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Acyl Transfer in Clorobiocin Biosynthesis: Involvement of Several Proteins in the Transfer of the Pyrrole-2-carboxyl Moiety to the Deoxysugar

Anja Freitag, Emmanuel Wemakor, Shu-Ming Li,* and Lutz Heide*[a]

Clorobiocin is an aminocoumarin antibiotic containing a pyrrole-2-carboxyl moiety, attached through an ester bond to a deoxysugar. The pyrrole moiety is important for the binding of the antibiotic to its biological target, gyrase. The complete biosynthetic gene cluster for clorobiocin has been cloned and sequenced from the natural producer, Streptomyces roseochromogenes *DS 12.976. In this study, the genes* cloN1 *and* cloN7 *were deleted separately from a cosmid containing the complete clorobiocin cluster. The modified cosmids were introduced into the genome of the heterologous host* Streptomyces coelicolor *M512 by using the integration functions of the ΦC31 phage. While a heterologous producer strain harbouring the intact clorobiocin biosynthetic gene cluster accumulated clorobiocin, the* cloN1- *and* cloN7*-defective integration mutants accumulated a clorobiocin derivative that lacked the pyrrole-2-carboxyl moiety, while also producing free pyrrole-2-carboxylic acid. The structures of these metabolites were confirmed by NMR and MS analysis. These results showed that CloN1 and CloN7, together with the previously investigated CloN2, are involved in the transfer of the pyrrole-2-carboxyl moiety to the deoxysugar of clorobiocin. A possible mechanism for the role of these three proteins in the acyl-transfer process is suggested.*

Introduction

The structurally related aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 are potent inhibitors of bacterial gyrase[1] and represent interesting starting compounds for the development of new antibacterial agents. X-ray crystallographic analysis has shown that the acyl moieties at the 3''-hydroxy groups of the deoxysugars of these antibiotics—the carbamoyl group in novobiocin and the 5-methylpyrrole-2-carboxyl moiety in clorobiocin and coumermycin A_1—are particularly important for the binding of these antibiotics to the biological target: the B subunit of gyrase.[2,3]

In the last few years we have cloned, sequenced and analysed the biosynthetic gene clusters of novobiocin,[4] clorobiocin[5] and coumermycin A_1.[6] Orthologous genes in these three clusters show high sequence similarity to each other (70–90% identity at the amino acid level), and the genes in all three clusters are organized in a remarkably similar order.[7] Heterologous expression experiments[8] confirmed that the DNA regions from *novE* to *gyrB*^{R} or from *cloE* to *parY*^{R} (Figures 1 and 2) contain the entire biosynthetic information for the formation of novobiocin or clorobiocin, respectively.

The clorobiocin cluster (Figures 1 and 2) contains a total of 29 open reading frames. Experimental investigations, carried out mostly with genes of the clorobiocin cluster and in some cases with the orthologous genes of the novobiocin or coumermycin cluster, have established the functions of most of these genes (Scheme 1): *cloSTUVW* are involved in the formation of the deoxysugar moiety of the antibiotic,[4,9] *cloHI*[10,11] and probably *cloJK*[12] are involved in the formation of the aminocoumarin ring, while *clo-hal* codes for a halogenase that attaches the chlorine atom to the aminocoumarin moiety,[13] and *cloQR*, and probably *cloF*, are responsible for the formation of the 3-dimethylallyl-4-hydroxybenzoic acid moiety,[11,14] which is subsequently attached to the aminocoumarin ring in a process catalysed by the amide synthethase encoded by *cloL*.[15,16] The glycosyl transferase encoded by *cloM* transfers the deoxysugar to the 7-OH group of the aminocoumarin ring,[17,18] the pyrrole-2-carboxyl moiety is formed from proline in a process catalysed by the gene products of *cloN3*, *cloN4* and *cloN5*,[19,20] and the gene product of *cloN2* is involved in the transfer of this moiety to the deoxysugar.[21] The 5-position in the pyrrole moiety is subsequently methylated with involvement of the gene product of *cloN6*.[22] The *cloP* gene codes for a methyltransferase responsible for the methylation of the 4-OH group of the deoxysugar,[23] *cloG*[24] and probably *cloE*[25] are regulatory genes, while *gyrB*^{R} and *parY*^{R} provide resistance to the producing organism against the antibiotic.[26] An inactivation experiment showed that *cloZ*, which has no orthologue in the novobiocin or coumermycin clusters, is not required for clorobiocin biosynthesis.[13]

[a] *Dipl. Biochem. A. Freitag, E. Wemakor, Priv. Doz. Dr. S.-M. Li, Prof. L. Heide*
Pharmazeutische Biologie, Pharmazeutisches Institut
Eberhard-Karls-Universität Tübingen
Auf der Morgenstelle 8, 72076 Tübingen (Germany)
Fax: (+49) 7071-295-250
E-mail: shuming.li@uni-tuebingen.de
heide@uni-tuebingen.de

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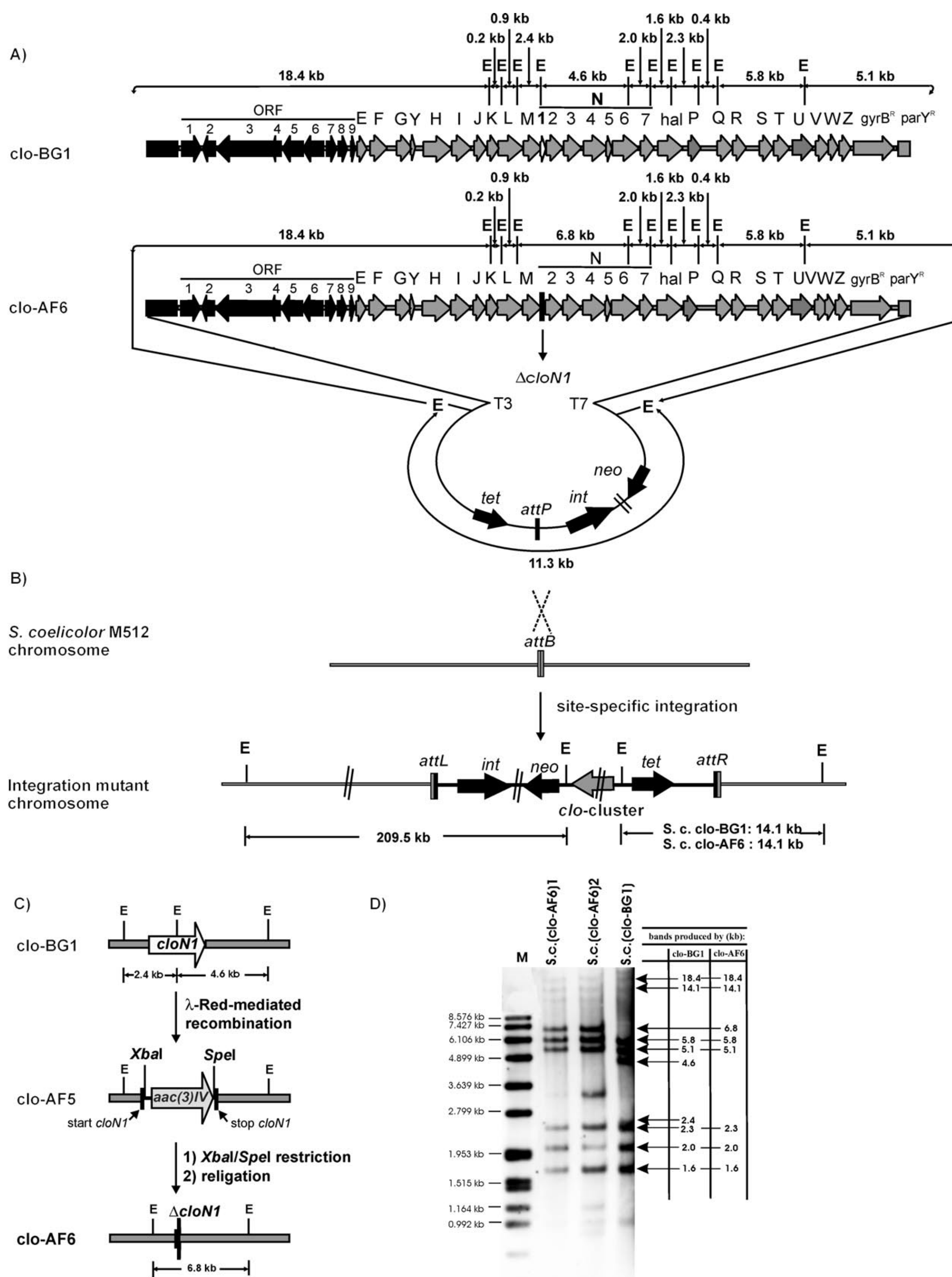

Figure 1. A) Cosmid constructs clo-BG1 (intact) and clo-AF6 (*cloN1*$^{-}$) containing the ΦC31 integration functions and the clorobiocin biosynthetic gene cluster. B) Schematic representation of site-specific integration of clo-AF6 into the *S. coelicolor* chromosome. E = EcoRI restriction site. T3, T7 = T3 and T7 promoters of the SuperCos1 vector. Fragment sizes resulting from digestion with EcoRI are indicated. Cosmid backbone out of scale. See ref. [44] for details of the integration mechanism. C) Detail of the replacement of *cloN1* by an apramycin resistance gene, flanked by XbaI and SpeI recognition sites. D) Southern blot analysis of *S. coelicolor* M512 integration mutant harbouring clo-AF6. M = DIG-labelled DNA Molecular Weight Marker VII (Roche). Genomic and cosmid DNA were digested with EcoRI. The DIG-labelled cosmid clo-BG1 was used as probe.

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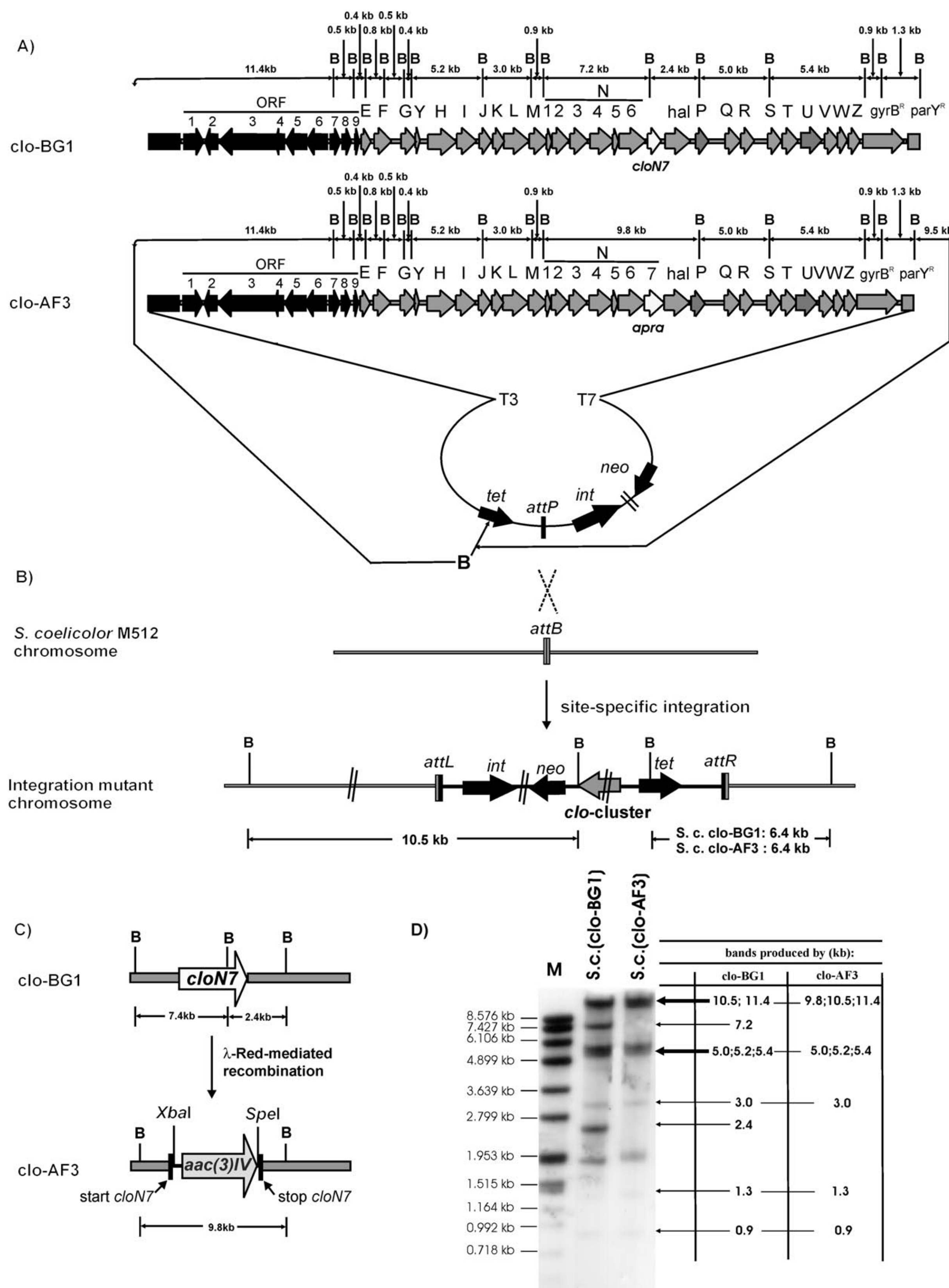

Figure 2. A) Cosmid constructs clo-BG1 (intact) and clo-AF3 (*cloN7*⁻) containing the ΦC31 integration functions and the clorobiocin biosynthetic gene cluster. B) Schematic representation of site-specific integration of clo-AF3 into the *S. coelicolor* chromosome. B = BamHI restriction site. T3, T7 = T3 and T7 promoters of the SuperCos1 vector. Fragment sizes resulting from digestion with BamHI are indicated. Cosmid backbone out of scale. C) Deletion of the *cloN7* gene by use of an apramycin resistance cassette containing flanking XbaI and SpeI recognition sites. D) Southern blot analysis of *S. coelicolor* M512 integration mutant harbouring clo-AF3. M = DIG-labelled DNA Molecular Weight Marker VII (Roche). Genomic and cosmid DNA were digested with BamHI. The DIG-labelled cosmid clo-BG1 was used as probe.

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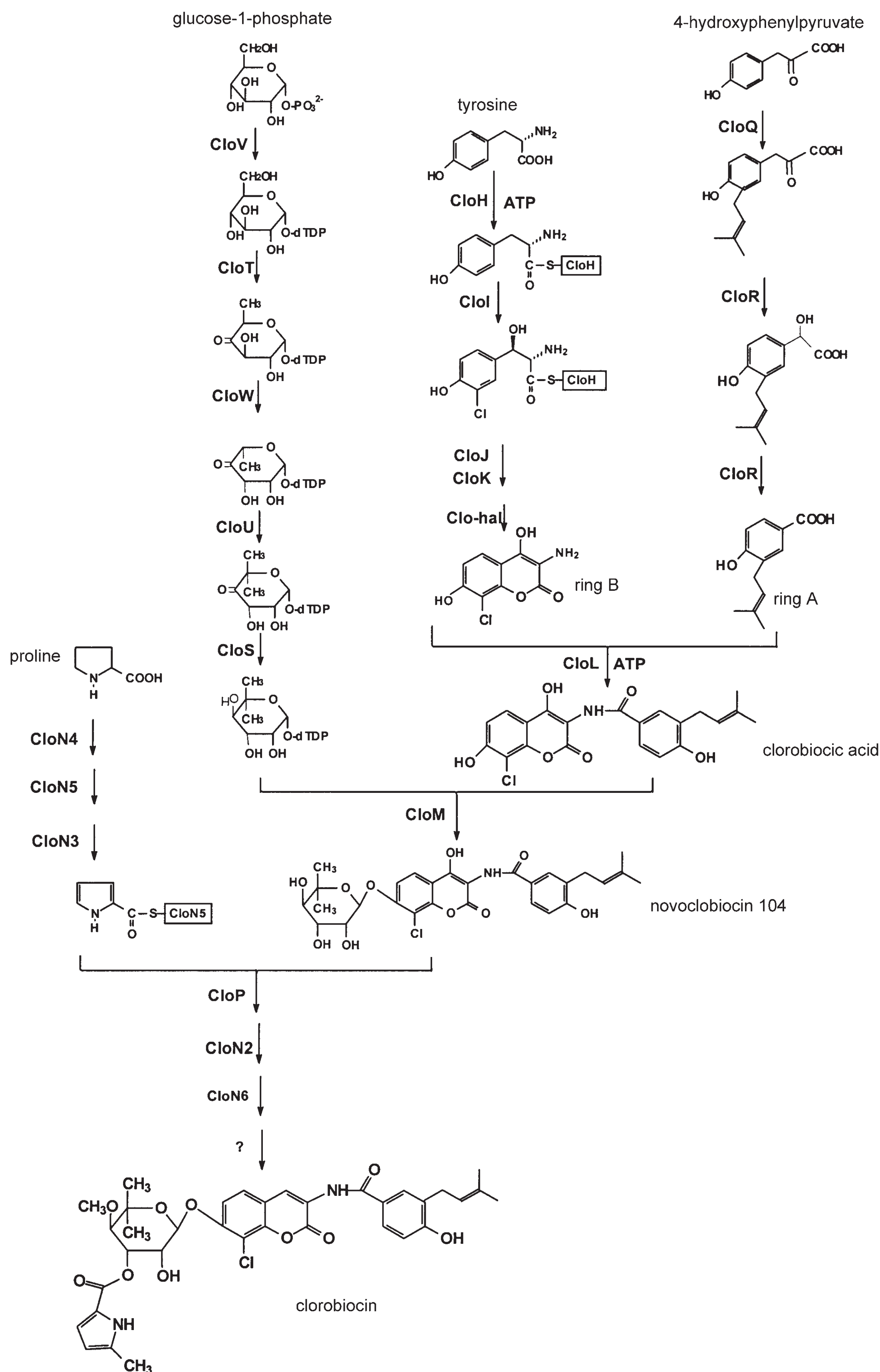

Scheme 1. Biosynthetic pathway of clorobiocin.

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This leaves only three ORFs for which no function has so far been suggested in the clorobiocin cluster: *cloY*, *cloN1* and *cloN7*. The aim of this study was the investigation of the functions of *cloN1* and *cloN7*. For this purpose we carried out gene inactivation experiments by deleting these genes from a cosmid containing, besides the complete biosynthetic gene cluster of clorobiocin, the integrase gene and the *attP* site of the bacteriophage ΦC31. The modified cosmids were introduced into the genome of the heterologous host *Streptomyces coelicolor*.[8] The secondary metabolites of the resulting *cloN1*$^-$ and *cloN7*$^-$ integration mutants were isolated and their structures were elucidated by spectroscopic methods. The result showed that *cloN1* and *cloN7*—in addition to *cloN2*—are also required for the transfer of the pyrrole-2-carboxylic acid moiety from the acyl carrier protein CloN5 to the deoxysugar moiety of clorobiocin.

Results

Sequence analysis of *cloN1* and *cloN7*

The *cloN1* gene is a small ORF coding for a protein of 95 amino acids. This protein shows 86% identity to CouN1 from the coumermycin cluster, but no similarity to other proteins in the database are revealed by BLAST searches. Closer inspection of the sequence, however, shows an Asp-Ser-Leu motif at positions 44–46 in both CloN1 and CouN1. Secondary structure prediction suggests that this motif is located at the N terminus of the second of four α-helices in the protein, suggestive of a 4′-phosphopantetheinyl (=4′-PP) cofactor attachment site, typically contained in the acyl carrier proteins (ACPs) involved in fatty acid biosynthesis[27] and in peptide and polyketide antibiotic biosynthesis.[28] Stachelhaus et al. have defined the consensus sequence of ACPs as (L/I)GxDS(L/I), which is perfectly matched by the LGVDSL motif contained both in CloN1 and in CouN1. An alignment of CloN1 and CouN1 with the ACPs CloN5 and CouN5,[20,29] which comprise 89 amino acids each, as well as with the similarly small acyl carrier proteins SCO2389, SCO5089, SCO5316 and SCO5877 from *S. coelicolor*, shows a nearly identical position of the Asp-Ser-Leu motif in all the proteins (the motif in CloN5 and CouN5 is Asn-Ser-Leu).

CloN1 may therefore represent an acyl carrier protein and be involved in an acyl transfer reaction. In clorobiocin biosynthesis, acyl transfer processes are expected to take part in the biosynthesis of the aminocoumarin ring via a β-hydroxytyrosyl-ACP,[10] in the amide bond formation,[16] in the formation of the pyrrole-2-carboxylic acid moiety[20] and in the transfer of this moiety to the deoxysugar moiety of clorobiocin.[21]

The *cloN7* gene, the second gene addressed in this study, codes for a protein of 278 amino acids and shows 82% identity (AA level) to *couN7* of the coumermycin cluster. A BLAST search shows similarity to many other entries in the database, mainly from bacterial genome sequencing projects. A search for conserved domains reveals a typical α/β-hydrolase fold in CloN7. The α/β-hydrolase superfamily[30] comprises a wide variety of enzymes, acting as hydrolases or acyltransferases. This family also includes thioesterases involved in the cleavage of peptidyl or β-ketoacyl residues from the 4′-PP cofactors of acyl carrier proteins during peptide antibiotic or polyketide antibiotic biosynthesis. Notably, CloN7 shows some similarity to the thioesterase genes *bhp* and PCZA361.30 of balhimycin and vancomycin biosynthesis, respectively.[31,32] These glycopeptide antibiotics contain β-hydroxytyrosyl moieties, formed in a reaction sequence similar to that involved in the formation of the β-hydroxy-tyrosyl-ACP intermediate in clorobiocin and novobiocin biosynthesis.[10,31,33]

Sequence analysis of *cloN1* and *cloN7* therefore indicated different possible ways in which these genes might be involved in clorobiocin biosynthesis. In order to establish their true functions, we decided to carry out inactivation experiments with both genes.

Construction of the *cloN1* and *cloN7* inactivation cosmids clo-AF6 and clo-AF3

Cosmid clo-BG1, containing the complete biosynthetic gene cluster of clorobiocin, the attachment site *att*P and the integrase gene of bacteriophage ΦC31, had been constructed previously.[8] Site-specific integration of this cosmid into the *att*B site of the *S. coelicolor* M512 genome had resulted in the production of clorobiocin by the integration mutant, in amounts comparable to the production in the wild-type producer strain.[8] Gene inactivation in cosmid clo-BG1, carried out in *E. coli* by PCR targeting, and heterologous expression of the modified cosmids in *S. coelicolor* has been successfully used to study gene function.[23,34] We decided to use this method for inactivation of the genes *cloN1* and *cloN7*.

The apramycin resistance gene *aac(3)IV* was amplified from plasmid pUG019[8] by PCR, with use of primers with 39 bp extensions homologous to the regions upstream and downstream of *cloN1* and *cloN7*. The PCR products were used to replace the entire open reading frames of *cloN1* and *cloN7*, respectively, in cosmid clo-BG1, leaving only the start and the stop codons intact. This gave the modified cosmids clo-AF5 (*cloN1*$^-$, apr^R) and clo-AF3 (*cloN7*$^-$, apr^R), respectively.

In these constructs, the *aac(3)IV* gene is placed between XbaI and SpeI restriction sites, which are rare in *Streptomyces* DNA. To avoid possible polar effects of the *cloN1* inactivation cassette on downstream genes, the apramycin resistance cassette was removed by digestion of clo-AF5 with XbaI and SpeI and religation of the compatible overhangs (see Figure 1C), leaving only an 18 bp in-frame "scar" sequence in place of the gene *cloN1*.[8] The resulting cosmid was termed clo-AF6 (*cloN1*$^-$, apr^S).

Since the *cloN7* inactivation construct was introduced at the end of a putative transcription unit, removal of this cassette for avoidance of polar effects was considered unnecessary. This was later confirmed by the secondary metabolite accumulation in the *cloN7*$^-$ integration mutant harbouring clo-AF3 (see below).

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Integration of cosmids clo-AF6 and clo-AF3 into the genome of *S. coelicolor* M512

The cosmids clo-AF6 and clo-AF3 were introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation as described in ref. [35]. Five mutants resulting from integration of cosmid clo-AF6 into the host genome were selected by kanamycin resistance, and ten clo-AF3 integration mutants were selected by apramycin and kanamycin resistance.

The correct genotype of the *cloN1*$^-$ and *cloN7*$^-$ mutants was confirmed by Southern blotting, with use of the entire cosmid clo-BG1 as probe (Figures 1 and 2).

The deletion of *cloN1* in *cloN1*$^-$ integration mutants was verified by the absence of the 2.4 kb and 4.6 kb EcoRI bands and the presence of the expected 6.8 kb band in the mutant (Figure 1). The replacement of *cloN7* by the apramycin resistance cassette in *cloN7*$^-$ mutants was shown by the presence of a 9.8 kb BamHI band, instead of the original 7.2 and 2.4 kb bands on clo-BG1 (Figure 2).

Analysis of secondary metabolite production in the *cloN1*$^-$ and *cloN7*$^-$ mutants

For analysis of secondary metabolites, the *S. coelicolor* M512 integration mutants, harbouring either the intact cluster (clo-BG1) or the *cloN1*$^-$ (clo-AF6) or *cloN7*$^-$ (clo-AF3) defective clusters, were cultured in production medium as described previously.[5,36] The ethyl acetate extracts of the cultures were analysed by HPLC and the structures of the metabolites were subsequently elucidated by mass spectroscopy and ^{1}H NMR after preparative isolation.

HPLC analysis showed that the integration mutants of *S. coelicolor* M512, carrying the unmodified cosmid clo-BG1, formed clorobiocin as the dominant product, accompanied by its structural isomer isoclorobiocin and its non-chlorinated derivative novclobiocin 101 (Figure 3 A). The same compounds have been observed in previous studies.[8] In contrast, both the *cloN1*$^-$ mutants and the *cloN7*$^-$ mutants produced no clorobiocin, but instead a compound with the retention time of 19 min as main product (Figure 3 B and C). This compound was subsequently identified as novclobiocin 104 (see Figure 3 B and C for structure).

For structural elucidation, a *cloN1*$^-$ mutant and a *cloN7*$^-$ mutant were cultured in 200 mL production medium. The cultures were extracted with ethyl acetate and the extracts were subjected to column chromatography on Sephadex LH 20, followed by purification by reversed-phase HPLC. The negative FAB-MS of the isolated compound showed a negative ion at 588 $[M-H]^-$, 107 mass units smaller than that of clorobiocin, corresponding to the lack of the 5-methylpyrrole-2-carboxyl moiety. The ^{1}H NMR spectrum showed the same signals as clorobiocin for the protons of the aminocoumarin moiety, the prenylated 4-hydroxybenzoate moiety and the deoxysugar moiety, but the signals for the protons of the methylated pyrrole unit were absent. This spectrum corresponded to that of novclobiocin 104 obtained from a *cloN2*-defective mutant in a previous study.[21]

The *cloN1*$^-$ mutant also produced an additional product (retention time 18 min), which was isolated in the same way. Negative FAB MS showed an ion at m/z 554 $[M-H]^-$, 35 mass units smaller than that of novclobiocin 104, corresponding to a lack of the chlorine atom. This substance was therefore identified as novclobiocin 107 (see Figure 3 B for structure) by comparison of its MS and ^{1}H NMR data with data obtained in a previous study.[37] HPLC analysis (Figure 3 C) suggested that this compound was also formed by the *cloN7*$^-$ mutant, albeit in lower amounts.

S. coelicolor harbouring clo-BG1 produced 26 mg L^{-1} clorobiocin. The *cloN1*$^-$ and the *cloN7*$^-$ mutants produced 35 mg mL^{-1} and 10 mg L^{-1} novclobiocin 104, respectively.

Identification of pyrrole-2-carboxylic acid by MS and ^{1}H NMR

The culture extracts of both the *cloN1*$^-$ and the *cloN7*$^-$ mutants were further analysed for the presence of free pyrrole-2-carboxylic acid by HPLC with detection at 262 nm. Pyrrole-2-carboxylic acid was unequivocally detected in both mutants. This was confirmed not only by HPLC but also by ^{1}H NMR and MS analysis after preparative isolation. The ^{1}H NMR spectrum showed three doublet of doublets signals at 6.91, 6.81 and 6.15 ppm, which were identical to those of authentic pyrrole-2-carboxylic acid and consistent with the literature data.[21,38]

Discussion

In this study we inactivated the *cloN1* and *cloN7* genes of the clorobiocin biosynthetic gene cluster. Both mutations resulted in the accumulation of the same secondary metabolites: novclobiocin 104 (Figure 3 B and C) and pyrrole-2-carboxylic acid. These findings show that neither *cloN1* nor *cloN7* has an essential role in any of the biosynthetic reactions producing novclobiocin 104, including the acyl transfer process involved in the aminocoumarin ring formation from L-tyrosine[10] and the amide bond formation between aminocoumarin and substituted benzoic acid.[16] At the same time, our findings have also demonstrated that neither *cloN1* nor *cloN7* is required for the biosynthesis of the pyrrole-2-carboxyl moiety. This is consistent with recent evidence from in vitro studies that CloN3, CloN4 and CloN5 are sufficient for the conversion of L-proline into pyrrole-2-carboxyl-*S*-CloN5.[20]

Unlike the wild type, however, the *cloN1*$^-$ and *cloN7*$^-$ mutants were unable to connect the pyrrole-2-carboxyl moiety to the 3''-OH group of novclobiocin 104. In the wild type, this reaction produces novclobiocin 109 (=5'''-desmethyl-clorobiocin), which is subsequently methylated by CloN6 at position 5 of the pyrrole unit to give clorobiocin.[21,22] Since the inactivation of *cloN2*[21] had likewise resulted in the accumulation of pyrrole-2-carboxylic acid and novclobiocin 104, it has to be concluded that each of the three genes *cloN1*, *cloN2* and *cloN7* is essential for the acyl transfer process that transfers the pyrrole-2-carboxyl moiety from the acyl carrier protein CloN5[20] to the deoxysugar moiety of novclobiocin 104.

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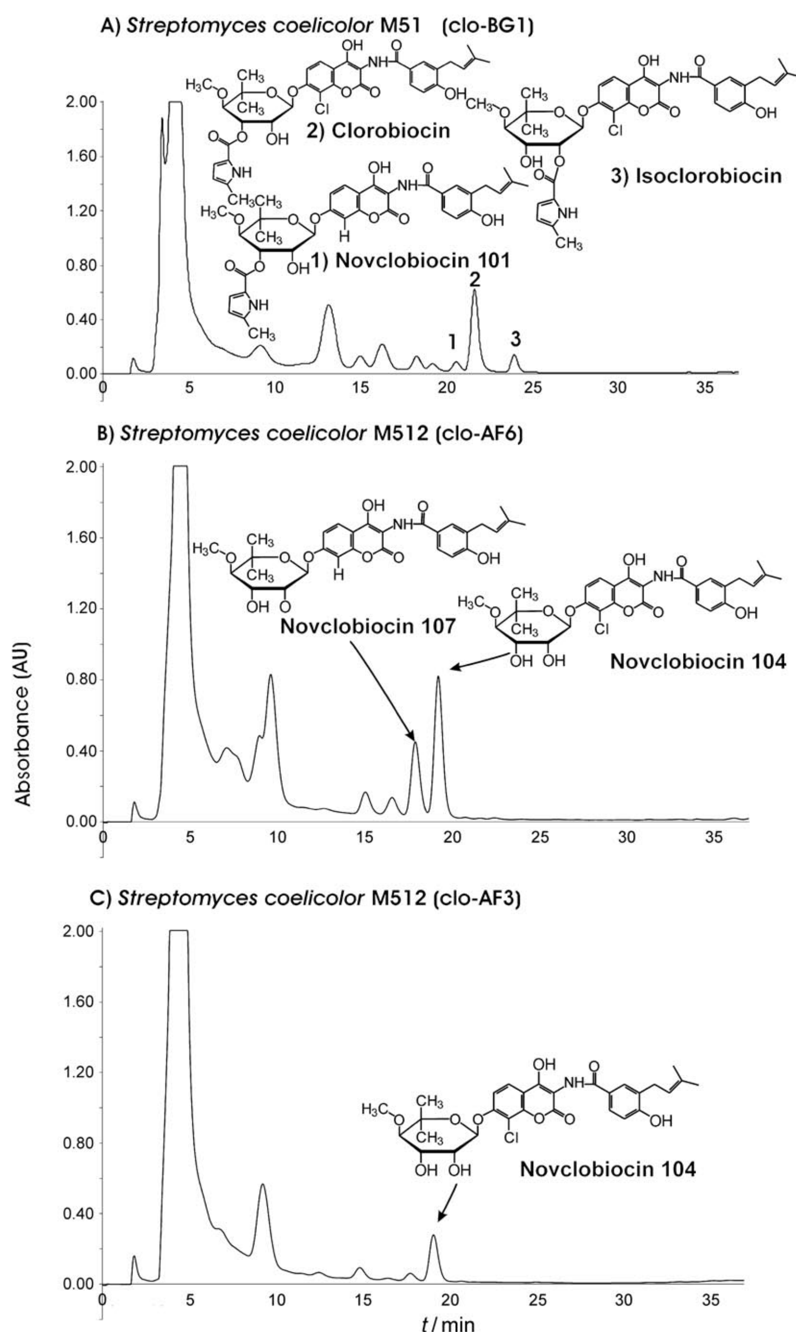

Figure 3. HPLC analysis of clorobiocin derivatives from heterologous production strains: A) *S. coelicolor* M512 harbouring cosmid clo-BG1 with the intact clorobiocin biosynthetic gene cluster. B) *S. coelicolor* M512 harbouring cosmid clo-AF6 with the *cloN1*$^{-}$ clorobiocin biosynthetic gene cluster. C) *S. coelicolor* M512 harbouring cosmid clo-AF3 with the *cloN7*$^{-}$ clorobiocin biosynthetic gene cluster.

Though this finding appeared surprising at first, sequence analysis of the three genes suggested a possible mechanism consistent with our experimental results (Scheme 2). CloN1 shows the typical structure of an acyl carrier protein (ACP), and after 4′-phosphopantetheinylation of the Asp44-Ser45-Leu46 cofactor attachment site, it may be acylated with the pyrrole-2-carboxyl moiety supplied by pyrrole-2-carboxyl-*S*-CloN5. The transfer of this acyl moiety from CloN5 to CloN1 might be catalysed by CloN2, which displays sequence similarity to many acyl-ACP-synthases, but especially to DpsC from *S. peuceticus*. This enzyme has been shown to catalyse the transfer of a propionyl moiety from propionyl-*S*-CoA to the 4′-PP cofactor of an ACP,[39] which is similar to the reaction we propose here for CloN2.

CloN7 is a member of the large and diverse family of α/β-hydrolases, which also include thioesterases involved in the biosynthesis of peptide and polyketide antibiotics. These thioesterases transfer acyl moieties from the 4′-PP cofactor of ACPs either to water (resulting in the free acid) or to hydroxy or amino groups, resulting in esters, lactones, amides or lactams. Correspondingly, CloN7 may transfer the pyrrole-2-carboxyl moiety from the 4′-PP cofactor of CloN1 to the 3′′-OH group of novclobiocin 104 (Scheme 2).

Consistently with this hypothesis, orthologues of *cloN1* and *cloN7* are found in the biosynthetic gene cluster of coumermycin A1 (i.e., *couN1* and *couN7*). This antibiotic also contains a pyrrole-2-carboxyl moiety attached to the deoxysugar. However, no similar genes are found in the gene cluster for novobiocin, which lacks this moiety.

While the suggested reaction mechanism is speculative at present (biochemical confirmation would require the active expression and purification of 4′-PP-CloN1, CloN2, CloN3, CloN4, 4′-PP-CloN5 and CloN7), our experiments clearly show that transfer of the pyrrole-2-carboxyl moiety from the acyl carrier protein CloN5 to novclobiocin 104 requires, besides the putative transferases CloN2 and CloN7, an additional putative acyl carrier protein: CloN1. Since there is no obvious chemical reason why two ACPs should be required for this reaction, it is tempting to speculate that the complete CloN3/CloN4/CloN5 pathway for pyrrole-2-carboxylic acid biosynthesis (see Scheme 2), which is catalysed by a highly similar three-enzyme system in pyoluteorin biosynthesis in *Pseudomonas fluorescens* and in undecylprodigine biosynthesis in *S. coelicolor*,[29] may have been acquired by the ancestor of the clorobiocin producer in the form of the *cloN3*/*cloN4*/*cloN5* gene fragment (Figures 1 and 2). The three enzymes encoded in this fragment may then have interacted with an existing ACP/acyl-ACP-synthase/thioesterase system, encoded by *cloN1*, *cloN2* and *cloN7*, to give the pathway to clorobiocin.

Methylation of position 5 of the pyrrole-2-carboxyl moiety by CloN6[22] takes place after transfer of this moiety to novclobiocin 104.[21] Correspondingly, the *cloN1*$^{-}$, *cloN2*$^{-}$ and *cloN7*$^{-}$ mutants accumulated pyrrole-2-carboxylic acid (probably released from the thioester with 4′-PP-CloN5 by hydrolysis), but no 5-methylpyrrole-2-carboxylic acid was detected. Also in correspondence with this hypothesis, a *cloN6*$^{-}$ mutant was found to produce novclobiocin 109 (=5′′′-desmethyl-clorobiocin) as the main product.[22]

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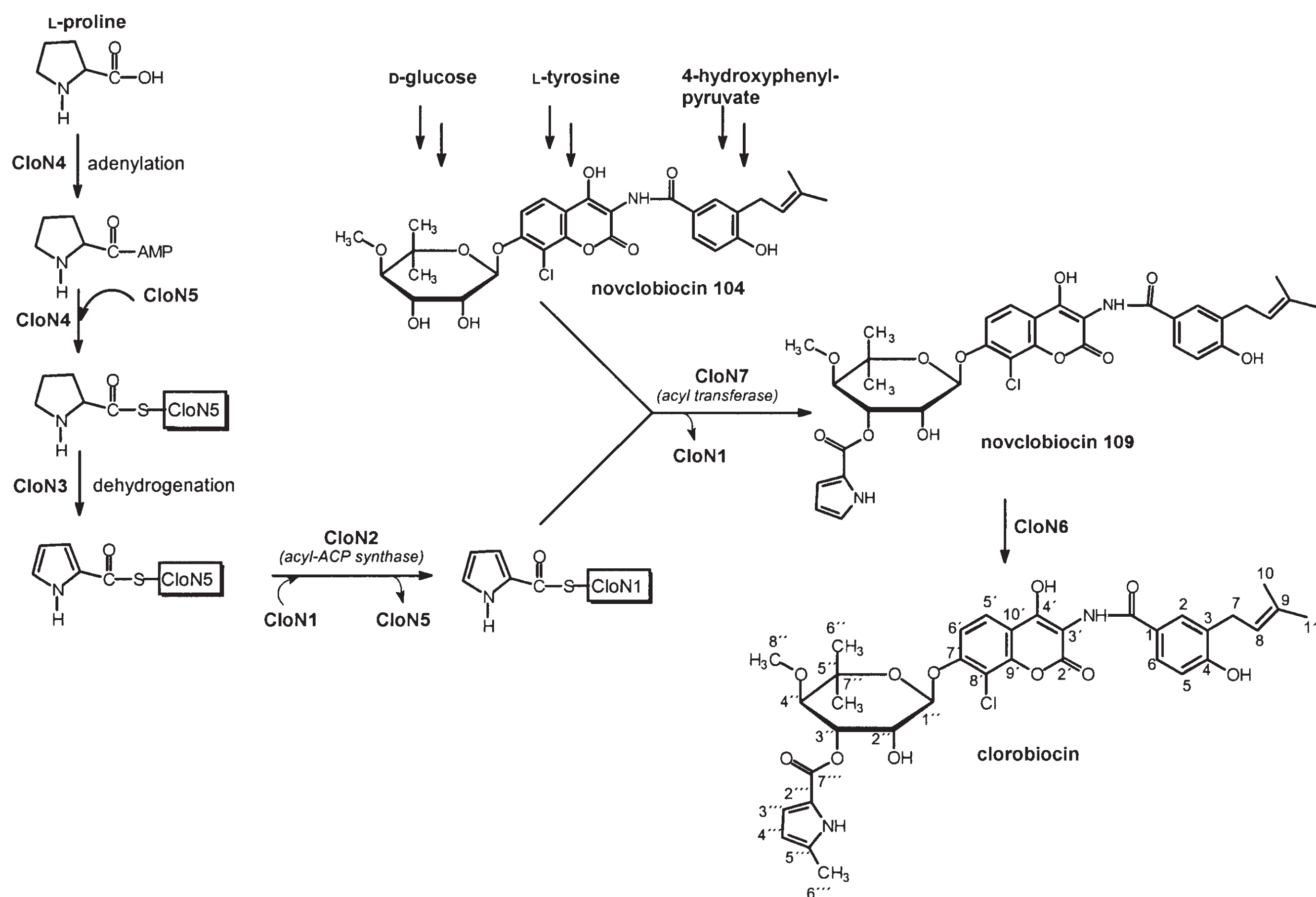

Scheme 2. Hypothetical scheme for the late steps of clorobiocin biosynthesis and the possible roles of CloN1, CloN2 and CloN7.

The fact that the *cloN1*⁻, *cloN2*⁻ and *cloN7*⁻ mutants accumulated novclobiocin 104, in which the 4-hydroxy group of the deoxysugar is methylated, confirms our earlier conclusion that 4′′-*O*-methylation precedes 3′′-*O*-acylation in clorobiocin biosynthesis.[23]

In this study we inactivated *cloN1* and *cloN7* in cosmids by use of the PCR targeting method, followed by heterologous expression of the modified cosmids in *S. coelicolor* M512. This provides a further example of the usefulness of this technique[8] for the study of gene functions.

Experimental Section

Bacterial strains, cosmids and culture conditions: *S. coelicolor* M512 (Δ*redD* Δ*actII-ORF4* SCP1⁻ SCP2⁻)[40] was kindly provided by E. Takano (Tübingen, Germany) and Janet White (Norwich, UK). The strains were cultured as described previously.[34,35] *E. coli* strains ET12567[41] and XL1 Blue MRF′ (Stratagene, Heidelberg, Germany) were used for DNA propagation and grown as described.[42] The REDIRECT© technology kit for PCR targeting[43] was obtained from Plant Bioscience Limited (Norwich, UK). Kanamycin (50 μg mL^{-1}), chloramphenicol (25–50 μg mL^{-1}) and apramycin (50 μg mL^{-1}) were used for selection of recombinant strains. Cosmid clo-BG1 and plasmid pUG019 have been described previously.[8]

DNA isolation, manipulation and cloning: Standard procedures for DNA isolation and manipulation were performed as described by Sambrook et al.[42] and Kieser et al.[35] Isolation of cosmids and plasmids was carried out with ion-exchange columns (Nucleobond AX kits, Macherey–Nagel, Düren, Germany) according to the manufacturer's protocol. Genomic DNA was isolated from *Streptomyces* strains by lysozyme treatment and phenol/chloroform extraction.[35]

Southern blot analysis was performed on Hybond-N nylon membrane (Amersham Biosciences, Freiburg, Germany) with a digoxigenin-labelled probe with use of the DIG high prime DNA labelling and detection starter kit II (Roche Applied Science, Mannheim, Germany).

Inactivation of cloN1 and cloN7 in cosmid clo-BG1: In cosmid clo-BG1, *cloN1* or *cloN7* were replaced, through λ-RED-mediated recombination,[8] with the apramycin resistance *(aac(3)IV)* cassette, which was excised from pUG019 by digestion with EcoRI and HindIII and is flanked by XbaI and SpeI recognition sites. The apramycin resistance cassette for replacement of *cloN1* was generated by PCR amplification with use of the excised template and the primer pair **cloN1-f** (5′-**CAC AGT GGA ACA CAA TCG ATG GGG GAA TTA CGC GAG ATG** ATT CCG GGG ATC *TCTAGA* TC-3′) and **cloN1-r** (5′-**GGC CAC GTA TAT ACC TGA TGT CCG CAT CTG TTG CGC CTA** *ACTAGT* CTG GAG CTG CTT C-3′). Bold letters represent the homologous extensions to the DNA regions immediately upstream and downstream of *cloN1*, including the putative start and stop codons of *cloN1*. The italic letters indicate the XbaI and SpeI restriction sites.

Similarly, the apramycin resistance cassette for replacement of *cloN7* was obtained by PCR amplification by use of the primer pair **cloN7-f** (5′-**GGC AGA CTC CCC AAC AGC AGA GAG GAC CAA CTG-AG-C ATG** ATT CCG GGG ATC *TCTAGA*TC-3′) and **cloN7-r** (5′-**AGT GTG-CGT GGT GCG CCA GCA CTC CGA CAA GCA CCG TTA** *ACTAGT* CTG G-AG CTG CTT C-3′). Bold letters represent the homologous extensions to the DNA regions immediately upstream and downstream of *cloN7*, including the putative start and stop codons of *cloN7*. The italic letters indicate the XbaI and SpeI restriction sites. PCR amplification was performed in 50 μL volume with template (50 ng), dNTPs (0.2 mM), each primer (50 pmol) and DMSO (5% *v/v*), with use of the Expand High Fidelity PCR system (Roche Molecular Biochemicals): denaturation at 94 °C for 2 min, then 10 cycles with denaturation at 94 °C for 45 s, annealing at 45 °C for 45 s and extension at 72 °C for 90 s , followed by 15 cycles with annealing at 48 °C, and the last elongation step at 72 °C for 5 min. The PCR products were introduced by electroporation into *E. coli* BW25113/pIJ790 harbouring cosmid clo-BG1.[43] The modified cosmids were isolated and analysed by restriction enyzme digestion. The cosmid resulting from the replacement of *cloN7* was termed clo-AF3 and the cosmid resulting from the replacement of *cloN1* was termed clo-AF5. For the excision of the apramycin resistance cassette from clo-AF5, cosmid DNA was isolated from *E. coli* ET12567, digested with XbaI and SpeI, and religated overnight at 16 °C to give cosmid clo-AF6.

Heterologous expression of clo-AF3 and clo-AF6 in *S. coelicolor* M512: Because of the potent methylation restriction system of *S. coelicolor*, cosmid DNA had to be passed through a non-methylating host. We used *E. coli* ET12567 for this purpose.[8] The modified cosmids clo-AF3 and cloAF-6, still carrying the kanamycin resistance gene *neo*, were then introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation.[35] Clones resistant to kanamycin and apramycin (clo-AF3), as well as only to kanamycin (clo-AF6), were selected, and checked for site-specific integration into the genome by Southern blot analysis.

Production and analysis of secondary metabolites: Transformants of *S. coelicolor*, harbouring clo-BG1, clo-AF6 and clo-AF3, respectively, were cultured and assayed for the production of clorobiocin derivatives by HPLC as described previously,[34] with use of a Multosphere RP18–5 column (250×4 mm, 5 μm, C+S Chromatographie Service, Düren, Germany) at a flow rate of 1 mL min^{-1}, with use of a linear gradient from 60 to 100% of solvent B in 28 min (solvent A = MeOH/H_2O/HCOOH 20:79:1; solvent B = MeOH/HCOOH 99:1) with detection at 340 nm. The column was then washed with 100% B for 3 min and equilibrated with 40% A in B for 6 min. Authentic clorobiocin (Aventis) was used as standard. For analysis of the pyrrole-2-carboxylic acid, a linear gradient from 0 to 100% of solvent B in 15 min (solvent A = H_2O/HCOOH 99:1; solvent B = MeOH/HCOOH 99:1) was used with the same HPLC equipment and the same column. The column was then washed with 100% B for 5 min and equilibrated with 100% A for 15 min. UV detection was carried out at 262 nm. Pyrrole-2-carboxylic acid was used as standard.

For preparative isolation, cultivation was carried out in 500 mL baffled flasks containing production medium (50 mL) as described above. The bacterial cultures of *cloN1*$^-$ and *cloN7*$^-$ mutants (a total of 200 mL each) were pooled, acidified with HCl to pH 4 and extracted with ethyl acetate after removal of the lipophilic components by treatment with petroleum ether. The residue from the ethyl acetate extract after evaporation of the solvent was dissolved in methanol (2–5 mL) and passed through a glass column (2.6 cm× 65 cm) filled with Sephadex LH 20 (Amersham Biosciences, Freiburg, Germany) and eluted with methanol. The fractions obtained after separation on Sephadex LH 20 were analysed by HPLC under the conditions described above. Fractions containing aminocoumarin derivatives and pyrrole-2-carboxylic acid were pooled and further purified on a preparative HPLC column (Multosphere 120 RP18–5, 5 μm, 250×10 mm, C&S Chromatographie Service, Düren, Germany) with use of the same solvents and gradient as for the analytical column, but with a flow of 2.5 mL min^{-1}. From the *cloN1*$^-$ mutant, two clorobiocin derivatives could be isolated, and these were identified as novclobiocin 104 and 107. Novclobiocin 104 was also isolated from the *cloN7*$^-$ mutant. Pyrrole-2-carboxylic acid was isolated from both *cloN1*$^-$ and *cloN7*$^-$ mutants. The structures of the purified compounds were confirmed by ^{1}H NMR and MS analysis.

Negative-ion FAB mass spectra were recorded on a TSQ70 spectrometer (Finnigan, Bremen, Germany) with diethanolamine as matrix. ^{1}H NMR spectra were measured on an AMX 400 spectrometer (Bruker, Karlsruhe, Germany), in CD_3OD as solvent.

Novclobiocin 104: ^{1}H NMR (CDO_3D): $\delta = 1.10$ (s, 3H; 3H-6′′), 1.30 (s, 3H; 3H-7′′), 1.73 (s, 6H; 3H-10, 3H-11), 3.33 (d, overlapping with the solvent signals, 2H; 2H-7), 3.40 (d, $J = 9.7$ Hz; H-4′′), 3.59 (s, 3H; 3H-8′′), 4.11 (dd, $J_1 = 1.8$ Hz, $J_2 = 3.3$ Hz; H-2′′), 4.18 (dd, $J_1 = 3.3$ Hz, $J_2 = 9.8$ Hz; H-3′′), 5.35 (t, $J = 7.5$ Hz; H-8), 5.63 (d, $J = 1.8$ Hz; H-1′′), 6.81 (d, $J = 8.3$ Hz; H-5), 7.23 (d, $J = 9.0$ Hz; H-6′), 7.71 (dd, $J_1 = 2.0$ Hz, $J_2 = 8.2$ Hz; H-6), 7.75 (d, $J = 2.5$ Hz; H-2), 7.87 ppm (d, $J = 8.9$ Hz; H-5′); negative FAB-MS characterized by ion peaks at *m/z* 588 ([*M*−H]$^-$), 554, 400 and 209. These data are consistent with those reported for novclobiocin 104.[21,22]

Novclobiocin 107: ^{1}H NMR (CDO_3D): $\delta = 1.12$ (s, 3H; 3H-6′′), 1.32 (s, 3H; 3H-7′′), 1.73 (s, 6H; 3H-10, 3H-11), 3.30 (d, overlapping with the solvent signals; 2H-7), 3.36 (d, $J = 9.4$ Hz; H-4′′), 3.58 (s, 3H; 3H-8′′), 4.01 (dd, $J_1 = 2.3$ Hz, $J_2 = 3.3$ Hz; H-2′′), 4.09 (dd, $J_1 = 3.3$ Hz, $J_2 = 9.3$ Hz; H-3′′), 5.35 (t, $J = 7.6$ Hz; H-8), 5.52 (d, $J = 2.3$ Hz; H-1′′), 6.80 (d, $J = 8.4$ Hz; H-5), 6.94 (overlapping signals; H-6′), 6.94 (overlapping signals; H-8′), 7.75 (br s; H-2), 7.74 (d, $J = 8.4$ Hz; H-6), 7.89 ppm (d, $J = 7.8$ Hz; H-5′); negative FAB-MS characterized by ion peaks at *m/z* 554 ([*M*−H]$^-$), 400 and 209. These data are consistent with those reported previously.[37]

Pyrrole-2-carboxylic acid: ^{1}H NMR (CDO_3D): $\delta = 6.15$ (dd, $J_1 = 2.5$ Hz, $J_2 = 3.6$ Hz; H-4), 6.81 (dd, $J_1 = 1.4$ Hz, $J_2 = 3.7$ Hz; H-3), 6.91 ppm (dd, $J_1 = 1.5$ Hz, $J_2 = 2.4$ Hz; H-5); negative FAB-MS characterized by the molecular ion at *m/z* 111 ([*M*−H]$^-$.

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