DOI: 10.1002/cbic.200700549

Heterologous Expression And Genetic Engineering of the Phenalinolactone Biosynthetic Gene Cluster by Using Red/ET Recombineering

Tina M. Binz,[a] Silke C. Wenzel,[a] Hans-Jörg Schnell,[b] Andreas Bechthold,[b] and Rolf Müller*[a]

The heterologous expression of natural product biosynthetic pathways is of increasing interest in biotechnology and drug discovery. This approach enables the production of complex metabolites in more amenable host organisms and provides the basis for the generation of novel analogues through genetic engineering. Here we describe a straightforward strategy for the heterologous expression of the highly complex phenalinolactone biosynthetic pathway, which was recently cloned from Streptomyces *sp. Tü6071. The biosynthetic gene cluster comprises at least 11 transcriptional units that harbor 35 genes, which together catalyze the assembly of structurally unique tricyclic terpene glycosides with antibacterial activity. By using Red/ET recombineering, the phenalinolactone pathway was reconstituted from two cosmids and heterologously expressed in several* Streptomyces *strains. The established expression system now provides a convenient platform for functional investigations of the biosynthetic genes and the generation of novel analogues, by genetic engineering of the pathway in* Escherichia coli*. Deletion of a modifying gene from the expression construct resulted in a novel, unglycosylated phenalinolactone derivative; this demonstrates the promise of this methodology.*

Introduction

Members of the genus *Streptomyces* are soil-dwelling bacteria, and are the most prolific producers of antibiotics yet discovered.[1,2] More than two-thirds of all natural-product anti-infectives and other secondary metabolites in clinical use are produced by *Streptomyces*, which underlines their importance in human medicine.[3] Over the last decade, many of the corresponding biosynthesis gene clusters have been cloned and sequenced, which enables a detailed analysis of the molecular principles that govern the pathways. Manipulation of these gene sets represents a promising tool both to obtain deeper insights into the biosynthesis, and to generate novel derivatives of these compounds for evaluation as drug leads.[4] This approach can be facilitated significantly by the expression of entire gene clusters in suitable heterologous hosts, particularly if the genetic manipulation of the producer strain is difficult. The strategies that are used for heterologous expression have recently been reviewed.[5] To date, heterologous expression of secondary metabolic pathways from *Streptomyces* has mainly been achieved in related actinomycete species. In most cases, the gene clusters were relatively small (less than 30 kbp[5]), and are located on a single cosmid or bacterial artificial chromosome (BAC) within a genomic library.[6–9] Expression of larger and more complex pathways remains more demanding, but can be facilitated by constructing several plasmids that harbor subsets of the biosynthetic genes.[10,11]

The phenalinolactones (PLs) are structurally intriguing tricyclic terpene glycosides that are produced by *Streptomyces* sp. Tü6071, which exhibit promising antibacterial activity. In addition to a highly oxidized γ-butyrolactone ring, which is most likely derived from pyruvate, the oxidatively functionalized terpenoid backbone is decorated with a 5-methylpyrrole-2-carboxylic acid as well as the rare deoxyhexose, 4-*O*-methyl-L-amicetose. The recent cloning and sequence analysis of the *pla* biosynthetic gene cluster from *Streptomyces* sp. Tü6071, revealed a highly complex genetic architecture that consists of 35 orfs that are organized into 11 putative operons.[12] Here, we have used the Red/ET recombineering technology (λ-mediated recombination)[13–15] to reconstitute the *pla* biosynthetic gene cluster from two cosmids. The complete gene cluster was then successfully expressed in three *Streptomyces* strains. This advance enables both functional studies of the pathway as well as attempts to generate novel analogues, by genetic engineering of the gene cluster in *Escherichia coli*. As proof-of-principle, we deleted a modifying gene (the glycosyl transferase that is encoded from *plaA6*) from the expression construct to generate a novel unglycosylated phenalinolactone derivative.

[a] *Dipl.-Biol. T. M. Binz, Dr. S. C. Wenzel, Prof. Dr. R. Müller*
Universität des Saarlandes, Institut für Pharmazeutische Biotechnologie
66123 Saarbrücken (Germany)
Fax: (+49) 681-302-5473
E-mail: rom@mx.uni-saarland.de

[b] *Apotheke H.-J. Schnell, Prof. Dr. A. Bechthold*
Albert-Ludwigs-Universität Freiburg
Institut für Pharmazeutische Wissenschaften,
Pharmazeutische Biologie und Biotechnologie
Stefan-Meier-Strasse 19, 79104 Freiburg (Germany)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

Results

Reconstitution of the complete phenalinolactone biosynthetic gene cluster

We employed Red/ET recombineering in *E. coli*[13–15] to rebuild the entire *pla* biosynthetic pathway on an integrative *E. coli*–*Streptomyces* shuttle vector (pOJ436[16]), and to introduce additional genetic elements for heterologous expression (Figure 1). Two pOJ436-derived cosmids (Cos3-1O12 and Cos10-4D08[12]) that contain overlapping regions of the *pla* gene cluster were used as starting points. First, Cos10-4D08 was modified by insertion of a gene cassette upstream of the *pla* gene cluster to generate construct CPhl9. In addition to a *Sca*I restriction site

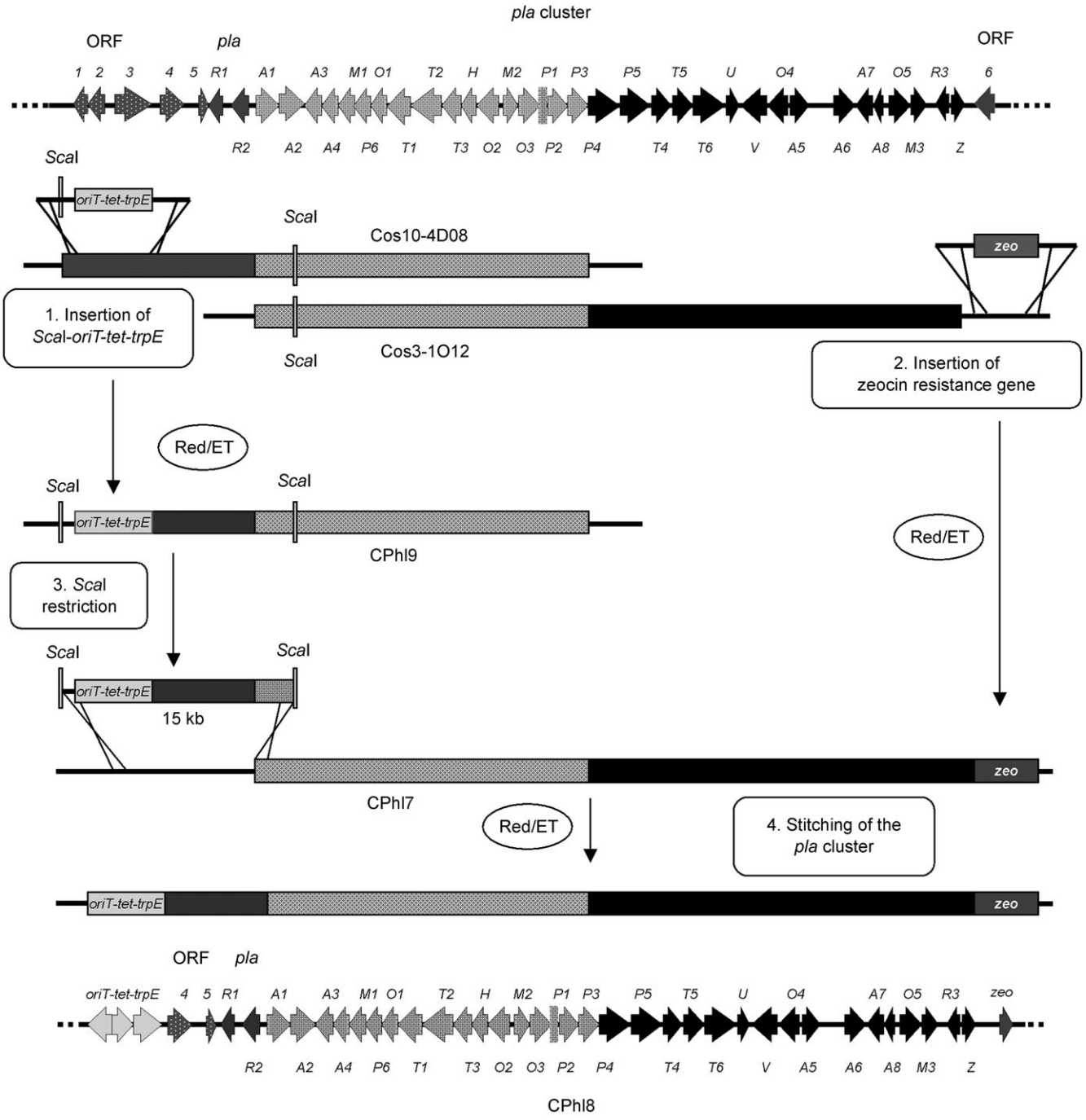

Figure 1. Description of the cloning strategy. The phenalinolactone biosynthetic gene cluster consists of 35 orfs, and was located on two overlapping cosmids (Cos3-1O12 and Cos10-4D08) that carry the pOJ436 backbone. In the first step, a *ScaI-oriT-tet-trpE* cassette was inserted via Red/ET recombineering into Cos10-4D08 at the upstream end of the gene cluster to generate the construct CPhl9. In the next step, introduction of the zeocin-resistance gene at the downstream end of the cluster led to the construct CPhl7. After restriction digest of CPhl9 with *Sca*I, the resulting 15 kb fragment was used to reassemble the whole cluster by Red/ET recombineering. The final expression construct, which contains the whole cluster was designated CPhl8.

and a tetracycline (*tet*)-resistance gene, the introduced cassette also contained an *oriT* as well as a portion of the *trpE* gene from *Pseudomonas putida*, which should enable the transfer of the expression construct into pseudomonads in the future. This step also resulted in the deletion of orfs 1–3, which are not involved in PL biosynthesis (Figure 1). In the second recombineering step to create construct CPhl7, a zeocin-resistance gene, was inserted into Cos3-1O12 at the opposite end of the gene cluster (downstream of *plaZ*); this resulted in the deletion of *orf6*. Finally, CPhl9 was digested with *Sca*I (a *Sca*I restriction site is also present in the overlapping region of the cosmids) to generate a linear 15 kb fragment, which was then recombined with CPhl7. The resulting construct, CPhl8, contains the complete *pla* biosynthetic pathway within the pOJ436 vector backbone. To confirm the individual cloning steps, each of the constructs that were generated by recombination was digested with a diagnostic set of restriction enzymes.

Heterologous expression of the phenalinolactone biosynthetic gene cluster in *Streptomyces*

Construct CPhl8, which harbors the complete *pla* biosynthetic gene cluster was introduced into the host organisms *Streptomyces lividans* TK24,[17] *S. coelicolor* A3(2)[18] and *S. coelicolor* M512[19] by conjugation. Because the pOJ436 vector contains the ϕC31 integrase gene, integration was expected to occur at a chromosomal *attB* site. Transconjugants that harbor the expression construct were identified by growth on SM agar plates that contained apramycin. The presence of the cosmid CPhl8 in the resulting strains was verified by PCR from genomic DNA by using oligonucleotides that are specific for the amplification of a 777 bp fragment from the apramycin-resistance gene. The recombinant strains *S. lividans*/CPhl8, *S. coelicolor* A3(2)/CPhl8 and *S. coelicolor* M512/CPhl8, which harbor the phenalinolactone pathway were then cultivated in production medium together with the corresponding wild-type strains, as well as with the natural phenalinolactone producer *Streptomyces* sp. Tü6071. Extracts from these cultures were analyzed by HPLC–MS and the obtained data were compared to MS/MS fingerprints of authentic phenalinolactone (PL) standards.

Phenalinolactones A and D were detected in the extracts of the natural producer *Streptomyces* sp. Tü6071 and in extracts of the recombinant *S. lividans* and *S. coelicolor* strains that harbor the *pla* gene cluster, but not in the extracts of the corresponding wild-type *S. lividans* and *S. coelicolor* strains. This result clearly demonstrates the successful heterologous production of the terpene glycosides (Figure 2). Production under standard conditions at 28 °C was much lower in the heterologous host strains than in the native host *Streptomyces* sp. Tü6071 (Table 1). In an attempt to optimize production in the heterologous hosts, different cultivation conditions (additional media and temperatures) were tested. Phenalinolactone production in *S. lividans* was successfully increased 100-fold after cultivation at 37 °C (compared to 28 °C), although PL production in the *S. coelicolor* mutants was completely abolished at the higher temperature (37 °C).

We also attempted to improve production by supplementation with a putative biosynthetic precursor. Because the core terpenoid backbone in the phenalinolactone biosynthesis is derived from isoprenoid building blocks, we administered mevalonolactone to the culture medium. Mevalonolactone is the lactonized form of mevalonate, a direct precursor in the mevalonate pathway for the construction of isoprenoid units.[20] Most *Streptomyces* strains employ the non-mevalonate pathway for the formation of isoprenoids, but some strains addi-

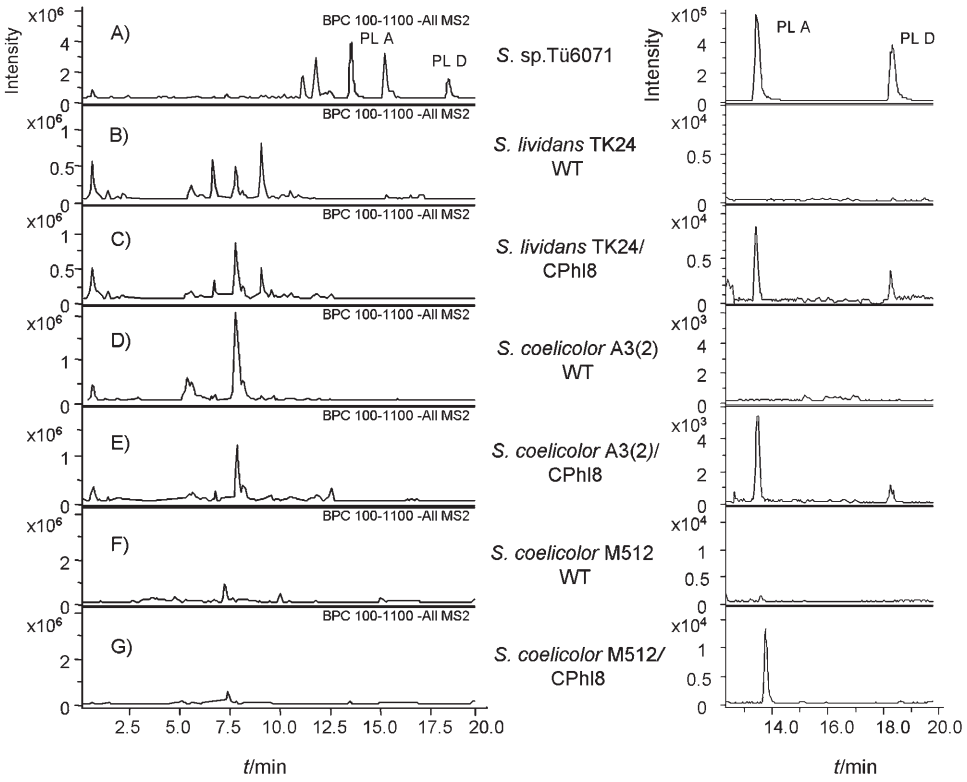

Figure 2. HPLC–MS analysis (Base peak chromatograms (BPCs) *m/z* 100–1100 -All MS^2) of A) Natural producer strain *Streptomyces* sp. Tü6071, B) *S. lividans* TK24 WT C) *S. lividans* TK24/CPhl8, D) *S. coelicolor* A3(2) WT, E) *S. coelicolor* A(3)2/CPhl8, F) *S. coelicolor* M512 WT and G) *S. coelicolor* M512/CPhl8. The chromatograms show the negative ions within a mass range of 100–1100 in auto MS^2 mode. CID fragmentation between retention times of 12–14 min and 18–20 min was monitored for the masses 714.5 and 698.3, which correspond to the phenalinolactone A and D standards. The section in each chromatogram between 12–20 min is expanded on the right. PL A and/or PL D were detected in the extracts of the recombinant strains.

Table 1. Phenalinolactone production by *Streptomyces* sp. Tü6071, *S. coelicolor* and *S. lividans*.

Strain	Production of phenalinolactone [μg L^{-1}]	
	28 °C	37 °C
Streptomyces sp. Tü6071	500	200
S. lividans TK24/CPhl8	0.13	10
S. coelicolor A3(2)/CPhl8	0.01	–
S. coelicolor M512/CPhl8	0.5	–

tionally use the mevalonate pathway to produce terpenoid antibiotics.[20] Previous experiments clearly demonstrated that the isoprene building blocks for phenalinolactone biosynthesis are derived from the nonmevalonate pathway.[12] This was corroborated by a feeding experiment that used 1 mM mevalonolactone, which neither increased phenalinolactone production in *Streptomyces* sp. Tü6071 nor in the heterologous host strains.

Genetic modification of the *pla* biosynthetic gene cluster

The heterologous expression system described here provides the basis for engineering the PL biosynthetic pathway to generate novel analogues. Based on the Red/ET recombineering technique,[14] single or multiple in-frame deletions at any desired position within the expression construct can be performed. Therefore, we aimed to modify the *pla* gene cluster by deletion of the glycosyl-transferase-encoding gene *plaA6* from the CPhl8 expression construct, to generate a phenalinolactone derivative that lacks the 4-*O*-methyl-L-amicetose moiety. For this, a kanamycin (*km*)-resistance cassette was amplified by PCR to incorporate flanking flippase (FLP) recombinase target sites (FRT sites), as well as 50 bp homology arms to enable double homologous recombination (Figure 3). After replacement of the target gene (*plaA6*) against the selection marker (*km*) by Red/ET recombineering, the *km* gene was excised from the expression construct by FLP-recombinase-catalyzed site-specific recombination to create a markerless in-frame deletion. The modified *pla* gene cluster was subsequently transformed into three heterologous host strains by conjugation, and integration of the construct (CPhl18) into the chromosome was confirmed by PCR analysis as described above.

The production profile of the mutant strains was analyzed by HPLC–MS, and compared to that of the wild-type strains, and the natural phenalinolactone producer *Streptomyces* sp. Tü6071. As expected, phenalinolactones A and D (*m/z* 714.5 [*M*−H]$^-$ at retention time 13.8 min, and *m/z* 698.3 [*M*−H]$^-$ at 18.1 min, respectively) were detected in the extract of the natural producer, but not in extracts of the heterologous hosts. Instead, a new compound (*m/z* 570.3 [*M*−H]$^-$ at 13.0 min), which was designated as PL E, was detected in the mutant *S. coelicolor* M512/CPhl18; it differs from PL D by 128 atomic mass units, which is consistent with the anticipated loss of the 4-*O*-methyl-L-amicetose moiety (Figure 4). This compound could not be detected in the *S. lividans* TK24/CPhl18 and *S. coelicolor* A3(2)/CPhl18 mutants. High-resolution LC-coupled Fourier transform-Orbitrap MS was performed to verify the elemental composition of $C_{31}H_{40}O_9N_1$ for PL E (*m/z*: calcd: 570.27086; found: 570.26733 [*M*−H]$^-$; deviation: 3.52166 mDa). Because the production yield of the new derivative (about 1 μg L)$^{-1}$ was

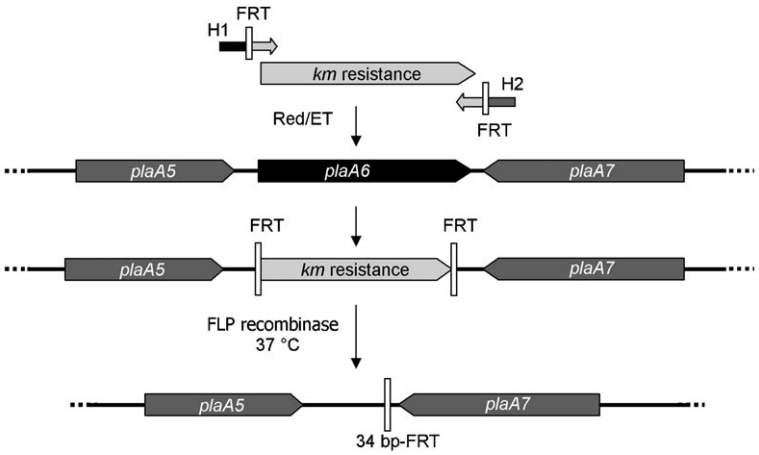

Figure 3. Deletion of the glycosyl-transferase-encoding gene *plaA6* by using a kanamycin-resistance cassette that was flanked by FRT recognition sites. Red/ET recombineering was used to replace *plaA6* with the kanamycin-resistance cassette. Incubation with FLP recombinase at 37 °C led to a markerless deletion of *plaA6* in the *pla* cluster (construct CPhl18).

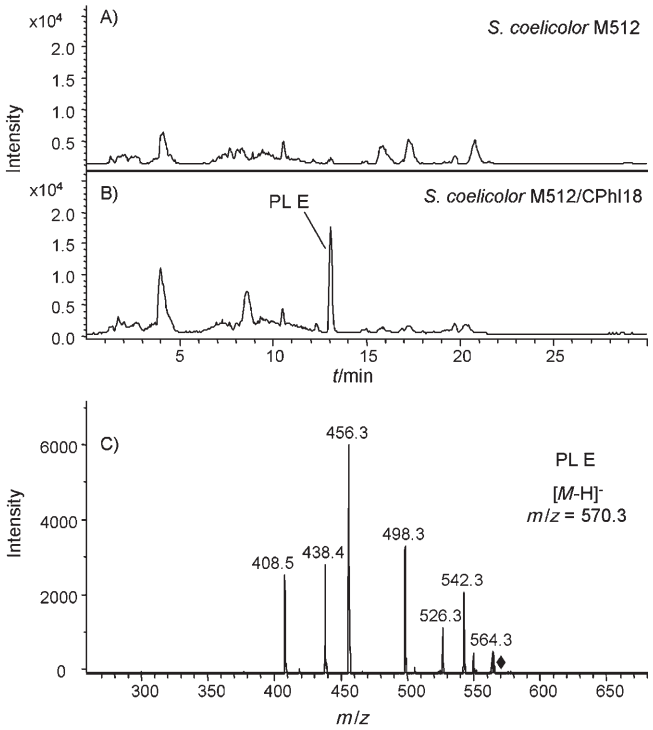

Figure 4. HPLC–MS analysis of A) parental strain *S. coelicolor* M512 and B) *S. coelicolor* M512/CPhl18 (*plaA6* deletion). A new compound, designated PL E, was detected in the extract of *S. coelicolor* M512/CPhl18 at a retention time of 13.0 min. C) Fragmentation pattern of PL E (molecular ion, *m/z* 570.3 [*M*−H]$^-$).

too low for NMR studies, additional structural information was obtained by using tandem-MS and subsequent comparison of the MS^2 fingerprints with those that were obtained for the glycosylated compound PL D. The principal collision-induced dissociation (CID) fragmentations for the standard substance PL D and the new derivative PL E, were assigned as summarized in (Table S2). Fragmentation of the terpenoid backbone as well as of the sugar and the pyrrole's carboxylic acid groups was not observed in the MS^2 spectra of PL D; this indicates that despite the loss of the sugar group, the fragmentation pattern of phenalinolactones D and E should be very similar. Indeed, comparison of the fragmentation pattern of both molecules showed that they exhibited the same set of peaks, but were offset by a constant mass difference that corresponds to 4-*O*-methyl-L-amicetose (Table S2 and Figure 5). These peaks were derived from the parent molecules by expulsion of CO, CO_2 and H_2O, although the precise locations of these losses remain to be elucidated. The expulsion of $C_4H_2O_4$, which occurred from both compounds can be attributed to the γ-butyrolactone. These data strongly suggest that PL E is the desired unglycosylated derivative of PL D.

Discussion

Heterologous expression of complex natural product biosynthetic gene clusters in amenable host strains has become an important tool in natural products research and drug discovery. This strategy provides an alternative to (over)producing structurally complex substances that would be difficult or impossible to access by other means, and can enable the generation of novel analogues by combinatorial biosynthesis approaches. The successful heterologous expression of a number of *Streptomyces* clusters has been reported, in which the pathways have typically been reconstituted in related actinomycete strains.[6–9,21,22] Because most of these gene clusters were relatively small (< 30 kbp), it was often possible to retrieve the entire gene set from a single cosmid within a genomic library of the natural producer strain.[5] In cases where the libraries were prepared in *E. coli–Streptomyces* shuttle vectors (for example, the medermycin and griseorhodin A biosynthetic gene clusters[6,23]), the transfer of the pathways into heterologous strains was straightforward.

However, many natural product biosynthetic gene clusters are larger than the average insert size of common cosmid vectors. One strategy for overcoming this size limitation is to use BAC shuttle vectors for library construction because they can accommodate inserts in excess of 100 kbp. This approach enabled the successful expression of the 128 kbp daptomycin biosynthetic gene cluster from *Streptomyces roseosporus* in a related *Streptomyces* strain.[24] Another option is the cloning of subsets of the biosynthetic gene cluster into compatible expression plasmids followed by their stepwise introduction and coexpression in suitable host strains.[10,11] Because the cloning procedure allows the introduction of artificial promoter regions, heterologous production can be performed even in non-related host organisms. By using this strategy, natural products from *Streptomyces* were produced in *E. coli*, and myxobacterial compounds were obtained from *Streptomyces* host strains.[25–27]

An alternative to these classical, time-consuming cloning and mutagenesis approaches is the reassembly of large natural product pathways on a single

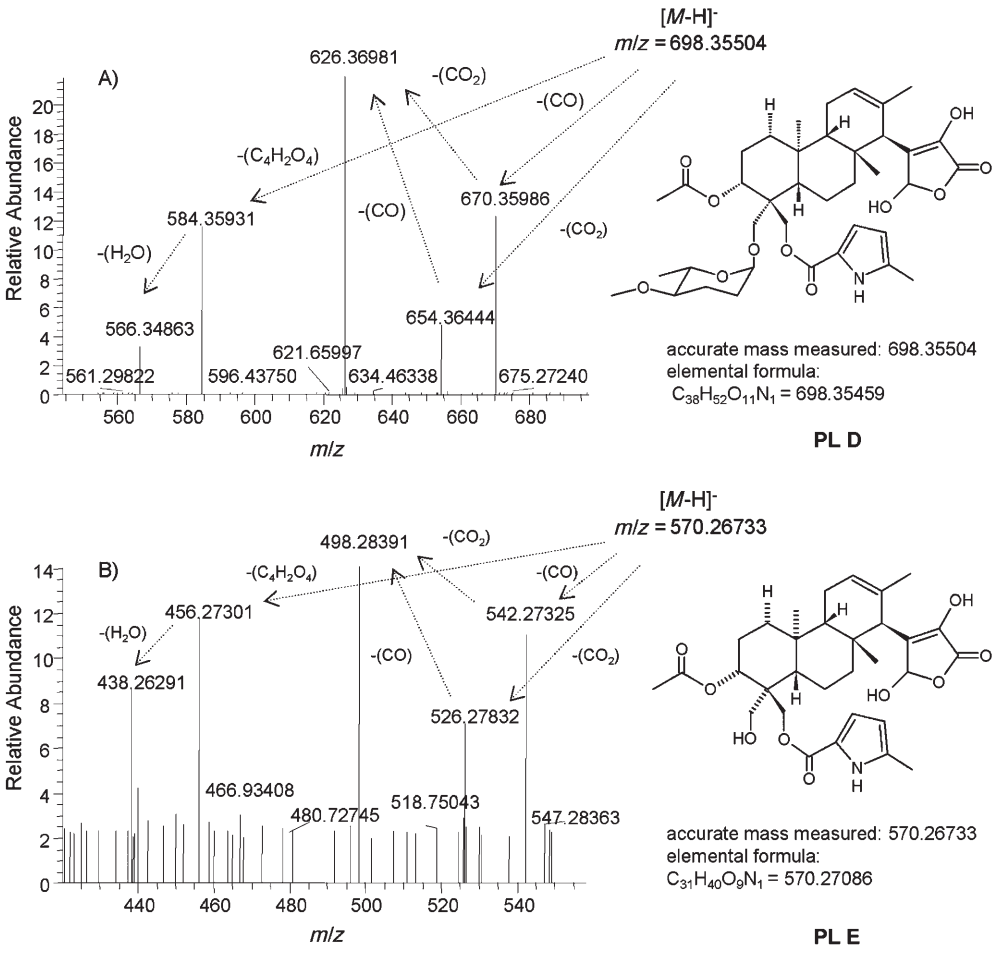

Figure 5. Verification of the unglycosylated derivative PL E. Accurate mass determination of A) the standard substance PL D and B) the unglycosylated compound PL E with high-resolution LC-coupled Orbitrap-MS yielded molecular ions of *m/z* 698.35504 $[M-H]^-$ and *m/z* 570.26733 $[M-H]^-$, respectively; these are suggestive of the elemental formulas $C_{38}H_{52}O_{11}N_1$ and $C_{31}H_{40}O_9N_1$, respectively. Additional support for structure assignment was provided by MS^2 studies, which showed that PL D and PL E have the same fragmentation patterns, but with the peaks offset by a constant mass, which corresponds to the 4-*O*-methyl-L-amicetose moiety that is missing in PL E.

transferable vector system, by using Red/ET technology. This recombination approach was developed by the Stewart group in 1998,[14] and is ideal for manipulating large pieces of DNA because it does not depend on the use of restriction enzymes. Phage-derived protein pairs (RecE/RecT from the Rac prophage or Redα/Redβ from the lambda phage) are employed to precisely alter target DNA molecules by homologous recombination within *E. coli* strains.[13] For the heterologous expression of biosynthetic pathways in *Streptomyces* strains, Red/ET cloning has been used to modify the backbone of cosmids that contain complete biosynthetic pathways; this enables the heterologous expression of the aminocoumarin antibiotics novobiocin and clorobiocin.[28] In these examples, all of the genes were oriented in the same direction, which is consistent with the presence of a single transcriptional unit. In comparison to the aminocoumarin pathways, the phenalinolactone gene cluster of *Streptomyces* sp. Tü6071 is more complex; it consists of 35 ORFs which appear to be organized into 11 individual transcriptional units.[12] This highly divergent architecture would seem to present a significant challenge to the regulatory apparatus of heterologous host strains. Thus, in attempting to achieve heterologous expression of the phenalinolactone pathway, we developed a Red/ET-based methodology to reconstitute the entire gene cluster on one construct. Whereas similar approaches have already been used to engineer myxobacterial systems,[29,30] the 'stitching together' of complex actinomycete pathways via Red/ET recombineering has not yet been described.

The Red/ET engineering of the PL pathway enabled the efficient transfer into several *Streptomyces* strains. The entire gene cluster was rebuilt from overlapping cosmids that are based on the integrative *E. coli–Streptomyces* shuttle vector pOJ436. The final 62 kbp construct was then successfully conjugated with high efficiency into the host strains *S. lividans* TK24 and *S. coelicolor* A3(2), and integration into the chromosomes was confirmed by PCR. The resulting mutant strains *S. lividans* TK24/CPhl8 and *S. coelicolor* A3(2)/CPhl8 were both shown to produce phenalinolactones A and D. This result strongly suggests that the inserted cluster contains all of the genes that are necessary for the biosynthesis of phenalinolactone, although participation of enzymes that are native to the heterologous hosts cannot be ruled out. Evidently, all of the regulatory elements required for transcription of the *pla* genes were recognized in the heterologous hosts, albeit at reduced efficiency relative to the native host strain (Table 1).

One potential complication of using *S. lividans* and *S. coelicolor* as heterologous hosts is their endogenous production of the antibiotics prodiginin (Red), actinorhodin (Act) and calcium-dependent antibiotic (CDA), which might hamper the simultaneous biosynthesis (and detection) of a desired new compound. This problem has been circumvented in several cases by using a mutant strain of *S. coelicolor* M512, in which the Act and Red gene clusters have been deleted.[28,31] Therefore, *S. coelicolor* M512 was also used here as an additional heterologous host for phenalinolactone biosynthesis. Indeed, after successful expression of the cluster in this strain, production of phenalinolactone was five- to 50-fold higher at 28 °C relative to that in *S. lividans* TK24 or *S. coelicolor* A3(2) (Table 1). Varying the culture conditions yielded a further enhancement in productivity (to 100-fold after cultivation of *S. lividans* at 37 °C), whereas no increase in phenalinolactone biosynthesis was obtained for the *S. coelicolor* strains. In fact, incubation at 37 °C completely abolished PL biosynthesis, which is consistent with the fact that production by the native host *Streptomyces* sp. Tü6071 is also significantly lower at this temperature (200 $\mu g\,L)^{-1}$. Thus, under optimized fermentation conditions, it is clear that *S. lividans* is a superior heterologous host for phenalinolactone biosynthesis than *S. coelicolor* strains, although the production is still 50-fold lower than from the natural producer. An attempt was also made to increase phenalinolactone production by feeding the putative precursor mevalonolactone, but no increase in the yield of PL was observed. Analysis of the *pla* cluster shows that it incorporates genes for producing isoprenoid building blocks via the mevalonate-independent pathway, and indeed, this biosynthetic origin has previously been demonstrated.[12] In addition, genes that belong to the mevalonate pathway have not yet been identified in either *S. lividans* or *S. coelicolor*.[32,33] Thus, it seems that the heterologous host strains that were used here lack the enzymes to metabolize mevalonate, which accounts for our failure to increase phenalinolactone yields by supplementation.

The expression system that is described here further enabled the generation of an unglycosylated phenalinolactone derivative. Red/ET technology was combined with the FRT/FLP recombinase system to construct a markerless deletion of the glycosyl transferase gene *plaA6*. Heterologous expression of the modified construct resulted in the unglycosylated derivative phenalinolactone E, which demonstrates that the glycosyl transferase Pla A6 catalyzes the attachment of 4-*O*-methyl-L-amicetose to the hydroxylated terpenoid backbone. Surprisingly, the biosynthesis of PL E was only observed in *S. coelicolor* M512, although yields of the parent glycosylated compound from *S. lividans* TK24 were much higher. In this case, the utility of *S. coelicolor* mutant strain M512 as a suitable host might stem from its overall lower background of secondary metabolism.

Although several *Streptomyces* gene clusters have been relocated to heterologous hosts by using Red/ET cloning, transcription in each case was under the control of a single or few promotors.[28] Thus, the *pla* cluster represents the most complicated gene set yet to be heterologously expressed in any *Streptomyces* sp. In the future, efforts to understand the regulatory factors that govern phenalinolactone biosynthesis should aid in efforts to optimize heterologous production, for example by overexpressing positive regulators. In addition, the results described here set the stage for further engineering the biosynthetic machinery in *E. coli* towards the generation of novel PL analogues.

Experimental Section

Bacterial strains and culture conditions: The *Streptomyces* strains that were used in this work were *Streptomyces* sp. Tü6071,[12] *S. lividans* TK24,[17] *S. coelicolor* A3(2)[18] and *S. coelicolor* M512 (Δ*redD*,

Δ*actII-ORF4* SCP1⁻SCP2⁻).[19] NL111 liquid medium was used for growth and production.[12] For phenalinolactone production, the cultures were cultivated under standard conditions at 28 °C and 180 rpm on a rotary shaker and harvested after 6–8 days, as described previously.[12] Studies on the optimization of PL production were performed under standard cultivation conditions in NL111 medium but at 37 °C. For genomic DNA isolation, the *Streptomyces* strains were grown in YEME medium.[16] Apramycin (60 μg mL^{-1}) was used for selection of recombinant *Streptomyces* strains. *E. coli* ET12567 that carried pUZ8002 was used for conjugation according to the procedure described by Kieser et al.[16] *E. coli* strains were cultured in LB medium at 37 °C, with the appropriate antibiotic selection: ampicillin (100 μg mL^{-1}), apramycin (60 μg mL^{-1}), tetracycline (12.5 μg mL^{-1}), zeocin (25 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}).

Cloning and genetic engineering of the phenalinolactone biosynthetic gene cluster: All PCRs were carried out in an Eppendorf Mastercycler by using Phusion Polymerase (Finnzymes, Espoo, Finland), TripleMaster Polymerase (Eppendorf, Hilden, Germany) or HotStarTaq Polymerase (Qiagene, Hamburg, Germany) according to the manufacturers' protocols. Amplification of the *ScaI-oriT-tet-trpE* cassette by using Phusion Polymerase was carried out by using CMch37[29] as template, and the oligonucleotides PhlET10 and PhlET11 (Table S1). Conditions on the Eppendorf Mastercycler were as follows: 30 s at 98 °C, denaturation at 98 °C (8 s), annealing at 57 °C (25 s), and extension at 72 °C (45 s); 30 cycles. The ~600 bp zeocin cassette was amplified by Phusion Polymerase with oligonucleotides PhlET6 and PhlET7 (Table S1), by using CMch37 as template. Conditions on the Eppendorf Mastercycler were as follows: 30 s at 98 °C, denaturation at 98 °C (8 s), annealing at 57 °C (25 s), and extension at 72 °C (15 s); 30 cycles. The ~1.1 kb kanamycin-resistance cassette flanked by FRT sites was amplified with Triple Master Polymerase by using the construct pR6K-amp-gb2-neo-loxP (Gene Bridges GmbH; http://www.genebridges.com, technical protocol) as a template with oligonucleotides PhlET22 and PhlET23 (Table S1). Conditions on the Eppendorf Mastercycler were as follows: 3 min at 95 °C, denaturation at 95 °C (1 min), annealing at 57 °C (1 min), and extension at 72 °C (1 min); 30 cycles. The PCR product was precipitated with sodium acetate and used for Red/ET recombination. Cloning of the phenalinolactone gene cluster was performed by using Red/ET recombination.[14] For Red/ET recombination, an aliquot (30 μL) of competent *E. coli* GB2005/pSC101-BAD-γβαA-amp (Gene Bridges GmbH; http://www.genebridges.com) cells were electroporated with a mixture of 0.3 μg of a linear fragment (either a PCR product or a fragment that was obtained from restriction) and the target cosmid. After electroporation, colonies that grew under selection for the antibiotic-resistance genes were examined for the intended Red/ET recombination product by restriction analysis with a set of enzymes. Site-specific recombination to remove the kanamycin-resistance marker was performed by using FLP recombinase (New England Biolabs) according to the manufacturer's protocol.

Transformation of the expression constructs into *Streptomyces* host strains: Transformation of the cosmids CPhl8 and CPhl18 into *S. lividans* TK24, *S. coelicolor* A3(2) and *S. coelicolor* M512 was performed by conjugation according to a standard protocol.[16] Transconjugants were transferred to SM agar plates that contained nalidixic acid (25 μg mL^{-1}) and apramycin (60 μg mL^{-1}) and successful integration of the constructs into the *Streptomyces* chromosome was checked by PCR. By using HotStarTaq Polymerase and the oligonucleotides apra for and apra rev (Table S1), the apramycin-resistance gene (777 bp) was amplified from genomic DNA of the mutant strains. PCR conditions by using an Eppendorf Mastercycler were as follows: initial denaturation and activation of the polymerase at 95 °C (15 min), denaturation at 94 °C (20 s), annealing at 54 °C (20 s) and extension at 72 °C (50 s); 30 cycles.

Expression and analysis: The *Streptomyces* mutants harboring the *pla* biosynthetic gene cluster (*S. lividans* TK24/CPhl8, *S. coelicolor* A3(2)/CPhl8 and *S. coelicolor* M512/CPhl8) were cultivated in 1-L baffled shake flasks containing NL111 medium (200 mL) that was supplemented with apramycin (60 μg mL^{-1}). The cultures were inoculated with a 3-day-old preculture (1:100) and incubated for 6–8 days at 180 rpm and 28 °C or 37 °C on a rotary shaker. The cultures were harvested by centrifugation and the supernatants were extracted with ethyl acetate. The organic phase was evaporated and redissolved in DMSO (500 μL, 50%). The extracts (10 μL) were then analyzed by HPLC–MS. Analysis was carried out by using an Agilent 1100 series solvent delivery system that was equipped with a photodiode array detector and coupled to a Bruker HCTplus ion trap mass spectrometer. Chromatographic separation was carried out on a Nucleodur C18/3 μm RP column (125×2 mm; Macherey & Nagel) that was equipped with an C18/5 precolumn (8×3 mm) by using a mobile phase system that consisted of 0.1% formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B). The following gradient was applied: 0–2 min 25% B, 2–22 min linear from 25% B to 95% B, 22–24 min isocratic at 95% B. Detection was carried out in negative-ionization mode at a scan range of *m/z* 100–1100. Phenalinolactones were identified by comparison to the retention times (t_R) and the MS data of authentic standards (Phenalinolactone A: t_R = 13.8 min, *m/z* 714.5 $[M-H]^-$; phenalinolactone D: t_R = 18.1 min, *m/z* 698.3 $[M-H]^-$).

S. lividans TK24/CPhl18, *S. coelicolor* A3(2)/CPhl18 and *S. coelicolor* M512/CPhl18 that contained the modified *pla* gene cluster (*plaA6* deletion) were cultivated in 1-L baffled shake flasks that contained NL111 medium (200 mL) that was supplemented with apramycin (60 μg mL^{-1}). The mutants were cultivated, harvested and extracted as described above. Phenalinolactone E was identified by comparison to the retention times and the MS data of phenalinolactone D (phenalinolactone E: t_R = 13.0 min, *m/z* 570.3 $[M-H]^-$). To verify the identity of the compound, a LC-coupled FT-Orbitrap-MS analysis was performed. Analysis was carried out with an Accella UPLC system (Thermo Electron Corporation) that was coupled to a LTQ Orbitrap Mass Spectrometer (Thermo Fisher Scientific) that was operating in negative-ionization mode at a scan range of *m/z* 100–2000. A Hypersil Gold column (2.1×50 mm; Thermo Fisher Scientific) was used for separation with a solvent system consisting of 0.1% formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B). A gradient of 5–95% B was applied over 10 min. Measurements were carried out in single-ion mode (SIM). Ions of the *m/z* 570.27 (PL E, new derivative) and *m/z* 698.35 (PL D, standard substance) were collected and subjected to collision-induced dissociation (CID).

Acknowledgements

The authors would like to thank K. Weissman for comments on this manuscript and D. Krug for help with HPLC–MS analysis. This work was supported by the Bundesministerium für Bildung und Forschung (BMB+F).

Keywords: biosynthesis · gene expression · phenalinolactone · Red/ET recombineering · *Streptomyces*

[1] G. L. Challis, D. A. Hopwood, *Proc. Natl. Acad. Sci. USA.* **2003**, *100*, 14555–14561.
[2] H. B. Bode, R. Müller, *Angew. Chem.* **2005**, *117*, 6988–7007; *Angew. Chem. Int. Ed.* **2005**, *44*, 6828–6846.
[3] R. H. Baltz, *Trends Microbiol.* **1998**, *6*, 76–83.
[4] D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2007**, *70*, 461–477.
[5] S. C. Wenzel, R. Müller, *Curr. Opin. Biotechnol.* **2005**, *16*, 594–606.
[6] K. Ichinose, M. Ozawa, K. Itou, K. Kunieda, Y. Ebizuka, *Microbiology* **2003**, *149*, 1633–1645.
[7] C. Y. Kim, H. J. Park, E. S. Kim, *J. Microbiol. Biotechnol.* **2003**, *13*, 819–822.
[8] Z. Xu, K. Jakobi, K. Welzel, C. Hertweck, *Chem. Biol.* **2005**, *12*, 579–588.
[9] U. von Mulert, A. Luzhetskyy, C. Hofmann, A. Mayer, A. Bechthold, *FEMS Microbiol. Lett.* **2004**, *230*, 91–97.
[10] L. Tang, S. Shah, L. Chung, J. Carney, L. Katz, C. Khosla, B. Julien, *Science* **2000**, *287*, 640–642.
[11] Q. Xue, G. Ashley, C. R. Hutchinson, D. V. Santi, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 11740–11745.
[12] C. Dürr, H. J. Schnell, A. Luzhetskyy, R. Murillo, M. Weber, K. Welzel, A. Vente, A. Bechthold, *Chem. Biol.* **2006**, *13*, 365–377.
[13] J. P. P. Muyrers, Y. Zhang, F. A. Stewart, *Trends Biochem. Sci.* **2001**, *26*, 325–331.
[14] Y. Zhang, F. Buchholz, J. P. P. Muyrers, A. F. Stewart, *Nat. Genet.* **1998**, *20*, 123–128.
[15] Y. Zhang, J. P. P. Muyrers, G. Testa, F. A. Stewart, *Nat. Biotechnol.* **2000**, *18*, 1314–1317.
[16] T. Kieser, M. Bibb, M. J. Buttner, K. F. Chater, D. A. Hopwood, *Practical Streptomyces Genetics*, The John Innes Foundation, Norwich, **2000**.
[17] X. H. Yin, C. Gerbaud, F. X. Francou, M. Guerineau, M. J. Virolle, *Gene* **1998**, *215*, 171–180.
[18] S. D. Bentley, K. F. Chater, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, D. A. Hopwood, *Nature* **2002**, *417*, 141–147.
[19] B. Floriano, M. Bibb, *Mol. Microbiol.* **1996**, *21*, 385–396.
[20] T. Kawasaki, T. Kuzuyama, K. Furihata, N. Itoh, H. Seto, T. Dairi, *J. Antibiot.* **2003**, *56*, 957–966.
[21] L. P. Thapa, T.-J. Oh, H. C. Lee, K. Liou, J. W. Park, Y. J. Yoon, J. K. Sohng, *Appl. Microbiol. Biotechnol.* **2007**, *76*, 1357–1364.
[22] A. Luzhetskyy, A. Mayer, J. Hoffmann, S. Pelzer, M. Holzenkämpfer, B. Schmitt, S. E. Wohlert, A. Vente, A. Bechthold, *ChemBioChem* **2007**, *8*, 599–602.
[23] A. Li, J. Piel, *Chem. Biol.* **2002**, *9*, 1017–1026.
[24] V. Miao, M. F. Coeffet-LeGal, P. Brian, R. Brost, J. Penn, A. Whiting, S. Martin, R. Ford, I. Parr, M. Bouchard, C. J. Silva, S. K. Wrigley, R. H. Baltz, *Microbiology* **2005**, *151*, 1507–1523.
[25] R. Zirkle, J. M. Ligon, I. Molnar, *Microbiology* **2004**, *150*, 2761–2774.
[26] B. A. Pfeifer, C. C. Wang, C. T. Walsh, C. Khosla, *Appl. Environ. Microbiol.* **2003**, *69*, 6698–6702.
[27] B. A. Pfeifer, S. J. Admiraal, H. Gramajo, D. E. Cane, C. Khosla, *Science* **2001**, *291*, 1790–1792.
[28] A. S. Eustaquio, B. Gust, U. Galm, S. M. Li, K. F. Chater, L. Heide, *Appl. Environ. Microbiol.* **2005**, *71*, 2452–2459.
[29] S. C. Wenzel, F. Gross, Y. Zhang, J. Fu, F. A. Stewart, R. Müller, *Chem. Biol.* **2005**, *12*, 349–356.
[30] O. Perlova, J. Fu, S. Kuhlmann, D. Krug, F. A. Stewart, Y. Zhang, R. Müller, *Appl. Environ. Microbiol.* **2006**, *72*, 7485–7494.
[31] A. Freitag, H. Rapp, L. Heide, S. M. Li, *ChemBioChem* **2005**, *6*, 1411–1418.
[32] T. Kuzuyama, T. Dairi, H. Yamashita, Y. Shoji, H. Seto, *Biosci. Biotechnol. Biochem.* **2004**, *68*, 931–934.
[33] D. E. Cane, C. Chow, A. Lillo, I. Kang, *Bioorg. Med. Chem.* **2001**, *9*, 1467–1477.

Received: September 14, 2007
Published online on December 21, 2007

