Effects of deletions of *mbtH*-like genes on clorobiocin biosynthesis in *Streptomyces coelicolor*

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In the biosynthetic gene cluster of the aminocoumarin antibiotic clorobiocin, the small ORF *cloY* encodes a 71 aa protein which shows significant sequence similarity to *mbtH* from the mycobactin biosynthetic gene cluster of *Mycobacterium tuberculosis*. *mbtH*-like genes are frequently found in the biosynthetic gene clusters of peptide antibiotics and siderophores, but their function has remained enigmatic. In a recent publication it has been suggested that these genes may have no function for secondary metabolite biosynthesis. An in-frame deletion of *cloY* in the clorobiocin cluster has now been carried out. When the modified cluster was expressed in the heterologous host *Streptomyces coelicolor* M512, clorobiocin was still formed. However, when the two further *mbtH*-like genes from elsewhere in the host genome were inactivated as well, clorobiocin formation was reduced dramatically. Complementation with *cloY* or with any of three other *mbtH*-like genes restored clorobiocin formation. This is the first report proving the requirement of an *mbtH*-like gene for secondary metabolite formation, and the first proof that different *mbtH*-like genes can functionally replace each other. Feeding of an *mbtH*-defective triple mutant strain with an intact 3-amino-4,7-dihydroxy-coumarin moiety restored antibiotic production, showing that *cloY* is specifically required for the formation of this moiety of the clorobiocin molecule.

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INTRODUCTION

The aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A₁ (Fig. 1) are potent inhibitors of DNA gyrase, thereby presenting interesting starting compounds for the development of new antibiotics by combinatorial biosynthesis and metabolic engineering (Flatman *et al.*, 2006; Li & Heide, 2004, 2005). Their characteristic structural moiety is a 3-amino-4,7-dihydroxycoumarin unit. The same unit is also found in simocyclinone D8, an antibiotic which otherwise has a completely different structure than the aforementioned compounds (Fig. 1). Interestingly, simocyclinone D8 has also been identified as a potent inhibitor of gyrase (Flatman *et al.*, 2005).

We have cloned and sequenced the biosynthetic gene clusters of these four aminocoumarin antibiotics (Galm *et al.*, 2002; Pojer *et al.*, 2002; Steffensky *et al.*, 2000; Wang *et al.*, 2000). Inactivation experiments as well as biochemical investigations allowed us to assign all ORFs contained in the novobiocin and the clorobiocin clusters to their functions in

catalysis, regulation or self-resistance (Freitag *et al.*, 2005; Li & Heide, 2004). The only gene of the clorobiocin cluster for which no functional assignment has been possible yet is the small ORF *cloY*.

As shown in Fig. 1, *cloY* is situated downstream of the regulatory gene *cloG* (Eustáquio *et al.*, 2005b) and upstream of the genes *cloHIJK* which encode enzymes involved in 3-amino-4,7-dihydroxycoumarin biosynthesis (Chen & Walsh, 2001; Pacholec *et al.*, 2005). A similar arrangement of genes is found in the coumermycin A₁ and the novobiocin gene clusters. However, while the coumermycin A₁ cluster contains an orthologue of *cloY* (i.e. *couY*), the novobiocin cluster does not contain such a gene. In the simocyclinone D8 cluster, no orthologue of the regulatory gene *cloG* is found, but orthologues of all other mentioned genes of aminocoumarin biosynthesis are found, including the small ORF *simY* with high sequence similarity to *cloY* (Fig. 1).

CloY, *CouY* and *SimY* are predicted proteins of 71, 71 and 70 aa, respectively. All three show high similarity to the *mbtH* gene in the gene cluster for mycobactin, a non-ribosomally formed peptide siderophore from *Mycobacterium tuberculosis* (Quadri *et al.*, 1998). Genes with high similarity to *mbtH* have been identified in many

Abbreviation: CDA, calcium-dependent antibiotic.

A table of PCR primers used in this study is available as supplementary data with the online version of this paper.

Fig. 1. (a) Chemical structures of the aminocoumarin antibiotics clorobiocin, coumermycin A₁, novobiocin and simocyclinone D8. The characteristic 3-amino-4,7-dihydroxycoumarin moieties are highlighted. (b) Organization of genes for the biosynthesis of the aminocoumarin moiety (depicted in grey) within the gene clusters of clorobiocin (clo), simocyclinone D8 (sim), coumermycin A₁ (cou) and novobiocin (nov).

biosynthetic gene clusters for antibiotics and siderophores from bacteria. However, the function of these genes is unknown, and a recent publication on the gene cluster for the siderophore ornibactin aptly concluded that ‘the role of the *mbtH*-like proteins remains a mystery’ (Agnoli *et al.*, 2006).

In a study using transposon mutagenesis in the biosynthetic gene cluster of the siderophore vicibactin in *Rhizobium leguminosarum*, insertion mutants of the *mbtH*-like gene *vbsG* were obtained. *vbsG* is the first gene of an operon which also comprises the NRPS gene *vbsS* and the mono-oxygenase gene *vbsO*. The latter enzyme has been suggested to hydroxylate ornithine to *N*⁵-hydroxyornithine (Carter *et al.*, 2002). Vicibactin formation was abolished in the *vbsG* insertion mutant, but since the transposon insertion may also have affected the expression of *vbsS* and *vbsO*, this does not represent unequivocal proof for an essential function of *vbsG* in vicibactin biosynthesis.

Only a single experimental study has specifically addressed the function of an *mbtH*-like gene: Stegmann *et al.* (2006)

recently reported the inactivation of the *mbtH*-like gene ORF1 from the biosynthetic gene cluster of the glycopeptide antibiotic balhimycin. The inactivation had no effect on balhimycin biosynthesis, and the authors concluded that ‘it can be assumed that MbtH-like proteins do not have an essential role in the specific secondary metabolite biosynthesis’. However, they also mentioned the possibility that *mbtH*-like genes situated elsewhere in the genome may take over the role of ORF1 in balhimycin biosynthesis.

We speculated that the strict conservation of *mbtH*-like genes throughout many secondary metabolic gene clusters of different bacterial taxa may suggest the existence of an evolutionary pressure favouring the maintenance of this ORF, and we therefore decided to investigate the importance of *cloY* for clorobiocin biosynthesis experimentally.

We have developed methods for the heterologous expression of the clorobiocin biosynthetic gene cluster in *Streptomyces coelicolor* M512 (Eustáquio *et al.*, 2005a). These methods not only allow the convenient investigation of gene replacements or in-frame deletions within the

biosynthetic gene cluster, but also the expression of the modified clusters in a completely sequenced host and the rapid modification of any gene of interest in the host genome. Using these methods, we could now show that *cloY* is indeed required for efficient clorobiocin biosynthesis, specifically for the formation of the aminocoumarin moiety, and that it can be functionally replaced by various other *mbtH*-like genes. This is the first study proving the functional importance of members of the *mbtH*-like gene family.

METHODS

Strains and culture conditions. *S. coelicolor* M512 ($\Delta redD \Delta actII$ -ORF4 SCP1[−] SCP2[−]) was kindly provided by E. Takano (Gröningen, The Netherlands) and J. White (Norwich, UK), and routinely cultured in 50 ml Trypticase Soy Broth (30 g l^{−1}) at 30 °C and 200 r.p.m. for 2 days. For the production of clorobiocin and novobiocin, a 3 ml sample from TSB cultures was precultured in

300 ml baffled flasks containing 50 ml corn starch medium (Kieser *et al.*, 2000). After growth for 2 days at 30 °C and 180 r.p.m., 5 ml of the preculture was inoculated into 300 ml baffled flasks containing 50 ml production medium (Eustáquio *et al.*, 2005a) and cultivated at 30 °C and 180 r.p.m. for 7 days.

For feeding experiments, 2 mg 3-amino-4,7-dihydroxy-8-methylcoumarin dissolved in 100 % ethanol was added to 50 ml cultures 2 days and again 4 days after inoculation. After cultivation for a further 3 days, cultures were harvested and analysed for secondary metabolite formation.

Escherichia coli strains, as listed in Table 1, were cultured in liquid or on solid Luria–Bertani medium at 37 °C (Sambrook & Russell, 2001). *E. coli* BW25113 carrying the temperature-sensitive recombination plasmid pIJ790 was cultivated in SOB medium at 30 °C (Gust *et al.*, 2004).

Kanamycin (50 µg ml^{−1}), chloramphenicol (25 µg ml^{−1}), apramycin (50 µg ml^{−1}), thiostrepton (8 µg ml^{−1}), carbenicillin (100 µg ml^{−1}) and viomycin (30 µg ml^{−1}) (all from Merck, except viomycin from the John Innes Centre, Norwich, UK) were used for selection of recombinant strains.

Table 1. Plasmids, cosmids and strains used in this study

Plasmid/cosmid/strain	Relevant genotype/comments*	Source/reference
Plasmids		
pIJ773	<i>aac(3)IV</i> (Am ^R) + <i>oriT</i>	Gust <i>et al.</i> (2003)
pIJ780	<i>vph</i> from Ω fragment (Vm ^R) + <i>oriT</i>	Gust <i>et al.</i> (2003)
pIJ790	λ RED (<i>gam bet exo cat</i>) (Cm ^R) <i>araC rep101</i> ^{ts}	Gust <i>et al.</i> (2003)
pUG019	<i>aac(3)IV</i> (Am ^R)	Eustáquio <i>et al.</i> (2005a)
pUZ8002	<i>tra neo</i> (Km ^R) RP4	Paget <i>et al.</i> (1999)
pUWL201	<i>trs</i> (Ts ^R) <i>bla</i> (Cb ^R) <i>PerME colE1</i>	Doumith <i>et al.</i> (2000)
pMW01	pUWL201 with <i>cloY</i> under control of <i>PerME</i>	This study
pMW02	pUWL201 with <i>cchK</i> under control of <i>PerME</i>	This study
pMW03	pUWL201 with CDA-ORFX under control of <i>PerME</i>	This study
pMW04	pUWL201 with <i>couY</i> under control of <i>PerME</i>	This study
SuperCos-1	Cosmid vector: <i>bla</i> (Cb ^R) <i>neo</i> (Km ^R) <i>cos</i>	Stratagene
Cosmids		
novBG01	Integrative cosmid containing complete novobiocin gene cluster: <i>neo</i> (Km ^R) <i>bla</i> (Cb ^R) <i>attP int tet oriT</i>	Eustáquio <i>et al.</i> (2005a)
cloBG01	Integrative cosmid containing complete clorobiocin gene cluster: <i>neo</i> (Km ^R) <i>bla</i> (Cb ^R) <i>attP int tet oriT</i>	Eustáquio <i>et al.</i> (2005a)
cloMW15	cloBG01 with <i>cloY</i> replaced by <i>aac(3)IV</i> (Am ^R)	This study
cloMW16	cloBG01 with in-frame deletion of <i>cloY</i>	This study
StE8	Cosmid containing <i>S. coelicolor</i> DNA comprising SCO03218 (CDA-ORFX)	Redenbach <i>et al.</i> (1996)
StF76	Cosmid containing <i>S. coelicolor</i> DNA comprising SCO0489 (<i>cchK</i>)	Redenbach <i>et al.</i> (1996)
StE8-vio ^R	StE8 with SCO3218 replaced by <i>vph</i> (Vm ^R)	This study
StF76-apra ^R	StF76 with SCO0489 replaced by <i>aac(3)IV</i> (Am ^R)	This study
<i>E. coli</i>		
BW25113	K12 derivative: $\Delta araBAD \Delta rhaBAD$	Datsenko & Wanner (2000)
ET12567	<i>dam dcm hsdS cat</i> (Cm ^R) <i>tet</i> (Tc ^R)	MacNeil <i>et al.</i> (1992)
DH5 α	<i>recA1 endA1 relA1</i>	Stratagene
<i>S. coelicolor</i>		
M512	M145 derivative: $\Delta actII$ -ORF4 $\Delta redD$ SCP1 [−] SCP2 [−]	Bentley <i>et al.</i> (2002)
MW01	M512 with SCO3218 (CDA-ORFX) replaced by <i>vph</i> (Vm ^R)	This study
MW03	M512 with SCO3218 replaced by <i>vph</i> (Vm ^R) and with SCO0489 replaced by <i>aac(3)IV</i> (Am ^R)	This study

*Am, Apramycin; Cb, carbenicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; Ts, thiostrepton; Vm, viomycin.

DNA isolation, manipulation and cloning. Standard procedures for DNA isolation and manipulation were performed as described previously (Sambrook & Russell, 2001). Isolation of DNA fragments from agarose gels and purification of PCR products were carried out with the NucleoSpin 2 in 1 Extract Kit (Macherey–Nagel). Genomic DNA isolation from *Streptomyces* strains, protoplast transformation and regeneration were carried out according to the procedures described by Kieser *et al.* (2000). REDIRECT technology for PCR targeting was obtained from Plant Bioscience Limited and used for inactivation experiments according to the PCR targeting protocol (Gust *et al.*, 2003).

Generation of *mbtH* mutants

Construction of cloMW15 and cloMW16. cloBG01 is a SuperCos1-based cosmid containing the intact clorobiocin biosynthetic gene cluster and the *attP/int* cassette from the bacteriophage ΦC31 for stable integration into the *S. coelicolor* M512 host genome (Eustáquio *et al.*, 2005a) (Fig. 2a). An apramycin resistance cassette [*aac(3)IV*] was amplified from plasmid pUG019 (Eustáquio *et al.*, 2005a) using primers cloYF_Xba and cloYR_Spe (primer sequences are given in Table S1, available as supplementary data with the online version of this paper). The primers contained restriction sites for *Xba*I and *Spe*I for removal of the cassette. The resulting 1077 bp

Fig. 2. (a) Cosmid cloBG01 containing the intact clorobiocin cluster and the integration functions of phage ΦC31. Genes of the clorobiocin cluster are depicted in grey. (b) Inactivation of *cloY*. Cosmids cloBG01 and cloMW16 were introduced into the genome of the heterologous host *S. coelicolor* M512, using the ΦC31 attachment site.

PCR product was used to replace the *cloY* gene from cosmid cloBG01 by using λ RED-mediated recombination as described previously, resulting in cosmid cloMW15 (Gust *et al.*, 2003) (Fig. 2b).

Deletion of *aac(3)IV* from cloMW15 was carried out by restriction with *Xba*I and *Spe*I and religation, resulting in cosmid cloMW16 (Fig. 2b).

Deletion of the *mbtH*-like homologues in *S. coelicolor*. For deletion of the *mbtH* orthologues from *S. coelicolor*, a 1576 bp gene disruption cassette, containing the viomycin resistance marker *vph* and *oriT* for conjugal transfer into *Streptomyces* strains, was amplified from plasmid pIJ780 using primers CDA_Spe_F and CDA_Xba_R (Table S1). This fragment was used to replace the gene SCO3218 (CDA-ORFX) in cosmid StE8 (kanamycin-resistant) (Redenbach *et al.*, 1996) using the λ RED-mediated recombination system for PCR targeting (Gust *et al.*, 2003), resulting in cosmid StE8 Δ CDA-ORFX (Fig. 3a).

Likewise, a 1461 bp gene disruption cassette containing *aac(3)IV* and *oriT* was amplified from plasmid pIJ773 using primers cchK_Spe_F and cchK_Xba_R and used for replacement of SCO0489 from cosmid StF76 (Redenbach *et al.*, 1996) as described above, resulting in StF76 Δ cchK (Fig. 3b).

After electroporation of cosmid StE8 Δ CDA-ORFX into *E. coli* ET12567/pUZ8002 for conjugal transfer into *Streptomyces*, recombinant *E. coli* strains carrying StE8 Δ CDA-ORFX were selected using 30 mg viomycin l⁻¹. Conjugal transfer of the cosmid into *S. coelicolor* M512 and screening for double cross-over mutants (viomycin-resistant, kanamycin-sensitive) was carried out as described previously (Freitag *et al.*, 2004), resulting in the Δ CDA-ORFX mutant *S. coelicolor* MW01. Subsequently, cosmid StF76 Δ cchK was introduced into *S. coelicolor* MW01 by conjugation, using apramycin (50 μ g ml⁻¹) and viomycin (30 μ g ml⁻¹) for selection. Screening for double cross-over mutants (apramycin- and viomycin-resistant; kanamycin-sensitive) resulted in the Δ CDA-ORFX Δ cchK double mutant *S. coelicolor* MW03.

Construction of plasmids for complementation of *S. coelicolor* MW01 and *S. coelicolor* MW03. For complementation of the Δ *mbtH* *S. coelicolor* mutants, the genes *cloY*, SCO0489 (*cchK*), SCO3218 (CDA-ORFX) and *couY* were amplified by PCR using the primer pairs *cloY*_Hind_F and *cloY*_Spe_R, *cchK*_Hind_F and *cchK*_Spe_R, CDA_Hind_F and CDA_Spe_R, *couY*_Hind_F and *couY*_Spe_R, respectively (primer sequences are given in Table S1, available as supplementary data with the online version of this paper).

The PCR products were purified using the Nucleospin Kit (Macherey-Nagel), incubated with *Hind*III and *Spe*I, and subsequently ligated into the *Hind*III/*Spe*I sites of expression vector pUWL201 (Douthett *et al.*, 2000). This resulted in plasmids pMW01, pMW02, pMW03 and pMW04, respectively. DNA sequencing of these plasmids using the sequencing primer ermE_seq confirmed the correct sequence of all constructs. For protoplast transformation, the four plasmids were transferred into the non-methylating *E. coli* strain ET12567 and DNA was isolated by standard procedures after growth at 37 °C overnight. Transformation of *S. coelicolor* strains was carried out by polyethylene glycol-mediated protoplast transformation (Kieser *et al.*, 2000). Selection of recombinant strains was performed on DNA solid medium containing 8 μ g thiostrepton ml⁻¹.

Analysis of secondary metabolites. Standard procedures for isolation of clorobiocin and novobiocin were used as described previously (Eustáquio *et al.*, 2003a, 2005a). A 1 ml sample of bacterial culture from production medium was acidified to pH 4 with HCl and extracted twice with an equal volume of ethyl acetate. After centrifugation and separation of the organic phase, the solvent was evaporated and the residue was dissolved in 200 μ l methanol. Metabolites were analysed by HPLC using a Multisphere RP 18-5 column (250 $\times$ 4 mm, 5 μ m; C+S Chromatographie Service, Düren, Germany) at a flow rate of 1 ml min⁻¹ using a linear gradient from 40 to 100% of solvent B in solvent A over 37 min (solvent A, MeOH/H₂O/HCOOH, 50:49:1; solvent B, MeOH/HCOOH, 99:1) with detection for clorobiocin at 340 nm.

Fig. 3. Inactivation of *mbtH*-like genes. CDA-ORFX from cosmid StE8 (a) and *cchK* from cosmid StF76 (b), resulting in the viomycin-resistant cosmid StE8 Δ CDA-ORFX and the apramycin-resistant cosmid StF76 Δ cchK, respectively. Introduction of both cosmids by conjugal transfer into *S. coelicolor* M512 and screening for double cross-over mutants resulted in the viomycin and apramycin-resistant Δ CDA-ORFX Δ cchK double mutant strain *S. coelicolor* MW03.

For HPLC analysis of novobiocin 102, a linear gradient from 60 to 100 % of solvent B in solvent A (solvent A, H₂O/HCOOH, 99:1; solvent B, MeOH/HCOOH, 99:1) and detection at 305 nm were used (Eustáquio *et al.*, 2003b). Authentic clorobiocin or novobiocin (Aventis) was used as the standard.

RESULTS

Deletion of *cloY* from the clorobiocin cluster

To determine whether *cloY* is essential for clorobiocin biosynthesis, we carried out an inactivation experiment. Clorobiocin can be produced by heterologous expression of the clorobiocin biosynthetic gene cluster in *S. coelicolor* M512, using the integrative cosmid cloBG01 (Eustáquio *et al.*, 2005a). For the inactivation of *cloY*, an apramycin resistance cassette was amplified by PCR and used to replace the entire ORF of *cloY* in cosmid cloBG01, resulting in cosmid cloMW15 (Fig. 2b).

To avoid a possible polar effect, the cassette was removed by *Xba*I/*Spe*I digestion and religation as described previously (Eustáquio *et al.*, 2005a). As shown in Fig. 2(b), the resulting cosmid, cloMW16, carries only a minimal 18 bp 'scar' sequence, situated in between the original start and stop codon of *cloY*.

The *cloY* replacements within cloMW15 and cloMW16 were verified both by restriction analysis and PCR. Transfer of cloMW16 into *S. coelicolor* M512 was carried out by conjugation. Three individual *Streptomyces* exconjugants were selected, and the integration of cloMW16 was verified by PCR with chromosomal DNA.

Subsequently, both *S. coelicolor* (cloBG01) and the Δ *cloY* strain *S. coelicolor* (cloMW16) were cultured and analysed for clorobiocin formation by HPLC (Fig. 4a). The strain carrying the intact cluster produced 54 mg clorobiocin l⁻¹, while the *cloY* deletion mutants produced on average 12 mg l⁻¹.

Previous inactivation experiments within the aminocoumarin biosynthetic gene clusters, deleting either biosynthetic or regulatory genes, had always resulted in complete or at least near-complete abolition of antibiotic production (Eustáquio *et al.*, 2005b; Li & Heide, 2005). The present result, however, was remarkably inconclusive. It neither proved that *cloY* was required nor that it was unnecessary for clorobiocin production. Therefore, we considered the hypothesis that, after deletion of *cloY*, this gene may have been functionally replaced by other genes contained in the genome of the producer strain. For an investigation of this hypothesis, our heterologous expression system offers a distinct advantage: the complete genome sequence of *S. coelicolor* has been determined (Bentley *et al.*, 2002) and very efficient methods for the genetic manipulation of this strain are available.

Deletion of *mbtH* homologues in *S. coelicolor*

An analysis of the *Streptomyces coelicolor* genome sequence revealed two ORFs that show high similarity to *cloY*, i.e.

SCO3218 (CDA-ORFX) from the calcium-dependent antibiotic (CDA) gene cluster (Hopwood & Wright, 1983) and SCO0489 (*cchK*) from the coelichelin gene cluster (Challis & Ravel, 2000). These two genes show 68 and 62 % identity to *cloY* at the amino acid level, respectively. Both CDA-ORFX and *cchK* are annotated in the database as *mbtH*-like genes, but the function of these genes is unknown. CDA-ORFX was inactivated in *S. coelicolor* M512 (see Methods), resulting in the desired Δ CDA-ORFX mutant *S. coelicolor* MW01. Three individual recombinants were isolated and the correct genotype was verified by PCR in all of them. Subsequently, *cchK* was inactivated in *S. coelicolor* MW01, resulting in the Δ CDA-ORFX Δ *cchK* double mutant MW03. The absence of the two *mbtH*-like genes in *S. coelicolor* MW03 was confirmed by PCR.

Cosmid cloMW16 was introduced into *S. coelicolor* MW03, resulting in the triple mutant *S. coelicolor* MW03(cloMW16), an integration mutant containing the Δ *cloY* clorobiocin cluster in a host lacking all *mbtH* homologues. Three individual mutants of this strain were cultivated in clorobiocin production medium and secondary metabolite formation was analysed by HPLC. All Δ *mbtH* triple mutants showed essentially negligible amounts of clorobiocin (Fig. 4a). While *S. coelicolor* M512(cloBG01) produced approximately 50 mg clorobiocin l⁻¹, the Δ *mbtH* triple mutants produced a mean of only 0.4 mg l⁻¹. This demonstrated that deletion of all three *mbtH*-like genes from this heterologous clorobiocin producer reduced clorobiocin production by 99 % in comparison to the reference strain.

Complementation of the Δ *mbtH* triple mutant with *cloY*, CDA-ORFX and *cchK*

To prove that the observed reduction of clorobiocin biosynthesis was indeed due to the inactivation of the three *mbtH*-like genes *cloY*, *cchK* and CDA-ORFX, we complemented the Δ *mbtH* triple mutant by expression of intact copies of these three genes, each cloned into the expression vector pUWL201 (Doumith *et al.*, 2000) (see Methods). The three plasmids were introduced into *S. coelicolor* MW03(cloMW16) and three independent transformants were obtained for each construct. Cultivation of the transformants in clorobiocin production medium and analysis of secondary metabolites by HPLC analysis showed that clorobiocin production was restored in all strains, reaching 8 mg l⁻¹ with the *cloY* expression plasmid, 4.1 mg l⁻¹ with the *cchK* plasmid and 5.1 mg l⁻¹ with the CDA-ORFX plasmid (Fig. 4b). In contrast, a control strain transformed with the empty vector pUWL201 produced <0.5 mg l⁻¹.

Therefore, complementation of the Δ *mbtH* triple mutant with each of the three *mbtH* homologues resulted in an at least 10-fold increase of clorobiocin production in the Δ *mbtH* triple mutant, showing for the first time that *mbtH*-like genes from different clusters and organisms can functionally replace each other. However, complementation did not fully restore clorobiocin production to the original

Fig. 4. (a) HPLC analysis of clorobiocin production in different *S. coelicolor* mutants: *S. coelicolor* M512(cloBG01) carrying the intact clorobiocin cluster; *S. coelicolor* M512(cloMW16) carrying the *cloY*-deficient clorobiocin cluster; *S. coelicolor* MW03(cloMW16) carrying the *cloY*-deficient clorobiocin cluster in an *mbtH*-deficient host strain. (b) HPLC analysis of clorobiocin production in the *mbtH*-triple mutant *S. coelicolor* MW03(cloMW16) complemented with *cloY*, *cchK*, CDA-ORFX or *couY*. As observed previously (Eustáquio *et al.*, 2005a), clorobiocin ($R_t = 20.6$ min) is accompanied by structural analogues, mainly the non-chlorinated compound novclorobiocin 101 ($R_t = 20.1$ min) and isoclорobiocin ($R_t = 22.7$ min). The ratio between clorobiocin and novclorobiocin 101 varied between different culture batches of the same strain.

level. This may be attributed to an inappropriate regulation of the transcription and translation of the *mbtH*-like genes from the multicopy vectors with constitutive promoters.

Complementation with *couY* from the coumermycin gene cluster

The *cloY* gene from the clorobiocin gene cluster shows 81 % identity at the amino acid level to *couY* from the gene cluster

for the aminocoumarin coumermycin A₁ (Fig. 1). To prove whether *couY* can also complement the $\Delta mbtH$ triple mutant, we cloned *couY* into the expression vector pUWL201. After introduction of the resulting plasmid into *S. coelicolor* MW03(cloMW16) three independent transformants were selected and examined for clorobiocin production. Again, clorobiocin production was restored, reaching a level of 5.3 mg l^{-1} (Fig. 4b). Therefore, also *couY* can functionally replace *cloY* in clorobiocin biosynthesis.

Proof of the involvement of *cloY* in the formation of the 3-amino-4,7-dihydroxycoumarin moiety

The experiments described above have shown that *cloY* is required for clorobiocin biosynthesis and that it can be functionally replaced by other *mbtH* orthologues. This raises the question whether it acts as a global regulator for the biosynthesis of clorobiocin, or whether it has a catalytic, regulatory or other function for the formation of a specific moiety of this antibiotic.

Since in coumermycin A₁ and simocyclinone D8 gene clusters *cloY* and its orthologues *couY* and *simY* are localized in close proximity to the genes of 3-amino-4,7-dihydroxycoumarin formation (Fig. 1), we speculated that the function of *cloY* may be related to the formation of this moiety. To test this hypothesis, we decided to feed the $\Delta mbtH$ triple mutant, which carries the *cloY*-deficient clorobiocin cluster in a completely *mbtH*-deficient host strain, with the aminocoumarin moiety of novobiocin. This moiety carries a methyl group instead of a chlorine atom at position 8 of the 3-amino-4,7-dihydroxycoumarin molecule (Fig. 1). Previous experiments had shown that this compound was readily accepted by the enzymes of clorobiocin biosynthesis, resulting in a structural analogue of clorobiocin, i.e. novclobiocin 102, which differs from clorobiocin only by the methyl group instead of the chlorine (Eustáquio *et al.*, 2003a, 2004).

A total of 2 mg 8-methyl-3-amino-4,7-dihydroxycoumarin was fed to a 50 ml culture of the $\Delta mbtH$ triple mutant *S. coelicolor* MW03(cloMW16) (see Methods). After cultivation for 7 days, secondary metabolite formation was analysed by HPLC and HPLC-MS. Without feeding, very low formation of clorobiocin was observed again (0.4 mg l⁻¹). After feeding, however, an accumulation of novclobiocin 102 was clearly observed, reaching 5.2 mg l⁻¹. The identity of this compound was confirmed by LC-MS analysis in comparison with an authentic reference sample. Therefore, our experiment showed that deletion of *mbtH* specifically affected the formation of the aminocoumarin moiety of clorobiocin, while the biosynthesis of the other structural moieties and their linkage reactions were still possible in the $\Delta mbtH$ triple mutant.

Heterologous expression of the novobiocin biosynthetic gene cluster in the $\Delta mbtH$ double mutant

The biosynthetic gene clusters of clorobiocin, coumermycin A₁ and simocyclinone D8 contain an *mbtH*-like gene (i.e. *cloY*, *couY* and *simY*, respectively), but no *mbtH*-like gene is found in the novobiocin gene cluster (Fig. 1). Novobiocin can be produced by heterologous expression of the novobiocin gene cluster in *Streptomyces coelicolor* M512, using the integrative cosmid novBG01 (Eustáquio *et al.*, 2005a). However, given our observation that *cloY* was required for clorobiocin biosynthesis, this raised the

question whether novobiocin biosynthesis really proceeds without involvement of an *mbtH* homologue, or whether *mbtH*-like genes encoded in the host genome cooperate with this process. To investigate this question we introduced the integrative cosmid novBG01 (Eustáquio *et al.*, 2005a) by site-specific integration into the $\Delta mbtH$ -double mutant strain *S. coelicolor* MW03. Four independent integration mutants were selected for further investigation, and the presence of the gene cluster for novobiocin biosynthesis was confirmed by PCR.

The *mbtH*⁺ strain *S. coelicolor* M512(novBG01) and the four $\Delta mbtH$ double mutant strains of *S. coelicolor* MW03-(novBG01) were cultured as described above and analysed by HPLC, using novobiocin as standard. The $\Delta mbtH$ double mutants containing the novobiocin gene cluster still produced novobiocin (5.7 mg l⁻¹), i.e. approximately 50% of the amounts found in the *mbtH*⁺ strain *S. coelicolor* M512(novBG01) (12.5 mg l⁻¹). These results show that novobiocin biosynthesis can take place even in the absence of *mbtH*-like genes, although it appears to be enhanced by the presence of an intact copy of *cchK* or CDA-ORFX.

DISCUSSION

The gene *cloY* of the clorobiocin biosynthetic gene cluster is a member of the family of *mbtH*-like genes, which consists of at least 106 entries in the database (Stegmann *et al.*, 2006). They are found in the biosynthetic gene clusters of peptide and aminocoumarin antibiotics and of siderophores from different bacterial taxa. Fig. 5 shows an alignment of the predicted amino acid sequences of 24 *MbtH*-like proteins from the gene clusters of well characterized compounds, including peptide and glycopeptide antibiotics, phosphonotricin, enterochelin, mycobactin and aminocoumarins. While *mbtH* genes mostly represent separate ORFs, there are three notable exceptions: the N terminus of the NikP1 protein of nikkomycin biosynthesis has obvious sequence similarity to *MbtH*-like proteins (Fig. 5). The enzyme NikP1 activates histidine by thioester formation, which is followed by β -hydroxylation of the amino acid under catalysis of the cytochrome P₄₅₀ enzyme NikQ (Chen *et al.*, 2002). The reaction sequence catalysed by NikP1 and NikQ, i.e. activation and β -hydroxylation of histidine, is very similar to the reactions catalysed by CloH and CloI in clorobiocin biosynthesis, i.e. activation and β -hydroxylation of tyrosine (Chen & Walsh, 2001). The second example where *MbtH* forms the N-terminal part of a larger protein is LtxB from lyngbyatoxin biosynthesis (Edwards & Gerwick, 2004). The predicted amino acid sequence of LtxB shows similarity to cytochrome P₄₅₀ enzymes. This protein has been suggested to catalyse an epoxidation of tryptophan during the non-ribosomal biosynthesis of the dipeptide lyngbyatoxin. The third example is the *mbtH* orthologue from the bleomycin cluster, but no functional data are available on this gene.

The alignment of the amino acid sequences predicted from *mbtH*-like genes, shown in Fig. 5, shows a remarkable

Fig. 5. Alignment of the predicted amino acid sequences from *mbtH*-like genes found in the biosynthetic gene clusters of different antibiotics and siderophores. *cloY*, clorobiocin (Pojer *et al.*, 2002); *couY*, coumermycin A₁ (Wang *et al.*, 2000); *simY*, simocyclinone (Galm *et al.*, 2002); *cchK*, coelichelin (Challis & Ravel, 2000); *ORFX*, CDA (Bentley *et al.*, 2002); *ORF1/van*, vancomycin (Zerbe *et al.*, 2002); *ORF1/bal*, balhimycin (Stegmann *et al.*, 2006); *ORF7/comE*, complestatin (Chiu *et al.*, 2001); *tcp13*, teicoplanin (Sosio *et al.*, 2000); *tcp17*, teicoplanin (Sosio *et al.*, 2000); *dbv15*, glycolipid antibiotic A40926 (Sosio *et al.*, 2003); *dptG*, daptomycin (Miao *et al.*, 2005); *mppT*, mannopeptimycin (Magarvey *et al.*, 2006); *lptG*, lipopeptide antibiotic A54145 (Miao *et al.*, 2006); *mbtH/myc*, mycobactin (Cole *et al.*, 1998); *nikP1**, nikkomycin (Wang *et al.*, 2003); *ltxB**, lyngbyatoxin (Edwards & Gerwick, 2004); *bleo**, bleomycin (Shen *et al.*, 2002); *nocI*, nocardicin (Gunsior *et al.*, 2004); *vbsG*, vicibactin (Carter *et al.*, 2002); *phpA*, phosphinotricin tripeptide (Blodgett *et al.*, 2005); *vioN*, viomycin (Thomas *et al.*, 2003); *ybdZ/ent*, enterobactin (Bentley *et al.*, 2002); *tioT*, thiocoraline (Lombo *et al.*, 2006). *For the proteins NikP1 (677aa), LtxB (460 aa) and Bleo (187 aa), only the *mbtH*-like region is depicted. The numbers on the right indicate the number of amino acids encoded by the ORF.

sequence conservation, most obvious from the three conserved tryptophan residues. In many predicted proteins there is a conservation of the sequence X₂₋₄NPF(D/E)(D/N)X₂GX(F/Y)LVLVNEXQHSLWPXFAXVPXGW(T/R)-X₇RX(D/E)CLX(Y/F)V(E/N)X₂WTDXRPSLX₂₋₁₂, or of similar amino acids. This strongly indicates the existence of an evolutionary pressure which favours the maintenance of this sequence, suggesting a definite function of the *mbtH*-like genes. But at present, neither database comparisons nor experimental data can suggest what this function may be. As for aminocoumarin antibiotic biosynthesis, the different enzymic steps for enterochelin formation (Crosa & Walsh, 2002), phosphinotricin formation (Schwartz *et al.*, 2004) and glycopeptide antibiotic formation (Sosio *et al.*, 2003) have been investigated in considerable detail *in vitro*, without revealing an essential catalytic function which may be assigned to the MbtH proteins encoded in the respective clusters. Therefore, it has been speculated that MbtH-like proteins may be involved in regulatory or transport processes (Yeats *et al.*, 2003) or in protein-protein interactions (Stegmann *et al.*, 2006), but no experimental support has been provided for these hypotheses.

The biosynthetic pathways of all compounds mentioned in the alignment in Fig. 5 involve the activation of amino acids, or of aromatic acids, by thioester formation with the 4'-phosphopantetheinyl cofactor of an enzyme, often followed by chemical modification of the activated acid by other enzymes and its subsequent transfer to amino or hydroxyl groups, forming peptide or ester bonds. Clorobiocin biosynthesis comprises two such reaction sequences: the activation and β -hydroxylation of tyrosine by CloH and CloI, which are the first steps of the biosynthesis of the aminocoumarin moiety (Chen *et al.*, 2002; Pacholec *et al.*, 2005), and the activation of proline and its subsequent oxidation to pyrrole-2-carboxyl-S-CloN5, catalysed by CloN3, CloN4 and CloN5 (Garneau *et al.*, 2005). Our study showed that feeding of an intact aminocoumarin moiety to a heterologous clorobiocin producer strain, defective in all three *mbtH*-like genes, restored formation of the antibiotic to a similar level to that obtained by complementation with *cloY* or other *mbtH* orthologues. This indicates that, except for the formation of the aminocoumarin moiety, all other biosynthetic steps were intact in the *mbtH*-defective mutant, including biosynthesis

and transfer of the pyrrole-2-carboxyl moiety. In contrast, earlier inactivation experiments of genes involved in the biosynthesis and transfer of the pyrrole-2-carboxyl moiety invariably led to strains accumulating novobiocin 102, i.e. a clorobiocin derivative lacking that moiety (Freitag *et al.*, 2005; Xu *et al.*, 2003). Therefore, we can conclude that *cloY* is required for the efficient formation of the aminocoumarin moiety of clorobiocin rather than for the formation or transfer of the pyrrole-2-carboxyl moiety. However, we cannot define whether *cloY* is involved in catalysis, in protein–protein interaction or in the regulation of transcription and translation.

The inactivation of *cloY* and two other *mbtH*-like genes in a heterologous producer of clorobiocin dramatically reduced clorobiocin formation. In contrast, Stegmann *et al.* (2006) found that inactivation of the *mbtH*-like gene ORF1 did not impair balhimycin biosynthesis. However, the authors mentioned that the genome of the balhimycin producer did contain at least two further *mbtH*-like genes, and they could not exclude the possibility that these genes may functionally replace ORF1 in their mutant. Our present results on clorobiocin biosynthesis suggests that different *mbtH*-like genes can functionally replace each other. Therefore, it will be necessary to inactivate all *mbtH*-like genes in the balhimycin producer strain before a conclusion can be drawn whether or not these genes are required for balhimycin biosynthesis. Any further investigation on the role of *mbtH*-like genes *in vivo* must consider the contribution of all copies of this gene family in the genome and should therefore be carried out only in completely sequenced strains.

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