activation strategy in the present investigation of a silent phenazine biosynthetic gene cluster from a *Streptomyces* strain.

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Phenazine natural products show antibacterial, antitumor and antimalarial activity. They are inhibitors of angiotensin-converting enzyme and steroid 5-α-reductase, and they act as virulence factors in pathogenesis.[11] Besides their role as antibiotics, phenazines have a variety of biological functions for the producing bacterium, often related to their capability to shuttle electrons by reversible oxidation and reduction.[12] Phenazine biosynthesis is limited to the actinobacteria (*e.g. Streptomyces*), certain groups of Gram-negative proteobacteria (*e.g. Pseudomonas*) and a few archaea.[13] *Pseudomonas* strains produce simple phenazines such as phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN). In contrast, *Streptomyces* strains can form more complex phenazines with very diverse substitution patterns of the phenazine core.

Many phenazine biosynthetic gene clusters have been identified in Gram-negative proteobacteria,[13] but so far only two have been described in *Streptomyces*.[14–16] Both these *Streptomyces* gene clusters show a core of seven biosynthetic genes (*phzBCDEFGA*) responsible for the formation of PCA. Additionally, they show genes involved in the modification of the phenazine core, *e.g.* by prenylation, methylation or oxidation. In the present study, we screened the genomic DNA of 190 strains for phenazine biosynthesis genes. Five strains were found to contain a putative phenazine biosynthetic gene cluster. In one of these strains, *Streptomyces tendae* Tü1028, we found additional genes which suggested the possible formation of a prenylated phenazine. This strain was selected for further investigation. However, this phenazine biosynthetic gene cluster was silent both in the genuine producer strain and in a heterologous producer strain, despite the use of many different culture media. Eventually, introduction of a constitutive *ermE** promoter upstream of the phenazine biosynthesis genes triggered the formation of two phenazine compounds. One of them is a new, previously undescribed phenazine derivative.

Results and discussion

Genomic screening for potential phenazine-producing strains

We screened a collection of 190 *Streptomyces* strains for the presence of phenazine biosynthesis genes. Degenerate primers for the gene *phzB* were developed (see Experimental procedures) and used to screen genomic DNA isolated from all 190 strains. The resulting PCR products were cloned and sequenced. Cosmid libraries of all investigated strains had been established previously using vector pOJ436.[17] From the strains containing a *phzB* ortholog, cosmids containing this gene were identified by Southern hybridization, and the presence of the *phzB* gene was additionally confirmed by PCR. The identified cosmids were re-screened with degenerate primers for the phenazine biosynthesis genes *phzD* and *phzF*.[13] In five of the investigated strains, cosmids were found which contained all three phenazine biosynthesis genes, as also confirmed by sequencing of the PCR products. These five strains were *Streptomyces olivaceus griseolus* Tü2353, *Streptomyces tendae* Tü1028, *Streptomyces* sp. 11412, *Streptomyces* Tü2873 and *Streptomyces* st. 2513/GT. None of these strains had been described previously as a producer of phenazines.

Screening of cosmids for isoprenoid biosynthesis genes

Our group is involved in the investigation of a recently discovered group of prenyltransferases with aromatic substrates, termed ABBA prenyltransferases.[18–20] Two previously identified gene clusters for prenylated phenazines were found to contain genes for ABBA prenyltransferases, as well as a group of genes of the mevalonate pathway required for the generation of the prenyl moiety.[14–16] Therefore, the cosmids identified in the screening described above were further screened for the genes for 3-hydroxy-3-methylglutaryl-CoA synthase (*hmgs*), 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgr*) and mevalonate diphosphate decarboxylase (*mdpd*), respectively. Only cosmids from *S. tendae* Tü1028 showed PCR products for these mevalonate biosynthesis genes, the cosmids from the other four strains did not. Sequencing of the corresponding PCR products from *S. tendae* Tü1028 confirmed the identity of these genes.

Screening of cosmids from *S. tendae* Tü1028 for ABBA prenyltransferase genes

17 cosmids from *S. tendae* Tü1028 had been found to contain *phzB*, *phzD* and *phzF*, and eight of these cosmids additionally contained the genes *hmgs*, *hmgr* and *mdpd*. All 17 cosmids were screened with degenerate primers for bacterial ABBA prenyltransferase (see Experimental procedures). Nine cosmids were found to contain the ABBA prenyltransferase gene, but only a single cosmid, termed CB15, showed the presence of all seven genes used in the present screening approach (*phzB*, *phzD*, *phzF*, *hmgs*, *hmgr*, *mdpd* and an ABBA prenyltransferase gene).

Sequencing of cosmid CB15

Cosmid CB15 from *S. tendae* Tü1028 was subjected to full-length automated sequencing using a shotgun library of DNA fragments. The insert (39 kb) showed an overall G + C content of 70.6% and comprised 31 putative coding sequences (Fig. 1). Table 1 shows the results of database comparisons for these 31 genes. Seven of the putative coding sequences, designated *phzBCDEFGA*, showed obvious similarities to the seven core phenazine biosynthesis genes commonly found in phenazine biosynthesis gene clusters.[13] Of these, *phzC* codes for a DAHP synthase, catalysing the first step of the shikimate pathway. The other six genes code for enzymes which catalyze all steps in the conversion of chorismic acid to 5,10-dihydro-phenazine-1-carboxylic acid, the immediate precursor of phenazine-1-carboxylic acid (PCA).[13] These seven genes in *S. tendae* Tü1028 appear to form a single operon. The intergenic regions between these genes do not exceed 80 bp, and in three instances the adjacent genes show overlapping stop and start codons, suggesting translational coupling. BLAST searches showed very close similarity of *phzBCDEFGA* to the phenazine biosynthetic gene clusters of *Streptomyces anulatus*[14] and *Streptomyces cinnamonensis*.[15,16] The seven core phenazine biosynthesis genes in *S. anulatus* are organized in the same order as in *S. tendae* Tü1028, and the entire operons from the two strains show 82% identity to each other on the nucleotide level, indicating a very close evolutionary relationship.

However, while both *S. anulatus* and *S. cinnamonensis* contain a methyltransferase gene similar to *phzM* of *Pseudomonas* for the

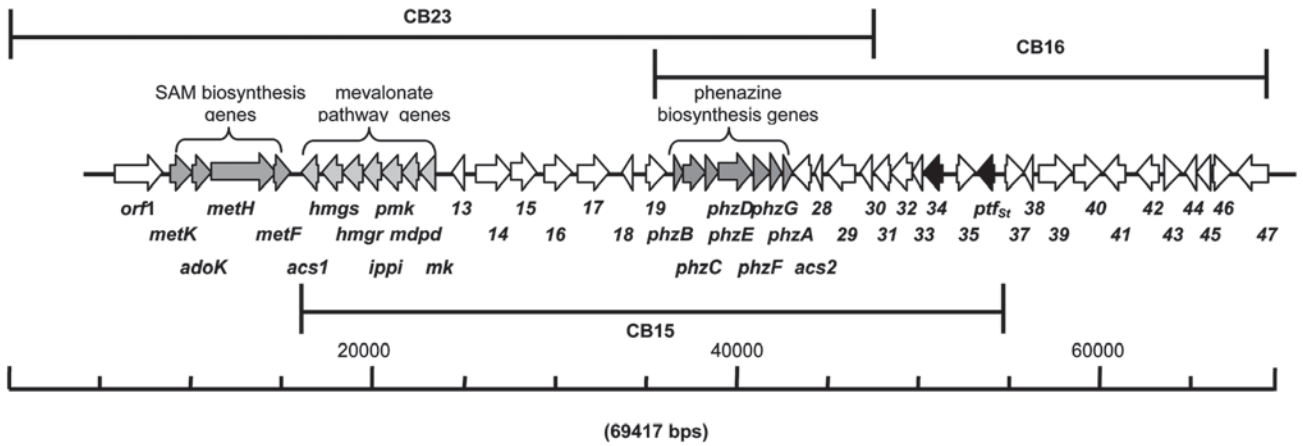


Fig. 1 Secondary metabolic gene cluster identified in the genome of *S. tendae* Tü1028. The relative positions of the inserts of cosmids CB15, CB16 and CB23 are indicated.

formation of *N*-methylated phenazines,[21] no ortholog of this gene is found in *S. tendae* Tü1028, indicating that this strain cannot synthesize *N*-methylated phenazines.

The left end of the insert of cosmid CB15 as depicted in Fig. 1 contained an operon of seven genes of the mevalonate pathway, encoding all enzymes required for the biosynthesis of the isoprenoid precursor dimethylallyldiphoshate (DMAPP). The first enzyme of this pathway has only recently been characterized in streptomycetes.[22] It catalyzes the conversion of acetyl-CoA and malonyl-CoA to acetoacetyl-CoA, a reaction which is different from the first step of the mevalonate pathway in eukaryotes. Again, this operon of mevalonate biosynthesis genes shows very high similarity to the corresponding operons in *S. anulatus* and *S. cinnamonensis*. In all three strains, the genes are organized in the same order, and the entire operons from the three strains show approximately 80% sequence identity to each other on the nucleotide level.

In contrast to *S. anulatus* and *S. cinnamonensis*, *S. tendae* Tü1028 contained an additional copy of the acetoacetyl-CoA synthase gene, termed *acs2* and located adjacent to the phenazine biosynthesis genes. *acs2* shows 100% identity on the nucleotide level to its paralog *acs1* which is located adjacent to the other mevalonate biosynthesis genes.

At the right end of the insert of cosmid CB15 (Fig. 1), a putative ABBA prenyltransferase gene was found and designated *ptf$_{St}$*. Its predicted product (305 aa) showed similarity to the phenazine prenyltransferase genes *ppzP* from *S. anulatus* and *epzP* from *S. cinnamonensis*, and to prenyltransferases involved in the biosynthesis of prenylated naphthalene derivatives such as NphB from *Streptomyces* sp. CL190 [23] or Fnq26 from *S. cinnamonensis*.[24]

Furthermore, the cluster contained a gene coding for a putative polyprenyl diphosphate synthase (*orf19*) and a putative type III polyketide synthase gene (*orf34*). Similar genes have been found in gene clusters for prenylated naphthalenes like naphterpin and furanonaphthoquinone I.[15,23]

Cultivation of *S. tendae* Tü1028 and analysis for phenazines and prenylated secondary metabolite

To investigate the production of secondary metabolites, *S. tendae* Tü1028 was cultivated in the medium described for the production of prenylated phenazines and prenylated polyketides in *S. cinnamonensis*[25] for 5, 7 and 9 days. However, HPLC-UV analysis of the culture supernatant and of mycelia extracts did not show any products with the typical UV absorption of phenazines. The strain was then cultivated in the media described for production of the phenazines aestivophoenin,[26] phenazoviridin,[27] saphenamycin[28] and griseolutein.[29] Finally, the strain was cultivated in 9 other media previously used for the production of secondary metabolites by *Streptomyces* strains (see ESI‡), but no phenazines could be detected. Likewise, no product with a similar UV absorption as naphterpin[30] or furanonaphthoquinone I [15] was observed. HPLC-MS was used to search for masses of known phenazines, prenylated phenazines and prenylated polyketides, but without success. Following a suggestion by Seto *et al.*,[56] we cultivated the strain in the presence and absence of the HMG-CoA reductase inhibitor pravastatin (3–14 mM) and compared the chromatograms in order to detect products formed under involvement of the mevalonate pathway, but again without success. The identified gene cluster apparently remained silent under all investigated conditions.

Heterologous expression of cosmid CB15 from *S. tendae* in *S. coelicolor* M512

In a previous study,[14] the heterologous expression of cosmid 18A9 from *S. anulatus* which comprised similar genes as CB15 from *S. tendae* Tü1028 had resulted in the production of both PCA and its prenylated derivative endophenazine A. Therefore, we decided to heterologously express cosmid CB15.

Cosmid CB15 carries an *oriT* for conjugal transfer into *Streptomyces* and the ΦC31 integration functions for integration into the host genome.[17] CB15 was introduced into *S. coelicolor* M512 by conjugation. Three independent exconjugants were selected and cultivated in the production media used for phenazine production by *S. anulatus*.[14] Extracts of the culture supernatant and of the mycelia were investigated for PCA and for PCA derivatives by HPLC-UV and HPLC-MS. However, we could not detect any phenazine formation. The same heterologous expression experiment was carried out with seven other cosmids from *S. tendae* Tü1028, containing phenazine biosynthesis genes, but again without success. This indicated that the

Table 1 Genes identified on cosmids CB15, CB16 and CB23 from *S. tendae* Tü1028. The overlap between the three cosmid inserts is depicted Fig. 1. The nucleotide sequence has been deposited in GenBank under accession number JQ659263

Orf	aa	Proposed function	Ortholog identified by BLAST search	Identity/ similarity%	Accession
Cosmid CB23					
orf1	880	Putative peptide synthase	*Streptomyces rochei*	46/56	NP_851498
metK	402	Putative *S*-adenosylmethionine synthetase	*Streptomyces pristinaespiralis* ATCC 25486	90/95	ZP_06913987
adoK	327	Putative adenosine kinase	*Streptomyces pristinaespiralis* ATCC 25486	74/81	ZP_06913988
metH	1167	Putative methionine synthase	*Streptomyces pristinaespiralis* ATCC 25486	86/92	ZP_06913989
metF	301	Putative 5,10-methylenetetrahydrofolate reductase	*Streptomyces aureofaciens*	85/91	ADM72814
Cosmid CB15					
acs1	325	Acetoacetyl-CoA synthase	PpzT, *Streptomyces anulatus*	74/82	CAX48662
hmgs	389	3-Hydroxy-3-methylglutaryl CoA synthase	HMGS, *Streptomyces* sp. CL190	83/90	BAB07795
hmgr	353	3-Hydroxy-3-methylglutaryl coenzyme A reductase	HMGR, *Streptomyces* sp. CL190	91/95	BAA70975
ippi	364	Isopentenyl diphosphate isomerase	*Streptomyces* sp. CL190	84/93	Q9KWG2
pmk	365	Phosphomevalonate kinase	PMEVK, *Kitasatospora griseola*	71/82	BAB07819
mdpd	299	Mevalonate diphosphate decarboxylase	MDPD, *Streptomyces cinnamonensis*	83/89	ADQ43374
mk	263	Mevalonate kinase	NapT6, *Streptomyces* sp. CNQ525	77/86	ABS50475
orf13	226	Conserved hypothetical protein, Ovm-Z like	NapU1 *Streptomyces* sp. CNQ525	65/76	ABS50476
orf14	666	Putative nitrogen regulatory protein	*Streptomyces ambofaciens*	71/79	CAK50991
orf15	480	Putative 3-carboxymuconate cycloisomerase	*Streptomyces ambofaciens*	77/83	CAK50990
orf16	516	Putative monooxygenase	Fnq24, *Streptomyces cinnamonensis*	63/74	CAL34102
orf17	576	Putative cytochrome B subunit	Fnq25, *Streptomyces cinnamonensis*	74/84	CAL34103
orf18	214	ECF subfamily RNA polymerase sigma factor	*Geodermatophilus obscurus* DSM 43160	43/54	YP_003409857
orf19	359	Polyprenyl diphosphate synthase	*Rhodococcus erythropolis* PR4	58/71	BAH34278
phzB	162	Putative enzyme of phenazine biosynthesis	*Streptomyces anulatus*	80/91	CAX48672
phzC	395	3-Deoxy-D-arabino-heptulosonic acid 7-phosphate synthase	*Streptomyces anulatus*	80/90	CAX48671
phzD	207	2,3-Dihydro-3-hydroxy-anthranilate (DHHA) synthase	*Streptomyces cinnamonensis*	88/92	CAL34109
phzE	640	2-Amino-2-desoxy-isochorismate synthase	*Streptomyces anulatus*	81/86	CAX48669
phzF	279	*trans*-2,3-Dihydro-3-hydroxyanthranilate isomerase	*Streptomyces anulatus*	87/91	CAX48668
phzG	233	FMN-dependent oxidase	*Streptomyces anulatus*	68/78	CAX48667
phzA	155	Putative enzyme of phenazine biosynthesis	*Streptomyces anulatus*	83/90	CAX48666
acs2	325	Acetoacetyl-CoA synthase	PpzT, *Streptomyces anulatus*	74/82	CAX48662
orf28	153	Putative transcriptional regulator	Fnq17, *Streptomyces cinnamonensis*	52/68	CAL34095
orf29	527	Putative fatty acid CoA-ligase	NapB4 *Streptomyces* sp. CNQ525	81/88	ABS50481
orf30	207	Putative WrbA NAD(P)H:quinone oxidoreductase	Fnq10, *Streptomyces cinnamonensis*	70/80	CAL34088
orf31	332	Putative methyltransferase	*Streptomyces* sp. KO-3988	73/85	BAE78972
orf32	385	Hypothetical protein	*Streptomyces* sp. KO-3988	79/87	BAE78971
orf33	188	Putative MomA-like oxygenase	*Streptomyces antibioticus*	86/90	BAD89290
orf34	355	Putative type III polyketide synthase	RppA, *Streptomyces antibioticus*	94/97	BAB91443
orf35	332	Sigma factor, includes region 2	*Rhodococcus jostii* RHA1	51/63	YP_700910
ptf_{St}	305	ABBA prenyltransferase	*Streptomyces* sp. CL190	83/90	BAE00106
Cosmid CB16					
orf37	322	Putative transcriptional regulator	*Thermomonospora curvata* DSM 43183	50/65	YP_003299085
orf38	148	Hypothetical protein	*Streptomyces hygroscopicus* ATCC 53653	88/92	ZP_07295901
orf39	590	Putative FAD-binding monooxygenase	*Salinispora tropica* CNB-440	60/75	YP_001159014
orf40	503	Putative drug resistance transporter	*Catenulispora acidiphila* DSM 44928	53/70	YP_003111195
orf41	424	Putative bilirubin oxidase	*Streptomyces pristinaespiralis* ATCC 25486	61/73	ZP_06914024
orf42	399	Hypothetical protein	*Anaeromyxobacter dehalogenans* 2CP-C	33/49	YP_463241
orf43	277	Hypothetical protein	NapU4, *Streptomyces aculeolatus*	53/67	ABS50471
orf44	198	Putative oxidoreductase	*Streptomyces avermitilis* MA-4680	90/94	NP_821545
orf45	272	Putative myo-inositol 1-monophosphatase	*Streptomyces avermitilis* MA-4680	93/96	YP_003485917
orf46	306	Putative LysR family transcriptional regulator	*Streptomyces avermitilis* MA-4680	93/98	NP_821547
orf47	594	Putative ABC transporter permease	*Streptosporangium roseum* DSM 43021	51/65	YP_003338959

phenazine biosynthetic gene cluster was not expressed in the heterologous host.

Introduction of a constitutive promoter upstream of the phenazine biosynthesis genes

Sequence analysis of the phenazine biosynthesis genes in cosmid CB15 gave no indication for any mutation which would impair the catalytic activity of the encoded enzymes. We therefore speculated that these genes were not expressed. The regulatory mechanisms of phenazine biosynthesis in *Pseudomonas* are quite complicated, as *e.g.* shown for the biosynthesis of phenazine-1-carboxamide in *P. chlororaphis* PCL 1391,[31] and the regulatory mechanisms of phenazine biosynthesis in *Streptomyces* have not been investigated at all. This made it difficult to activate the gene cluster by the use of genuine regulatory genes of the pathway. Therefore, we decided to introduce the strong constitutive promoter *ermE** [32] upstream of the operon of phenazine biosynthesis genes into cosmid CB15, using RED/ET-mediated recombination. In previous experiments for gene replacement by this method, we had used gene cassettes containing an *oriT* for conjugal transfer of the resulting constructs.[33,34] Since the cosmid CB15 already contains *oriT*, we constructed a new cassette without *oriT* in order to avoid an undesired recombination with the cosmid backbone (see Experimental procedures). This new cassette contained the *ermE** promoter as well as the hygromycin resistance gene (under the

control of the *lac* promoter) as a selective marker (Fig. 2A). RED/ET-mediated recombination was used to introduce this cassette into cosmid CB15 (Fig. 2A). The recombinants were selected with hygromycin B and verified by restriction analysis. The resulting construct CB15–ermE* was then introduced into the genome of *S. coelicolor* M512 using biparental conjugation. Seven exconjugants were selected with hygromycin B and apramycin, and cultivated in a medium used previously for the heterologous production of phenazines.[14] Extracts of the culture supernatant were analyzed by HPLC. This readily showed the production of two compounds with the typical absorption spectrum of phenazines (Fig. 2B), indicating that the activation of the silent gene cluster by introduction of the *ermE** promoter upstream of the *phzBCDEFGA* operon had been successful.

Isolation of a new phenazine derivative formed by heterologous expression of the activated phenazine biosynthetic gene cluster

One of the two compounds formed after heterologous expression of the phenazine cluster (Fig. 2B) showed a molecular ion at *m*/*z* 225 ([M + H]$^+$) and was readily identified as phenazine-1-carboxylic acid in comparison to an authentic reference substance. The other compound showed a molecular ion at *m*/*z* 353 ([M + H]$^+$). Positive ion mode high resolution mass spectrometry showed an exact mass of 353.1243310 Dalton, indicating a molecular formula of $C_{18}H_{16}N_4O_4$ (calculated mass 352.1244315 Dalton, Δ 0.28 ppm), different from any phenazine derivative described previously.

To identify the structure of the new product, the heterologous expression strain *S. coelicolor* M512 carrying cosmid CB15–ermE* was cultivated in a 10 litre fermenter. From the culture medium, the compound was isolated using XAD-16 resin and purified by chromatography on Sephadex LH-20 and by preparative reversed phase HPLC. 35 mg of a red solid compound was obtained and 5 mg were investigated by unidimensional (^{1}H and ^{13}C) and multidimensional (^{1}H-^{1}H COSY, HSQC and HMBC) NMR spectroscopy, in comparison to PCA. This showed that the new compound represented a conjugate of phenazine-1-carboxylic acid, attached *via* an amide bond to the amino group of glutamine (Fig. 2B). The ^{1}H and ^{13}C NMR data of the compound are summarized in Table 2, and the ^{1}H-^{1}H-COSY, HSQC and HMBC correlations are depicted in Fig. S1 (ESI‡). It will be termed α-*N*-(phenazine-1-carbonyl)-glutamine (PCA-Gln) hereafter.

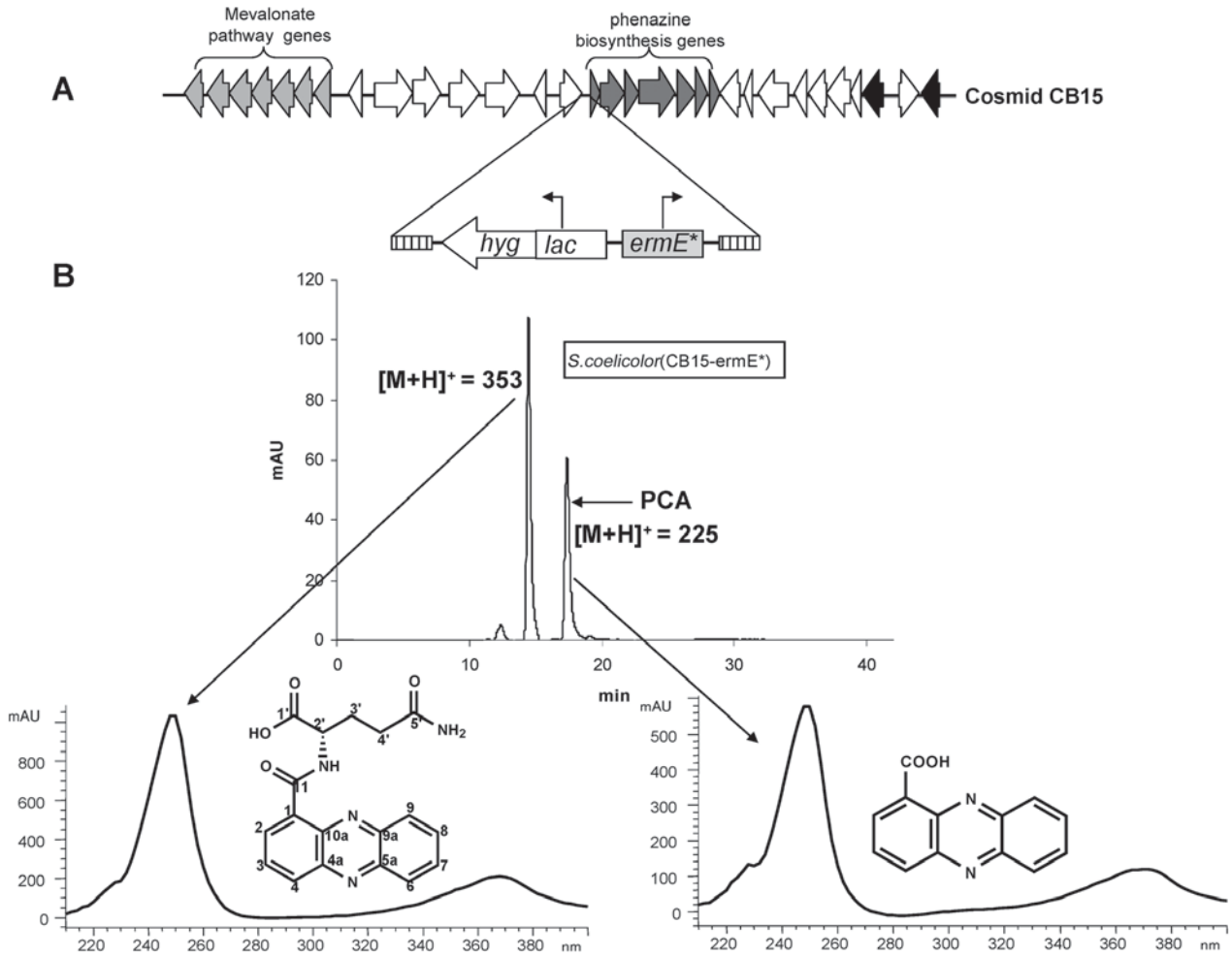


Fig. 2 (A) Introduction of the constitutive *ermE** promoter into cosmid CB15, resulting in cosmid CB15–ermE*. (B) HPLC analysis of the culture supernatant of the heterologous expression strain *S. coelicolor* (CB15–ermE*); detection: 365 nm. Below the chromatogram, the UV spectra of the two main products are shown.

Table 2 ^{1}H and ^{13}C NMR spectroscopic data of α-*N*-(phenazine-1-carbonyl)-L-glutamine. Chemical shifts are expressed in δ values using the solvent as an internal standard (400 MHz, d_6-DMSO). Assignments were made using 2D NMR data, shown in Fig. S1.‡ Numbering of the structure is also given in Fig. 2

Position	^{13}C NMR (100.6 MHz, d_6-DMSO) δ_C [ppm]	^{1}H NMR (400 MHz, d_6-DMSO) δ_H [ppm]
1	129.0	
2	134.5	8.73, 1H, (dd, 7.1, 1.5)
3	130.4	8.10, 1H, (dd, 8.8, 7.1)
4	133.4	8.46, 1H, (dd, 8.8, 1.5)
4a	142.6	
5a	142.9	
6	129.4	8.36, 1H, (ddd, 7.8, 1.7, 0.6)
7	131.8	8.06, 1H, (ddd, 7.8, 6.7, 1.6)
8	132.4	8.10, 1H, (ddd, 8.4, 6.7, 1.7)
9	129.3	8.32, 1H, (ddd, 8.4, 1.6, 0.6)
9a	141.0	
10a	140.1	
11	163.7	
1′	173.3	
2′	52.6	4.69, 1H, (dddd, 7.2, 7.2, 2.2, 2.2)
2′–NH		11.18, 1H, (d, 7.2)
3′	27.5	2.08–2.20, $1H_a$, m 2.20–2.39, $1H_b$, m
4′	31.2	2.20–2.39, 2H, m
5′	173.3	
5′–NH_2		6.78, $1H_a$, s 7.34, $1H_b$, s

Identification of the stereochemical configuration of α-*N*-(phenazine-1-carbonyl)-glutamine

The stereochemical configuration of the amino acid glutamine in the structure of PCA-Gln was determined by enantioselective HPLC analysis.[35] We therefore synthesized α-*N*-(phenazine-1-carbonyl)-L-glutamine and α-*N*-(phenazine-1-carbonyl)-D-glutamine (see Experimental procedures). The isolated PCA-Gln and the two synthesized compounds were analyzed by HPLC using two complementary chiral columns which contained as chiral selectors either quinine (QN) or quinidine (QD) derivatives. As expected, on the QN column the L-Gln derivative was more retained ($R_t = 14.6$ min) in comparison to the D-Gln derivative ($R_t = 6.9$ min). The isolated PCA-Gln showed the same retention time as the synthesized L-Gln derivative. Using the QD column, the D-Gln derivative was more retained in comparison to the L-derivative ($R_t = 14.4$ min and $R_t = 6.3$ min, respectively). Again, the isolated PCA-Gln product showed the same retention time as the L-Gln derivative. These results prove that in the structure of the isolated PCA-Gln, glutamine has the L-configuration.

The conjugation of phenazine-1-carboxylic acid to L-glutamine is catalyzed by enzymes of the expression host *Streptomyces coelicolor* M512

Except for phenazine-1-carboxamide in *Pseudomonas* and sendomycin A and B from *Streptomyces endus* subsp. *aureus* DO-59,[11] very few amides of PCA have been described in nature. Mechanistically, the conjugation of PCA to glutamine requires an activation of the carboxyl group of PCA, *e.g.* in the form of a coenzyme A ester or of an acyl adenylate, similar to the biosynthesis of phenazine-1-carboxamide under catalysis of PhzH in *Pseudomonas*.[21] Surprisingly, no candidate gene for such an amide synthetase was found in the insert of cosmid CB15. The only adenylate-forming enzyme encoded in CB15 is Orf29, a putative fatty acid CoA ligase. However, this gene shows high similarity to genes found in the biosynthetic gene clusters of the prenylated polyketides napyradiomycin[36] and furaquinocin[37] which are unrelated to phenazines. In order to test whether the conjugation of PCA to glutamine may be carried out by enzymes of the expression host *S. coelicolor* M512 rather than by enzymes encoded in cosmid CB15, we added PCA (final concentration 0.1 mM) to cultures of the expression host *S. coelicolor* M512 (not containing the CB15 cosmid). After 3 days of cultivation, the culture supernatant was extracted and analyzed by HPLC. In two parallel control experiments, PCA was added to cultures of *S. tendae* Tü1028 or to sterile culture medium. The result was strikingly clear (Fig. 3): PCA remained unchanged in the sterile medium and in cultures of *S. tendae*, but was nearly quantitatively (97%) converted to PCA-Gln in the cultures of *S. coelicolor* M512. This conversion is therefore carried out by enzymes encoded in the genome of *S. coelicolor* M512.

Enzymes which can catalyze the transfer of an acyl moiety to the α-amino group of glutamic acid are *e.g.* FolC of folate biosynthesis[38] or MurD of murein biosynthesis.[39] Orthologs of these genes are encoded by the genes *sco2614* and *sco2086* of *S. coelicolor*. These or similar enzymes may be responsible for the observed conjugation of PCA to glutamate.

The conjugation of phenazine-1-carboxylic acid to L-glutamine is likely to represent a resistance mechanism

Phenazines possess antibiotic activity, owing to the fact that they can reduce molecular oxygen to toxic, highly reactive oxygen species.[12] *Pseudomonas aeroginosa*, a producer of phenazines, protects itself from this toxic effect of phenazines by production of superoxide dismutases and catalase.[40] *Enterobacter agglomerans* forms a phenazine binding protein which facilitates the export of the toxic molecule.[41] The resistance mechanisms of phenazine-producing actinobacteria have not been examined. In order to investigate whether the conjugation of PCA to glutamate may offer a mechanism to detoxify PCA, and/or to facilitate its export, we compared the antibiotic activity of PCA and of PCA-Gln against *E. coli*, *Bacillus subtilis* and *Streptomyces coelicolor* M512 in disk diffusion assays. Both compounds showed no effect on *E. coli* in the tested concentrations.

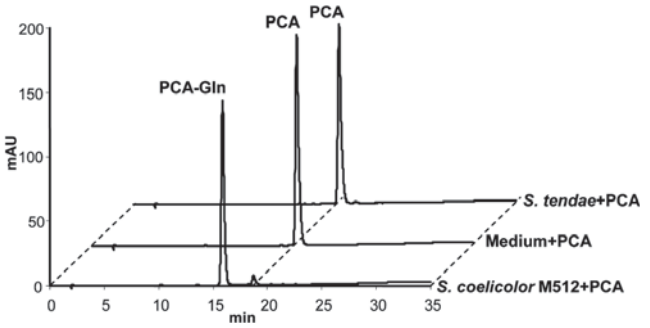


Fig. 3 HPLC analysis of the feeding of phenazine-1-carboxylic acid (PCA) to cultures of *Streptomyces coelicolor* M512, to cultures of *S. tendae*, or to sterile culture medium. Only *S. coelicolor* M512 converted PCA to α-*N*-(phenazine-1-carbonyl)-L-glutamine (PCA-Gln).

However, PCA had a strong antibiotic effect against *Bacillus subtilis*, while PCA-Gln had not (Fig. 4a). As shown above, *Streptomyces coelicolor* M512 rapidly conjugates PCA to L-glutamine, and correspondingly this strain was resistant to both PCA and PCA-Gln (Fig. 4b). These results suggest that glutamination of PCA may represent a resistance mechanism. Notably, in soil which is the natural habitat of *Streptomyces* strains PCA is produced by *Pseudomonas* strains and can reach growth-inhibitory concentrations.[42] Therefore, a resistance mechanism against PCA may offer a competitive advantage to a *Streptomyces* strain in nature.

To our knowledge, this is the first report of the conjugation of PCA to glutamine or to any other amino acid *via* an amide bond. However, the conjugation of the phenazine–dicarboxylic acid derivative SB 212021 to *N*-acetyl-cysteine has been described.[43] In that case, conjugation occurred *via* the thiol group of cysteine and also resulted in the loss of the (weak) antibacterial activity of the phenazine. A similar *N*-acetyl-cysteine adduct has been described for a polyketide antibiotic, also leading to a loss of biological activity.[44]

Biochemical investigation of the prenyltransferase Ptf_{St}

Cosmid CB15 from *S. tendae* contained phenazine biosynthesis genes, mevalonate pathway genes and a gene for an aromatic prenyltransferase, ptf_{St}. However, in the present study we could not detect any prenylated phenazines, in contrast to a previous study on a gene cluster from *S. anulatus* which contained similar genes.[14] We therefore speculated that the prenyltransferase gene ptf_{St} may not code for a phenazine prenyltransferase such as PpzP or EpzP,[14,16] but for a hydroxynaphthalene prenyltransferase such as NphB or Fnq26.[23,24] Phenazine and hydroxynaphthalene prenyltransferases are similar in their amino acid sequence, but different in their specificity for the aromatic substrate.

We therefore expressed Ptf_{St} in *E. coli* as a His-tagged protein and purified it by Ni^{2+} affinity chromatography (see Experimental procedures). In contrast to PpzP and EpzP, Ptf_{St} did not show product formation using 5,10-dihydro-PCA and either DMAPP or GPP as substrates. In contrast, prenylated products were readily obtained when Ptf_{St} was incubated with GPP and different phenolic substrates including 1,6-dihydroxynaphthalene (1,6-DHN), 2,7-dihydroxynaphthalene (2,7-DHN) and the flavonoids apigenin, genistein or naringenin (Table 3). DMAPP was not accepted as an isoprenoid substrate. The K_m values of Ptf_{St} for 1,6-DHN and genistein were determined to be 0.36 and 0.16 mM, respectively. Using 1,6-DHN as an aromatic substrate, the K_m for GPP was determined as 0.13 mM. In contrast to most other prenyltransferases of the ABBA superfamily,[20,45] Ptf_{St} requires the presence of Mg^{2+} ions for its catalytic activity. These biochemical characteristics (substrate and product specificity, K_m values and magnesium dependency) are very similar to those of NphB, which is involved in the biosynthesis of the prenylated naphthalene derivative naphterpin.[23,46] This indicates that Ptf_{St} may be involved in the biosynthesis of a prenylated naphthalene derivative, most likely in the prenylation of a polyketide formed from 1,3,5,8-tetrahydroxynaphthalene (THN), the presumed product of the type III polyketide synthase Orf34 (Table 1).[47] A mixed biosynthetic gene cluster for prenylated THN derivatives and phenazines was also found in *S. cinnamonensis*, and many of the genes found in the

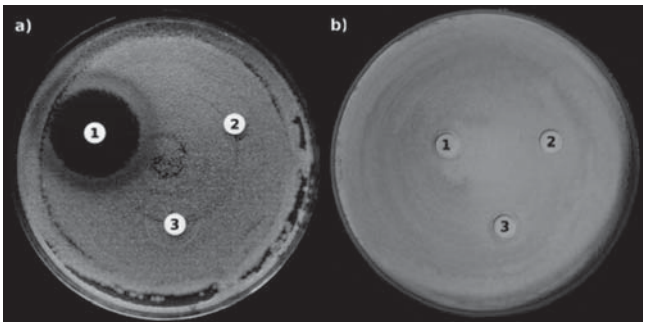
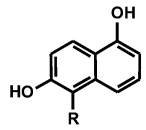
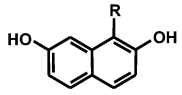
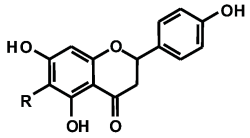
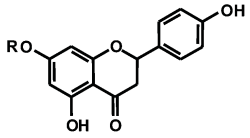
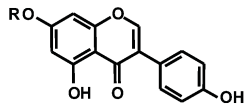
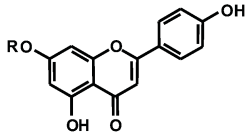
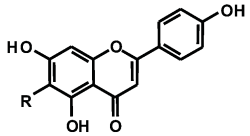


Fig. 4 Antibiotic activity of PCA and of PCA-Gln against (a) *Bacillus subtilis* and (b) *Streptomyces coelicolor* M512. To the paper disks, either 0.5 μmol PCA (1) or 0.5 μmol PCA-Gln (2) or solvent (3) were applied. In parallel experiments, no growth inhibition on *Streptomyces coelicolor* M512 was exerted by PCA or PCA-Gln in amounts of up to 5 μmol (data not shown).

Table 3 Prenylation of phenolic compounds under catalysis of Ptf_{St}. GPP (1 mM) was used as an isoprenoid substrate. DHN = dihydroxynaphthalene

Substrate	Product	R =	k_{cat} (10^{-3} s^{-1})
1,6-DHN	5-Geranyl-1,6-DHN		5.96
2,7-DHN	1-Geranyl-2,7-DHN		4.96
Naringenin	6-Geranyl-naringenin		0.35
	7-*O*-Geranyl-naringenin		0.29
Genistein	7-*O*-Geranyl-genistein		0.42
Apigenin	7-*O*-Geranyl-apigenin		0.13
	6-Geranyl-apigenin		0.07

presently described gene cluster of *S. tendae* Tü1028 have orthologs in the cluster of *S. cinnamonensis*. In contrast to the cluster in *S. cinnamonensis*, however, the cluster in *S. tendae* Tü1028 remained silent under many different culture conditions and the structure of the possibly encoded prenylated naphthalene remains unknown.

Sequence analysis of the border regions of the identified gene cluster

In order to check for the presence of further genes for the biosynthesis of prenylated naphthalenes and/or phenazines, we sequenced two cosmids overlapping with the insert of cosmid CB15 (Fig. 1). This revealed on the right end of the cluster (Fig. 1 and Table 1) genes coding for putative regulators and redox enzymes, an ABC transporter gene as well as a gene (*orf43*) with similarity to the hypothetical protein NapU4 from the napyradiomycin cluster.[36] On the left end of the cluster, we found four genes likely to be involved in the recycling of *S*-adenosylhomocysteine (SAH) to *S*-adenosylmethionine (SAM), *i.e. metK*, *adoK*, *metH* and *metF*. The function of these genes may be the supply of SAM for methylation reactions within the biosynthetic pathway, but may also include a role in the regulation of secondary metabolism.[48] Notably, in the silent cluster in *S. tendae* Tü1028, one of the genes required for the recycling of SAH to SAM, *i.e.* the *S*-adenosylhomocysteinase gene *sahH*, was missing, in contrast to the active clusters found in *S. cinnamonensis* and two other organisms.[48] The genes further upstream of *metK* showed similarity to primary metabolic genes, suggesting that this gene may mark the border of the identified secondary metabolic gene cluster.

Conclusions

The phenazine biosynthetic gene cluster of *S. tendae* Tü1028 was successfully activated by introduction of the constitutive, strong *ermE** promoter upstream of the *phzBCDEFGA* operon. The resulting heterologous expression strain produced 15 mg l^{-1} of phenazine derivatives, showing that the 6.5 kb operon was efficiently transcribed from this promoter. In drug discovery by genome mining, the activation of the silent clusters will present one of the principal challenges. Our study shows that the introduction of a constitutive promoter in front of biosynthetic gene operons may provide a useful tool to meet this challenge. Tetracycline-inducible versions of the *ermE** promoter have been developed,[49] and may allow the controlled expression of secondary metabolic gene clusters.[50]

Heterologous expression of biosynthetic gene clusters in host strains which are completely sequenced, easily cultivatable and amenable for genetic manipulation is another important tool in genomic mining[51] and was also used in the present study. Unexpectedly, we found that the expression host enzymatically modified the compound formed under direction of the heterologously introduced genes: phenazine-1-carboxylic acid, formed under catalysis of *phzBCDEFGA*, was conjugated to the α-amino group of glutamine in the form of an amide. This compound is a new, previously undescribed phenazine derivative, and its formation is likely to represent a resistance mechanism against the antibiotic effect of phenazine-1-carboxylic acid.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The 190 *Streptomyces* strains including *S. tendae* Tü1028 were obtained from strain collection of Combinature Biopharm AG (now Merlion Pharmaceuticals GmbH). Strains were grown in liquid YMG medium (4 g of Bacto yeast extract, 10 g of malt extract, 4 g of glucose-monohydrate per l; pH 7.3) or on solid MS medium. For production of phenazine secondary metabolites, the medium described by Sedmera *et al.*[57] was used.

Escherichia coli XL1 Blue MRF, *E. coli* SURE (Stratagene, Heidelberg, Germany), *E. coli* BW 25113, and *E. coli* ET12567 (pUB307) were used for cloning and were grown in liquid or on solid (1.5% agar) Luria–Bertani or SOB medium at 37 °C. The REDIRECT technology kit for PCR targeting was obtained from Plant Bioscience Limited (Norwich, UK). Carbenicillin (50–100 μg ml^{-1}), apramycin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), chloramphenicol (25 μg ml^{-1}), nalidixic acid (20 μg ml^{-1}) and hygromycin B (40–100 μg ml^{-1}) were used for selection of recombinant strains.

Chemicals

Dimethylallyl diphosphate (DMAPP) and geranyl diphosphate (GPP) were synthesized according to Woodside *et al.*[52] Kanamycin, carbenicillin and hygromycin were purchased from Genaxxon BioSciences GmbH (Biberach, Germany) and phenazine-1-carboxylic acid was from InFarmatik. IPTG, Tris, NaCl, glycerol, dithiothreitol, $MgCl_2$, formic acid, sodium dodecyl sulfate, polyacrylamide, and EDTA were from Carl Roth, Karlsruhe, Germany. Apramycin, nalidixic acid, 1,6-dihydroxynaphthalene (1,6-DHN), methanol, Tween 20, imidazole, *N*-*N*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide were from Sigma Aldrich, Steinheim, Germany. 2,7-Dihydroxynaphthalene (2,7-DHN) was from AcrosOrganics. Merck supplied chloramphenicol, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium carbonate, sodium hydrogen carbonate and β-mercaptoethanol. Lysozyme was from Boehringer Ingelheim, Heidelberg, Germany.

Genetic procedures

Standard methods for DNA isolation and manipulation were performed as described by Kieser *et al.*[53] and Sambrook and Russell[54] DNA fragments were isolated from agarose gels by using a PCR purification kit (Amersham Biosciences). The genomic DNA of the 190 streptomycetes strains was isolated with the NucleoSpin® 96 Tissue Core Kit from Macherey-Nagel, Düren, Germany.

Construction and screening of the cosmid library

Cosmid libraries from all investigated strains had been established prior to the present study, using the vector pOJ436.[17] The genomic DNA of the 190 *Streptomyces* strains was initially screened for the gene *phzB* using the following degenerate primers: *phzB_For_1*: 5′-CT(G/C)TGGAC(G/C)AC(G/C)GA(C/T)AC(G/C)GG-3′ and *phzB_R_1*: 5′-GAA(G/C)(G/C)(A/T)(A/G)TG(G/C)AGGAA(A/G)TG(A/G)TT-3′. The resulting PCR

products were verified by sequencing. The amplified PCR product was used as a probe for screening of the cosmid libraries by Southern hybridization. The positive cosmids were screened by PCR screening for the presence of *phzD*, *phzF*, *hmgr* (3-hydroxy-3-methyl-glutaryl-CoA reductase), *hmgs* (3-hydroxy-3-methyl-glutaryl-CoA synthase), *mdpd* (mevalonate diphosphate decarboxylase) and for a putative ABBA prenyltransferase gene.[20] The following primers were used: *PhzD_for* (5′-CGC GCC GTC CTG (A/G)TN CA(C/T) GA(C/T) (A/C/T)T-3′) and *phzD_rev* (5′-CGG TGG TGG TCC CGG (G/C)(A/T)(A/G) AA(A/G) TCN (G/C)-3′); *phzF_for* (5′-CAT CCG GAT CTT GAC CCC NGT NAA (C/T)GA-3′) and *phzF_rev* (5′-GAG GGG CGC CCC AT(C/T) TCN CAN CC-3′); *HMGR_for* (5′-GGG CAT CGC CGC GAC CCT CGT GGA GGA GGG-3′) and *HMGR_rev* (5′-GCG ATG ACG GGG AGG CGC CGG GCG TTC TC-3′); *HMGS_for* (5′-GCC AAG TCC GCC GGN GTN TA(C/T) GT-3′) and *HMGS_rev* (5′-AGC CGG AAG GGG CCN GTN GT(C/T) TG-3′); *MDPD_for* (5′-GAC CCT GGA CGT CTT CCC NAC NAC NAC-3′) *MDPD_rev* (5′-GCG TTC CGC TCG GC(A/G/T) AT(C/T) TCN-3′), *PT_for* 5′-A(G/C)GT(G/C) CCGCT(G/C)GCCAC(G/C)TACGAG-3′ and *PT_rev* 5′-C(G/C)GC(G/C)AG(G/C)CG(G/C)CGGGCGTTCT-3′. Only one cosmid, CB15 from *S. tendae* Tü1028, was found to contain all the investigated phenazine, mevalonate and prenyltransferase genes.

Heterologous expression of cosmid CB15

Cosmid CB15 was first transformed into the nonmethylating host *E. coli* ET12567 containing plasmid pUZ8002, and the nonmethylated DNA was introduced into *Streptomyces coelicolor* M512 *via* biparental conjugation.[53]

Construction of the cassette for the activation of the silent phenazine biosynthesis gene cluster

The hygromycin resistance gene together with the *lac* promoter was amplified from plasmid pIJ797 with the following primers: *pLac_kpn_F*: GAC TCA CTA TAG GGC GAA TTG GGTACC GGG CCC CCC CTC GAG GTCGA and *Plac_kpn_R*: GTA ACA TCA AGG CCC GAT CCT T GGTACC CTT GCC CTC CCG CAC GAT GAT CG. The introduced KpnI restriction sites are underlined.

The PCR product was then digested with KpnI and ligated into the KpnI site of pUWL201. The resulting plasmids were transformed into *E. coli* XL1 blue and selected with carbenicillin and hygromycin.

Restriction analysis was used to identify a plasmid which contained the *ermE** and the *lac* promoters in opposite transcriptional directions. This plasmid was used as a template to amplify the cassette with two long primers with homology to the intergenic region upstream of the phenazine biosynthesis gene *phzB: Plac_4_R*: GCC GCG AAA ACC CGT GAC GAC CGT GCG GCC GGG TCC GGA TCG ATA AGC TTG ATT GTA GG and *ErmE_4_R*: CTC TGC GTT CTC GGA AGG CGT GTT CTC GGA AGG CGT GTT GCT TCT AGA ACT AGT GGA TC.

λ-RED recombination was used to introduce the resulting cassette into cosmid CB15 resulting in cosmid CB15–ermE*. Cosmid CB15–ermE* was then transformed into *E. coli* ET12567/pUZ8002 and conjugated with *S. coelicolor* M512. The exconjugants were selected with hygromycin B and apramycin. Seven exconjugants were selected for cultivation.

Production and analysis of secondary metabolites

The exconjugants as well as wild type *S. tendae* Tü1028 were precultured for 48 h in liquid YMG medium (50 ml). 50 ml of production medium was then inoculated with 2.5 ml of the precultures. The flasks were agitated on a rotary shaker at 30 °C and 200 rpm for 120 h.

For isolation of secondary metabolites, cultures (50 ml) were centrifuged at 3500 × *g* for 10 min, and supernatant and mycelia were analysed separately. Mycelia were extracted with methanol (10 ml) by vortexing. 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 redissolved in methanol (0.5 ml).

The supernatant was adjusted to pH 4.0 using 1 M HCl, and extracted with the same volume of ethylacetate. The organic phase was evaporated and the residue was redissolved in methanol.

Extracts were analyzed by 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 40 to 100% of solvent B in 20 min (solvent A: water–formic acid (999 : 1); solvent B, methanol) and detection at 252 and 365 nm. Additionally, UV spectra were acquired from 200 to 400 nm by a photodiode array detector. The absorbance at 365 nm was used for quantitative analysis, employing an authentic reference sample of PCA as an external standard.

Analysis by LC-MS

The extracts were examined with LC-MS and LC-MS^2 analyses using a Nucleosil 100-C18 column (2 × 100 mm, 3 μm) coupled to an ESI mass spectrometer (LC/MSD Ultra Trap System XCT 6330; Agilent Technology). Analysis was carried out at a flow rate of 0.4 ml min^{-1} with a linear gradient from 10 to 100% of solvent B in 15 min (solvent A: water–formic acid (999 : 1); solvent B: acetonitrile–formic acid (999.4 : 0.6)). Detection was carried out at 230, 260, 280, 360, and 435 nm. Electrospray ionization (positive and negative ionization) in Ultra Scan mode with a capillary voltage of 3.5 kV and a drying gas temperature of 350 °C was used for LC-MS analysis. For LC-MS^2 and LC-MS^3, the analysis was carried out in positive ionization mode with a capillary voltage of 3.5 kV at 350 °C.

Preparative isolation of PCA-Gln

The strain *S. coelicolor* (CB15–ermE*) was precultured in 500 ml of production medium for 48 h at 27 °C. This culture was inoculated into a 10 litre fermenter containing the same production medium and grown at 27 °C for 96 h. The cultures were then filtrated under vacuum using 3% celite. The mycelia were discarded and the culture filtrate was adjusted to pH 7.0 and applied to XDA-16 macro-porous adsorptive resins chromatographic column and eluated with 70% methanol. After concentration, the aqueous solution was extracted with dichloromethane and the separated organic phase was discarded. The aqueous solution

was adjusted to pH 3.0 and extracted three times with ethyl acetate. The ethyl acetate phase was evaporated and the residue was redissolved in methanol and fractioned using a liquid chromatography system with a Sephadex LH20 column (2.5 × 90 cm) and methanol as the mobile phase. The fractions containing PCA-Gln as the main product were pooled and the solvents were evaporated. The residue was redissolved in dimethylsulfoxide (DMSO) and applied to a preparative HPLC system with a Reprosil Basic C18 column (250 × 20 mm). The separation was carried out with a linear gradient from 60 to 70% of solvent B in 15 min (solvent A: water–formic acid (999 : 1); solvent B: methanol) and the fractions containing pure PCA-Gln were pooled and dried using lyophilisation. 35 mg of pure PCA-Gln could be extracted.

Stereochemical identification of *N*-(phenazine-1-carbonyl)-L-glutamine

For the synthesis of the reference substances, 9.9 mg of PCA were suspended in 400 µl of acetonitrile, added to 49.9 mg of *N*,*N*-dicyclohexylcarbodiimide dissolved in 300 µl of acetonitrile and incubated at 60 °C. After 60 min, 10.5 mg of *N*-hydroxysuccinimide dissolved in 300 µl of acetonitrile were added to the reaction mixture and incubated for further 24 h. The solution was then filled up with acetonitrile to 1 ml and the reaction mixture was divided into three tubes, each containing 300 µl. We added 1 mg of D-glutamine, dissolved in 200 µl of carbonate buffer (0.1 M $NaHCO_3$/0.1 M Na_2CO_3; 2 : 1, (v/v)) to the first tube; 1 mg of L-glutamine dissolved in the same buffer to the second tube and the third tube was mixed with 200 µl of the carbonate buffer. The tubes were incubated for 8 days at 25 °C. 20 µl of each reaction solution were diluted with 180 µl of methanol, and 20 µl were injected into an enantioselective HPLC system (LaChrom) with a DAD-detector and Chiralpak QN-AX or QD-AX columns (5 µm, 150 × 4 mm ID, Chiral Technologies Europe, Illkirch, France). We used a mixture of methanol–acetic acid–ammonium acetate (98 : 2 : 0.5; v/v/w) as the mobile phase with an isocratic flow rate of 1 ml min^{-1}. The detection was carried out at 210 nm, 250 nm, 280 nm and 340 nm.

Feeding experiments with PCA

For feeding experiments we used deep-well plates.[55] 2 × 3 ml production medium was inoculated with *S. coelicolor* spores and incubated at 30 °C on a rotary shaker at 250 rpm. After 48 h, PCA was added to the medium to a final concentration of 0.1 mM. As negative control, PCA was added in the same concentration to 2 × 3 ml sterile medium in the same deep-well plate. Another control was done by feeding PCA to two *S. tendae* Tü1028 cultures under the same conditions. 100 µl from each culture were extracted and analyzed by HPLC as described above.

Antibiotic activity of PCA and of PCA-Gln

Antibacterial activity of PCA and PCA-Gln was tested using *E. coli* K12, *Bacillus subtilis* ATCC 14893 and *Streptomyces coelicolor* M512. For the bioassays, 0.5 µmol of the respective substance (as potassium salt) in 10 µl of 0.1 M Tris HCl pH 8.0 were applied to filter paper disks (6 mm diameter). In tests against *E. coli* K12 or *Bacillus subtilis*, the filter disks were placed on LB nutrient agar inoculated with either *E. coli* or *Bacillus subtilis*. After culturing overnight at 37 °C, the diameter of the growth inhibition zone was determined. In tests against *Streptomyces coelicolor* M512, the filter disks were placed on MS nutrient agar[53] inoculated with a spore suspension of the test strain. After culturing for 48 hours at 30 °C, the diameter of the growth inhibition zone was determined.

Expression and purification of Ptf_{St}

For the construction of the expression plasmid pET28a-OS02, ptf_{St} was amplified with Phusion® DNA polymerase (Finnzymes, Woburn, MA) using the cosmid CB15 as a template. The following primers were used: *F*: GTC AGA ACC AAC GCA TG CATATG TCA ATG TCC GGA GCC GCT GAT G and *R*: GCT CTG CTG CGG CGGCTCGAG TCA GTC CTC CAG CGC GTC G. The underlined letters represent NdeI and XhoI restriction sites, respectively. The resulting PCR fragment was digested with NdeI and XhoI and ligated into plasmid pET28a digested with the same restriction enzymes. The resulting plasmid pET28a-OS02 was verified by restriction analysis and sequencing.

The plasmid pET28a-OS02 was transformed into *E. coli* BL21 (DE3) pLysS (Promega) and a pre-culture of 100 ml of liquid LB medium was cultured overnight at 37 °C and 200 rpm. 35 ml of the pre-culture were inoculated into 1 l of TB medium 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 6 h of cultivation, the cells were harvested by centrifugation for 10 min at 2700 × *g* at 4 °C. The cells (17 g from 1 l of culture) were resuspended in 43 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). 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). The protein was eluted using imidazole buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 250 mM imidazole). The buffer was changed using PD-10 desalting columns (GE Healthcare Life Sciences), equilibrated with 50 mM Tris pH 8, 10% (v/v) glycerol, 2 mM DTT. 60 mg of His_6–Ptf_{St} could be purified from 1 litre of culture.

Assay for prenyltransferase activity

The reaction mixture (200 µl) contained 50 mM Tris–HCl pH 9.0, 2.5 mM aromatic substrate, 1 mM DMAPP or GPP, 5 mM magnesium chloride and 0.3 nmol of Ptf_{St}. After incubation of the assay for 15 min at 30 °C (for 1,6-DHN and 2,7-DHN) or 45 min (for apigenin, genistein and naringenin), the reaction was stopped by adding 200 µl of ethylacetate : formic acid (39 : 1). After vortexing and centrifugation, 160 µl of the organic phase was evaporated. The residue was dissolved in 50 µl of methanol. 20 µl were analyzed by HPLC-UV and HPLC-MS. HPLC-UV analysis was carried out as described above, with detection at

260 nm for genistein, 290 nm for naringenin, 339 nm for apigenin and 268 nm for 1,3-DHN, 1,6-DHN and 2,7-DHN. LC-MS analysis was carried out as described above, with negative ionization.

For determination of the K_m values for 1,6-DHN and genistein, GPP was kept at a constant concentration of 1 mM. For determination of the K_m value for GPP, 1,6-DHN was kept at 2.5 mM.

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