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Five gene products are required for assembly of the central pyrrole moiety of coumermycin A_1

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Abstract Coumermycin A_1 is an aminocoumarin antibiotic produced by *Streptomyces rishiriensis*. It exhibits potent antibacterial and anticancer activity. The coumermycin A_1 molecule contains two terminal 5-methyl-pyrrole-2-carboxylic acid moieties and one central 3-methylpyrrole-2,4-dicarboxylic acid moiety (CPM). While the biosynthesis of the terminal moieties has been elucidated in detail, the pathway leading to the CPM remains poorly understood. In this work, the minimal set of genes required for the generation of the CPM scaffold was identified. It comprises the five genes *couR1*, *couR2a*, *couR2b*, *couR3*, and *couR4* which are grouped together in a contiguous 4.7 kb region within the coumermycin A_1 biosynthetic gene cluster. The DNA fragment containing these genes was cloned into an expression plasmid and heterologously expressed in *Streptomyces coelicolor* M1146. Thereupon, the formation of CPM could be shown by HPLC and by HPLC-MS/MS, in comparison to an authentic CPM standard. This proves that the genes *couR1–couR4* are sufficient to direct the biosynthesis of CPM, and that the adjacent genes *couR5* and *couR6* are not required for this pathway. The enzyme CouR3 was expressed in *Escherichia coli* and purified to near homogeneity. The protein exhibited an ATPase activity similar to that reported for its close ortholog, the threonine kinase PduX. However, we could not show a threonine kinase activity of CouR3, and; therefore, the substrate of CouR3 in CPM biosynthesis is still unknown and may be different from threonine.

Keywords *Streptomyces* · Coumermycin · Pyrrole · Biosynthesis · Heterologous expression

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Introduction

Coumermycin A_1 (Fig. 1), hereinafter called coumermycin, is a member of the aminocoumarin group of antibiotics. It strongly inhibits bacterial DNA replication through binding to the GyrB subunit of gyrase [19]. Coumermycin also exhibits a potent antiproliferative activity in mammalian cells resulting from inhibition of heat shock protein 90 which plays a key role in the stability of multiple cell-signaling molecules including several oncogenic kinases [18]. Therefore, coumermycin represents an interesting lead for generation of new antibacterial and anticancer drugs.

Structurally, coumermycin is unique among the aminocoumarins in containing a central pyrrole unit, i.e., 3-methylpyrrole-2,4-dicarboxylic acid (hereinafter called central pyrrole moiety, CPM). Two 3-amino-4,7-dihydroxycoumarin moieties are connected via amide bonds to the CPM. The 7-hydroxy group of each aminocoumarin moiety is glycosidically linked to an unusual deoxysugar,

noviose, which is acylated at its 3-hydroxy group with a 5-methylpyrrole-2-carboxyl acid (hereinafter called terminal pyrrole moiety).

The biosynthetic pathway leading to the terminal pyrrole moieties of coumermycin has been examined in detail [2, 6–8, 26]. These moieties are derived from proline, which is activated as prolyl-AMP, loaded as thioester onto a specific carrier protein, oxidized in two discrete two-electron steps to a pyrrole derivative, methylated and transferred to the deoxysugar moiety of the antibiotic in a two-step acyl transfer process.

By contrast, the data on the biosynthetic pathway leading to the central pyrrole moiety are rather fragmentary. From the results of a feeding study with [U–^{14}C]L-proline [21], it was suggested that proline is the precursor of all three pyrrole moieties of the coumermycin molecule. However, the method of degradation and analysis used in that study did not differentiate between the central and the terminal pyrrole moieties; and therefore, the study did not provide indisputable evidence for the origin of both types of pyrroles. Recently, a feeding experiment with [U–^{13}C, ^{15}N]L-threonine showed that the central pyrrole moiety of coumermycin is derived from threonine rather than from proline, and that the intact threonine skeleton with its four carbons and its nitrogen atom is incorporated into CPM [24].

Furthermore, inactivation of the seven genes *couN1–couN7*, which are involved in the conversion of proline to the terminal pyrrole moiety, showed that these genes are not required for the formation of the central pyrrole moiety. Therefore, the central pyrrole moiety formation must be formed by a different pathway than the terminal pyrrole moieties [24, 27]. A search in the chemical abstracts database (CAS) did not yield any other natural product containing a 3-methylpyrrole-2,4-dicarboxylic acid skeleton; therefore, no biosynthetic pathway leading to this structural moiety has been investigated previously. However, previous studies showed that differences and similarities of the structures of aminocoumarin antibiotics were always reflected by differences and similarities of the respective biosynthetic gene clusters [16]. Only the coumermycin structure contains the CPM moiety, and the coumermycin gene cluster contains a subcluster of genes, termed *couR1–couR6*, for which there are no orthologues in the gene cluster of the other aminocoumarin antibiotics, including the clusters of the closely related compounds clorobiocin and novobiocin. Recently, individual inactivations of the genes *couR1–couR4* resulted either in an abolishment or in a strong reduction of coumermycin production. The *ΔcouR1* and *ΔcouR4* mutants were complemented with intact copies of the deleted genes, which restored coumermycin production, albeit only to a low production level. Also, external feeding of the central pyrrole moiety to the individual *couR1–couR4* deficient

Fig. 2 Strategy for sequential deletion of the genes adjacent to the genes *couR1–couR4* and cloning of the resulting gene cassette into the pUWL201 vector. In the first step, the genes from *couR5* to *parY* were replaced by an apramycin resistance cassette, generating couMW16/ΔR5-parY. In a parallel experiment, the genes from *couS* to *parY* were replaced by the same cassette, generating couMW16/ΔS-parY (not shown). *aac(3)IV*, apramycin resistance cassette; *aadA*, spectinomycin/streptomycin resistance cassette; P-*ermE**, strong constitutive *ermE** promoter. The size of the genes is out of scale

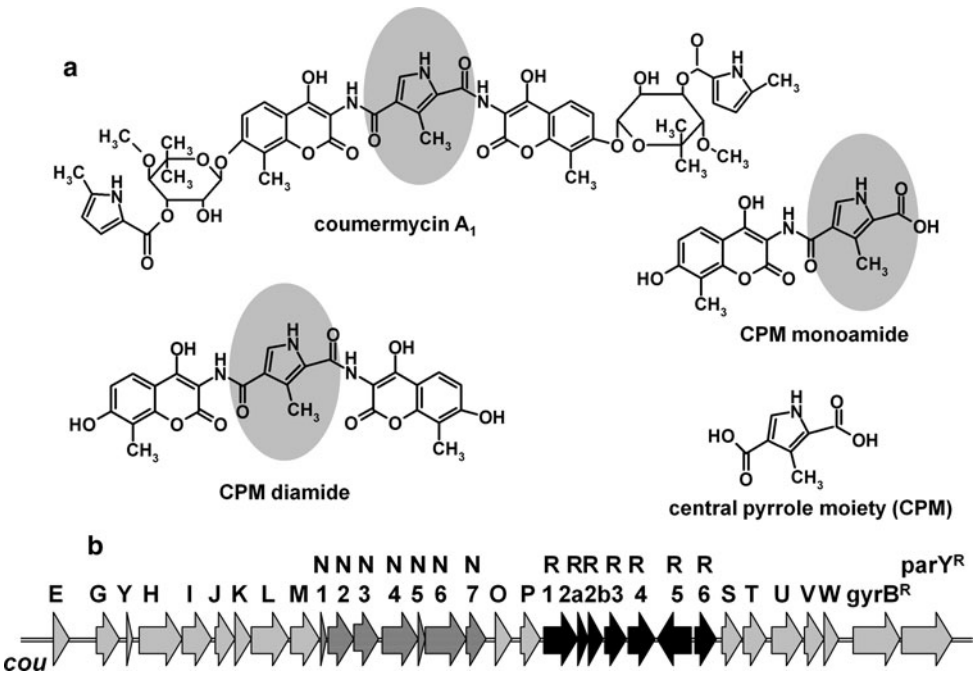

Fig. 1 **a** Structure of the aminocoumarin antibiotic coumermycin A_1 and precursors thereof. The central pyrrole moiety of the coumermycin molecule is highlighted in *grey*. **b** The biosynthetic gene cluster of coumermycin. The genes investigated in this study for their participation in the biosynthesis of the central pyrrole moiety are *shaded* in *black*

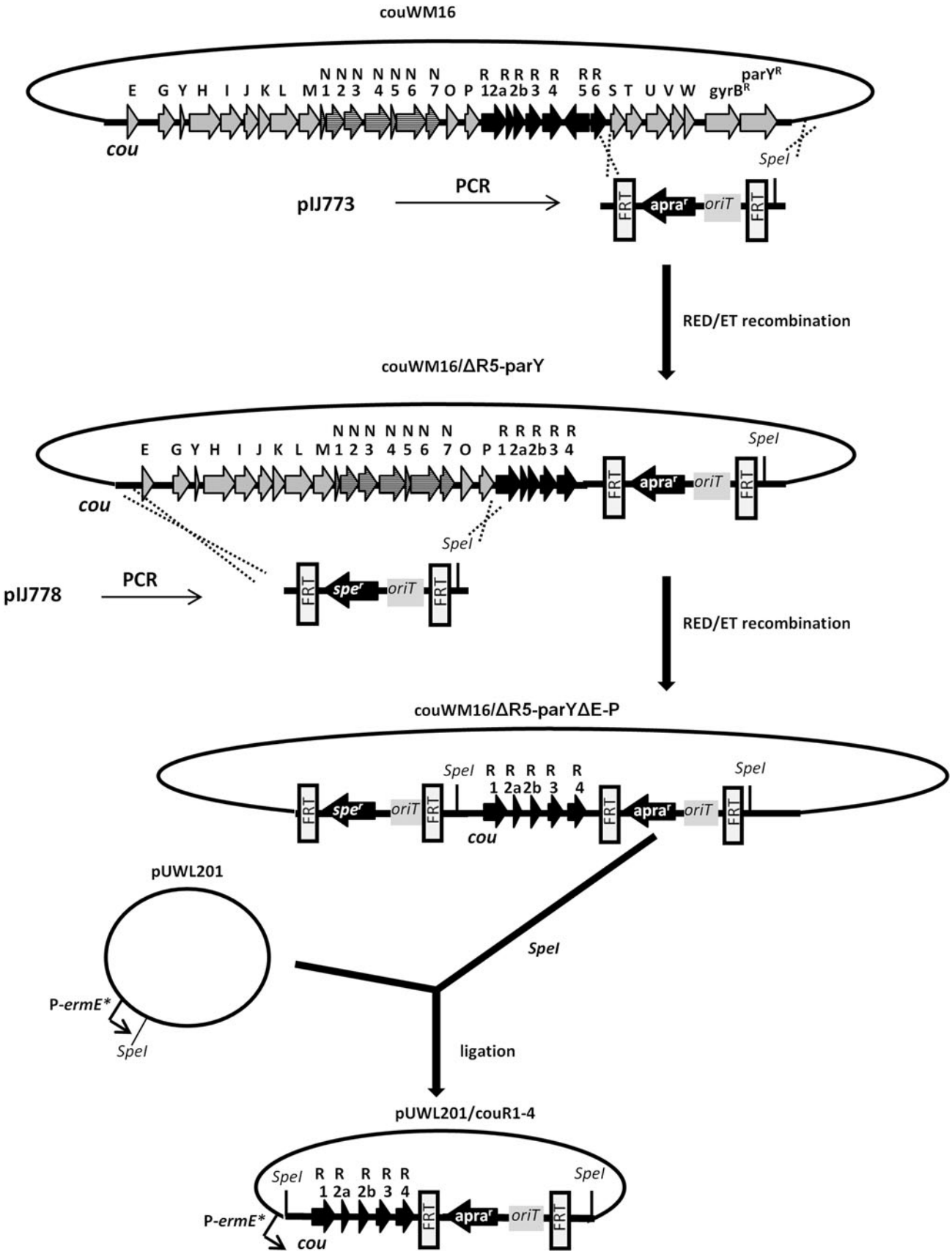
couWM16
E G Y H I J K L M N N N N N N N 1 2 3 4 5 6 7 O P R R R R R R R 12a 2b 3 4 5 6 R R S T U V W parY^R gyrB^R
cou
SpeI
pIJ773
PCR
FRT
apra^r
oriT
FRT
RED/ET recombination
couWM16/ΔR5-parY
E G Y H I J K L M N N N N N N N 1 2 3 4 5 6 7 O P R R R R 1 2a 2b 3 4
SpeI
cou
FRT
apra^r
oriT
FRT
SpeI
pIJ778
PCR
FRT
spe^r
oriT
FRT
RED/ET recombination
couWM16/ΔR5-parYΔE-P
SpeI
R R R R R 1 2a 2b 3 4
SpeI
FRT
spe^r
oriT
FRT
cou
FRT
apra^r
oriT
FRT
pUWL201
SpeI
P-ermE*
SpeI
ligation
pUWL201/couR1-4
SpeI
R R R R R 1 2a 2b 3 4
SpeI
P-ermE*
cou
FRT
apra^r
oriT
FRT

Table 1 PCR primers used in this study

Name	Sequence (5′–3′)[a]	Restriction site	Position[b]
R5rightend_F	CATCGCCGATCTCGGCCGGGACTGGCTCATCCCCGGGT AGTCTAGAATTCCGGGGATCCGTCGACC	*Xba*I	26,338–26,377
Sright end_F	GCTCAACCTGCTCACCGTCGCACAGGCCGGGACCGTCTG ATCTAGAATTCCGGGGATCCGTCGACC	*Xba*I	28,877–28,916
R5rightend_R	GACGATGACGGCGCTGGAAACGATGAACCCCGACTGGTGT ACTAGTTGTAGGCTGGAGCTGCTTC	*Spe*I	40,891–40,930
leftendR1_F	TCGGTTCACTCATCACAGCCTCATCACTTCCGTCGGAAGCA TTCCGGGGATCCGTCGACC	–	221–260
leftendR1_R	CAGAGCCCTGCACGGACCGATGTTCTGAGTGAGTACACATA CTAGTTGTAGGCTGGAGCTGCTTC	*Spe*I	21,656–21,695

[a] Restriction sites (*Xba*I and *Spe*I) introduced into the sequence are underlined in the primer sequences

[b] Position corresponds to that in the sequence of the couMW16 cosmid [25]

mutants restored coumermycin production showing that the respective genes take part in the biosynthesis of the central pyrrole moiety, and a pathway for CPM formation was suggested [24].

In this study, the minimal set of genes required for generation of the CPM scaffold was identified. Heterologous expression of a DNA fragment comprising the genes *couR1–couR4* led to the formation of CPM which was identified by HPLC and mass spectrometry in comparison to an authentic reference substrate. Furthermore, the putative kinase CouR3 was expressed in *E. coli* and purified. The protein was found to show a clear ATPase activity, comparable to its close ortholog, PduX, which has been proven to act as a threonine kinase. However, we could not show a specific threonine kinase activity for CouR3, and we, therefore, speculate that the substrate of CouR3 in the biosynthesis of CPM may be different from threonine.

Materials and methods

Bacterial strains, plasmids, cosmids, and culture conditions

All chemicals used were of analytical grade. For standard cloning procedures *Escherichia coli* XL1 Blue MRF′ (Stratagene, Heidelberg, Germany) was used. *E. coli* ET12567 [17] was used for isolation of non-methylated plasmid and cosmid DNA prior to transformation into *Streptomyces coelicolor* M1146 to bypass methyl-sensing restriction. The REDIRECT technology kit for PCR targeting [10], containing *E. coli* ET12567, *E. coli* ET12567/pUZ8002, *E. coli* BW25113/pIJ790, *E. coli* DH5α/pIJ778 and *E. coli* DH5α/pIJ773, *E. coli* DH5α/pIJ780 was obtained from Plant Bioscience Limited (Norwich, UK). *E. coli* strains were grown in liquid or solid Luria–Bertanni medium [20] with appropriate antibiotics at 37 °C.

Streptomyces coelicolor M1146 [9] was used as a host strain for heterologous expression of the CPM gene subcluster. The M1146 lacks the biosynthetic genes for the production of actinorhodin, the prodiginines, coelimycin, methylenomycin and the calcium-dependent antibiotic. The deletion of these gene clusters removes unwanted competition for precursors, and prevents potential cross-talk between different biosynthetic pathways. Furthermore, the strain has a much simplified extracellular metabolite profile, facilitating analysis of culture supernatants. *S. coelicolor* M1146 and integration mutants derived thereof were routinely cultured either on MS agar [13] or in 250 ml baffled Erlenmeyer flasks containing a stainless steel spring and 50 ml of TSB medium (BD Biosciences, Franklin

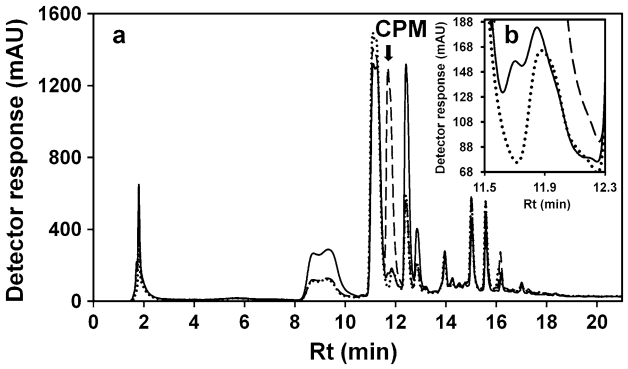

Fig. 3 *a* HPLC analysis of cultures of the heterologous expression strain *S. coelicolor* M1146/pUWL201/*couR1–4* after 8 days of cultivation. *b* Zoom of part of the chromatogram (from R_t 11.5 to 12.3 min). *Solid line S. coelicolor* M1146/pUWL201/couR1–4, *dotted line* negative control (*S. coelicolor* M1146), *dashed line* positive control (*S. coelicolor* M1146 culture with authentic CPM added just prior to extraction). All measurements were performed in triplicate and confirmed the presence of a peak with the retention time of CPM (11.7 min) in the *S. coelicolor* M1146/pUWL201/*couR1–4* strain and in the positive control, and the absence of this peak in the negative control

Lakes, USA). The cultivation was carried out at 30 °C and 200 rpm.

Kanamycin (15 μg ml^{-1} in liquid medium and 50 μg ml^{-1} in solid medium for *Streptomyces*; 50 μg ml^{-1} for *E. coli*), chloramphenicol (25–50 μg ml^{-1}), apramycin (50 μg ml^{-1}), carbenicillin (100 μg ml^{-1}), nalidixic acid (25 μg ml^{-1}) and thiostrepton (8 μg ml^{-1}) were used for selection of recombinant strains.

The pUWL201 [3] is an *E. coli–Streptomyces* shuttle vector containing the strong constitutive *ermE** promoter as well as an ampicillin and a thiostreptone resistance gene. It was kindly provided by A. Bechthold (Freiburg, Germany) and was originally obtained from U. Wehmeier (Wuppertal, Germany). Cosmid couMW16, harbouring the entire coumermycin gene cluster, has been described previously [25]. The backbone of this cosmid contains the integrase gene (*int*) and the attachment site (*attP*) of phage_ΦC31, as well as tetracycline and neomycin resistance genes.

DNA isolation, manipulation, and cloning

Standard procedures followed those of Sambrook and Russell [3] and Kieser et al. [25]. The DNA fragments were isolated from agarose gels using a QIAquick Gel Extraction Kit (Quiagen, Hilden, Germany) according to the manufacturer's protocol.

Gene deletions

For preparation of the respective cosmid constructs (Fig. 2) the λ RED recombination [11, 28] was used. The apramycin resistance cassette, *aac(3)IV*, was excised from pIJ773 by digestion with *Eco*RI and *Hind*III. For the deletion of all the genes downstream of the *couR4* and *couR6,* the cassette was amplified with primer pair R5rightend_F and R5rightend_R and couSrightend _F and R5rightend_R (Table 1), respectively. The resulting PCR products were employed for the deletion of the respective genes on cosmid couMW16 by PCR targeting [10]. The genotype of the resulting cosmids, couMW16_ΔR5-parY and couMW16_ΔS-parY, was verified by restriction analysis.

The spectinomycin/streptomycin resistance cassette, *aadA*, was excised from pIJ778 by digestion with *Eco*RI and *Hind*III. For the deletion of all the genes upstream of the *couR1*, the cassette was amplified with primer pair leftendR1_F and leftendR1_R (Table 1). The resulting PCR product was employed for the deletion of the respective genes on cosmids couMW16_ΔS-parY and couMW16_ΔR5-parY by PCR targeting [10]. Then, the genotype of the resulting cosmids, couMW16_ΔS-parYΔE-P and couMW16_ΔR5-parYΔE-P, was verified by restriction analysis.

Construction of expression plasmids containing the putative CPM biosynthetic gene subcluster from *S. rishiriensis*

The *Streptomyces* expression vector pUWL201, containing the *ermE** promoter, was used for construction of the plasmids pUWL201/couR1–4 and pUWL201/couR1–6. The couMW16_ΔS-parYΔE-P and couMW16_ΔR5-parYΔE-P cosmids were digested by *Spe*I and the resulting 8,683 and 6,144 bp *Spe*I fragments were ligated into the *Spe*I site of the pUWL201 vector to yield the pUWL201/couR1–6 and pUWL201/couR1–4 plasmids, respectively. The correct orientation of the respective inserts in the two plasmids was confirmed by restriction analysis.

Introduction of plasmid and cosmid DNA into *S. coelicolor* M1146

The expression plasmids pUWL201/couR1–4 and pUWL201/couR1–6, and the mutated cosmids couMW16ΔR5-parY and couMW16ΔS-parY contained the *ori*T of the *aac(3)IV* cassette from pIJ773, which had been used for PCR targeting to generate them. Therefore, the constructs can be introduced into *S. coelicolor* M1146 by conjugation.

Cosmid DNA was first transferred to *E. coli* ET12567 and then introduced into *S. coelicolor* M1146 by triparental intergeneric conjugation with the help of *E. coli* ET12567/pUB307 [5]. Apramycin and kanamycin resistant clones were selected.

Plasmid DNA was introduced into *S. coelicolor* M1146 by conjugation with *E. coli* ET12567/pUZ8002 [13]. Apramycin resistant clones were selected.

The respective exconjugants were then sporulated on MS agar plates. Single spores were isolated, germinated and used for generation of spore suspension.

Heterologous expression of the putative CPM biosynthetic gene subcluster in *S. coelicolor* M1146

To investigate secondary metabolite production, homogenized frozen inoculum was prepared as described by Siebenberg et al. [23] except that precultivation was carried out in TSB medium (BD Biosciences, Franklin Lakes, USA) with kanamycin (15 μg ml^{-1}) until an OD_{600} 0.5–0.7 was reached.

Fifty μl of frozen inoculum were mixed with 10 ml CDM medium [14] supplemented with thiamine (10 μg ml^{-1}), pyridoxine (8 μg ml^{-1}), siloxylated ethylene oxide/propylene oxide copolymer Q2-5247 (Dow Corning, Auburn, MI, USA) (0.6 % w/v) and $CoCl_2$ (0.2 μg ml^{-1}) without addition of antibiotics [23]. In the experiment presented in Fig. 3, siloxylated ethylene oxide/

propylene oxide copolymer Q2-5247 and $CoCl_2$ were omitted. Then, 3 ml aliquots of this mixture were distributed to individual wells of the 24-square deep-well plates [23]. The deep-well plates were shaken at 300 rpm and 30 °C for 8 days before harvest. In the experiments depicted in Fig. 5, samples were harvested 1–5 days after inoculation.

Analysis of secondary metabolite production

For investigation of secondary metabolite production, three individual wells of the 24-square deep-well plates were analyzed separately. For analysis of the formation of the free central pyrrole moiety (CPM), 1 ml of the culture was transferred from the well into an Eppendorf tube, and the remaining 2 ml of culture were discarded. The cells were lyzed by lysozyme (1.9 mg ml^{-1}) treatment for 60 min at 30 °C. Then the cultures were adjusted to pH 2 by addition of HCl and extracted three times with an equal volume of ethyl acetate. The organic phases were pooled, concentrated in vacuo to dryness and the residue was dissolved in 100 µl of ethanol. For analysis of the formation of the monoamide or the diamide of the central pyrrole moiety, cells were not lyzed by lysozyme but cultures were directly acidified and extracted with ethylacetate as described above.

The central pyrrole moiety was analyzed on an Agilent HPLC system with a photodiode array detector. The analysis was performed by using a Reprospher C 18-DE column (5 µm, 150 × 3 mm, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) at a flow rate of 0.6 ml min^{-1}. A linear gradient from 5 to 100 % solvent B (methanol + 0.1 % formic acid) in solvent A (H_2O + 0.1 % formic acid) over 20 min was employed. The UV absorption was recorded at 259 nm. Authentic 3-methylpyrrole-2,4-dicarboxylic acid was used as standard [22].

The monoamide and the diamide of the central pyrrole moiety were analyzed by using the same HPLC column at a flow rate of 0.6 ml min^{-1}. A linear gradient from 60 to 100 % solvent B (acetonitrile + 0.1 % formic acid) in solvent A (H_2O + 0.1 % formic acid) over 20 min was employed. The UV absorption was recorded at 345 nm. Authentic novobiocin and coumermycin (Sigma-Aldrich, St. Louis, USA) were used as standards.

Liquid chromatography-mass spectrometry (LC–MS) analysis was used to confirm the identity of the central pyrrole moiety formed by the cultures. The extracts were examined with LC–MS and LC–MS^2 analysis by using a Nucleosil 100 C_{18} column (100 × 2 mm, 3 µm, equipped with a precolumn 10 × 2 mm containing the same stationary phase) 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 5 to 100 % of solvent B in 20 min [solvent A: water/formic acid (999:1); solvent B: methanol/formic acid (999.4:0.6)]. Detection was carried out at 259 nm (±10 nm). Electrospray ionization in Ultra Scan mode with capillary voltage of 3.5 kV and a drying gas temperature of 350 °C was used for LC–MS analysis. For LC–MS^2, the analysis was carried out in positive ionization mode with a capillary voltage of 3.5 kV at 350 °C. Injection volume was 5 µl.

Cloning of *couR3*

The nucleotide sequence of *couR3* was optimized for expression in *E. coli* with the GeneDesigner Tool and synthesized commercially by Eurofins MWG Operon (Ebersberg, Germany). The sequence of *couR3* was synthesized including the His8 tag and the linker region as found in pHis8 [12]. It was supplied as insert in the pCR2.1 vector (Invitrogen) and used without further cloning. The expression construct was named pCR2.1/His8/couR3.

Protein expression and purification

Escherichia coli Rosetta 2 (DE3) pLysS cells harboring the pCR2.1/His8/couR3 expression construct were cultured in Terrific Medium [20] supplemented with kanamycin (50 µg ml^{-1}) and chloramphenicol (25 µg ml^{-1}).The cells were grown at 37 °C and 250 rpm to an A_{600} of 0.8. Temperature was lowered to 16 °C, and IPTG was added to a final concentration of 0.5 mM. Synthesis of recombinant protein was allowed to proceed for 20 h. Cells were harvested by centrifugation for 20 min at 2,700*g* at 4 °C, and the pellet was stored at −20 °C. For lysis the cells were thawed and resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 % glycerol, 50 mM imidazole, 0.5 mg ml^{-1} lysozyme) using a ratio of 25 ml lysis buffer for 10 g of pellet. After stirring for 30 min at 4 °C, cells were ruptured with a Branson sonifier. The lysate was cleared by centrifugation (38,720*g*, 4 °C, 45 min).The supernatant was applied to a nickel-nitrilotriacetic acid-agarose resin column (GE Healthcare) according to the manufacturer's instructions, using a linear gradient of 0–100 % 300 mM imidazole (in 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 % glycerol) in 60 min for elution. The eluate was passed through PD-10 columns (Amersham Biosciences, Germany) equilibrated and eluted with 50 mM Tris–HCl, pH 7.5, 10 mM NaCl, 15 % glycerol. The protein was stored in this buffer at −80 °C. The quality of the enzyme preparation was analyzed by SDS-PAGE according to the method of Laemmli [15] in 10 % polyacrylamide gels.

CouR3 activity determination

The CouR3 activity was measured by using the ADP Quest assay kit according to the manufacturer's instructions (DiscoveRx, Birmingham, UK). The assay uses a coupled enzyme reaction system to generate hydrogen peroxide from ADP (ADP reacts with PEP to form pyruvate by pyruvate kinase, and then pyruvate oxidase generates hydrogen peroxide). Hydrogen peroxide when combined with acetyl dihydroxyphenoxazine in the presence of peroxidase generates the fluorescent resorufin dye. Standard assay mixtures contained 15 mM HEPES, pH 7.5, 20 mM NaCl, 10 mM $MgCl_2$ in a total volume of 0.1 ml. Activity was determined by monitoring the fluorescence intensity with an Infinite 200 (Tecan, Germany) microplate reader. The excitation wavelength was 530 nm and emission wavelength was 590 nm. To minimize background fluorescence, 96-well black microplates (Greiner, Germany) were used. Quantitation was based on standard curves made with ADP. The measurements were performed in triplicates.

DNA computer-assisted sequence analysis

All sequence similarity searches were carried out on the amino acid level in the GenBank database by help of the BLAST program (release 2.2.4; [1]).

Results and discussion

Construction of expression plasmids containing the candidate genes for CPM biosynthesis

We identified seven genes (*couR1*, *couR2a*, *couR2b*, *couR3*, *couR4*, *couR5*, and *couR6*) which are present in the coumermycin cluster but not in any of the other aminocoumarin clusters. The genes seem to be candidates which may direct the biosynthesis of CPM. In previous experiments, the genes *couR1–couR4* were knocked out individually and the resulting *couR1–couR4* mutants were complemented with intact copies of the respective genes [24]. In contrast, *couR5* and *couR6* have not been examined yet.

We now carried out deletions of the genes upstream and downstream of the *couR1–couR6* gene set. Two deletions were made sequentially, starting from the complete coumermycin biosynthetic gene cluster which is contained in the integrative cosmid couMW16 [25]. First, the genes downstream of the *couR* genes were replaced by an apramycin resistance cassette via RED/ET mediated recombination (Fig. 2). Since the roles of *couR5* and *couR6* in CPM biosynthesis were not clear, we deleted in two independent experiments either the 14.5 kb fragment comprising the genes from *couR5* to *parY*, or the 12 kb fragment comprising the genes from *couS* to *parY*. The resulting cosmids were named couMW16/ΔR5-parY and couMW16/ΔS-parY, respectively.

Subsequently, the genes upstream of the group of *couR* genes were replaced by a spectinomycin resistance cassette via RED/ET mediated recombination. The 21.4 kb fragment comprising the genes from *couE* to *couP* in either couMW16/ΔR5-parY or couMW16/ΔR5-parY was deleted to yield the cosmids couMW16/ΔR5-parYΔE-P and couMW16/ΔS-parYΔE-P (Fig. 2), respectively.

Finally, the 6.1 kb *SpeI* fragment of couMW16/ΔR5-parYΔE-P, containing the *couR1–4* genes as well as the *aac(IV)3* cassette, was transferred to pUWL201 to yield pUWL201/couR1–4 (Fig. 2). The pUWL201 vector contains the strong constitutive *ermE** promoter for transcription of the *couR1–4* genes, and this construct was generated in order to test whether expression of these genes results in CPM production. The presence of the apramycin resistance gene in the *aac*(IV)3 cassette enabled positive selection in the final cloning step. Moreover, the presence of an *oriT* enabled the use of conjugation instead of the less efficient transformation for the introduction of the large expression plasmid pUWL201/couR1–4 into the *S. coelicolor* M1146 host.

Genes *couR1–couR4* are sufficient for the biosynthesis of the central pyrrole moiety of coumermycin

To investigate whether any of the genes downstream of *couR4* are involved in CPM biosynthesis, the integrative cosmids couMW16/ΔR5-parY and couMW16/ΔS-parY were introduced into *S. coelicolor* M1146 by conjugation.

Table 2 Production of the central pyrrole moiety (CPM) in form of its monoamide and its diamide in heterologous host strains expressing modifications of the coumermycin biosynthetic gene cluster

Strain	CPM monoamide		CPM diamide	
	Production (mg ml^{-1})	SD	Production (mg ml^{-1})	SD
S. coelicolor M1146/couSS-01	5.69	±0.98	5.60	±0.49
S. coelicolor M1146/couMW16/ΔS-parY	4.13	±0.71	6.20	±0.22
S. coelicolor M1146/couMW16/ΔR5-parY	2.81	±0.29	5.95	±0.57

SD standard deviation

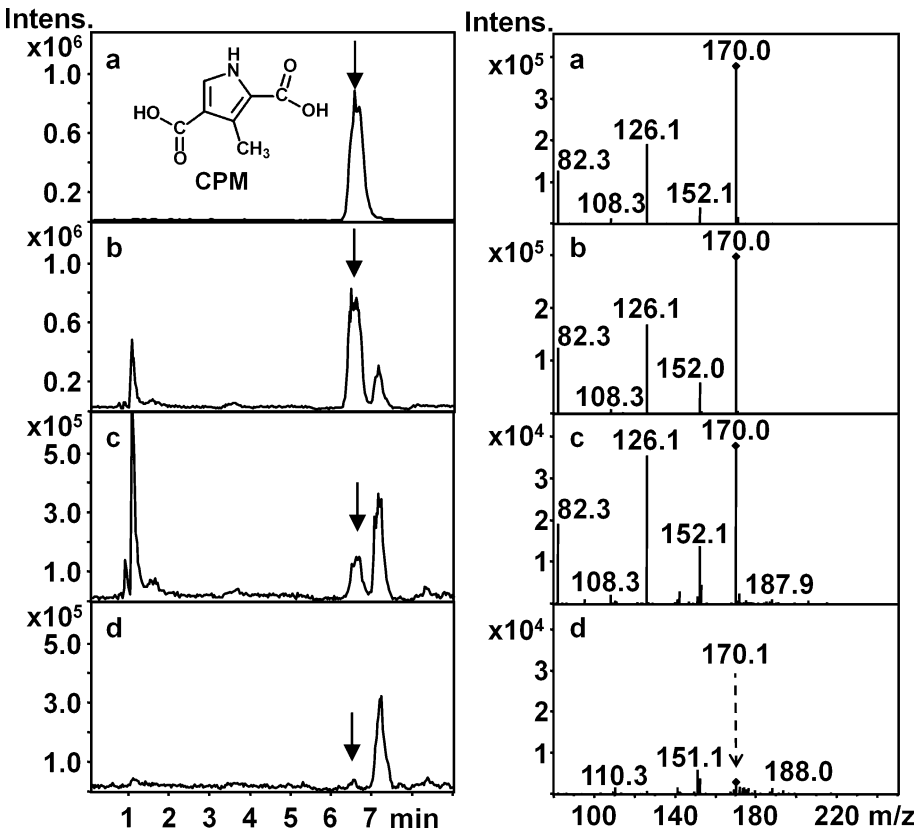

Fig. 4 LC–MS–MS identification of CPM (= 3-methylpyrrole-2,4-dicarboxylic acid) produced by the cultures of *S. coelicolor* M1146/pUWL201/*couR1–4*. **a** CPM standard, **b** positive control (*S. coelicolor* M1146 culture with authentic CPM added just prior to extraction), **c** *S. coelicolor* M1146/pUWL201/couR1–4, **d** negative control (*S. coelicolor* M1146). *Left* extracted ion chromatograms (m/z 170.1; positive mode). *Right* fragmentation pattern of the pseudo molecular ion m/z 170.1 at the retention time of the authentic CPM reference substance (6.5 min under these chromatographic conditions). The peak eluting at 7.2 min showed a mass spectrum different from CPM and, therefore, is a different compound formed by the *S. coelicolor* M1146 cultures

Clones of the resulting integration mutants were then cultivated in a chemically defined production medium [14] and production of the respective coumermycin intermediates was monitored by HPLC analysis.

Integration of the intact coumermycin gene cluster (contained in cosmid couMW16) into the ΦC31 attachment site of the genome of *S. coelicolor* M1146 results in the heterologous production of coumermycin [25]. Deletion of the genes *couSTUVW*, all of which are responsible for deoxysugar biosynthesis [16], was expected to result in the accumulation of the coumermycin aglycone (i.e., CPM diamide, Fig. 1) and possibly of its immediate precursor, CPM monoamide (Fig. 1). Therefore, the *S. coelicolor* M1146/couSS01strain, which has been shown to accumulate these two coumermycin precursors [24], was used as a positive control in this study. This strain contains, in the

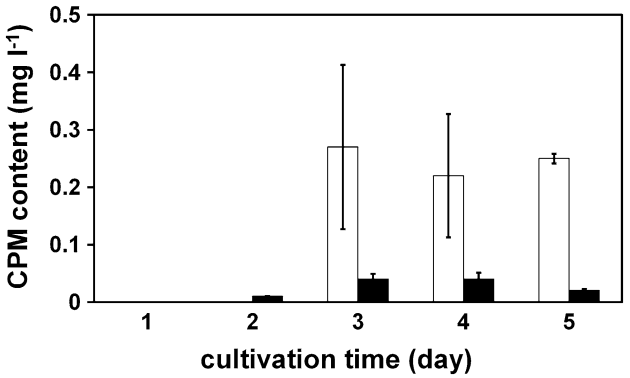

Fig. 5 Time course of the accumulation of CPM in mycelium and in medium of cultures of *S. coelicolor* M1146/pUWL201/couR1–4. *Columns shaded* in *black* represent CPM content in the mycelium; *white columns* represent CPM content in the medium. The measurements were performed in triplicates

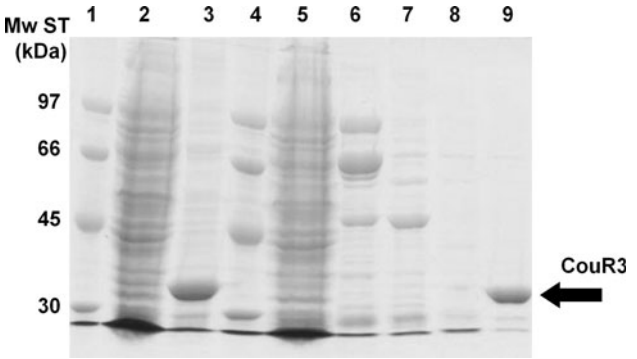

Fig. 6 SDS PAGE analysis of the expression and purification of CouR3 protein. *Lanes 1* and *4* molecular weight standards, *lane 2* soluble protein after induction, *lane 3* insoluble protein after induction, *lane 5* flow through upon washing with 50 mM imidazole, *lanes 6–9*, fractions resulting from elution with a linear gradient from 50 to 300 mM imidazole. The calculated molecular mass of the CouR3 protein is 34.8 kDa. 10 % (w/v) polyacrylamid gel, stained with Coomassie Blue

heterologous expression host *S. coelicolor* M1146 described in the Experimental Section, a coumermycin biosynthetic gene cluster from which the genes *couM–couN7* have been deleted. Gene *couM* codes for the glycosyltransferase, which is responsible for the transfer of the deoxysugar moiety to the aminocoumarin ring. Deletion of *couM–couN7*, therefore, resulted in accumulation of CPM diamide and CPM monoamide. *S. coelicolor* M1146 was used as a negative control.

In accordance with the above mentioned expectation, compounds with the same HPLC retention time as CPM monoamide and CPM diamide were detected in the spent medium of the mutants tested (Table 2). Since both the CPM diamide and CPM monoamide contain the complete CPM of coumermycin, this experiment provided evidence that none of the genes downstream of the *couR1–6* gene set (from couS to parY) is required for the formation of CPM. In addition, also in the absence of the genes *couR5* and *couR6* CPM, production was clearly observed; therefore, these two genes are not required for CPM biosynthesis. This finding is consistent with the functions predicted from sequence comparisons of the gene products of *couR5* and *couR6*. CouR5 shows sequence similarity to transporters and CouR6 is similar to transcriptional regulators.

In order to investigate also the possible involvement of the genes upstream of the *couR1* gene in CPM biosynthesis, the plasmid pUWL201/couR1–4 was introduced into *S.coelicolor* M1146. The resulting transformant was cultivated in CDM medium and the production of CPM was determined by HPLC and HPLC-MS. Indeed, *S. coelicolor* M1146 cells expressing pUWL201/couR1–4 produced a small amount of a compound with an HPLC retention time identical to that of chemically synthesized CPM. The extract of the host cells without the *couR1–couR4* construct did not yield any peak with the same retention time (Fig. 3). The new compound produced by *S. coelicolor* M1146 cells expressing pUWL201/couR1–4 showed a pseudomolecular ion $[M+H]^+$ at m/z 170.1 identical to that of chemically synthesized CPM. The LC–MS–MS analysis of this peak clearly showed the same retention time and fragmentation pattern for the new compound as for the authentic reference compound (Fig. 4). No peak with that fragmentation pattern was found in the negative control, i.e., in the strain lacking the *couR1–couR4* construct.

The fragments obtained from the ion peak at m/z 170.1 $[M+H]^+$ were interpreted as products of decarboxylation (m/z 126.1 $[M+H]^+–CO_2$), dehydration (m/z 152.1 $[M+H]^+–H_2O$), and both dehydration and decarboxylation (m/z 108.1 $[M+H]^+–H_2O–CO_2$). The same mass spectra were obtained from the chemically synthesized CPM standard, from the positive control strain to which synthetic CPM had been added immediately prior to analysis, and from the strain expressing the *couR1–couR4* construct. The negative control strain did not produce such a compound (Fig. 4).

We also determined the time course of CPM production in the strain harboring pUWL201/couR1–4. The strain was cultivated in CDM medium, and CPM accumulation in the cells and in the medium was monitored by HPLC. *S. coelicolor* M1146 and the same strain with external addition of CPM (2.2 mg ml^{-1}) at the time of inoculation were used as negative and positive controls, respectively.

No peak corresponding to that of the CPM standard was found in the extract of the negative control strain. In contrast, the *S. coelicolor* M1146 strain carrying the pUWL201/couR1–4 construct produced CPM. The level of the newly formed metabolite reached its maximum after three days and did not change significantly thereafter (Fig. 5). Externally added CPM could be recovered almost completely if extracted immediately after its addition to the culture. However, after 3 days of culture, the total amount of CPM recovered was only 0.45 mg, i.e., 20 % of the originally added amount. It, therefore, seems that CPM is not stable in the culture and may be degraded or modified. This may explain the relatively low amount of CPM found in the strain expression the *couR1–couR4* construct.

The above mentioned experiments prove that heterologous expression of the genes *couR1–couR4* results in the formation of CPM, and; therefore, they support the hypothesis that the enzymes encoded by these genes together catalyze the complete secondary metabolic pathway to this key precursor of coumermycin. In contrast, *couR5* and *couR6* are not required for this pathway.

Investigation of the enzymatic activity of CouR3

The previously suggested pathway to CPM begins with the *O*-phosphorylation of threonine [24]. The gene product of *couR3* shows significant similarity to kinases of the GHMP family, for example, it shows 34 % identity to the threonine kinase PduX operating in adenosylcobalamine biosynthesis in *Salmonella enterica* [4]. Threonine was shown to be a CPM precursor, providing the heterocyclic nitrogen as well as four of the seven carbons of the CPM moiety [24]. Therefore, we decided to investigate the kinase activity of CouR3. The *couR3* gene was synthesized commercially with optimized codon usage for heterologous expression in *E. coli* and with the promoter region and the affinity tag sequence of vector pHis8. The resulting construct, pCR2.1/His8/couR3, contained in the pCR2.1 vector (Invitrogen), was directly used for CouR3 expression in *E. coli*. The CouR3 was expressed as a histidine fusion protein. The protein was mostly insoluble (Fig. 6, lane 3), despite the low post-induction temperature of 16 °C. Still,

from 1 liter of culture, 7.6 mg of soluble CouR3 could be purified by metalloaffinity chromatography to near homogeneity (Fig. 6, lane 9).

To test whether the purified CouR3 exhibited a threonine kinase activity, we used a coupled fluorometric assay that measured the formation of ADP from ATP (described under "Materials and methods"). With ATP as substrate, the specific activity of purified CouR3 was 45 nmol min^{-1} mg of $protein^{-1}$ which is similar to the value of 56 nmol min^{-1} mg of $protein^{-1}$ observed for PduX [4]. In the absence of ATP, no ADP formation was detected. In the absence of CouR3, the formation of ADP decreased by 94 %. The remaining 6 % may have resulted from ATP-dependent background fluorescence, similar to the observation by Fan and Bobik [4] in their investigation of PduX. Unexpectedly, and in contrast to the results reported for PduX, omission of L-threonine from the assay did not reduce the ADP formation catalyzed by CouR3. Such a high intrinsic ATPase activity may either indicate that free threonine is not the genuine substrate of CouR3, or that the assay conditions are quite different from the optimal conditions of the enzyme. However, both the assay conditions, and the method of detection by a coupled spectrophotometric assay, were identical to the assay conditions used in the investigation of PduX.

Our biochemical experiment, therefore, could not provide evidence that CouR3 catalyzes the phosphorylation of threonine which has been suggested as the first step of the CPM biosynthetic pathway by Siebenberg et al. [24]. It should be noted that feeding *O*-phosphothreonine to a *couR3* defective mutant increased coumermycin production only 1.4-fold, while feeding CPM to the same mutant increased coumermycin production more than 50-fold [24]. Also, the result of that feeding experiment questions whether threonine phosphorylation is the first step in the CPM biosynthesis. There is no doubt that L-threonine provides the heterocyclic nitrogen as well as four of the seven carbons of the CPM moiety; however, the recruitment of threonine into the CPM biosynthetic pathway may start with a reaction different from the threonine kinase reaction catalyzed by PduX.

In this study, we have confirmed that the genes *couR1*, *couR2a*, *couR2b*, *couR3*, and *couR4* are sufficient for the CPM biosynthesis and the genes *couR5* and *couR6* are not required for this pathway. Also, we found that the enzyme CouR3 exhibits an ATPase activity; however, we could not show a threonine kinase activity of CouR3. Therefore, the possibility has to be considered that the substrate of CouR3 may be different from threonine.

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