Biosynthesis of aminocoumarin antibiotics in *Streptomyces*: Generation of structural analogues by genetic engineering and insights into the regulation of antibiotic production

Biosynthese von Aminocoumarinantibiotika in *Streptomyces*: Herstellung von Analoga durch genetische Manipulation und Einblick in die Regulation der Antibiotikaproduktion

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CONTENTS

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ABBREVIATIONS

°C	degree celsius
µ	micro
aa	amino acids
aac(3)IV	apramycin resistance gene
Amp	ampicillin
APS	ammonium persulphate
Apra	apramycin
ATP	adenosine triphosphate
bp	base pair
cccDNA	covalently closed circular DNA
CFU	colony forming unit
Cm	chloramphenicol
CSPD	chemiluminescence substrate
Da	dalton
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5´-triphosphate
dsDNA	double-stranded DNA
DTT	1,4-dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
FAB	fast atom bombardment
Fig.	figure
FRT	FLP recognition target
g	gram
GyrB	gyrase B subunit
h	hour
HCl	hydrochloric acid
HCOOH	formic acid
His_6	hexahistidine
HPLC	high performance liquid chromatography
Hyg	hygromycin
Hz	hertz
IPTG	isopropyl-*ß*-thiogalactoside
k	kilo
KAc	potassium acetate
Kan	kanamycin
kb	kilo base pairs
kDa	kilo dalton
l	litre
lacZα	gene portion for α-complementation of *ß*-galactosidase
M	molar
m	milli
Mb	mega base pairs
min	minute
MS	mass spectroscopy
MW	molecular weight
n	nano

NaAc	sodium acetate
NaOH	sodium hydroxide
Nd	nalidixic acid
neo	neomycin/kanamycin resistance gene
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
nt	nucleotide
OD_{600}	optical density at 600 nm
ORF	open reading frame
oriT	origin of transfer from RK2
p	pico
PCR	polymerase chain reaction
PCP	peptidyl carrier protein
PEG	polyethylene glycol
^{R}	resistant
RBS	ribosome binding site
Ring A	3-dimethylallyl-4-hydroxybenzoic acid
RNase	ribonuclease
RP	reverse phase
rpm	rotation per minute
RT	room temperature
s	second
s.	see
S.	*Streptomyces*
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S. roseochromogenes	*S. roseochromogenes* var. *oscitans*
ssDNA	single-stranded DNA
TEMED	N,N,N´,N´-tetramethylethylenediamine
TES	N-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid
Thio	thiostrepton
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris-maleate	Tris-(hydroxymethyl)-aminomethane-maleate
Topo	topoisomerase
U	unit
UV	ultraviolet
WT	wild-type
×*g*	ground acceleration
X-gal	5-bromo-4-chloro-3-indolyl-*ß*-D-galactopyranoside

SUMMARY

The aminocoumarin antibiotics novobiocin (*nov*) and clorobiocin (*clo*) are potent inhibitors of bacterial DNA gyrase produced by different *Streptomyces* strains. Since they are closely related in structure, and their biosynthetic gene clusters have been cloned and sequenced, they represent interesting starting compounds for the development of new antibacterial agents by genetic engineering. However, the functional analysis of biosynthetic genes and the availability of genetic tools for manipulation of producer strains are pre-requisites for such approaches.

Clorobiocin is more potent than novobiocin. It differs from the latter, clinically approved drug in the substitution pattern at C-8´ of the aminocoumarin moiety, carrying a chlorine atom instead of a methyl group, and in the presence of a 5-methylpyrrole-2-carboxyl moiety instead of a carbamoyl group at 3´´-OH of the deoxysugar unit. One aim of this thesis was the production of hybrid antibiotics, combining structural features of these two compounds. By gene inactivation, *clo-hal* was identified as the gene of the halogenase responsible for the introduction of the chlorine atom of clorobiocin. Inactivation of *cloZ* did not affect clorobiocin formation, showing that this ORF is not involved in the halogenation, nor is it essential for clorobiocin biosynthesis under the present culture conditions.

Expression of the methyltransferase gene *novO* in the *clo-hal*$^-$ mutant of the clorobiocin producer *S. roseochromogenes* led to the very efficient formation of a clorobiocin analog with $-CH_3$ instead of –Cl at C-8´ (= novclobiocin 102). However, earlier attempts to generate a hybrid antibiotic with a chlorine atom at C-8´ and a carbamoyl group at 3´´-OH (novclobiocin 114) by conventional gene inactivation/gene expression experiments in the novobiocin producer *S. spheroides* had remained unsuccessful (Pojer and Heide, unpublished results), due to difficulties in the genetic manipulation of this natural producer. In order to circumvent this problem, the entire biosynthetic gene cluster of novobiocin was now expressed in *S. coelicolor* and *S. lividans* which are well characterized and easy to manipulate. By the same method, the clorobiocin cluster was also heterologously expressed. For this purpose, cosmids containing the integration functions of the *Streptomyces* phage ϕC31 and the complete novobiocin or clorobiocin clusters were introduced into the heterologous

hosts, respectively. *S. coelicolor* M512 derivatives produced at least five times more of the respective antibiotic than *S. lividans* TK24 and further investigations were carried out using the former strain as host.

Inactivation of the methyltransferase gene *novO* could be rapidly achieved in the cosmid by λ-Red-mediated recombination in *Escherichia coli*. Subsequent integration of the *novO*$^-$ cosmid into the *S. coelicolor* genome, followed by co-expression of *clo-hal*, readily allowed the production of the desired hybrid antibiotic novclobiocin 114. BhaA, responsible for 3-chlorination of the *b*-hydroxy-tyrosyl moieties of the glycopeptide antibiotic balhimycin, was unable to replace Clo-hal, suggesting that the two halogenases have different substrate specificities.

Furthermore, shortening of the cosmids' inserts proved, for the first time, that the DNA regions from *cloE* to *gyrB*^{R} and from *novE* to *gyrB*^{R} contain all genes which are essential for clorobiocin and novobiocin production, respectively.

Comparison of the antibacterial activity of clorobiocin and novobiocin analogues with –Cl, –H or –CH_3 at C-8´ showed that substitution with either –CH_3 or –Cl was important for biological activity. –CH_3 or –Cl led to comparable activity, whereas –H led to a strong loss in activity.

Moreover, functional proof for the role of NovG as a positive regulator of novobiocin biosynthesis was provided by genetic and biochemical approaches. Using the method described above, Δ*novG* mutants were created. These Δ*novG* mutants produced only 2% of the novobiocin amount formed by the *S. coelicolor* strains carrying the intact novobiocin cluster. The production could be restored by introducing an intact copy of *novG* into the mutant. The introduction of *novG* on a multicopy plasmid into the strain carrying the intact novobiocin cluster led to almost three-fold overproduction of the antibiotic, suggesting that novobiocin biosynthesis is limited by the availability of NovG protein. Furthermore, purified N-terminal His_6 tagged NovG showed specific DNA-binding activity for the *novG-novH* and for the *cloG-cloY* intergenic regions of the novobiocin and clorobiocin biosynthetic gene cluster, respectively. By comparing the DNA sequences of the fragments binding NovG, conserved inverted repeats were found in both fragments, similar to those identified as the binding sites for StrR, the well-studied pathway-specific

transcriptional activator of streptomycin biosynthesis. The consensus sequence for the StrR and the putative NovG binding sites is GTTCRACTG$(N)_{11}$CRGTYGAAC. Therefore, NovG and StrR apparently belong to the same family of DNA-binding regulatory proteins.

Furthermore, inactivation of *novE* by gene replacement using PCR targeting proved that *novE* does not have an essential catalytic role in novobiocin biosynthesis, since some novobiocin was still produced by the mutants. This gene is likely to have a regulatory function, as a 25–fold drop in productivity was observed in the mutants.

ZUSAMMENFASSUNG

Die Aminocoumarinantibiotika Novobiocin (*nov*) und Clorobiocin (*clo*) sind potente Gyrasehemmer, die von unterschiedlichen *Streptomyces*-Stämmen gebildet werden. Da sie strukturell eng verwandt sind und ihre Biosynthese-Gencluster kloniert und sequenziert wurden, stellen sie interessante Ausgangssubstanzen für die Entwicklung neuer Wirkstoffe durch genetische Manipulation der Produzenten dar. Der Funktionsnachweis der Gene und die genetische Manipulierbarkeit von Produzenten sind jedoch Voraussetzungen dafür.

Clorobiocin ist potenter als Novobiocin. Es unterscheidet sich vom klinisch untersuchten Novobiocin durch die Substitution an C-8´ des Aminocoumarinrings (–Cl anstatt von $–CH_3$), und durch Anwesenheit einer 5-Methylpyrrol-2-carboxyl Einheit anstatt einer Carbamoylgruppe an 3´´-OH des Desoxyzuckers. Ein Ziel dieser Dissertation war die Produktion von Hybrid-Antibiotika, in denen die strukturellen Eigenschaften dieser beiden Stoffe kombiniert sind. Durch Geninaktivierung wurde *clo-hal* als das Gen nachgewiesen, das für die Halogenierung von Clorobiocin zuständig ist. Die Inaktivierung von *cloZ* hatte keinen Einfluss auf die Clorobiocin-Produktion. Dies zeigte, dass *cloZ* nicht an der Halogenierung beteiligt ist sowie nicht essentiell für die Clorobiocin-Biosynthese unter den verwendeten Kulturbedingungen ist.

Die Expression des Methyltransferasegens *novO* in der *clo-hal*$^-$ Mutante des Clorobiocinproduzenten *S. roseochromogenes* führte zur effizienten Bildung eines Clorobiocin-Analogon mit $–CH_3$ anstatt von –Cl an C-8´ (= novclobiocin 102). Jedoch waren frühere Versuche zur Herstellung des mit einem Chloratom an C-8´ und einer Carbamoylgruppe an 3´´-OH (= novclobiocin 114) versehenen Hybridantibiotikums im natürlichen Novobiocinproduzenten *S. spheroides* gescheitert (Pojer und Heide, unveröffentlichte Daten), weil dieser Stamm genetisch nur schwer zu manipulieren ist. Um dieses Problem zu umgehen, wurde das komplette Novobiocin-Cluster nun in den gut bekannten und einfach zu manipulierenden *S. coelicolor* und *S. lividans* Stämmen exprimiert. In gleicher Weise wurde auch das Clorobiocin-Cluster heterolog exprimiert. Dafür wurden Cosmide, die die Integrationsfunktionen des *Streptomyces*-Phagen ϕC31 sowie das komplette Novobiocin- bzw. Clorobiocin-Cluster beinhalten,

jeweils in die heterologen Wirte eingebracht. *S. coelicolor* M512 Stämme produzierten mindestens fünfmal mehr des entsprechenden Antibiotikums als *S. lividans* TK24. Deshalb wurden die folgenden Untersuchungen in *S. coelicolor* M512 durchgeführt.

Die Inaktivierung von *novO* wurde im Cosmid mittels λ-Red-vermittelter Rekombination in *Escherichia coli* erzielt. Anschließende Integration des *novO*$^-$ Cosmids in das *S. coelicolor* Genom und Expression von *clo-hal* (in einem replikativen Expressionsplasmid) erlaubten rasch die Produktion des gewünschten Hybrid-Antibiotikums Novclobiocin 114. Die Halogenase BhaA ist für die 3-Chlorierung der *b*-Hydroxy-tyrosyl Einheiten des Glykopeptid-Antibiotikums Balhimycin verantwortlich. BhaA war nicht in der Lage, Clo-hal funktionell zu ersetzen. Dies spricht dafür, dass die zwei Halogenasen unterschiedliche Substratspezifität besitzen.

Außerdem konnte durch die Verkürzung der Cosmid-Inserts zum ersten Mal nachgewiesen werden, dass die DNA-Regionen von *cloE* bis *gyrB*^{R} bzw. von *novE* bis *gyrB*^{R} alle für die Clorobiocin- bzw. Novobiocin-Produktion essentiellen Gene beinhalten.

Der Vergleich der antibakteriellen Aktivität von Clorobiocin- und Novobiocin-Analoga mit –Cl, –H oder –CH_3 an C-8´ zeigte, dass Substitution mit –CH_3 bzw. –Cl in beiden Fällen wichtig für die biologische Aktivität war. Während –CH_3 bzw. –Cl zu vergleichbarer Aktivität führten, verursachte –H einen starken Aktivitätsverlust.

Zudem wurde mittels genetischer und biochemischer Methoden bewiesen, dass NovG ein positiver Regulator der Novobiocin-Biosynthese ist. *S. coelicolor* Stämme mit einem *novG*-defekten Novobiocincluster wurden hergestellt. Diese Δ*novG* Mutanten produzierten nur noch 2% Novobiocin im Vergleich zum Stamm mit dem intakten Cluster. Die Produktion konnte durch Expression einer intakten Kopie von *novG* wiederhergestellt werden. Die Einführung von *novG* in einem Multicopy-Plasmid in den *S. coelicolor* Stamm mit dem intakten Novobiocin-Cluster führte zu fast dreifacher Überproduktion des Antibiotikums. Dies deutet darauf hin, dass die Novobiocin-Biosynthese durch die Verfügbarkeit von NovG-Protein begrenzt wird. Außerdem zeigte gereinigtes NovG-Protein mit einem N-terminalen His_6-Tag

spezifische DNA-bindende Aktivität für die *novG-novH* bzw. *cloG-cloY* intergenetischen Regionen des Novobiocin- bzw. Clorobiocin-Clusters. Durch Vergleich der DNA-Sequenzen dieser Fragmente wurden konservierte palindromische Sequenzen in beiden Fragmenten gefunden. Diese palindromischen Sequenzen sind ähnlich zu den für StrR identifizierten Bindungsstellen im Streptomycin-Cluster. Die Consensus-Sequenz für die StrR- und für die putative NovG-Bindungsstelle ist GTTCRACTG$(N)_{11}$CRGTYGAAC. Daher gehören NovG und StrR wahrscheinlich zur gleichen Familie von DNA-bindenden, regulatorischen Proteinen.

Außerdem bewies die Inaktivierung von *novE* mittels Genaustausch durch PCR-Targeting, dass *novE* keine essentielle katalytische Funktion in der Novobiocin-Biosynthese hat, da Novobiocin immer noch gebildet wird. Eine regulatorische Rolle kann für dieses Gen wegen der 25-fachen Produktivitätsverringerung in der Mutante vermutet werden.

I. INTRODUCTION

I.1. The search for new antibiotics: an overview of genetic approaches

Antibiotic resistance has developed to every class of antibiotics, both natural and synthetic, over the course of one year to more than a decade after the first clinical use (see Table I.1). For example, virtually all gram-positive infections were susceptible to penicillin in the 1940s, but in hospitals today, the vast majority of infections caused by important bacterial agents like *Staphylococcus aureus* are penicillin-resistant, and up to 50% are resistant to methicillin. Also vancomycin, the principal alternative treatment for methicillin-resistant infections, has been overcome by some of the most frequent infection agents in hospitals. Resistance, then, has proven not to be a matter of "if", but a matter of "when" (Palumbi, 2001; Walsh, 2003). Therefore, there is a constant need for the development of new antibacterial drugs in order to reduce the impact of resistance in antibacterial therapy, as emphasized by the World Health Organization (2001).

TABLE I.1: Dates of deployment of representative antibiotics, and the evolution of resistance. (Source (Palumbi, 2001))

Antibiotic	Year deployed	Resistance observed
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	late 1960s

Natural products play a dominant role in the discovery of new drugs. This is particularly evident in the areas of cancer and infectious diseases, where 62% and 78% of these drugs, respectively, were shown to be of natural origin, based on the numbers of new drugs approved by regulatory agencies (e.g., the United States Food and Drug Administration, FDA) from 1981 to 2002 (Newman *et al.*, 2003).

Structural modifications of these natural products are often necessary for improvements in efficacy and pharmacokinetics. Optimization of drug leads can be achieved using modern organic chemistry. Yet, introduction of structural changes by chemical synthesis is not always easy, especially if the compound's scaffold is very complex (Wohlleben and Pelzer, 2002).

In 1985, Hopwood *et al.* (1985) reported for the first time the production of "hybrid" antibiotics by genetic engineering of *Streptomyces* strains, i.e. through the transfer of biosynthetic genes between strains producing different members of the same class of antibiotics in order to combine structural features of both compounds.

In the meantime, different authors, e.g. (Kunnari *et al.*, 2000; Madduri *et al.*, 1998; Yanai *et al.*, 2004; Zhao *et al.*, 1999), have demonstrated that rational and directed generation of structurally modified antibiotics can be achieved by metabolic engineering of biosynthetic pathways, i.e. by the modification of specific biochemical reactions or the introduction of new ones using recombinant DNA technology (Stephanopoulos, 1999).

Another approach to create new compounds, which has been applied especially for polyketides and nonribosomal peptides, is the so-called combinatorial biosynthesis, namely the shuffling of biosynthetic genes with related functions but different specificities via genetic engineering to create combinatorial libraries of novel natural product-like molecules (Cane *et al.*, 1998; Khosla, 1998; McDaniel *et al.*, 1999; Walsh, 2002).

The last few years have shown that the manipulation of the genes which encode the enzymes in the biosynthetic pathways represents a promising alternative approach for redesign of antibiotic structures to create new activities and overcome bacterial resistance to existing drugs (Walsh, 2002).

The functional analysis of biosynthetic genes is, however, a pre-requisite for such approaches. Moreover, a principal limitation is the challenge associated with developing suitable protocols for the genetic manipulation of natural producers. The expression of biosynthetic genes in heterologous hosts which are genetically more amenable can be used to circumvent this problem (Eppelmann *et al.*, 2001; Sánchez *et al.*, 2002; Tang *et al.*, 2000).

I.2. *Streptomyces* – the largest antibiotic-producing genus

Streptomycetes are among the most numerous and ubiquitous soil bacteria, where they play a central role in carbon recycling. Unusually for bacteria, streptomycetes exhibit complex multi-cellular development: a branching, filamentous vegetative growth gives rise to aerial hyphae bearing long chains of reproductive spores (Bentley *et al.*, 2002; Kieser *et al.*, 2000).

They belong to the order Actinomycetales, which includes, besides a large number of antibiotic-producing species, also major pathogens like *Mycobacterium tuberculosis*, *M. leprae* and *Corynebacterium diphtheriae* (reviewed in (Embley and Stackebrandt, 1994)).

Streptomycetes produces over two thirds of the clinically useful antibiotics of natural origin (Kieser *et al.*, 2000). The history of antibiotics derived from this genus begins with the discovery of streptothricin, in 1942. It was streptomycin, however, discovered two years later that triggered systematic screening of antibiotics from *Streptomyces*. Between 1955 and 1962, about 80% of the antibiotics discovered originated from actinomycetes, among which the major contributor was *Streptomyces* (Watve *et al.*, 2001).

S. coelicolor A3(2) is genetically the best known representative of the genus. This strain is able to produce four known antibiotics, i.e. the tri-pyrroles prodiginines, the polyketide actinorhodin, the acidic lipopeptide calcium-dependent antibiotic (CDA) and the unusual exomethylene cyclopentanone methylenomycin. The biosynthetic gene clusters for the first three are found in the chromosome and for the latter, in plasmid SCP1.

The complete genome sequence of strain M145, which is a prototrophic derivative of strain A3(2) lacking its two plasmids (SCP1, linear, 365 kb; and SCP2, circular, 31 kb) has been reported (Bentley *et al.*, 2002). The single chromosome of 8.7 Mb is linear with a centrally located origin of replication and a G+C content of 72%. Interestingly, the distribution of different types of genes reveals a central core, comprising approximately half the chromosome and a pair of chromosome arms. Nearly all genes likely to be unconditionally essential are located in the core. Non-essential functions, such as secondary metabolites, lie in the arms (Bentley *et al.*, 2002).

Comparison of the *S. coelicolor* genome with those of two pathogenic actinomycetes, *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*, indicated a conserved gene arrangement between the whole genomes of the pathogens and just the core of the *S. coelicolor* chromosome. This suggested descent of the core from a common actinomycete ancestor, with the arms being acquired subsequently by horizontal gene transfer (Bentley *et al.*, 2002; Hopwood, 2003).

The complete genome of *S. avermitilis*, which produces the anti-parasitic agent avermectin, has also been sequenced recently (Ikeda *et al.*, 2003). Its genome (linear, 9 Mb, G+C content of 70.7%) shows a similar division between core and arm regions and provides further support for the idea that the arms have a separate origin from the core and an adaptive function (Hopwood, 2003; Ikeda *et al.*, 2003).

In the context of biotechnology, the most interesting finding in the two *Streptomyces* genomes is the abundance of genes that would encode enzymes for secondary metabolism. Before the genome was sequenced, three antibiotics and a spore pigment were known to be encoded in the *S. coelicolor* chromosome, but the sequence revealed 24 clusters for secondary metabolites. For *S. avermitilis*, 30 such clusters, nearly all encoding different compounds in the two species, were described (Hopwood, 2003).

Thus, there is increasing evidence that the full capacity for secondary metabolite production in soil microorganisms is not expressed under typical conditions used for antibiotic screening in the laboratory. Furthermore, only a small portion of microbes are culturable by present methods, indicating that a large number of pathways still awaits discovery in the actinomycetes. This lends encouraging support to use genetic approaches to antibiotic discovery, like heterologous expression of biosynthetic gene clusters in culturable and genetic manipulable strains and combinatorial biosynthesis or metabolic engineering as methods to increase the natural diversity (Hopwood, 2003; Walsh, 2003).

I.3. Aminocoumarin antibiotics

The aminocoumarin antibiotics novobiocin, clorobiocin (=chlorobiocin), and coumermycin A_1 (Fig. I.1) are potent inhibitors of bacterial DNA gyrase produced by different *Streptomyces* strains (Lewis *et al.*, 1996b; Maxwell, 1993; Maxwell, 1997). Novobiocin was first reported in the middle of 1950s (called then streptonivicin) (Hoeksema *et al.*, 1955; Smith *et al.*, 1956), whereas coumermycin and clorobiocin were found between the 1960s and the 1970s (Kawaguchi *et al.*, 1965; Ninet *et al.*, 1972). In the literature, two producers of novobiocin are described, i.e. *S. spheroides* NCIMB 11891 (Steffensky *et al.*, 2000b) and *S. niveus* (Smith *et al.*, 1956). However, Lanoot *et al.* (2002) have proposed that these two strains are synonyms of *S. caeruleus* LMG 19399T. Clorobiocin is produced by *S. hygroscopicus*, *S. albocinerescens* and *S. roseochromogenes* var. *oscitans* (Mancy *et al.*, 1974; Ninet *et al.*, 1972). Coumermycin A_1 is obtained from several *Streptomyces* strains, i.e. *S. rishiriensis*, *S. hazeliensis* var. *hazeliensis*, *S. spinichromogenes* and *S. spinicoumarensis* (Berger and Batcho, 1978). So far, two further aminocoumarin antibiotics, simocyclinones of the D class (Schimana *et al.*, 2000) and rubradirin (Sohng *et al.*, 1997), have been discovered which are also produced by different *Streptomyces* strains.

I.3.1. Chemical structure

The characteristic structural moiety of the aminocoumarin antibiotics is a 3-amino-4,7-dihydroxycoumarin unit (= Ring B), which is attached to a deoxysugar (= Ring C) at position 7´, and to an acyl component at position 3´ (Fig. I.1). Novobiocin and clorobiocin carry the same acyl substituent, 3-dimethylallyl-4-hydroxybenzoyl moiety (= Ring A). In contrast, the acyl component in coumermycin A_1 is a 3-methylpyrrole-2,4-dicarboxylic acid. To both carboxyl groups, the same aminocoumarin-deoxysugar moiety is attached, making the molecule nearly, but not completely symmetrical in structure. The 3´´-OH group of the deoxysugar is acylated, in novobiocin with a carbamoyl group, in the other two with a 5-methylpyrrole-2-carboxylic acid. Clorobiocin, as indicated by its name, carries a chlorine atom at position 8´ of the aminocoumarin unit, whereas novobiocin and coumermycin A_1 show a methyl group at the corresponding positions.

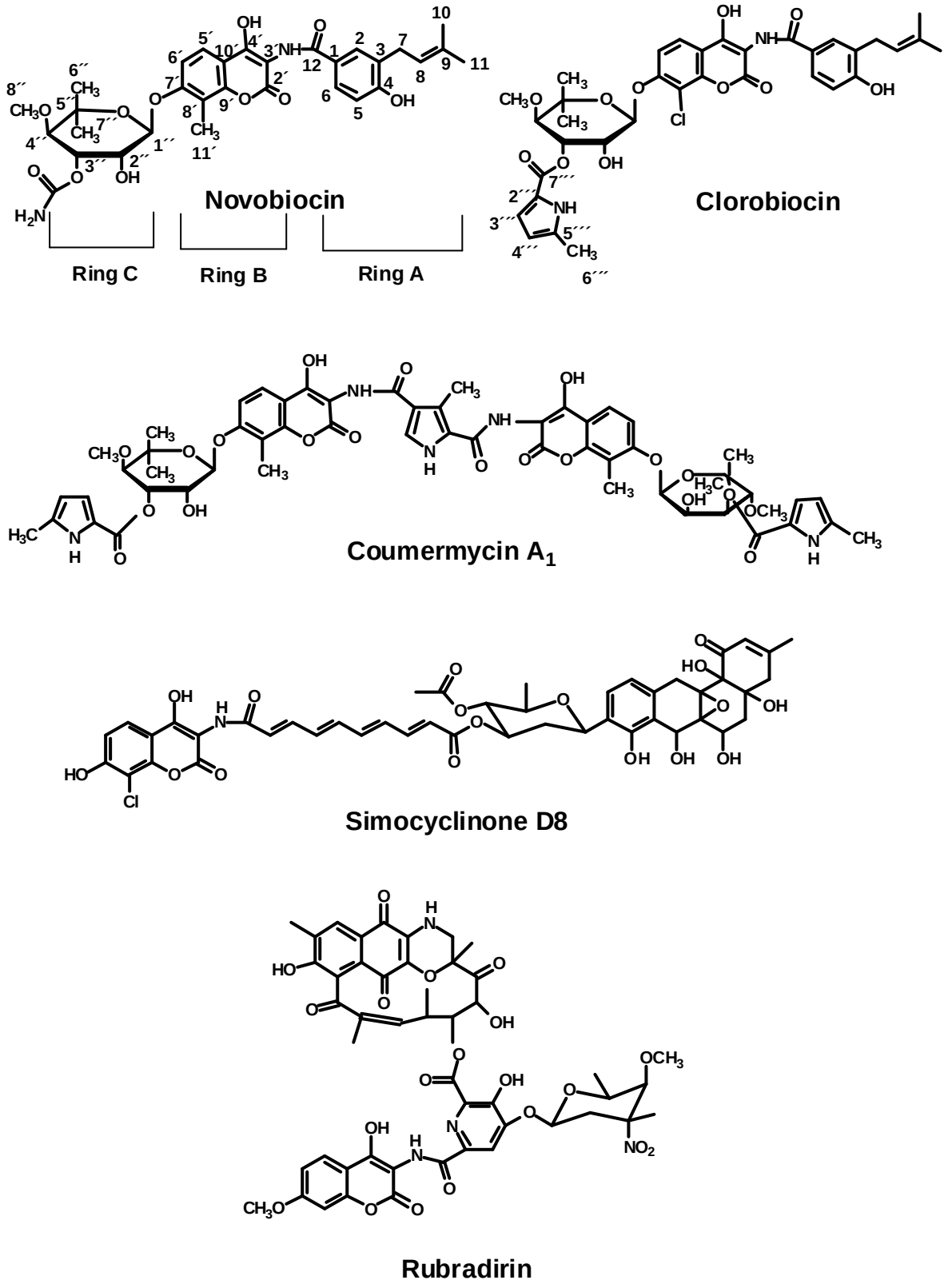


Fig. I.1: Structure of the aminocoumarin antibiotics.

The simocyclinones of the D class and rubradirin have in common with the “classical” aminocoumarins described above only the aminocoumarin ring, which is substituted at position 8 in simocyclinone D8 with a chlorine atom, just as in clorobiocin (Fig. I.1).

I.3.2. Mechanism of action and clinical application

The molecular basis of action of novobiocin, clorobiocin and coumermycin A_1 has been examined by Maxwell (1993; 1999), by Lewis *et al.* (1996b) and more recently by Maxwell and Lawson (2003). These drugs are very potent inhibitors of bacterial DNA gyrase. Their equilibrium dissociation constants are in the 10 nM range, i.e. two orders of magnitude lower than those of modern fluorochinolones. Their affinity for gyrase is therefore considerably higher than that of fluorochinolones.

Gyrase is a type II topoisomerase, which consists of two subunits, GyrA and GyrB, with the active enzyme being an A_2B_2 complex. DNA topoisomerases (enzymes which control the topological state of DNA) are found in all cell types and are essential for cell viability, since they are implicated in DNA transcription and replication. Gyrase is found in all bacteria but not in eukaryotes and is therefore a good target for antibacterial agents. It has the unique ability to introduce negative supercoils into closed circular DNA, playing a role in the maintenance of the level of intracellular DNA supercoiling and removing supercoils that build up ahead of and behind transcription and replication complexes. The supercoiling reaction involves the wrapping of DNA around the A_2B_2 complex, cleavage of this DNA in both strands and passage of a segment of DNA through this double-stranded break. Resealing of the break results in the introduction of two negative supercoils. Catalytic supercoiling requires the hydrolysis of ATP to complete enzyme turnover. GyrA is involved in DNA breakage-resealing reactions (N-terminus) and DNA wrapping (C-terminus), whereas GyrB catalyses ATP hydrolysis (N-terminus, 43 kDa domain) and interacts with GyrA and DNA (C-terminus).

Novobiocin, clorobiocin and coumermycin A_1 are competitive inhibitors of the ATPase reaction catalysed by GyrB. In comparison, quinolones inhibit gyrase-catalysed supercoiling by disrupting the DNA breakage-reunion reaction on GyrA. X-ray crystallographic studies using the 24 kDa N-terminal subdomain of *Escherichia coli* GyrB showed that the complexes between novobiocin (Lewis *et al.*, 1996a) or clorobiocin (Tsai *et al.*, 1997) and the protein involve hydrophobic interactions and a network of hydrogen bonds. Key hydrogen bonds include those between Arg136 and the coumarin ring, Asp73 and the 3´´-acyl group on the deoxysugar, and Asn46 and the 2´´-hydroxyl group on the deoxysugar (Fig. I. 2). The drugs do not occupy the

same binding pocket as ATP, but the sites for the two ligands overlap: the deoxysugar moiety overlaps the binding site for the adenine ring of ATP.

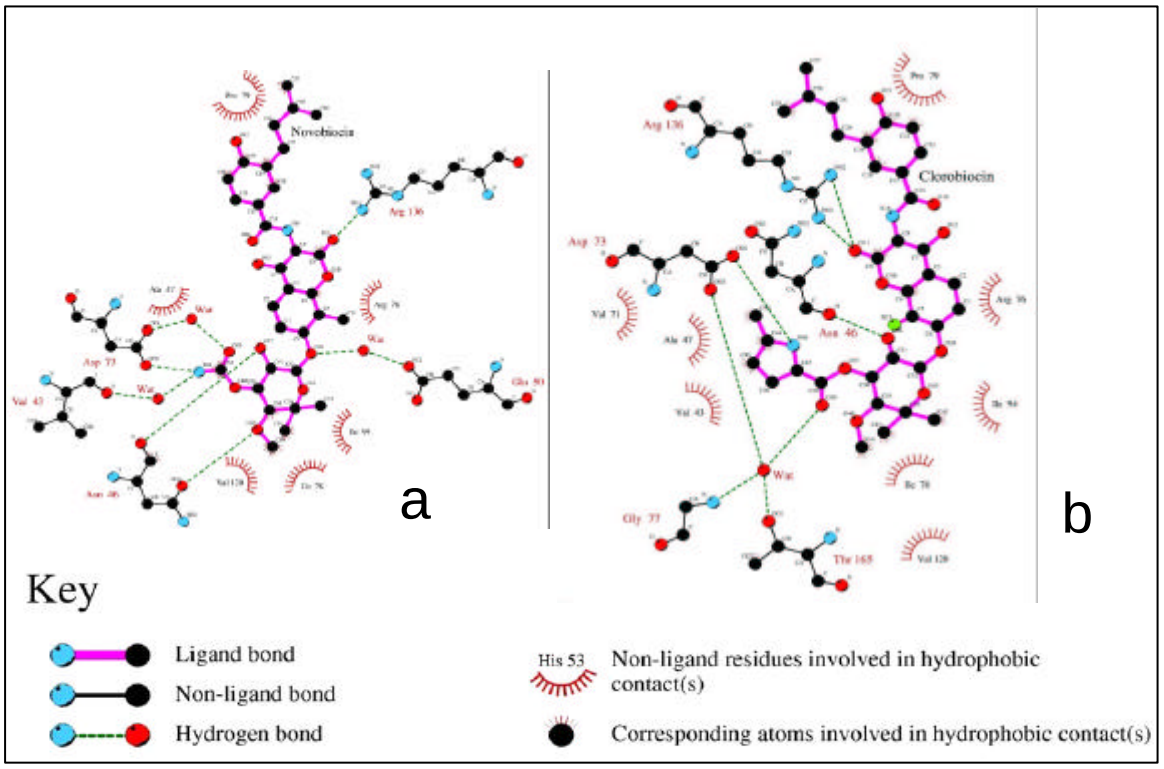


Fig. I.2: Interaction between *Escherichia coli* GyrB and novobiocin (a) as well as between GyrB and clorobiocin (b), adapted from (Maxwell and Lawson, 2003).

Coumermycin A_1 has been shown to stabilize a dimmer form of the 43 kDa N-terminal fragment of GyrB. This is consistent with the pseudo-dimeric structure of coumermycin (Fig. I.1) (Maxwell and Lawson, 2003).

Analysis of aminocoumarin-resistant bacterial strains from several species has identified point mutations that map to the 24 kDa N-terminal subdomain of GyrB. The most prevalent of these are mutations of an arginine residue (Arg136 in *E. coli* GyrB). GyrB proteins that bear mutations at Arg136 show low supercoiling and ATPase activities. It is likely that other mutations in GyrB would also confer coumarin resistance but would lead to inactive protein, i.e. they would not be selected in a bacterial screen (Maxwell, 1999).

Recently, Hardy and Cozzarelli (2003) proposed that topoisomerase IV (topo IV), another essential bacterial type II topoisomerase, is a secondary target of novobiocin.

The primary function of topo IV is the ATP-dependent decatenation of daughter chromosomes following DNA replication. The topo IV of *E. coli* has a similar structure as gyrase: two ParC and two ParE subunits which form a C_2E_2 complex. In a similar way, ParC is responsible for DNA breakage and reunion, and ParE contains the ATP-binding site. The authors concluded, by mutational experiments in the ParE subunit, that novobiocin likely inhibits topo IV by the same mechanism as DNA gyrase is inhibited.

Novobiocin (Albamycin®, Pharmacia & Upjohn) was licensed in the USA for the treatment of human infections with multi-resistant gram-positive bacteria such as *Staphylococcus aureus* and *S. epidermidis*. Its efficacy has been demonstrated in preclinical and clinical trials (Raad *et al.*, 1995; Raad *et al.*, 1998; Walsh *et al.*, 1993). However, due to some adverse reactions (principally urticaria and dermatitis), their poor solubility in water, and their low activity against Gram-negative bacteria (resulting from poor permeability), clinical use of these antibiotics remains restricted (Maxwell, 1993). Clorobiocin and coumermycin have not been used clinically. Therefore it is of interest to test whether new, structurally modified aminocoumarin antibiotics may be able to overcome the limitations of the known compounds (Maxwell and Lawson, 2003).

In addition, novobiocin and its derivatives have also been investigated as potential anticancer drugs. Novobiocin acts synergistically with etoposide and could be used in combination therapies to overcome drug resistance. The increase in etoposide cytotoxicity produced by novobiocin was not due to the combined effects of these agents on topoisomerase II, but to inhibition by novobiocin of etoposide efflux (Rappa *et al.*, 1992; Rappa *et al.*, 2000a; Rappa *et al.*, 2000b). Furthermore, novobiocin, clorobiocin and coumermycin A_1 were shown to interact with eukaryotic heat shock protein 90 (Hsp90), which plays a key role in the stability and function of multiple cell-signalling molecules, e.g. several oncogenic tyrosine and serine-threonine kinases, being expressed at two- to ten-fold higher levels in tumour cells than in their normal counterparts. Hsp90 is therefore considered to be a novel molecular target for anticancer therapeutics. Aminocoumarins markedly reduced cellular levels of oncogenic kinases *in vitro* and *in vivo* (mice) by interacting with Hsp90 (Marcu *et al.*, 2000). Recently, novobiocin was found to increase CD38 expression on cells of myeloid leukaemia lines. Human myelogenous leukaemia cells are unable to

differentiate to functional mature cells. This arrest in maturation is accompanied by uncontrolled cell proliferation. Novobiocin showed anti-proliferative properties and the ability to induce myeloid leukaemia cell lines to differentiate along the monocytic path. The latter was concluded by measuring the expression of a range of differentiation markers, like the surface antigen CD38 in the cell lines (Thiele *et al.*, 2002).

I.3.3. Structure-activity relationships

Hooper *et al.* (1982) have investigated aminocoumarin antibiotics and their analogues for their inhibitory activity towards *E. coli* DNA gyrase *in vitro* and for their antibacterial activity against *E. coli* cells. Important for activity was the aminocoumarin moiety linked to the substituted deoxysugar; lack of the pyrrole or carbamoyl substituent resulted in loss of inhibitory activity. Reusser and Dolak (1986) have also reported the novenamine, i.e. aminocoumarin moiety plus substituted deoxysugar, to represent the minimal structural entity of novobiocin necessary to interact with DNA gyrase. This is in accordance with X-ray data (see above). The aminocoumarin or the deoxysugar alone were inactive in antibacterial and anti-gyrase activities (Althaus *et al.*, 1988; Hooper *et al.*, 1982; Reusser and Dolak, 1986). Coumermycin A_1 was the most active compound (Hooper *et al.*, 1982). As mentioned above, the pseudo-dimeric structure of coumermycin may be responsible for such effect (Maxwell and Lawson, 2003). Besides, of many aryl and alkyl substituents tested, the prenylated benzoic acid moiety of novobiocin and clorobiocin, i.e. Ring A, was the most effective (Hooper *et al.*, 1982). This was also confirmed by a recent study (Galm *et al.*, 2004b). Ring A was supposed to be unimportant in DNA gyrase interactions and to only facilitate the absorption of aminocoumarin compounds (Lewis *et al.*, 1996a), which, however, was disproved by recent studies (Galm *et al.*, 2004b; Lafitte *et al.*, 2002).

Clorobiocin has been reported to show a higher inhibition of *Escherichia coli* gyrase and bacterial growth than novobiocin (Hooper *et al.*, 1982), and to bind more efficiently to isolated gyrase (Lafitte *et al.*, 2002; Lewis *et al.*, 1996a; Tsai *et al.*, 1997). Most authors have attributed the higher activity of clorobiocin primarily to the pyrrole moiety at C-3´´ (Berger and Batcho, 1978; Tsai *et al.*, 1997). Interestingly, however, clorobiocic acid, the aglycon of clorobiocin, but not novobiocic acid, was found to

inhibit both DNA synthesis *in vivo* and gyrase activity *in vitro* (Althaus *et al.*, 1988; Reusser and Dolak, 1986), suggesting that the chlorine atom makes an important contribution to the biological activity of this molecule.

I.3.4. Biosynthesis and identification of the biosynthetic gene clusters

The biosynthesis of novobiocin was firstly studied by feeding experiments in the 1960s and 1970s. Birch *et al.* (1962) proved that the deoxysugar is derived from intact glucose. Bunton *et al.* (1963) showed that tyrosine is the precursor of both the aminocoumarin moiety and the acyl component Ring A, and concluded by feeding of [carboxyl-^{18}O] labelled tyrosine that the ring oxygen of the aminocoumarin moiety comes from the carboxyl group of tyrosine. However, it has been shown recently by Holzenkämpfer and Zeeck (2002) that the ring oxygen of the aminocoumarin moiety of simocyclinone D8 is derived from molecular oxygen, not from the carboxyl group of tyrosine. The amino group in position 3´ of the coumarin unit was found to be derived from the nitrogen of tyrosine (Calvert *et al.*, 1972). Feeding experiments with ^{13}C-glucose provided evidence for the biosynthesis of the dimethylallyl moiety of novobiocin via the non-mevalonate (= methyl-erythritol-phosphate) pathway (Li *et al.*, 1998). Feeding of ^{14}C-labelled L-proline to a coumermycin producer led to the incorporation of radioactivity into the pyrrole groups of coumermycin A_1 (Scannell and Kong, 1969). However, the degradation method used in this experiment did not allow distinction between the central and the two terminal pyrrole moieties of this compound.

In 2000, cloning and sequencing of the novobiocin (*nov*) biosynthetic gene cluster from *S. spheroides* NCIMB 11981 provided the basis for molecular biological investigations of aminocoumarin antibiotics biosynthesis (Steffensky *et al.*, 2000b). Subsequently, the biosynthetic gene cluster for coumermycin A_1 (*cou*) from *S. rishiriensis* DSM 40489 (Wang *et al.*, 2000) and for clorobiocin (*clo*) from *S. roseochromogenes* var. *oscitans* DS 12.976 (Pojer *et al.*, 2002) were also identified. Comparison of the three gene clusters revealed a strikingly stringent correspondence between the structures of the antibiotics and the organization of the biosynthetic genes (Fig. I.3). The order of the genes coding for each structural moiety are perfectly identical for the three clusters.

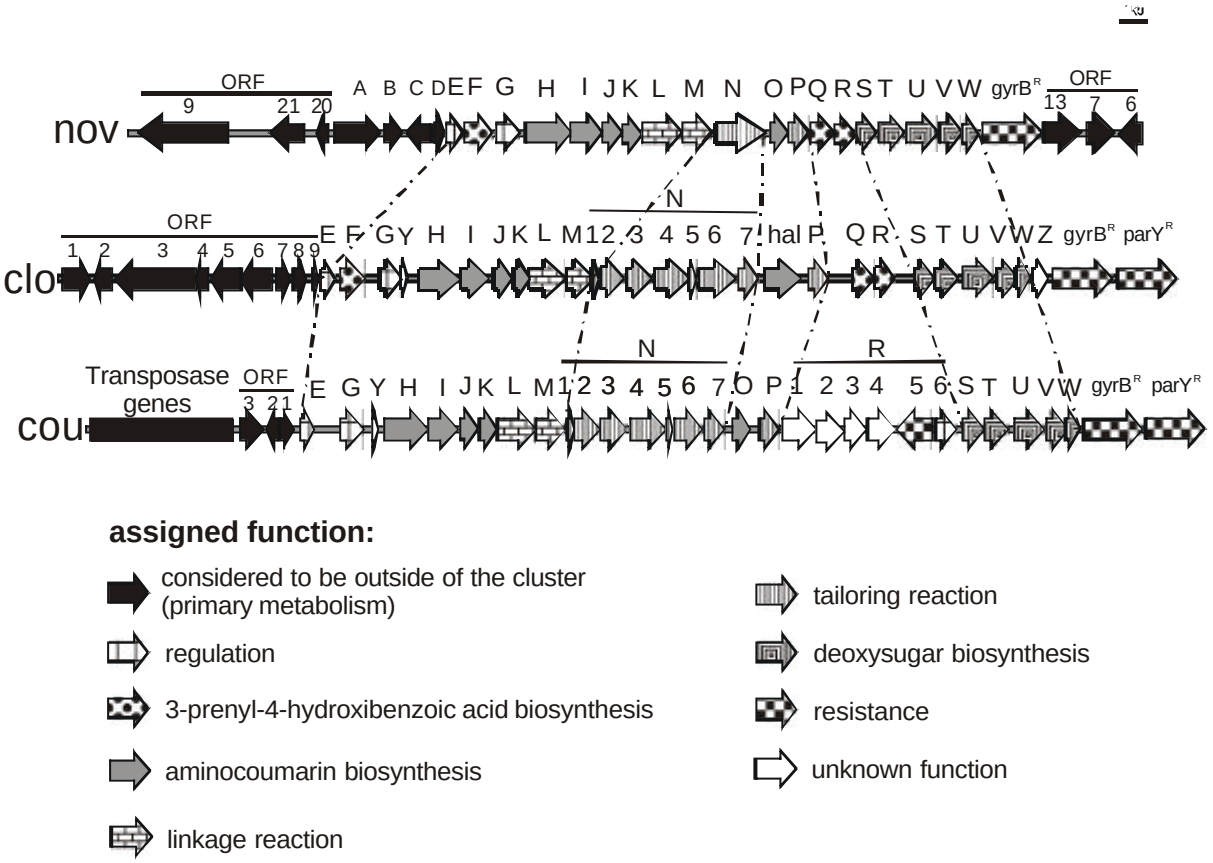


Fig. I.3: The biosynthetic gene clusters of novobiocin (*nov*), clorobiocin (*clo*) and coumermycin A_1 (*cou*). Accession numbers: novobiocin cluster AF170880, clorobiocin cluster AF329398, coumermycin cluster AF235050 (Li and Heide, 2004).

Downstream of *novE*/*cloE*/*couE*, the three clusters show very high similarity between each other, whereas upstream of these genes, the genomic sequences of the three organisms are completely different, containing different genes which may code for primary metabolic enzymes as shown by database comparison analysis. Therefore, it has been suggested but not proven that *novE*, *cloE* and *couE* may represent the left borders of the clusters, respectively (Eustáquio *et al.*, 2003b; Pojer *et al.*, 2002). Likewise, the aminocoumarin resistance genes $gyrB^R$ and $parY^R$ may delineate the right border of these clusters (Schmutz *et al.*, 2003a).

A $gyrB^R$ gene, coding for a resistant GyrB subunit, is found in all three clusters, as expected since the producers must obviously protect their gyrases from the inhibitory effect of aminocoumarins during antibiotic production. Furthermore, the clorobiocin and the coumermycin A_1 but not the novobiocin clusters were shown to contain an additional similar gene, $parY^R$. Expression of $gyrB^R$ and $parY^R$ in *S. lividans* resulted

in resistance against aminocoumarin antibiotics, proving that the function of these genes consists in confer resistance to aminocoumarins (Schmutz *et al.*, 2003a). Recently, Schmutz *et al.* (2004) have overexpressed and purified the encoded proteins, as well as GyrA and ParX subunits. The complex of GyrA and $GyrB^R$ was found to catalyse ATP-dependent supercoiling of DNA, i.e. to function as a gyrase, whereas the complex of ParX and $ParY^R$ catalysed ATP-dependent decatenation and relaxation, i.e. the functions of topo IV. This finding gives additional support to the hypothesis that topo IV is a secondary biological target of aminocoumarins (see above). The high affinity of coumermycin and clorobiocin to both their primary and secondary targets may explain the need of the antibiotic producers to create a resistance mechanism protecting both targets. In addition, this is believed to represent the first topo IV identified in actinobacteria. As explained above, topo IV is found in most bacteria and consists of a $(ParC)_2(ParE)_2$ complex. However, some members of the class of actinobacteria, like species of the genus *Corynebacterium* and *Mycobacterium*, contain only genes for gyrase, which seems to carry out the functions of both gyrase and topo IV. Recently, however, the complete genome sequence of *S. coelicolor* showed that this organism contains in addition to genes coding for gyrase subunits, two genes for another type II topoisomerase. These genes, annotated as “putative gyrase subunit A” (SCO5822) and “subunit B” (SCO5836), were called *parX* and *parY* by our group, respectively, since in a sequence comparison with type II topoisomerases from Gram-positive organisms, these genes could neither be classified as gyrase nor as topo IV (Schmutz *et al.*, 2004).

The hypothetical biosynthetic pathway of aminocoumarin antibiotics is presented in Fig. I.4, exemplified for clorobiocin.

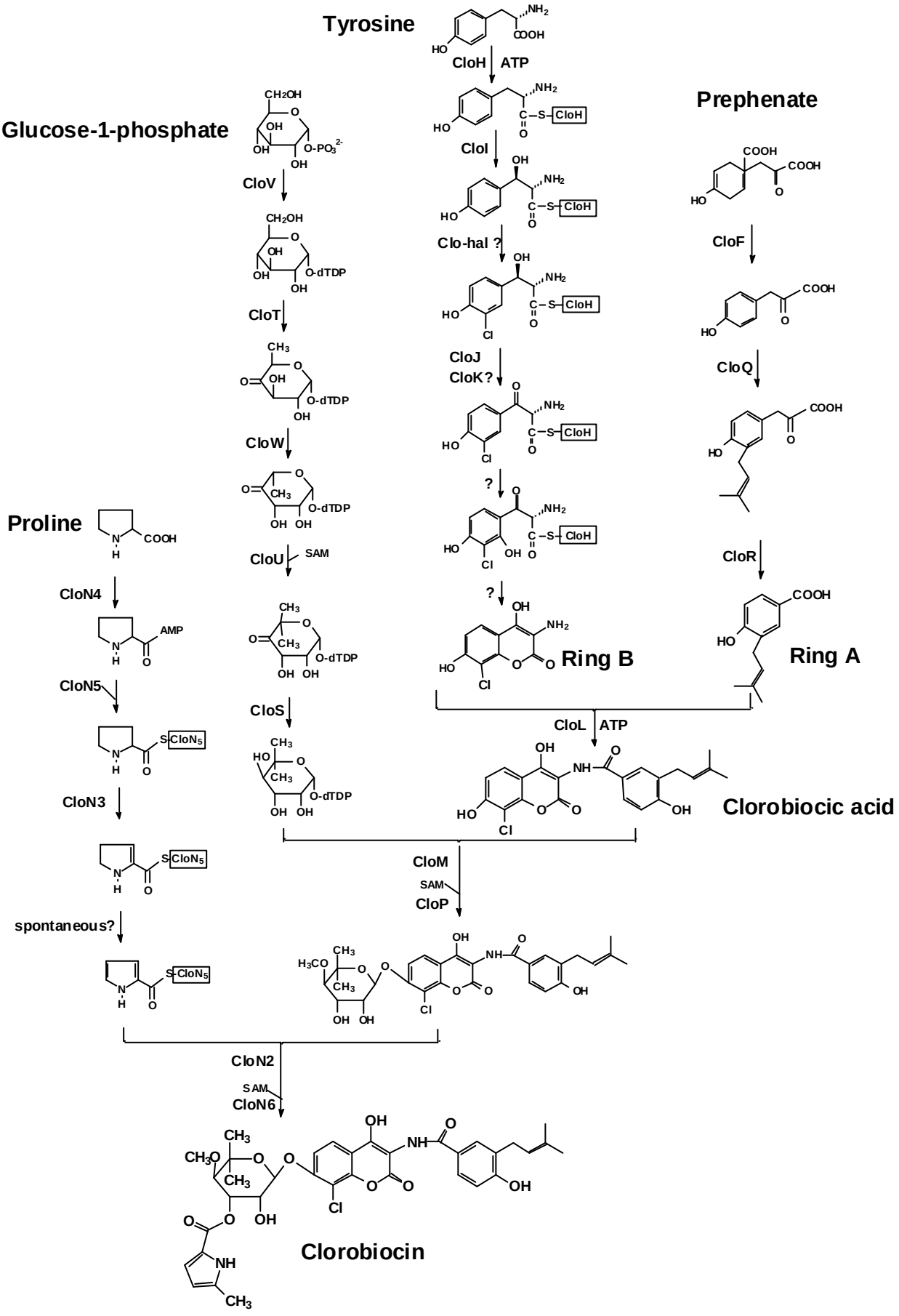


Fig. I.4: Hypothetical biosynthetic pathway of clorobiocin.

It is not clear at which exact step during the biosynthesis halogenation takes place.

Chen and Walsh (2001) have investigated the biosynthesis of the aminocoumarin unit of novobiocin by biochemical methods. The authors demonstrated that within this pathway, the enzyme NovH activates tyrosine by thioester formation with the 4'-phosphopantetheinyl cofactor, and subsequently the cytochrome P_{450} enzyme NovI hydroxylates the activated tyrosine in the b-position (Fig. I.4). It was proposed that NovJ and NovK may act together in the oxidation of b-hydroxytyrosine to b-ketotyrosine, however, no evidences were provided. The same functions may be expected for *clo*HIJK and *cou*HIJK, which show very high homology with each other and with *nov*HIJK.

Holzenkämpfer and Zeeck (2002) recently suggested that the ring oxygen of the aminocoumarin moiety of simocyclinone of the D class (Fig. I.1), which is similar to the aminocoumarin moiety of novobiocin, clorobiocin and coumermycin A_1, is introduced by the action of a monooxygenase. Chen and Walsh (2001) speculated that the product of *novC*, which is situated 5 kb upstream of *novHIJK* in the novobiocin cluster, may encode this monooxygenase. However, no homologue to *novC* is present in the clorobiocin or coumermycin clusters, casting doubts on this suggestion (Eustáquio *et al.*, 2003b; Pojer *et al.*, 2002). It remains to be shown, which gene codes for this monooxygenase, and whether such an enzyme is indeed involved in the aminocoumarin ring formation.

novL has been shown to encode an amide synthetase, linking the substituted benzoyl moiety (= Ring A) of novobiocin to the amino group of the aminocoumarin ring (Steffensky *et al.*, 2000a). The same function is expected for *cloL*. *couL* encodes an unusual amide synthetase, which catalyses the formation of both amide bonds of coumermycin A_1 (Fig. I.1), i.e. between the central pyrrole moiety and the two aminocoumarins (Schmutz *et al.*, 2003b).

Pojer *et al.* (2002; 2003b; 2003a) have elucidated the biosynthesis of Ring A by biochemical and genetic studies. Gene inactivation experiments showed that *cloR* and *cloQ* are essential for Ring A biosynthesis (Pojer *et al.*, 2002; Pojer *et al.*, 2003b). The precursor of this substituted benzoyl moiety is 4-hydroxy-phenylpyruvate (Pojer *et al.*, 2003b), rather than L-tyrosine, as reported by Bunton *et al.* (1963). CloQ is a prenyltransferase, which prenylates 4-hydroxy-phenylpyruvate (Pojer *et al.*, 2003b). CloR, a bifunctional non-heme iron oxygenase, catalyses the conversion of 3-

dimethylallyl-4-hydroxyphenylpyruvate to Ring A in two consecutive oxidative decarboxylation steps, with 3-dimethylallyl-4-hydroxymandelic acid as intermediate (Pojer *et al.*, 2003a). The same functions may be postulated for the corresponding genes of the novobiocin gene cluster, i.e. *nov*QR. NovF and CloF show sequence similarity to prephenate dehydrogenases and are likely to supply 4-hydroxyphenylpyruvate for the biosynthesis of Ring A. No orthologue of *nov/cloF* is found in the coumermycin cluster, consistent with the structure of this antibiotic (Eustáquio *et al.*, 2003b).

Inactivation of *couN3* and *couN4* showed the involvement of these genes in the biosynthesis of the terminal pyrrole-2-carboxylic acid moieties of coumermycin A_1, which were known to be derived from pyrrole (see above), and proved, for the first time, that the central 3-methylpyrrole-2,4-dicarboxylic acid unit of coumermycin is formed by a biosynthetic pathway that differs from that used to assemble the terminal pyrrole moieties (Xu *et al.*, 2002), indicating the need of additional genes which may be responsible for this pathway. *cou*R1-R6 are found in the coumermycin cluster at the same relative position as *nov/clo*QR in the novobiocin and clorobiocin clusters, respectively. It was speculated that *cou*R1-R4 could be involved in the biosynthesis of the central pyrrole unit of coumermycin A_1 (Wang *et al.*, 2000). However, no experimental evidence is available yet. *couR5*, a putative transporter gene, confers moderate levels of resistance against novobiocin and coumermycin A_1 when heterologously expressed in *S. lividans*, suggesting that it may be involved in antibiotic transport (Schmutz *et al.*, 2003a). CouR6 has a putative helix-turn-helix (HTH) motif in its N-terminal region, which is characteristic of bacterial regulatory proteins, and shows sequence similarity to transcriptional regulators of the LysR-type.

Five ORFs are found at the right end of all three gene clusters, i.e. *nov*STUVW, *clo*STUVW and *cou*STUVW, respectively. Based upon their homology to known genes of deoxysugar biosynthesis, they were assigned to the first five steps required for the biosynthesis of the deoxysugar moiety (Pojer *et al.*, 2002; Steffensky *et al.*, 2000b; Wang *et al.*, 2000). A functional proof for this hypothesis was provided by an inactivation experiment with *novT*, since *novT*$^-$ mutants accumulated the aglycon novobiocic acid (Steffensky *et al.*, 2000b).

NovM catalyses the formation of the glycosidic bond between the deoxysugar and the aminocoumarin ring. The aglycone novobiocic acid and TDP-L-noviose were shown to be the preferred substrates (Freel Meyers *et al.*, 2003). CloM and CouM may have the same function.

novN codes for the carbamoyltransferase responsible for the introduction of the carbamoyl group of novobiocin (Freel Meyers *et al.*, 2004; Xu *et al.*, 2004). At the corresponding position in the clorobiocin and coumermycin A_1 clusters, the genes *clo*/*cou*N1-N7 are found. Clo/CouN3-N5 are involved in the biosynthesis of the pyrrole-2-carboxyl moiety from proline (Thomas *et al.*, 2002; Xu *et al.*, 2002), whereas Clo/CouN2 are the acyltransferases involved in the attachment of this moiety to the deoxysugar of clorobiocin and coumermycin. Since only pyrrole-2-carboxylic acid, but not its 5-methyl derivative was found in *cloN2*-defective mutants, the methylation of the pyrrole unit must occur only after its transfer to the deoxysugar moiety (Xu *et al.*, 2003). An inactivation experiment provided evidence that CloN6 catalyzes the 5-methylation of the pyrrole group of clorobiocin (Westrich *et al.*, 2003). The same function may be assigned to CouN6. The functions of clo/couN1 and clo/couN7 remain to be elucidated.

Gene inactivation experiments showed that *couO* encodes a C-methyltransferase responsible for the transfer of the methyl group to C-8´ of the aminocoumarin ring, and *couP* an O-methyltransferase for methylation of 4´´-OH of the deoxysugar (Li *et al.*, 2002). Homologues of *couP* are found in the clorobiocin and novobiocin clusters (*cloP* and *novP*, respectively). However, no gene homologous to *novO* and *couO* exists in the clorobiocin cluster. Instead, the gene *clo-hal*, which shows sequence similarity to $FADH_2$-dependent halogenases, is located in the same relative position. In addition, the clorobiocin cluster also contains the ORF *cloZ*, which shows no sequence similarity to known genes. For all genes of the clorobiocin cluster, with the exception of *clo-hal* and *cloZ*, homologues exist in the novobiocin and/or coumermycin cluster. Since the halogen atom represents the only structural characteristic of clorobiocin absent from both novobiocin and coumermycin, it can be questioned whether *cloZ*, together with *clo-hal*, may play a role in the halogenation of clorobiocin.

Besides the genes above, the gene clusters of clorobiocin and coumermycin, but not of novobiocin, contain *cloY* and *cou*Y, respectively, of which the functions are still unknown.

I.4. Regulation of antibiotic production

Considerable efforts are directed towards understanding how streptomycetes synthesize antibiotics, in order to manipulate the gene clusters and produce novel compounds with improved properties. In addition, the identification and characterization of regulatory genes is crucial for the rational engineering of strains with enhanced antibiotic production.

Biosynthesis of secondary metabolites by *Streptomyces* is a complex process involving several levels of regulation. In this respect, two phylogenetically distant species have received the most extensive attention so far: *S. coelicolor* A3(2) and *S. griseus*. The former because of its early development as an excellent genetic system, which allowed the analysis of morphological differentiation and secondary metabolites formation, and the latter because it provided the first well-studied bacterial example of extra cellular signalling by an hormone-like acylated lactone (the γ-butyrolactone A-factor) (Chater and Horinouchi, 2003). In both cases, pleiotropic regulatory genes affect antibiotic biosynthesis by influencing the expression of pathway-specific regulatory genes, which are usually clustered with the structural biosynthetic genes (Chater and Bibb, 1997).

In *S. griseus*, StrR has been characterized as a pathway-specific regulatory protein. It activates the expression of streptomycin biosynthetic genes by binding to DNA loci which are characterized by an inverted repeat with the consensus sequence GTTCGActG$(N)_{11}$CagTcGAAc and which are located upstream of respective promoter regions (Retzlaff and Distler, 1995). StrR has a putative helix-turn-helix (HTH) motif in the central region of its primary structure (Retzlaff and Distler, 1995), which is typical for a family of bacterial and phage DNA-binding proteins (Pabo and Sauer, 1992).

Analysis of ActII-ORF4 from *S. coelicolor* (Arias *et al.*, 1999; Wietzorrek and Bibb, 1997) and of DnrI from *S. peucetius* (Sheldon *et al.*, 2002; Wietzorrek and Bibb, 1997) revealed another family of *Streptomyces* antibiotic regulatory proteins, termed

SARPs. They show in their predicted secondary structure a OmpR-like DNA-binding domain with a different structure than the typical HTH motif (Sheldon *et al.*, 2002; Wietzorrek and Bibb, 1997). These proteins activate transcription of target genes by binding to direct (rather than inverted) heptameric repeats with the consensus sequence TCGAGCG/C located close to the transcriptional start sites (Arias *et al.*, 1999; Sheldon *et al.*, 2002; Wietzorrek and Bibb, 1997).

Furthermore, other types of pathway-specific regulatory genes have been identified from different *Streptomyces* strains, like *srmR* of the spiramycin cluster in *S. ambofaciens*, the predicted product of which shows no significant sequence similarity to any other known regulatory protein, *mmyR* of the methylenomycin cluster in *S. coelicolor*, representing the first negative pathway-specific regulator of antibiotic production identified, and response regulator genes of putative two-component systems like *dnrN* of the daunorubicin cluster in *S. peucetius* and *redZ* of the undecylprodigiosin cluster in *S. coelicolor* (Chater and Bibb, 1997).

Extensive investigation of the biosynthetic pathways of the aminocoumarin antibiotics (see above) as well as the generation of novel antibiotics by metabolic engineering (this thesis (Eustáquio *et al.*, 2003a; Eustáquio *et al.*, 2004)), chemo-enzymatic synthesis (Xu *et al.*, 2004) and precursor-directed biosynthesis (Galm *et al.*, 2004a) have been carried out.

In contrast, little is known about how aminocoumarin antibiotic production is regulated. Two candidate genes may be involved in the regulation of aminocoumarin antibiotic biosynthesis, i.e. *nov/clo/couG* and *nov/clo/couE*.

NovG shows sequence similarity to StrR, the well-studied pathway-specific activator of streptomycin biosynthesis in *S. griseus* (see above). NovE shows sequence similarity only to an unknown protein, LmbU, of the lincomycin biosynthetic gene cluster. Lincomycin, like the aminocoumarin moiety of novobiocin, is formed from tyrosine, but the reaction sequence is unknown. Peschke *et al.* (1995) speculated that *lmbU* may encode a regulatory protein, but a catalytic function for LmbU could not be excluded.

I.5. Aims of this study

The principal aims of this study were:

1. To provide experimental evidence for the function of the genes involved in the chlorination and methylation reactions at position 8 of the aminocoumarin rings of the antibiotics clorobiocin and novobiocin, respectively.
2. To generate structural analogues of clorobiocin and novobiocin for an investigation of the structure-activity relationships.
3. To produce these antibiotics in a heterologous host, by transfer of the biosynthetic gene clusters to a different organism.
4. To investigate the regulation of novobiocin biosynthesis and to develop methods to increase its productivity by genetic means.

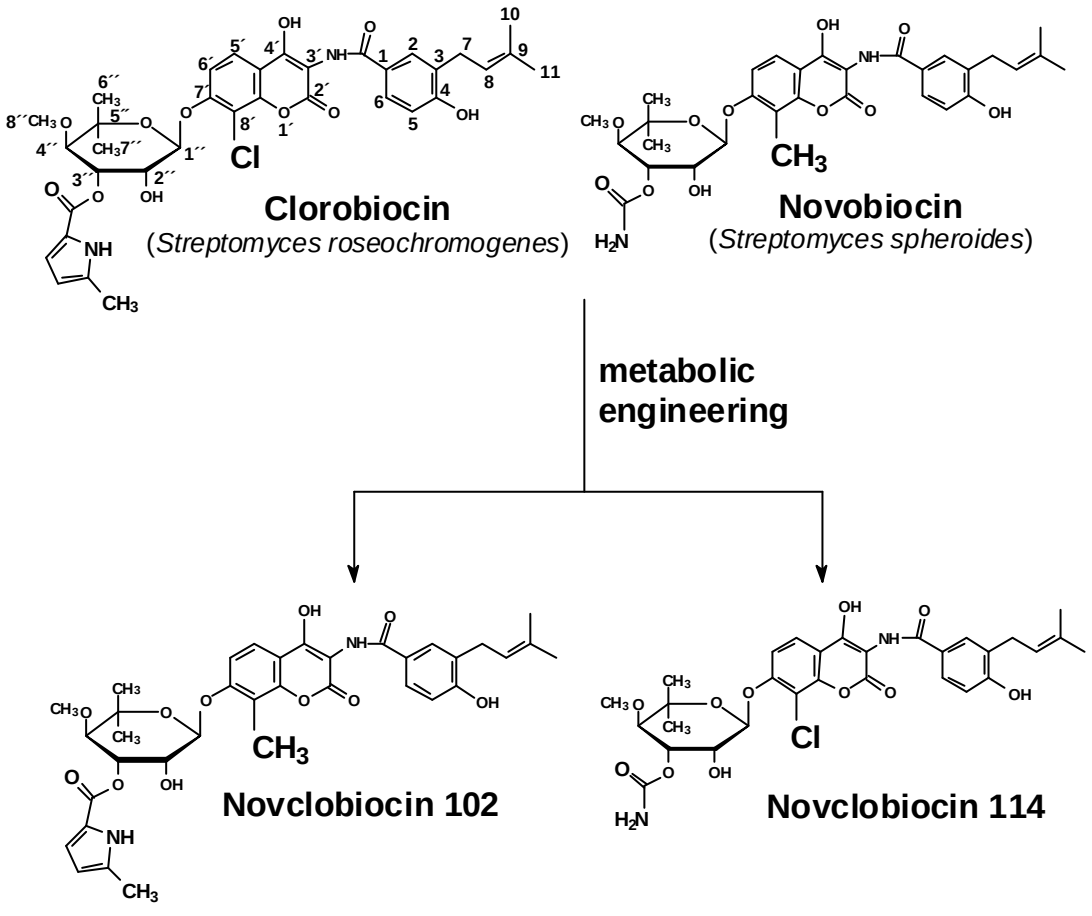


Fig. I.5: Structures of the aminocoumarin antibiotics clorobiocin and novobiocin and of the hybrid antibiotics novclobiocin 102 and 114, which should be generated by metabolic engineering of the biosynthetic gene clusters.

The first aim of the present study was to provide functional proof for the role of the putative halogenase gene *clo-hal* and of the gene *cloZ* in clorobiocin biosynthesis by gene inactivation.

The aminocoumarin antibiotic clorobiocin and the glycopeptide antibiotic balhimycin both contain 3-chloro-*b*-hydroxy-tyrosyl moieties, and the biosynthesis of these moieties show striking similarities. However, for both antibiotics it is not clear at which step of biosynthesis the halogenation reaction takes place (Eustáquio *et al.*, 2003a; Puk *et al.*, 2002; Süssmuth and Wohlleben, 2004). In order to gain more insights about the substrate specifity of the halogenases Clo-hal of clorobiocin biosynthesis and BhaA of balhimycin biosynthesis, it was therefore examined whether *bhaA* could functionally replace *clo-hal*.

Clorobiocin (Fig. I.5) has been reported to show a stronger inhibition of *Escherichia coli* gyrase and of bacterial growth than novobiocin (Hooper *et al.*, 1982), and to bind more tightly to isolated gyrase (Lafitte *et al.*, 2002; Lewis *et al.*, 1996a; Tsai *et al.*, 1997). The relative contribution of the 5-methylpyrrole-2-carboxyl moiety at 3´´-OH of clorobiocin and of the chlorine atom at C-8´ to this effect is not known (Berger and Batcho, 1978; Eustáquio *et al.*, 2003a; Tsai *et al.*, 1997). Hybrid antibiotics combining structural features of these two compounds (Fig. I.5) and derivatives lacking the substitution at C-8´ should allow an investigation of these structure-activity relationships. Generation of novclobiocin 102 (Fig. I.5) should be attempted by inactivation of *clo-hal* in the clorobiocin producer *S. roseochromogenes* and heterologous expression of the putative C-methyltransferase gene *novO* from the novobiocin producer *S. spheroides* in the *clo-hal*$^-$ mutant.

On the other hand, earlier attempts to express the halogenase *clo-hal* in a *novO*$^-$ mutant of the natural novobiocin producer *S. spheroides* in order to generate novclobiocin 114 had remained unsuccessful (Pojer and Heide, unpublished results). *S. spheroides* is very difficult to manipulate genetically (Hussain and Ritchie, 1991), and this may have been the cause for the failure of the experiment.

In *Escherichia coli*, rapid methods for gene inactivation are available, including a recently developed technique which uses the λ-Red recombination system (Datsenko and Wanner, 2000). In streptomycetes, genetic manipulation is more difficult. Reasonably efficient methods are established for *Streptomyces coelicolor*, the best-

characterized species of its genus and for *S. lividans*, a close relative of *S. coelicolor* (Bentley *et al.*, 2002; Kieser *et al.*, 2000). In contrast, the development of suitable protocols for the genetic manipulation of *Streptomyces* strains which produce useful antibiotics is time-consuming and often, e.g. for the novobiocin producer *S. spheroides*, gives unsatisfactory results.

In the present study it was therefore attempted to circumvent the problem of genetic manipulation in *S. spheroides* by heterologous expression of the entire biosynthetic gene cluster of novobiocin in *S. coelicolor* and *S. lividans*. The successful heterologous expression of this biosynthetic cluster should also provide, for the first time, proof that all genes which are essential for novobiocin biosynthesis are included in this single cluster. For the same purpose, heterologous expression of the clorobiocin cluster was also attempted. For these experiments, cosmids containing the integration system of the *Streptomyces* phage ϕC31 and the complete novobiocin or clorobiocin clusters should be introduced into the heterologous hosts. Prior to heterologous expression, the DNA sequence of the cosmids' inserts should be easily modified in *E. coli*, allowing, e.g., the production of the desired novclobiocin 114.

It had been suggested that the genes *novE*/*cloE* and *gyrB*^{R}/*parY*^{R} represent the borders of the novobiocin and clorobiocin clusters, respectively. Removal of the DNA regions outside of these borders should be attempted, in order to provide experimental evidence that they are non-essential for biosynthesis.

The last part of this study concerned investigations of regulatory processes. The identification of genes that regulate antibiotic production in streptomycetes can help to rationally engineer novel strains which show higher productivity.

Therefore, the role of *novG* as a regulator of novobiocin biosynthesis was investigated by genetic and biochemical approaches. It was also examined whether overexpression of *novG* can be used to increase novobiocin production. Furthermore, in order to gain insights into the role of the putative regulatory gene *novE*, a *novE*$^{-}$ mutant was generated and examined.

II. MATERIALS AND METHODS

II.1. Chemicals

Chemicals and components of the media used in this thesis are listed in Table II.1.

TABLE II.1: Chemicals and media components

Supplier	Chemical / Media component
Amersham Biosciences, Freiburg, Germany	Agarose
Aventis, Bad Soden, Germany	Clorobiocin
Bacto-Difco, Heidelberg, Germany	Agar Casaminoacids Corn starch (soluble) Malt extract Nutrient agar Peptone Tryptic soy broth Tryptone Yeast extract
Calbiochem-Novabiochem, Bad Soden, Germany	L-Proline Thiostrepton
Fluka, Ulm, Germany	Apramycin Novobiocin
FMC BioProducts, Rockland, USA	NuSieve® GTG® Agarose
Merck, Darmstadt, Germany	Chloramphenicol EDTA Ethanol Glucose Meat extract ß-Mercaptoethanol Methanol Phosphorus pentoxide Uvasol® methanol-d4
Roth, Karlsruhe, Germany	Ammonium persulphate (APS) 5-Bromo-4-chlor-3-indolyl-ß-D-galactopyranoside (X-Gal) Carbenicillin 1,4-Dithiothreitol (DTT) Glacial acetic acid Glycine Isopropanol Isopropyl-ß-thiogalactoside (IPTG)

	Maleic acid Phenol/Chloroform/Isoamylalkohol(25:24:1) Polyethyleneglycol (PEG) 1000 Rotiphorese® Gel 30 (30% acrylamid, 0.8% bisacrylamide) Sodium dodecyl sulphate (SDS) N,N,N´,N´-Tetramethylethylenediamine (TEMED) Tris-(hydroxymethyl)-aminomethane-maleate (Tris-maleate) N-Tris-(Hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES)
Serva, Heidelberg, Germany	Coomassie Brilliant Blue R250 N-Lauroylsarcosine (Na-Salt, 35%)
Sigma-Aldrich, Deisenhofen, Germany	Bromophenol blue Dimethyl formamide (DMF) Dimethyl sulfoxide (DMSO) Distillers grains and solubles Ethyleneglycol Imidazol Kanamycin Polyoxyethlenesorbitan monolaurate (Tween 20) Tetracycline Tris base
Südzucker, Mannheim, Germany	Sucrose

II.2. Materials for Chromatography

The liquid chromatography media were obtained as dry beads, suspensions or commercial columns. The storage of the media or columns was carried out according to the manufacturers' instructions.

TABLE II.2: Liquid chromatography media

Supplier	Medium
Amersham Biosciences, Freiburg, Germany	Sephadex® LH-20 (dry beads) Sephadex® G-25 NAP-10 (commercial column)
C+S Chromatographie Service, Düren, Germany	Multosphere® RP 18-5 (commercial colum, 5 µm, 250×4 mm) Multosphere® 100 RP 18-5 (commercial colum, 5 µm, 250×20 mm)
Qiagen, Hilden, Germany	Ni-NTA Agarose (Suspension)

II.3. Enzymes and kits

TABLE II.3: Enzymes and kits

Supplier	Enzymes and kits
Amersham Biosciences, Freiburg, Germany	Low Molecular Weight Calibration Kit for SDS-Electrophoresis Restriction endonucleases T4 DNA Ligase
Fluka, Ulm, Germany	Lysozyme (76 000 U/mg)
GibcoBRL Life Technologies, Karlsruhe, Germany	1 kb DNA Ladder High Molecular Weight DNA Marker
Macherey-Nagel, Düren, Germany	Nucleobond® AX100 NucleoSpin® Extract 2 in 1
New England Biolabs, Schwalbach, Germany	Restriction endonucleases 100 bp DNA Ladder
Plant Bioscience Limited, Norwich, UK	REDIRECT© technology: PCR-targeting system in *Streptomyces coelicolor*
Promega, Madison, WI, USA	*Taq* DNA polymerase *Pfu* DNA polymerase
Qiagen, Hilden, Germany	RNase A
Roche, Mannheim, Germany	DIG Gel Shift Kit, 2nd Generation DIG-High Prime DNA Labelling and Detection Starter Kit II DNA Molecular Weight Marker VII, DIG-Labelled Expand High Fidelity PCR System GC-Rich PCR System Kit
Strategene, Taufkirchen, Germany	Restriction endonucleases

II.4. Media, buffers and solutions

II.4.1. Media for bacterial cultivation

The media used in this study were as follows. Unless otherwise stated, the media were prepared with distilled water and autoclaved for 20 min at 121 °C (15 psi). To obtain solid media, 2% (w/v) agar was added before autoclaving. If necessary, sterile supplementary components like antibiotics and other heat-labile substances were added in the sterile media at time of use. The media were stored at RT or at 4 °C.

II.4.1.1. Cultivation of *E. coli*

LB (Luria-Bertani) Medium (Sambrook and Russell, 2001)

NaCl	10.0 g
Tryptone	10.0 g
Yeast extract	5.0 g

Dissolve the ingredients in about 900 ml water, adjust the pH to 7.0, and adjust the volume to 1 litre with water. Sterilize by autoclaving.

SOB Medium

Tryptone	20.0 g
Yeast extract	5.0 g
NaCl	0.5 g

Dissolve the ingredients in about 900 ml water, adjust the pH to 7.0, and adjust the volume to 1 litre with water. Sterilize by autoclaving. Add 10 ml of a sterile solution of 1 M $MgCl_2$.

II.4.1.2. Cultivation of *Streptomyces*

YMG (Yeast-Malt-Glucose) Medium

Yeast extract	4.0 g
Malt extract	10.0 g
Glucose	4.0 g

Dissolve the ingredients in about 900 ml water, adjust the pH to 7.3, and add water to make up to 1 litre. Sterilize by autoclaving.

TSB (Tryptone Soya Broth) Medium (Kieser *et al.*, 2000)

Tryptone Soya Broth	30.0 g

Dissolve the ingredient in up to 1 litre water, and sterilize by autoclaving.

YEME (Yeast Extract – Malt Extract) Medium (Kieser *et al.*, 2000)

Yeast extract	3.0 g
Peptone	5.0 g
Malt extract	3.0 g
Glucose	10.0 g
Sucrose	340 g

Dissolve the ingredients in up to 1 litre water. Sterilize by autoclaving. Add 5 ml of a sterile solution of 1 M $MgCl_2$.

MS (Mannitol Soya flour) Agar (Kieser *et al.*, 2000)

Mannitol	20.0 g
Soya flour	20.0 g
Agar	20.0 g

Dissolve the mannitol in up to 1 litre tap water and pour 100 ml into 300 ml Erlenmeyer flasks each containing 2 g agar and 2 g soya flour. Sterilize by autoclaving twice (115 ºC, 15 min) with gentle shaking between the two runs.

Note: This medium is appropriate for *S. coelicolor*, *S. lividans* and *S. spheroides* sporulation, but not for *S. roseochromogenes*.

DNA (Difco Nutrient Agar) (Kieser *et al.*, 2000)

Difco Nutrient Agar	4.6 g

Place the ingredient into 300 ml Erlenmeyer flasks and add 200 ml water. Sterilize by autoclaving.

Note: *S. coelicolor*, *S. lividans*, *S. roseochromogenes* and *S. spheroides* do not sporulate on this medium.

Grundy starch agar

Soluble starch	5.0 g
$(NH_4)_2HPO_4$	1.0 g
KCl	0.2 g
$Mg_2Cl \cdot 6H_2O$	0.2 g

Dissolve the ingredients in about 900 ml water, and adjust the pH to 7.3. Adjust the volume with water to 1 litre and add 15 g Agar. Sterilize by autoclaving. Use high petry dishes (9 cm), in which 50 ml agar can be poured; otherwise they will dry out during the long incubation period which is necessary for *S. roseochromogenes* var. *oscitans* sporulation.

Note: For sporulation of *S. roseochromogenes*, mix 2-3 ml 2-day-old YMG-culture with 1 g sterile powdered soil and plate the mixture on two grundy starch agar plates. Let dry for about 30 min under the laminar flow, and incubate at 28 ºC for four to six weeks till sporulation is observed (the spores have a light pinkish colour).

II.4.1.3. Clorobiocin production medium

Corn starch medium (pre-culture medium) (Mancy *et al.*, 1974)

Corn starch	10.0 g
Peptone	10.0 g
Meat extract	5.0 g

Dissolve the ingredients in about 900 ml water, adjust the pH to 7.0, and add water to make up to 1 litre. Sterilize by autoclaving.

Distillers solubles medium (production medium) (Mancy *et al.*, 1974)

Solution A	Distillers solubles	48.0 g
	Glucose	12.0 g
	Cobalt chloride	24 mg

Dissolve the ingredients in about 800 ml water, adjust the pH to 7.8, add 6.0 g calcium carbonate and then add water to make up to 887 ml.

Solution B Ammonium sulphate 32.0 g

Dissolve in 200 ml water and autoclave.

Solution C Glucose 75.0 g

Dissolve in 300 ml water and autoclave.

After autoclaving, add 13 ml solution B and 100 ml solution C to solution A.

II.4.1.4. Novobiocin production medium

CDM medium (Kominek, 1972)

tri-sodium citrate $\cdot 2H_20$	6.0 g
L-proline	6.0 g
$K_2HPO_4 \cdot 3H_2O$	2.0 g
$(NH_4)_2SO_4$	1.5 g
NaCl	5.0 g
$MgSO_4 \cdot 7H_2O$	2.05 g

$CaCl_2 \cdot 2H_2O$	0.4 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
Glucose (30% (w/v))	100 ml

Dissolve the ingredients till NaCl in water, adjust the pH to 7.2, dissolve the remaining ingredients except the glucose solution, and add water to make up to 900 ml. Sterilize by autoclaving. Add the sterile glucose solution.

II.4.1.5. Protoplast transformation of *Streptomyces*

CRM medium

Sucrose	103.0 g
Tryptic soy broth	20.0 g
$MgCl_2 \cdot 6H_2O$	10.12 g
Yeast extract	10.0 g

Dissolve in about 900 ml water, adjust the pH to 7.0 and adjust the volume to 1 litre. Sterilize by autoclaving. To 100 ml sterile medium, add 1 ml $CaCl_2$ (1 M) and a final concentration of 0.75% glycine (for *S. roseochromogenes*).

YEME medium with glycine (Kieser *et al.*, 2000)

For preparation of protoplasts of *S. coelicolor* and *S. lividans*, 25 ml Glycine 20% (w/v) are added to 1 litre YEME medium (s. II.4.1.2); 0.5% final concentration.

Trace elements solution

$ZnCl_2$	40 mg
$FeCl_3 \cdot 6H_2O$	200 mg
$CuCl_2 \cdot 2H_2O$	10 mg
$MnCl_2 \cdot 4H_2O$	10 mg
$Na_2B_4O_6 \cdot 10H_2O$	10 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10 mg

Dissolve in 1 litre distilled water and autoclave.

R2YE Medium (Kieser *et al.*, 2000)

Sucrose	103.0 g
K_2SO_4	0.25 g
$MgCl_2 \cdot 6H_2O$	10.12 g
Glucose	10.0 g

Casaminoacids	0.1 g
Trace elements solution	2.0 ml
Yeast extract	5.0 g
TES	5.73 g
Agar (plates)	23.0 g

Dissolve in water to a final volume of 1 litre and sterilize by autoclaving. To prepare soft agar, 6 g Agar instead of 23 g, were added.

After autoclaving, add:

KH_2PO_4 (0.5%)	10 ml
$CaCl_2 \cdot 2H_2O$ (5 M)	4 ml
L-Proline (20% (w/v))	15 ml
NaOH (1M)	7 ml

R3 soft agar

Sucrose	171.0 g
Glucose	10.0 g
Peptone	4.0 g
KCl	0.5 g
$CaCl_2 \cdot 2H_2O$	2.2 g
$MgCl_2 \cdot 6H_2O$	8.1 g
Agar	8.0 g

Dissolve in water to a final volume of 1 litre and autoclave. After that, add:

KH_2PO_4 (0.5%)	40 ml
TES (0.25 M, pH 7.2)	100 ml

Soft nutrient agar (Kieser *et al.*, 2000)

Nutrient broth	8 g
Agar	5 g

Dissolve in water to a final volume of 1 litre and autoclave.

II.4.2. Antibiotic solutions

Antibiotics were dissolved in appropriate solvents as stock solutions and kept at -20 °C. The aqueous solutions were sterilized by passing through a 0.22 µm filter. The solutions in ethanol and DMSO were autosterile. For antibiotic selection, the required antibiotics were added to the cooled media (RT to 60 °C) in appropriate concentration.

TABLE II.4: Solutions of antibiotics

Antibiotic	Concentration in		Solvent
	stock solution (mg/ml)	media (µg/ml)	
Apramycin	50	15-50[a]	H_2O
Carbenicillin	50-100	50-100	H_2O
Chloramphenicol	25-50	25-50	ethanol
Kanamycin	50	15-50[a]	H_2O
Tetracycline	12	12	ethanol
Thiostrepton	50	15-50[a]	DMSO
[a]15 µg/ml in liquid and 50 µg/ml in solid media for selection of *Streptomyces* strains; otherwise, 50 µg/ml.			

II.4.3. Buffers and solutions

Unless otherwise stated, the buffers and solutions were prepared with distilled water, autoclaved and stored at room temperature (RT).

II.4.3.1. Buffers and Solutions for DNA isolation

TABLE II.5: Buffers and solutions for plasmid and cosmid isolation from *E.coli*

Buffer	Components	Final concentration	Preparation
Solution MP1	Tris-HCl EDTA RNase A	50 mM 10 mM 100 µg/ml	Adjust the pH to 8.0. Add RNase A just before use.
Solution MP2	NaOH SDS	0.2 M 1% (w/v)	
Solution MP3	$KAc \cdot 3H_2O$	3 M	Adjust the pH to 4.8. Store at 4 °C.

TABLE II.6: Buffers and solutions for plasmid isolation from *Streptomyces*

Buffer	Components	Final concentration	Preparation
Solution MP1GL	Glucose Tris-HCl EDTA RNase A Lysozyme	50 mM 25 mM 10 mM 100 µg/ml 2-4 mg/ml	Adjust the pH to 8.0. Add RNase A and lysozyme just before use.

Solution MP2	NaOH SDS	0.2 M 1% (w/v)	
Solution MP3	$KAc \cdot 3H_2O$	5 M	Adjust the pH to 4.8. Store at 4 °C.

TABLE II.7: Buffers for isolation of genomic DNA from *Streptomyces*

Buffer	Components	Final concntration	Preparation
TSE buffer	Sucrose Tris-HCl EDTA RNase A Lysozyme	10.3% 25 mM 25 mM 100 µg/ml 3 mg/ml	Adjust the pH to 8.0. Add RNase A and lysozyme just before use.
2×Kirby mix	SDS Sodium 4-aminosalicilate 2 M Tris-HCl pH 8 equilibrated phenol pH 8.0	2 g 12 g 5 ml 6 ml	Dissolve the SDS and the sodium 4-aminosalicilate in up to 89 ml distilled water, add the Tris-HCl buffer and the phenol. Do not autoclave. Store protected from light at 4 ºC.
TE buffer	Tris-HCl EDTA	10 mM 1 mM	Adjust the pH to 7.5.

II.4.3.2. Buffers for DNA gel electrophoresis

TABLE II.8: Buffers for DNA gel electrophoresis

Buffer/solution	Components	Final concentration	Preparation
50×TAE	Tris base EDTA Glacial acetic acid	2 M 0.05 M 57.1 ml/l	Adjust the pH to 8.0 with glacial acetic acid.
Load buffer	Glycerol Bromophenol blue	30% (w/v) 0.25% (w/v)	Store at 4 °C
Ethidium bromide solution for staining the agarose gel	Ethidium bromide	1 µg/ml	

II.4.3.3. Buffers and solutions for Southern blot analysis

TABLE II.9: Buffers and solutions for Southern blot analysis

Buffer/solution	Components	Final concentration	Preparation
Denaturing solution	NaOH NaCl	0.5 M 1.5 M	
Neutralizing buffer	Tris-HCl NaCl	0.5 M 3 M	Adjust the pH to 7.5.
20×SSC buffer	Trisodium citrate NaCl	0.3 M 3 M	Adjust the pH to 7.0
Pre-hybridizing solution	Creamed milk powder SDS (10% (w/v) in H_2O) N-lauroylsarcosine (35% (w/v) in H_2O)	3% 0.02% 0.1%	Add to 5×SSC buffer before use.
Hybridizing solution	Creamed milk powder SDS (10% (w/v) in H_2O) N-lauroylsarcosine (35% (w/v) in H_2O)	1.5% 0.02% 0.1%	Add to 5×SSC buffer before use. Add appropriate probe (5-25 ng/ml)
2×Washing buffer	SDS (10% (w/v) in H_2O)	0.1%	Add to 2×SSC buffer before use.
0.5×Washing buffer	SDS (10% (w/v) in H_2O)	0.1%	Add to 0.5×SSC buffer before use.
Maleic acid buffer	Maleic acid NaCl	0.1 M 0.15 M	Adjust the pH to 7.5
Tween® wash buffer	Tween® 20	0.3%	Add to the maleic acid buffer before use.
Blocking solution	Creamed milk powder	3%	Add to the maleic acid buffer just before use
Detection buffer	Tris-HCl NaCl	0.1 M 0.1 M	Adjust the pH to 9.5
Antibody solution	Blocking solution Anti-DIG-AP-conjugate	40 ml 4 µl	Mix just before use.
Stripping solution for blot	NaOH SDS (10% (w/v) in H_2O)	0.2 M 0.1%	

II.4.3.4. Solutions for blue/white selection of *E. coli*

The storage was carried out at -20°C.

TABLE II.10: Stock solutions for blue/white selection.

Solution	Composition	Per plate
IPTG	80 mg/ml in distilled water, sterilize by filtering	15 µl
X-Gal	20 mg/ml in DMF, autosterile	60 µl

II.4.3.5. Buffers for preparation of protoplasts and transformation of *Streptomyces*

The following sterile solutions were prepared separately. To obtain P-buffer and T-buffer, they were mixed according to the description and stored at -20°C.

TABLE II.11: Buffers for preparation of protoplasts and transformation of *Streptomyces*

Buffer	Components	Amount (ml)
P(protoplast)-buffer (Kieser *et al.*, 2000)	Sucrose (12% (w/v) in H_2O)	85.5
	$MgCl_2 \cdot 6H_2O$ (1M)	1.0
	K_2SO_4 (140 mM)	1.0
	Trace elements solution	0.2
	KH_2PO_4 (40 mM)	1.0
	$CaCl_2 \cdot 2H_2O$ (250 mM)	1.0
	TES (0.25M, pH 7.2)	10.0
T(transformation)-buffer (Kieser *et al.*, 2000)	Sucrose (25% (w/v) in H_2O)	1.0
	Trace elements solution	0.03
	K_2SO_4 (140 mM)	0.1
	KH_2PO_4 (40 mM)	0.1
	$MgCl_2 \cdot 6H_2O$ (1 M)	0.1
	$CaCl_2 \cdot 2H_2O$ (5 M)	1.0
	Tris-maleate (0.5 M, pH 8.0)	1.0
	For *S. coelicolor*, *S. lividans*, *S. spheroides*, and *S. rishiriensis*, add PEG 1000 (50% (w/v) in H_2O);	5.0
	or for *S. roseochromogenes*, add PEG 1000 (68% (w/v) in H_2O)	6.6
		adjust the volume with distilled water to 10 ml.

II.4.3.6. Buffers for protein purification by nickel affinity chromatography

The buffers for protein purification were prepared with distilled water, autoclaved and stored at 4 °C. If required, lysozyme, imidazol and DTT were added just before use.

TABLE II.12: Buffers for protein purification

Buffer	Components	Amount
Lysis buffer	NaH_2PO_4/Na_2HPO_4 NaCl Lysozyme Imidazol	50 mM (pH 8.0) 300 mM 2 mg/ml 10 mM
Wash buffer	NaH_2PO_4/Na_2HPO_4 NaCl Imidazol	50 mM (pH 8.0) 300 mM 20-50 mM
Elution buffer	NaH_2PO_4/Na_2HPO_4 NaCl Imidazol	50 mM (pH 8.0) 300 mM 250 mM
Storage buffer	Tris-HCl Glycerol DTT	25 mM (pH 7.5) 10% (w/v) 2 mM

II.4.3.7. Buffers and solutions for protein gel electrophoresis (SDS-PAGE) and for Coomassie staining

All the buffers and solutions were prepared according to the user manual for protein gel electrophoresis of Bio-Rad (Bio-Rad, München, Germany).

TABLE II.13: Buffers and solutions for SDS-PAGE and Coomassie staining

Buffer/solution	Components	Amount	Preparation
Stacking gel (4%)	Distilled water 0.5 M Tris-HCl (pH 6.8) 10% (w/v) SDS Rotiphorese® Gel 30 10% (w/v) APS TEMED	6.1 ml 2.5 ml 0.1 ml 1.3 ml 0.05 ml 0.01 ml	Combine all the components, except APS and TEMED; degas under vacuum for about 15 min. Add APS and TEMED just before pouring the gel.
Resolving gel (12%)	Distilled water 1.5 M Tris-HCl (pH 8.8) 10% (w/v) SDS Rotiphorese® Gel 30 10% (w/v) APS TEMED	3.35 ml 2.5 ml 0.1 ml 4.0 ml 0.05 ml 0.005 ml	(See above).

Sample buffer	Distilled water 0.5 M Tris-HCl (pH 6.8) Glycerol SDS (10% (w/v) in H_2O) Bromophenol blue (0.5% (w/v) in H_2O)	3.55 ml 1.25 ml 2.5 ml 2.0 ml 0.2 ml	Add 5 µl ß-mercapto-ethanol to 95 µl sample buffer prior to each use. Store at 4 °C.
10×running buffer	Tris base Glycine SDS	30.0 g 144.0 g 10.0 g	Dissolve in distilled water to a final volume of 1 litre. Store at 4 °C.
Fixing buffer	Distilled water Acetic acid Methanol	70% (w/v) 10% (w/v) 20% (w/v)	Store at RT.
Coomassie Brilliant Blue G-250 solution	Coomassie Brilliant Blue G-250 Distilled water Acetic acid Methanol	0.25% (w/v) 45% (w/v) 10% (w/v) 45% (w/v)	Store at RT.
Bleaching solution for SDS-PAGE	Distilled water Acetic acid Methanol	45% (w/v) 10% (w/v) 45% (w/v)	Store at RT.

II.4.3.8. Buffers and solutions for gel mobility-shift assay

The buffers for gel mobility-shift assay were stored at RT.

TABLE II.14: Buffers and solutions for gel mobility-shift assay

Solution	Components	Amount	Preparation
5×TBE buffer	Tris base Boric acid EDTA (0.5 M, pH 8.0)	54.0 g 27.5 g 20 ml	Dissolve all components in 1 l distilled water, adjust to pH 8.0, and autoclave.
Native polyacrylamid gel (6%)	5×TBE Distilled water 10% (w/v) APS TEMED	1 ml 9 ml 0.1 ml 0.01 ml	Combine all the components, except APS and TEMED; degas under vacuum for about 15 min. Add APS and TEMED just before pouring the gel (85 x 75 x 0.75 mm).

5×assay buffer	Tris-HCl (pH 7.5) Glycerol DTT	62.5 mM 25% (w/v) 4 mM	Dissolve Tris base and glycerol in distilled water and adjust to pH 7.5. Autoclave. Add DTT from a 200 mM solution prior to each use.

II.5. Plasmids, bacterial strains, primers and probes

II.5.1. Vectors, cosmids and plasmids

TABLE II.14: Vectors, cosmids and plasmids

Name	Description	Source or reference
Vector		
pBluescript SK(-)	Cloning vector, *lacZα*, ColE1-origin, f1(-)-origin, Amp^R	Stratagene
pBSKT	pBluescript SK(+) derivative, *lacZα*, ColE1-origin, non-replicative in *Streptomyces*, Amp^R, $Thio^R$	(Lombó *et al.*, 1997)
pcDNA 2.1	Cloning vector, *lacZα*, ColE1-origin, f1-origin, Amp^R	Invitrogen
pEM4	*E. coli* - *Streptomyces* shuttle vector, *lacZα*, *ermE** promoter, Amp^R, $Thio^R$	(Quirós *et al.*, 1998)
pGEM-T	Linearized vector with T-overhang for direct cloning of PCR fragments with A-overhang, *lacZα*, ori, f1-origin, Amp^R	Promega
pIJ82	pSET152-derivative, *lacZα*, pUC-origin, *oriT* (RK2), *int* and *attP* of ϕC31, Hyg^R	Mike Butler (John Innes Centre, Norwich, UK)
pKC1132	*lacZα*, pUC-origin, non-replicative in *Streptomyces*, *oriT*, $Apra^R$	(Bierman *et al.*, 1992)
pLitmus28	Cloning vector, *lacZα*, ColE1-origin, M13-origin, Amp^R	New England Biolabs
pRSET B	Expression vector for *E. coli*, pUC-origin, T7 promotor, N-terminal 6×His-tag, Amp^R	Invitrogen
pSL1180	Cloning vector, blue/white selection not possible, pUC-origin, M13-origin, Amp^R	Amersham

pSPORT1	Cloning vector, *lacZα*, ori, Amp^R	Invitrogen
pUWL 201	*E. coli* - *Streptomyces* shuttle vector, *ermE** promoter, Amp^R, $Thio^R$	(Doumith *et al.*, 2000)
pWHM3	*E. coli-Streptomyces* shuttle vector, *lacZα*, Amp^R, $Thio^R$	(Vara *et al.*, 1989)
Cosmid		
Cosmid 9-6G	SuperCos1-based cosmid containing the novobiocin biosynthetic gene cluster, Amp^R, Kan^R	(Steffensky *et al.*, 2000b)
Cosmid 10-9C	SuperCos1-based cosmid containing the novobiocin biosynthetic gene cluster, Amp^R, Kan^R	(Steffensky *et al.*, 2000b)
Cosmid nov-BG1	From cosmid 10-9C, *bla* gene replaced by cassette from pIJ787 (*oriT*, *tet*, *attP*, *int* ϕC31), Kan^R. See Fig. III.6	Bertolt Gust (Eustáquio *et al.*, 2004)
Cosmid K1F2	SuperCos1-based cosmid containing the clorobiocin biosynthetic gene cluster without the resistance genes $gyrB^R$ and $parY^R$ (sequence AF 329398), Amp^R, Kan^R.	(Pojer *et al.*, 2002)
Cosmid D1A8	SuperCos1-based cosmid containing the clorobiocin biosynthetic gene cluster with the resistance gene $gyrB^R$, Amp^R, Kan^R	(Pojer *et al.*, 2002)
Cosmid clo-BG1	From cosmid D1A8, *bla* gene replaced by cassette from pIJ787 (*oriT*, *tet*, *attP*, *int* ϕC31), Kan^R. See Fig. III.7	Bertolt Gust (Eustáquio *et al.*, 2005)
Plasmid		
pcha	1.6 kb *Bam*HI-*Xba*I fragment (*clo-hal*) amplified by PCR from cosmid K1F2 into the same sites of pSETbhaA (*clo-hal* instead of *bhaA*), $Apra^R$	Florence Pojer, unpublished
pIJ787	SuperCos1-derivative, *bla* gene replaced by a cassette containing *oriT*, *tet*, *attP*, *int* ϕC31, Kan^R	Bertolt Gust (Eustáquio *et al.*, 2004)
pMS32	1.78 kb *Bam*HI fragment of cosmid 9-6G in pBluescript SK(-), containing (T7) the end of *novC*, *novD*, and the beginning of *novE* (T3), Amp^R	Marion Steffensky's Thesis
pMS33	1.43 kb *Bam*HI-*Eco*RI fragment of cosmid 9-6G in pBluescript SK(-),containing (T3) the end of *novF*, and *novG* (T7), Amp^R	Marion Steffensky's Thesis
pMS61	3.84 kb *Bgl*II fragment of cosmid 9-6G in pLitmus28, containing (T3) *novO*, *novP*, *novQ*, and the beginning of *novR* (T7), Amp^R	Marion Steffensky's Thesis
pMS62	5.58 kb *Bgl*II fragment of cosmid 9-6G in the *Bam*HI site of pBC SK(-), containing the end of *novS*, *novT-W*, and the start of $gyrB^R$, Amp^R	Marion Steffensky's Thesis

pMS63	11.83 kb *Not*I-*Hind*III fragment of cosmid 9-6G in pBlueskript SK(-), containing (T3) *ORF21* to *novG* (T7), Amp^R	Marion Steffensky's Thesis
pSETbhaA	*ermE** promoter and *bhaA* gene (halogenase from balhimycin cluster) in pSET152, $Apra^R$	(Puk *et al.*, 2002)
pTLO1	0.7 kb *Hind*III-*Xho*I fragment (*novO* and natural RBS) amplified by PCR from cosmid 9-6G into the same sites of pGEM7Zf(-), Amp^R	Thomas Luft (Eustáquio *et al.*, 2003a)
pTLO5	0.7 kb *Hind*III-*Xba*I fragment (*novO* and natural RBS) from pTLO1 into the same sites of pUWL201, Amp^R, $Thio^R$	Thomas Luft (Eustáquio *et al.*, 2003a)
pUG019	pBlueskript SK(-)-derivative containing an apramycin resistance (*aac(3)IV*) cassette flanked by *Xba*I and *Spe*I restriction sites, Amp^R	Ute Galm (Eustáquio *et al.*, 2004)
pZW10	12.748 kb *Bgl*II fragment (position 15 530 (T7) to 28 278 (T3) of AF235050) in the *Bam*HI site of pBluescript SK(-), Amp^R	(Wang *et al.*, 2000)
pZW11	14.255 kb *Bgl*II fragment (position 1 275 (T7) to 15 530 (T3) of AF235050) in the *Bam*HI site of pBluescript SK(-), Amp^R	(Wang *et al.*, 2000)

TABLE II.15: Plasmids and cosmids produced in this study

Name	Description
Plasmid	
pAE1	1 266 bp *Apa*I-*Xho*I fragment (position 9 705 – 10 971 of AF235050) from pZW11 into the same sites of pSL1180, containing *couN2*
pAE2	1 360 bp *Xho*I-*Apa*I fragment (position 3 655 – 5 015 of AF235050) from pZW11 into the same sites of pSL1180, containing the end of *couI* and beginning of *couJ*
pAE3	1.44 kb *Hind*III-*Eco*RI fragment of pAE2 into the same sites of pBluescript SK(-)
pAE4	1.48 kb *Eco*RI fragment (position 5 533 – 7 017 of AF235050) from pZW11 in the same site of pAE3
pAE5	2.93 kb *Hind*III-*Xba*I fragment of pAE4 into the same sites of pKC1132. Construct for *in frame* inactivation of *couJ*
pAE6	2.93 kb *Hind*III-*Xba*I fragment of pAE4 into the same sites of pBSKT. Construct for *in frame* inactivation of *couJ*
pAE7	209 bp *Eco*RI-*Sal*I fragment of pMS33 (position 7 613 – 7 821, AF170880) in the same sites of pBluescript SK(-), containing the NovG binding site (*novH*)

pAE8	1.43 kb *Bam*HI-*Eco*RI fragment of pMS33 (*novG*, position 6 393 – 7 821, AF170880) in the same sites of pWHM3, containing 336 bp before the putative start codon of *novG*
pAE9	235 bp *Not*I-*Mlu*I fragment of cosmid K1F2 (position 12 654 – 12 888, AF329398) in the same sites of pSPORT1, containing the NovG binding site (*cloY*)
pAE-B1	1.6 kb *Bam*HI-*Xba*I fragment (*bhaA*) from pSETbhaA into the same sites of pSPORT1
pAE-B2	1.6 kb *Eco*RI-*Bam*HI fragment (*bhaA*) from pAE-B1 into the same sites of pEM4
pAE-B3	1.6 kb *Bam*HI-*Xba*I fragment (*bhaA*) from pAE-B1 into the same sites of pUWL201
pAE-E1	1 037 bp *Sal*I fragment (position 4 571 – 5 608 of AF170880) from pMS63 into the *Xho*I site of pLitmus28, containing *novE*, the end of *novD* and beginning of *novF*
pAE-E2	1 kb *Xba*I-*Bgl*II fragment from pAE-E1 (*novE*) into the *Xba*I-*Bam*HI sites of pSPORT1
pAE-E4_1	1 kb *Eco*RI fragment from pAE-E2 (*novE*) into the same site of pWHM3 (against the *lacZα* orientation)
pAE-E4_3	1 kb *Eco*RI fragment from pAE-E2 (*novE*) into the same site of pWHM3 (same orientation as *lacZα*)
pAE-E5_1	1 kb *Xba*I-*Spe*I fragment from pAE-E1 (*novE*) into the *Xba*I site of pIJ82 (same orientation as *lacZα*)
pAE-E5_2	1 kb *Xba*I-*Spe*I fragment from pAE-E1 (*novE*) into the *Xba*I site of pIJ82 (against the *lacZα* orientation)
pAE-G1	1.19 kb *Aat*II-*Eco*RI fragment (*novG*, position 6 628 – 7 821, AF170880) of pMS33 in the same sites of pLitmus28, containing 103 bp before the putative start codon of *novG*
pAE-G2_1	1.20 kb *Xba*I-*Spe*I fragment of pAE-G1 (*novG*) in the *Xba*I site of pWHM3 (against *lacZα* orientation)
pAE-G2_2	1.20 kb *Xba*I-*Spe*I fragment of pAE-G1 (*novG*) in the *Xba*I site of pWHM3 (same orientation as *lacZα*)
pAE-G4	1 kb PCR fragment (*novG*, amplified using cosmid nov-BG1 as template and primers PnovG_f and PnovG_r) in pGEM-T
pAE-G5	0.96 kb *Bam*HI-*Hind*III fragment from pAE-G4 into the same sites of pRSET B. Construct for expression of *novG* from *S. spheroides* in *E. coli*
pAE-ha1	1 010 bp *Pst*I-*Xba*I fragment (position 27 100 – 28 110 of AF329398) amplified by PCR (primers Pha1 and Pha2) from cosmid K1F2 into the same sites of pcDNA2.1, containing the end of *cloN7*, und beginning of *clo-hal*

pAE-ha2	1 004 bp *Xba*I-*Hind*III fragment (position 29 613 – 30 617 of AF329398) amplified by PCR (primers Pha3 and Pha4) from cosmid K1F2 into the same sites of pBSKT, containing the end of *clo-hal* and beginning of *cloP*
pAE-ha3	1 052 bp *Sac*I-*Xba*I fragment from pAE-ha1 into the same sites of pAE-ha2. Construct for *in frame* inactivation of *clo-hal*
pAE-ha4	2 014 bp *Pst*I-*Hind*III fragment from pAE-ha3 into the same sites of pKC1132. Construct for *in frame* inactivation of *clo-hal*
pAE-ha5	1.6 kb *Bam*HI-*Xba*I fragment (*clo-hal* and natural RBS) from pcha into the same sites of pSPORT1
pAE-ha6	1.6 kb *Eco*RI-*Bam*HI fragment (*clo-hal* and natural RBS) from pAE-ha5 into the same sites of pEM4
pAE-ha7	1.6 kb *Bam*HI-*Xba*I fragment (*clo-hal* and natural RBS) from pAE-ha5 into the same sites of pUWL201
pAE-K1	1 045 bp *Sac*I-*Xba*I fragment (position 16 394 – 17 439 of AF329398) amplified by PCR (primers PK1 and PK2) from cosmid K1F2 into the same sites of pcDNA2.1, containing the end of *cloI*, all *cloJ*, and beginning of *cloK*
pAE-K2	992 bp *Xba*I-*Pst*I fragment (position 17 994 – 18 986 of AF329398) amplified by PCR (primers PK3 and PK4) from cosmid K1F2 into the same sites of pSPORT1, containing the end of *cloK* and beginning of *cloL*
pAE-K3	1 045 bp *Sac*I-*Xba*I fragment from pAE-K1 into the sames sites of pAE-K2
pAE-R1 (=pLcouR1-6)	7 976 bp *Eco*RI-*Hind*III fragment (position 17 745 – 25 721 of AF 235050) from pZW10 into the same sites of pLitmus28, containing the end of *couP* and *couR1-couR6*
pAE-R2 (=pScouR1-6)	8.0 kb *Xba*I-*Bgl*II fragment from pAE-R1 into the *Xba*I-*Bam*HI sites of pSPORT1, containing the end of *couP* and *couR1-couR6*
pAE-R3 (=pEMcouR1-6)	8.0 kb *Eco*RI fragment from pAE-R2 into the same site of pEM4, containing the end of *couP* and *couR1-couR6* downstream of the *ermE** promoter
pAE-Z1	1 018 bp *Hind*III-*Pst*I fragment (position 38 450 – 39 468 of AF329398) amplified by PCR (primers PZ1 and PZ2) from cosmid K1F2 into the same sites of pBluescript SK(-), containing the end of *cloV*, all *cloW* and beginning of *cloZ*
pAE-Z2	1 015 bp *Pst*I-*Xba*I fragment (position 40 146 – 41 161 of AF329398) amplified by PCR (primers PK3 and PK4) from cosmid K1F2 into the same sites of pBSKT, containing the end of *cloZ* and beginning of $gyrB^R$
pAE-Z3	1 018 bp *Hind*III-*Pst*I fragment from pAE-Z1 into the same sites of pAE-Z2. Construct for *in frame* inactivation of *cloZ*

pAE-Z4	2 033 bp *Hind*III-*Xba*I fragment from pAE-Z3 into the same sites of pKC1132. Construct for *in frame* inactivation of *cloZ*
Cosmid	
D1A8-h-773 (= H10)	From D1A8, *clo-hal* replaced by the apramycin resistance cassette from pIJ773
D1A8-Z-773 (= Z3)	From D1A8, *cloZ* replaced by the apramycin resistance cassette from pIJ773
10-9C-E-773 (= E1)	From 10-9C, *novE* replaced by the apramycin resistance cassette from pIJ773
clo-AE1	From clo-BG1, DNA sequence upstream of *cloE* was replaced by the apramycin resistance cassette from pUG019
clo-AE2	From clo-AE1, cassette excised by restriction and religation; the insert of clo-AE2 comprises the genes from *cloE* to *gyrB*^{R}, i.e. DNA sequence from position 9 200 to 40 573 in Gene Bank entry AF329398, and from position 1 to 2 238 in Gene Bank entry AY136281. The insert of clo-AE2 starts 148 bp upstream of the translational start codon of *cloE* (Fig. III.7)
nov-AE1	From nov-BG1, DNA sequence downstream of *gyrB*^{R} was replaced by the apramycin resistance cassette from pUG019
nov-AE2	From nov-AE1, cassette excised by restriction and religation
nov-AE3	From nov-AE2, DNA sequence upstream of *novE* was replaced by the apramycin cassette from pUG019
nov-AE4	From nov-AE3, cassette excised by restriction and religation; the insert of nov-AE4 comprises the genes from *novE* to *gyrB*^{R}, i.e. DNA sequence from position 4 628 to 25 617 in Gene Bank entry AF170880, and from position 1 to 2 490 in Gene Bank entry AF205854. The insert of nov-AE4 starts 180 bp upstream of the translational start codon of *novE* (Fig. III.6)
nov-AE5	From nov-AE2, DNA sequence downstream of *ORF20* was replaced by the apramycin resistance cassette from pUG019
nov-AE6	From nov-AE5, cassette excised by restriction and religation; the insert of nov-AE6 comprises the genes from *ORF20* to *gyrB*^{R}, i.e. DNA sequence from position 5 619 to 6 339 in Gene Bank entry AY227005, all sequence from Gene Bank entry AF170880 (1 – 25 617), and from position 1 to 2 490 in Gene Bank entry AF205854 (Fig. III.6)
nov-AE7	From nov-BG1, *novO* replaced by the apramycin resistance cassette from pUG019
nov-AE8	From nov-AE7, cassette excised by restriction and religation; Δ*novO* cosmid (Fig. III.10)
nov-AE9	From nov-BG1, *novG* replaced by the apramycin resistance cassette from pUG019

nov-AE10	From nov-AE9, cassette excised by restriction and religation; Δ*novG* cosmid (Fig. III.15)

II.5.2. PCR primers used for construction of plasmids

TABLE II.16: Primers used for construction of plasmids

Name	Sequence (5'-3')[a]	Restriction site	Positions (Acession number)	Plasmid
PnovG_f	TGGGG**A**T**CC**CATGACCAACAG	*Bam*HI	6720-6740 (AF170880)	pAE-G4
PnovG_r	GATTC**A**AGC**TT**TTGAACGTCAGG	*Hind*III	7704-7682 (AF170880)	
Pha1	TACGAGATCCAAG**CT**GCAGGTC	*Pst*I	27083-27104 (AF329398)	pAE-ha1
Pha2	GTCATATTC**TA**G**A**TTTTCGGACAC	*Xba*I	28112-28099 (AF329398)	
Pha3	ACAGTGAGGATC**TA**G**A**CCGAAGC	*Xba*I	29603-29625 (AF329398)	pAE-ha2
Pha4	GTCCATTCG**A**AGC**TT**GGCGAGC	*Hind*III	30630-30609 (AF329398)	
PK1	TTGCACGGGATGAGCTCACTGC	*Sac*I	16378-16399 (AF329398)	pAE-K1
PK2	TTCGGTGTC**T**AG**A**AGGGCGACG	*Xba*I	17450-17429 (AF329398)	
PK3	GACGGAGTTTCT**A**G**A**CGACGAGC	*Xba*I	17984-18006 (AF329398)	pAE-K2
PK4	CAGCACGCCAGGCAACACTGTG	---	19016-18995 (AF329398)	
PZ1	AGGAGAAGC**TT**GCCGATCCCAA	*Hind*III	38445-38467 (AF329398)	pAE-Z1
PZ2	CACGC**T**GC**A**GACCATCATCGAC	*Pst*I	39473-39452 (AF329398)	
PZ3	AGCGCAAC**T**G**C**A**G**ACGCGTGATG	*Pst*I	40135-40157 (AF329398)	pAE-Z2
PZ4	GTGGTCT**A**GAAGATGTCGCCGTC	*Xba*I	41169-41147 (AF329398)	

[a]Letters shown in bold represent mutations inserted in the original sequence to give desired restriction sites (underlined).

II.5.3. Bacterial strains

TABLE II.17: Bacterial strains of *E. coli* and *Streptomyces*

Strain	Relevant characteristics	Source or reference
E. coli XL1Blue MRF'	General cloning host (*rec*A1 *end*A1 *gyr*A96 thi-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F´ *pro*AB *lacI*^{q}ZDM15 Tn10 (Tet^R)]), Tet^R	Stratagene
E. coli ET 12567	Strain triply defective in DNA methylation (*dam*$^-$ *dcm*$^-$ *hsdM*$^-$), Tet^R, Cm^R	(MacNeil *et al.*, 1992)
E. coli BL21(DE3)pLysS	Host for the heterologous expression of His$_6$ tagged NovG (F$^-$ *omp*T *hsd*S$_B$($r_B^-m_B^-$) *gal dcm* (DE3)pLysS), Cm^R	Invitrogen
S. coelicolor M512	Δ*redD* Δ*actII-ORF4* SCP1$^-$ SCP2$^-$ (no production of actinorhodin, undecylprodigiosin, and methylenomycin)	(Floriano and Bibb, 1996)
S. coelicolor (clo-BG1)	*S. coelicolor* M512 containing the clorobiocin cluster and flanking DNA regions, Kan^R	This thesis
S. coelicolor (clo-AE2)	*S. coelicolor* M512 containing the clorobiocin cluster (from *cloE* to *gyrB*^{R}), Kan^R	This thesis
S. coelicolor (nov-BG1)	*S. coelicolor* M512 containing the novobiocin cluster and flanking DNA regions, Kan^R	This thesis
S. coelicolor (nov-AE4)	*S. coelicolor* M512 containing the novobiocin cluster (from *novE* to *gyrB*^{R}), Kan^R	This thesis
S. coelicolor (nov-AE6)	*S. coelicolor* M512 containing the novobiocin cluster (from *ORF20* to *gyrB*^{R}), Kan^R	This thesis
S. coelicolor (nov-BG1)/pAE-G2_1	Kan^R, Thio^R	This thesis
S. coelicolor (nov-BG1)/pAE-G2_2	Kan^R, Thio^R	This thesis
S. coelicolor (nov-BG1)/pAE8	Kan^R, Thio^R	This thesis
S. coelicolor (nov-BG1)/pWHM3	Kan^R, Thio^R	This thesis
S. coelicolor (nov-AE10)	*S. coelicolor* M512 containing a *novG*-defective novobiocin cluster, Kan^R	This thesis
S. coelicolor (nov-AE10)/pAE-G2_1	Kan^R, Thio^R	This thesis

S. coelicolor (nov-AE10)/pAE-G2_2	Kan^R, $Thio^R$	This thesis
S. coelicolor (nov-AE10)/pAE8	Kan^R, $Thio^R$	This thesis
S. coelicolor (nov-AE10)/pWHM3	Kan^R, $Thio^R$	This thesis
S. coelicolor (nov-AE8)	*S. coelicolor* M512 containing a *novO*-defective novobiocin cluster, Kan^R	This thesis
S. coelicolor (nov-AE8)/pTLO5	Kan^R, $Thio^R$	This thesis
S. coelicolor (nov-AE8)/pAE-ha7	Kan^R, $Thio^R$	This thesis
S. coelicolor (nov-AE8)/pAE-B3	Kan^R, $Thio^R$	This thesis
S. coelicolor (nov-AE8)/pUWL201	Kan^R, $Thio^R$	This thesis
S. lividans TK24	*str-6* $SLP2^-$ $SLP3^-$	(Kieser *et al.*, 2000; Shima *et al.*, 1996)
S. lividans (clo-BG1)	*S. lividans* TK24 containing the clorobiocin cluster and flanking DNA regions, Kan^R	This thesis
S. lividans (nov-BG1)	*S. lividans* TK24 containing the novobiocin cluster and flanking DNA regions, Kan^R	This thesis
S. roseochromogenes var. *oscitans* DS 12.976	Wild-type, clorobiocin producer	Aventis (Mancy *et al.*, 1974)
S. roseochromogenes AE-h2, AE-h10 and AE-h11	Three independent *clo-hal*$^-$ mutants of *S. roseochromogenes*, $Apra^R$	This thesis
S. roseochromogenes AE-h10/pAE-ha7	*clo-hal*$^-$ mutant complemented with *clo-hal*, $Apra^R$, $Thio^R$	This thesis
S. roseochromogenes AE-h10/pTLO5	*clo-hal*$^-$ mutant complemented with *novO*, $Apra^R$, $Thio^R$	This thesis
S. roseochromogenes AE-h10/pAE-B3	*clo-hal*$^-$ mutant transformed with *bhaA* construct, $Apra^R$, $Thio^R$	This thesis
S. roseochromogenes AE-h10/pUWL201	*clo-hal*$^-$ mutant transformed with empty vector pUWL201, $Apra^R$, $Thio^R$	This thesis

S. roseochromogenes AE-Z4, AE-Z25, and AE-Z40	Three independent *cloZ*$^-$ mutants of *S. roseochromogenes*, Apra^R	This thesis
S. spheroides NCIMB 11897	Wild-tpye, novobiocin producer	The National Collection of Industrial, Food & Marine Bacteria, Ltd, Aberdeen Scottland
S. spheroides AE-E2, AE-E3, and AE-E4	Three independent *novE*$^-$ mutants of *S. spheroides*, Apra^R	This thesis

II.5.4. Probes used in Southern blot analysis

TABLE II.18: DIG-labelled probes

Probes	Description
clo-hal	946 bp *Pvu*II-*Xba*I fragment (position 27 164 – 28 110, AF329398) from plasmid pAE-ha1
cloZ	1 027 bp *Bln*I-*Bam*HI fragment (position 38 909 – 39 468 and 40 146 – 40 614, AF329398) from plasmid pAE-Z3
Cosmid clo-BG1	Cosmid clo-BG1 digested with *Pst*I
Cosmid E1	Cosmid E1 digested with *Bam*HI
Cosmid H10	Cosmid H10 digested with *Bam*HI
Cosmid nov-BG1	Cosmid nov-BG1 digested with *Pst*I
couJ	1.36 kb *Xho*I-*Apa*I fragment (position 3 657 – 5 015, AF235050) from plasmid pAE2
novE	962 bp *Bam*HI fragment (position 5 431 – 6 392, AF170880) from plasmid pMS63

II.6. Culture conditions

II.6.1. Cultivation of *E. coli*

For cloning experiments, *E. coli* strains were grown overnight (16-18 h) in liquid or solid LB medium with appropriate antibiotic(s) at 37 °C (Sambrook and Russell, 2001).

For NovG overexpression, the incubation temperature was 15 to 30 ºC.

Permanent cultures of *E. coli* were prepared by mixing 600 µl of overnight culture with 400 µl of glycerol solution (50% (w/v) in distilled water) and stored at -70°C.

II.6.2. Cultivation of *Streptomyces*

II.6.2.1. General cultivation

Streptomyces strains were routinely cultured in liquid or solid YMG medium. Liquid cultures were carried out in baffled Erlenmeyer flasks containing a stainless steel spring at 180-200 rpm and 28-30 °C for 2 to 3 days. For preparation of protoplasts, *S. coelicolor* and *S. lividans* were cultured in YEME medium containing 0.5% glycine; *S. roseochromogenes*, in CRM medium containing 0.75% glycine; and *S. spheroides*, in TSB medium containing 10% sucrose and 0.4% glycine. For isolation of genomic DNA, *S. coelicolor*, *S. lividans* and *S. roseochromogenes* were cultured in YEME medium; and *S. spheroides* in YMG medium. An appropriate concentration of antibiotic(s) was added, if required.

II.6.2.2. Production of secondary metabolites

For the production of clorobiocin and derivatives, 1 ml of a two-day-old YMG culture of wild-type or mutant strains of *S. roseochromogenes* was inoculated into 50 ml corn starch medium and grown at 33 °C and 210 rpm for 2 days. 5 ml of this pre-culture were inoculated into 500 ml baffled flasks containing 50 ml of production medium, Distillers solubles-medium. Cultivation was carried out at 33 °C and 210 rpm for 5 to 8 days (adapted from (Mancy *et al.*, 1974)).

For the production of novobiocin and derivatives, about 10^8 spores or 1 ml of a two-day-old YMG culture of wild-type or mutant strains of *S. spheroides* were inoculated into 50 ml CDM medium and grown at 28 °C and 180 rpm for 4 days. 1 ml of this pre-culture was inoculated into 50 ml of CDM medium and grown for 3 to 10 days.

S. coelicolor and *S. lividans* parental and mutant strains containing the clorobiocin cluster were cultured as described above for *S. roseochromogenes*.

S. coelicolor and *S. lividans* parental and mutant strains containing the complete or modified novobiocin cluster were cultured as described above for *S. spheroides*. No spore suspension of transformants containing muti-copy recombinant plasmids was made. In this case, it was observed that the higher productivity was achieved by carrying out the cultivation as follows: 1 ml cells from an YMG pre-culture were inoculated into 50 ml CDM production medium and cultured at 30 °C and 200 rpm for 7 days; the cultivation in production medium was carried out without addition of antibiotics.

II.6.2.3. Preparation of mycelia for storage and of spore suspensions of *Streptomyces*

For preparation of mycelia for storage, 1 ml 2-day-old YMG culture was harvested by centrifugation and the cells were resuspended in 0.5 ml 20% glycerol. The storage was carried out at -70°C.

To prepare spore suspensions, *Streptomyces* strains were spread on YMG agar (*S. spheroides*) or on MS agar (*S. coelicolor* and *S. lividans*) and incubated at 30 °C for about a week. *S. roseochromogenes* var. *oscitans* sporulates not always and if, only very slowly (Mancy *et al.*, 1974). Its sporulation media normally contain starch and ammonium salts, like Grundy starch medium, which was used during this study. 2-3 ml 2-day-old YMG culture of *S. roseochromogenes* strains were mixed with 1 g sterile powdered soil and plated on two Grundy starch agar plates. The plates were incubated at 28 ºC for four to six weeks (the spores have a light pinkish colour).

The plates (one to two plates for good sporulaters, four for more sparely sporulating strains) were grown till they were well sporulated. 4 ml of Tween® 20 (0.1% (w/v)) were added to each plate and the spores scraped off of the top of the plates and into suspension. The resulting spore suspension was poured into a falcon tube and vortexed vigorously (about 1 min). The spores were separated from the mycelium by passing the suspension through sterile cotton pluged in a disposable syringe. Spores were collected by centrifugation (2,100×g, 10 min, 4 °C), and resuspended in 1-3 ml of 20% glycerol. The spore suspensions were kept at -70 °C.

II.6.2.4. Sensitivity of *S. rishiriensis*, *S. roseochromogenes* and *S. spheroides* to different antibiotics

The sensitivity of *S. rishiriensis*, *S. roseochromogenes* and *S. spheroides* to kanamycin, neomycin, and a mixture of streptomycin (strep) and spectinomycin (spec) was tested by (1) platting 100 µl of a 2-day-old YMG culture on YMG agar plates containing increasing concentrations of the respective antibiotic; and (2) by a spot test, i.e. six spots were made by dropping suspensions containing 10^2 to 10^7 spores (or alternatively, dilutions of a liquid culture) on YMG agar plates containing increasing concentrations of the respective antibiotic. After incubation at 30 ºC for five days, the plates were evaluated (Table II.19).

TABLE II.19: Sensitivity of *S. rishiriensis*, *S. roseochromogenes* and *S. spheroides* to different antibiotics

S. rishiriensis						
Kanamycin (µg/ml)	0	**20***	50	100	200	500
CFU	+++	0	0	0	0	0
Neomycin (µg/ml)	0	10	20	**50***	100	200
CFU	+++	+	+	0	0	0
Strep/Spec (µg/ml)	0	**5/100***	10/200	20/400		
CFU	+++	0	0	0		
S. roseochromogenes						
Kanamycin (µg/ml)	0	**20***	50	100	200	500
CFU	+++	0	0	0	0	0
Neomycin (µg/ml)	0	10	20	**50***	100	200
CFU	+++	15	9	0	0	0
S. spheroides						
Kanamycin (µg/ml)	0	20	**50***	100	200	500
CFU	+++	1	0	0	0	0
Neomycin (µg/ml)	0	**10***	20	50	100	200
CFU	+++	0	0	0	0	0

* Minimal antibiotic concentration, which inhibits growth completely.
+++ Confluent growth.
+ Growth observed by the spot test, CFU counting not possible.

II.7. Methods of molecular biology

II.7.1. Purification, concentration and quantification of DNA

Standard methods for DNA isolation and manipulation were performed as described elsewhere (Kieser *et al.*, 2000; Sambrook and Russell, 2001).

Phenol/chloroform extraction and ion exchange column chromatography were used for purification of DNA. Ethanol or isopropanol precipitation was used for concentration.

Quantification of DNA was carried out by using a GeneQuant photometer (Pharmacia, Freiburg, Germany) at 260 nm as well as by comparing the fluorescent intensity with DNA markers on agarose gels.

II.7.2. Agarose gel electophoresis of DNA

Gel electrophoresis with 0.8-1.5% (w/v) agarose was used to separate DNA fragments between 0.5 and 50 kb, and with 2-2.5% NuSieve®GTG® agarose to separate DNA fragments between 0.1 and 0.5 kb. The buffer system employed was 1×TAE buffer (Table II.8). After running the gels, they were stained with the fluorescent dye ethidium bromide, detected under the UV light at 312 nm and photographed by using Eagle Eye II System (Strategene, Heidelberg, Germany) (Sambrook and Russell, 2001).

DNA fragments were isolated from agarose gels using a NucleoSpin® 2 in 1 extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol.

II.7.3. DNA manipulation with enzymes

Restriction of DNA with endonucleases was carried out according to the manufacturer' instructions.

DNA ligation was achieved by using T4-DNA ligase. The ligation preparation, containing 1U T4 DNA ligase, 1×ligation buffer and a 1:1 (mole ratio) mixture of insert

and linearized vector (about 100 ng) in a final volume of 10 µl, was incubated at RT for 2 h or at 16 °C or 4 °C overnight.

II.7.4. DNA isolation

II.7.4.1. Isolation of plasmids from *E. coli*

Mini-preps employing alkaline lysis were used to isolate recombinant plasmids from *E. coli* for routine screening. 3 ml LB-medium was inoculated with a single colony and grown overnight at 37 °C, 170 rpm. 2 ml of this culture were harvested by centrifugation (10,000×g, 4 °C, 1 min) and resuspended in 250 µl solution MP1 by vortexing. The suspension was mixed with 250 µl solution MP2 by inversion and incubated at RT for 1-5 min. 250 µl solution MP3 was added and the mixture was incubated on ice for 5 min. After centrifugation (20,000×g, 4 °C, 15 min), the supernatant was poured into a fresh microfuge tube. The DNA was precipitated by addition of 0.8-fold isopropanol and centrifugation (20,000×g, 4 °C, 30 min). The DNA pellet was washed with 500 µl 70% ethanol, air dried and resuspended in 50 µl distilled water or TE buffer. The mentioned solutions are listed in Table II.5.

Preparative isolation of plasmids from *E. coli* was carried out with ion-exchange columns (Nucleobond® AX100, Macherey-Nagel, Düren, Germany) according to the manufacter´s protocol.

II.7.4.2. Isolation of plasmids from *Streptomyces*

Isolation of plasmid DNA from *Streptomyces* strains was carried out by alkaline lysis and potassium acetate precipitation, adapted from procedure D (Kieser *et al.*, 2000). 2 ml of a 2-day-old culture in YEME or YMG medium were harvested by centrifugation (17,000×g, 4 °C, 1 min). After washing with 1ml of solution MP1, the cells were resuspended in 500 µl of solution MP1GL by vortexing. The suspension was incubated at 37 °C for 30-60 min, then mixed with 500 µl of solution MP2 by inversion and incubated at RT for 10 min. 400 µl solution MP3 and 40 µl Rotiphenol® were added and mixed by inversion. The mixture was incubated on ice for 5 min. After 20 min centrifugation (20,000×g, 4 °C), the supernatant was poured into a fresh microfuge tube and extracted twice with 300 µl Rotiphenol®. The DNA was precipitated by addition of 0.8-fold volume of isopropanol and centrifugation

(20,000×g, 4 °C, 20 min). The DNA pellet was washed with 500 µl 70% ethanol, air dried and resuspended in 20-50 µl distilled water or TE buffer. If required, the plasmid DNA isolated from *Streptomyces* was amplified in *E. coli* XL1 blue MRF´ before restriction analysis. The mentioned solutions are listed in Tables II.5 and II.6.

II.7.4.3. Isolation of genomic DNA from *Streptomyces*

Genomic DNA was isolated from *S. spheroides* by lysozyme treatment and phenol-chloroform extraction (Kieser *et al.*, 2000). 2 ml of a 2-day-old culture in YMG medium were harvested by centrifugation (10,000×g, 4 °C, 1 min). The cells were washed with 1 ml TSE buffer and resuspended in 500 µl TSE buffer with lysozym (3 mg/ml) and RNase A (100 µg/ml) by vortexing. The suspension was incubated for 30 min at 37 °C and in between mixed well every ten minutes. 250 µl of 2% SDS solution were added and the mixture was incubated for 10 min at 60 °C, and for 10 min at RT. The lysate was extracted three times with 250 µl Rotiphenol®. The third extraction was carried out with addition of 70 µl NaAc (pH 4.8). The aqueous phase was separated and genomic DNA was precipitated by addition of 0.8-1.0 volume of isopropanol and centrifugation (20,000×g, 4 °C, 30 min). The DNA pellet was washed with 500 µl 70% ethanol, air dried and resuspended in 50 µl TE buffer.

Genomic DNA was isolated from *S. coelicolor*, *S. lividans and S. roseochromogenes* by the Kirby mix procedure (Kieser *et al.*, 2000). 2 ml of a 2-day-old culture in YEME medium were harvested by centrifugation (17,000×g, 4 °C, 1 min). The cells were washed with 1 ml TSE buffer and resuspended in 500 µl TSE buffer with lysozym (3 mg/ml) and RNase A (100 µg/ml) by vortexing. The suspension was incubated for 15 min at 37 °C. 400 µl of 2×Kirby mix were added and the mixture was vortexed vigorously for 1 min. 800 µl phenol/chloroform/isoamyl alcohol were added and the mixture was vortexed vigorously for 15 s and centrifuged at 17,000×g and 4 °C for 10 min. The supernatant was poured into a fresh microfuge tube and extracted a second time with addition of 70 µl “unbuffered” 3 M NaAc and 300 µl phenol/chloroform/isoamyl alcohol (1 min vigorous vortexing). The aqueous phase was separated and genomic DNA was precipitated by addition of 0.7-fold volume of isopropanol and centrifugation (20,000×g, 4 °C, 30 min). The DNA pellet was washed with 500 µl 70% ethanol, air dried and resuspended in 50 to 100 µl TE buffer.

II.7.5. DNA denaturation for ssDNA transformation in *Streptomyces*

II.7.5.1. Alkaline treatment

9 µl dsDNA in H_2O was mixed with 2 µl 1 M NaOH and incubated for 10 min at 37 °C. The mixture was replaced on ice and the reaction was terminated with addition of 2 µl 1 M HCl (Oh and Chater, 1997).

II.7.6. PCR amplification

II.7.6.1. General conditions

PCR amplifications were carried out with the GeneAmp PCR System 2400 (Perkin-Eimer, Weiterstadt, Germany). The amplification conditions for PCR reactions using the Expand High Fidelity PCR system (Roche) are given in Table II.20, and using the GC-Rich PCR system (Roche) in Table II.21.

TABLE II.20: PCR reaction and amplification conditions using the Expand High Fidelity PCR system

Substance	Final concentration
Reaction buffer (10×)	1×
DMSO	5% (v/v)
Template DNA	about 100 ng
dNTPs	0.2 mM each
Primer	50 pmol each
DNA-Polymerase	2.5 U
Add distilled water to make up to 50 µl	

Cyclus	Temperature	Time	Cycles
Hot start	94 °C	2 min	1
Denaturing	94 °C	45 s	30
Annealing	**50-60 °C**	45 s	
Elongation	72 °C	90 s	
Final elongation	72 °C	5 min	1
End	4 °C	∞	1

TABLE II.21: PCR reaction and amplification conditions using the GC-Rich PCR system

Substance	Final concentration
GC-Rich PCR reaction buffer with DMSO (5×)	1×
GC-Rich resolution solution (5 M)	0.5 mM
dNTP-Mix	0.2 mM each
Primer 1	20 pmol
Primer 2	20 pmol
Template DNA	about 100 ng
GC-Rich PCR System enzyme mix	2 U
Add distilled water to make up to 100 µl	

Cyclus	Temperature	Time	Cycles
Hot start	95 °C	5 min	1
Denaturing	95 °C	90 s	30
Annealing	55-60 °C	90 s	
Elongation	72 °C	45s/ 1 kb	
Final elongation	72 °C	5 min	1
End	4 °C	∞	1

For PCR amplification with *Taq* or *Pfu* polymerase, the PCR mixture (50-100 µl) contained 50 pmol each primer, 100-300 ng template DNA, 0.2 mM each dNTP, 1× reaction buffer, 5% (v/v) DMSO and 2-3 U polymerase. Amplification conditions were according to the supplier's instructions.

In general, 5 to 10 µl of the PCR reaction were analysed by gel electrophoresis.

II.7.6.2. Conditions for amplification of the apramycin resistance cassette from pIJ773 and from pUG019

The conditions for amplification of the apramycin resistance cassette from pIJ773 (REDIRECT© technology kit for PCR targeting (Gust *et al.*, 2003)) using the Expand High Fidelity PCR system (Roche) are given in Table II.22. Template DNA was prepared by digesting about 10 µg of pIJ773 with *Eco*RI and *Hind*III, and by isolating the 1.4 kb cassette fragment from an agarose gel (Gust *et al.*, 2003).

TABLE II.22: Conditions for amplification of the apramycin resistance cassette from pIJ773

Substance	Final concentration
Reaction buffer (10×)	1×
DMSO	5% (v/v)
Template DNA	about 100 ng
dNTPs	0.2 mM each
Primer	50 pmol each
DNA-Polymerase	2.5 U
Add distilled water to make up to 50 µl	

Cyclus	Temperature	Time	Cycles
Hot start	94 °C	2 min	1
Denaturing	94 °C	45 s	10
Annealing	**50 °C**	45 s	
Elongation	72 °C	90 s	
Denaturing	94 °C	45 s	15
Annealing	**55 °C**	45 s	
Elongation	72 °C	90 s	
Final elongation	72 °C	5 min	1
End	4 °C	∞	1

For amplification of the apramycin resistance cassette from **pUG019**, the first annealing temperature was 45 ºC instead of 50 ºC and the second, 48 ºC instead of 55 ºC; otherwise the conditions were the same as listed in Table II.22. Template DNA was prepared by digesting about 2 µg of pUG019 with *Eco*RI, *Hind*III and *Dra*I (*Dra*I cuts three times in the vector backbone, and it is used to guarantee the destruction of traces of cccDNA and to avoid purification of the cassette by gel electrophoresis). After digestion, the mixture was purified and concentrated by isopropanol precipitation.

II.7.7. Southern blot analysis

Southern blot analysis was performed on Hybond-N membranes (Amersham) by using the DIG high prime DNA labelling and detection starter kit II (Roche). Buffers and solutions are given in Table II.9.

Note: powder-free gloves were always worn to handle the used plastic or glass ware and the membrane (which should, if possible, only be handled with a pair of clean tweezers on the edges). Plastic and glass ware were thoroughly washed with distilled water before use.

II.7.7.1. Probe preparation

Probe for Southern hybridization was prepared by the random priming method using the DIG high prime DNA labelling and detection starter Kit II (Roche) according to the user's manual. The probes used in this thesis are listed in Table II.18.

II.7.7.2. Southern blot preparation

An agarose gel with DNA digested with appropriated enzymes and the DIG Molecular Weight Marker VII (Roche) was run. The gel was stained with ethidium bromide and photographed. The DNA was denaturized by soaking the gel in denaturing buffer for 2×15 min, and then neutralized by soaking in neutralizing buffer for 2×15 min, with gentle agitation. Southern blot was carried out by capillary transfer using 20×SSC buffer. For this purpose, the gel was placed on pre-wetted (in 20×SSC buffer) filter paper and overlaid with pre-wetted (in 2×SSC buffer) Hybond-N nylon membrane and pre-wetted (in 20×SSC buffer) filter paper. About 7 cm paper towels and a 1 kg weight were stacked on the top. The transfer was carried out overnight. The membrane was then crosslinked with UV light (312 nm, 60 s on the front side and 30 s on the backside) and washed with sterile, distilled water. The membrane was used immediately or stored at 4 ºC after air drying.

II.7.7.3. Prehybridization and hybridization

The membrane was incubated in prehybridization solution (20 ml/100 cm^2) for 4 h at 68 °C with gentle rotation. Appropiate probe was added to the hybridization solution (5-25 ng/ml). This was denaturized by heating (10 min in a boiling water bath), and immediately chilled in liquid nitrogen. The prehybridization solution was replaced by the hybridization solution containing the appropriate probe (about 7 ml/100 cm^2) and incubated overnight at 68 °C.

II.7.7.4. Detection

The membrane was washed twice with 2×washing buffer for 10 min at RT, and afterwards twice with 0.5×washing buffer at 68 °C for 20 min, with gentle rotation. After equilibration in maleic acid buffer for 5 min, the membrane was incubated with blocking solution for 30 min and then with antibody solution for 30 min. To remove

excess of antibody, the membrane was washed twice in Tween® washing buffer, 15 min each at RT. Finally, the membrane was equilibrated in detection buffer for 5 min and immediately placed between two plastic sheets (which were sealed in two sides) with the backside of the membrane placed on one sheet, and the front side remaining free by lifting the second sheet. A 1:100 dilution of CSPD stock solution in detection buffer was dropped onto the membrane (0.5 ml/100cm^2) and spread over it by letting down the second sheet. It was incubated at RT for 2-5 min protected from light. Excess of solution and air bubbles were removed by wiping the cover sheet with a clean paper tower. The membrane was exposed to a Hyperfilm ECL-X-ray film (Amersham Biosciences, Freiburg, Germany) at 37 °C for 30 min to 3 h, depending on the strength of the signal. The film was developed using standard methods.

II.7.7.5. Removal of probe

If necessary, probe was removed by washing the membrane twice with stripping solution for 15 min at 37 °C, followed by washing with 2×SSC solution for 30 min at RT. After that, the membrane could be used for further hybridization or stored in 2×SSC solution at 4 °C.

II.7.8. Introduction of DNA in *E. coli*

The methods described in this section were modified from Sambrook and Russel (2001).

II.7.8.1. $CaCl_2$-mediated transformation

Preparation of competent cells: 100 ml LB-medium was inoculated with 1 ml of an overnight culture of *E. coli* and cultivated at 37 °C, 170 rpm till the OD_{600} reached 0.6 (2.5-4 h). The cells were harvested by centrifugation (3,000×g, 4 °C, 5 min), resuspended in 30 ml ice-cold 0.1 M $MgCl_2$ and again centrifuged as above. The cell pellet was suspended in 20 ml ice-cold $CaCl_2$ (0.1 M) and incubated on ice for 20 min. After centrifugation, the pellet was suspended in 5 ml of $CaCl_2$ (0.1 M) solution containing 15% glycerol. Competent cells could be used immediately or dispensed in 200-µl aliquots in 1.5-ml microfuge tubes, and stored at -70 °C.

Note: Resuspension of cells should not be done by vortexing. Therefore, it is easier and quicker to resuspend the cells first in the remaining drops (after discarding the supernatant) by tapping the tube, and only afterwards to add the required solution and mix gently by inversion.

Transformation: DNA (0.1-1 µg in 1-5 µl) was added to 100-200 µl competent cells in 1.5-ml microfuge tube and incubated on ice for 30 min. The tube was then incubated at 42 °C for 2 min and cooled down on ice (about 5 min). 1 ml LB medium was pipetted into the tube, and the suspension was incubated on a water bath or on a shaker (170 rpm) for 1 h at 37 °C. 200 µl of the mixture were spread on a LB agar plate containing the appropriate(s) antibiotic(s) and the rest was centrifuged (17,000×g, 4 °C, 30 s), resuspended in 200 µl LB and spread on another LB agar plate. The plates were incubated at 37 °C. For transformation of *E. coli* XL1 Blue MRF' cells with a circular plasmid (cccDNA), the incubation on ice might be shortened to 10 min and the incubation with LB medium at 37 °C might be omitted, since the transformation efficiency is otherwise too high to allow growth of single colonies. However, for *E. coli* ET12567, all the procedure as described above should be carried out, as the transformation efficiency of this strain is lower (about 100-fold).

Blue/white selection: If a *lacZa*-containing cloning vector was used to prepare the recombinant plasmid, blue/white selection can facilitate the identification of the expected clones. For this purpose, first 15 µl of IPTG solution (80 mg/ml) in up to 100 µl H_2O (sterile) were pipetted on the top of the plates and spread evenly, and then 60 µl of X-Gal solution (20 mg/ml in DMF) was plated in the same way. The plates were air dried under the laminar flow for 30-45 min in order to evaporate the toxic DMF. Colonies containing the recombinant plasmid lack *b*-galactosidase activity and remain white.

II.7.8.2. Electroporation

Preparation of electro-competent cells: 50 ml LB-medium was inoculated with 1 ml of an overnight culture of *E. coli* and cultivated at 30 to 37 °C (see note below), 170 rpm till the OD_{600} reached 0.6 (2.5-4 h). The cells were harvested by centrifugation (3,000×g, 4°C, 5 min), and washed twice with 50 and 25 ml ice-cold 10% (w/v) glycerol solution, respectively. The cell pellet was suspended in the remaining drops

after discarding the supernatant. Competent cells could be used immediately or dispensed in 50-µl aliquots in 1.5-ml microfuge tubes, and stored at -70 °C.

Electroporation: DNA (about 100 ng in 1-2 µl distilled water) was added to 50 µl competent cells in 1.5-ml microfuge tube and incubated on ice for about 1 min. The mixture was then carefully transferred to an ice-cold electroporation cuvette (0.2 cm), avoiding formation of air bubbles, and electroporation was carried out using a BioRad electroporator set to 2.5 kV. The optimal time constant is 4.5 – 5.0 ms. 1 ml LB medium was immediately pipetted into the cuvette, and the suspension was transferred to a microfuge tube and incubated on a water bath or on a shaker (170 rpm) for 1 h at 30 to 37 °C. The mixture was spread on LB agar plates containing the appropriate(s) antibiotic(s) (no more than 200 µl per plate) and the plates were incubated at 30 to 37 °C.

Note: To maintain plasmid pIJ790 in *E. coli* BW25113 (REDIRECT© technology kit for PCR targeting (Gust *et al.*, 2003)), cells must be cultured at 30 °C, since pIJ790 contains a temperature sensitive origin of replication. Otherwise, the cultivation temperature was 37 °C.

II.7.9. Introduction of DNA in *Streptomyces*

II.7.9.1. PEG-mediated protoplast transformation

Relevant buffers and media are listed in Table II.11 and in section II.4.1.5, respectively.

Preparation of protoplasts:

- from *S. coelicolor* and *S. lividans*: slightly modified from (Kieser *et al.*, 2000). Mycelium from a 40 h old culture (50 ml YEME medium containing 0.5% glycine) was washed twice with 15 ml of a 10.3% sucrose solution, resuspended in 10 ml of lysozyme solution (2 mg/ml in P buffer) and incubated at 30 ºC for 15-60 min with gentle agitation. Protoplast formation was monitored using the microscope. After most cells became protoplasts, the reaction was stopped by incubation on ice. The following steps were carried out on ice. 10 ml of ice-cold P buffer were added and the

suspension was drawn in and out of a 10 ml pipette three times and filtered through glass wool. Protoplasts were sedimented gently by centrifugation (e.g. 1,000xg, 7 min). The supernatant was discarded, the pellet was first carefully resuspended in the remaining drop of liquid by tapping the tube, and then in 1 ml P buffer. The protoplast suspension can be immediately used for transformation or 100 µl aliquots can be stored at -70 ºC. To freeze protoplasts for storage, tubes were placed in ice contained in a plastic beaker, and the beaker was placed at -70 ºC overnight. To assess the protoplast regeneration, dilution series of the protoplast suspension in P buffer were prepared and plated on R2YE agar plates (see note below). The plates were incubated at 30 °C for 3-7 days. The regenerable protoplasts per ml suspension were calculated. To assess the proportion of non-protoplasted units in the suspension, samples were also diluted in distilled water and plated on regeneration plates (R2YE agar).

Note: platting of protoplasts was always done by overlaying with R2YE soft agar instead of spreading in order to avoid mechanical stress and lysis.

- from *S. roseochromogenes* and *S. spheroides*: as described above with the following modifications: mycelium from CRM cultures or TSB cultures containing 10% sucrose and 0.4% glycine, respectively; 1 mg lysozyme/ml P buffer for *S. roseochromogenes*.

Transformation: The transformation of *Streptomyces* strains was carried out by a modification of the method described by Kieser *et al.* (2000).

Before transformation of *Streptomyces* strains the plasmids were propagated in *E. coli* ET 12567 to bypass methyl-sensing restriction. *S. lividans* does not carry a methyl-sensing restriction system and DNA from common *E. coli* strains, such as XL1 Blue MRF' or DH5α can be used instead.

1-20 µg DNA (in maximal 10-20 µl TE buffer) were added to 100-200 µl of a protoplast suspension, containing at least 10^8 protoplasts per ml; 400-500 µl T-buffer containing PEG 1000 (45% (w/v) for *S. roseochromogenes*; 25% (w/v) for other strains) were immediately added, mixed by pipetting carefully three times and incubated at RT for 1 min. Increasing volumes of the resulting suspension (e.g. 10 µl, 100 µl, 200 µl, rest) were mixed with warm R3 or R2YE soft agar (about 50 °C, 4×3

ml) and plated on four R2YE plates. After 16-24 h incubation at 28 °C - 30 °C, the plates were overlaid with 3 ml of soft nutrient agar including the required antibiotics for selection of mutants, and incubation was continued for further 3-7 days. In order to enhance the efficiency of homologous recombination between inactivation constructs and the genome of *Streptomyces*, denatured DNA was used to transform *S. roseochromogenes* during inactivation via the standard method for *in frame* deletion (Kieser *et al.*, 2000). Transformation with multi-copy plasmids and with cosmids containing the site specific integration system of ϕC31 was carried out without denaturing the DNA.

II.7.9.2. Conjugation from *E. coli*

The introduction of DNA by conjugative transfer from *E. coli* was carried out as described by (Kieser *et al.*, 2000) with some modifications. For conjugative transfer, the vectors must contain *oriT* (origin of transfer), and transfer functions must be supplied *in trans* by the *E. coli* donor strain. In this study, the donor strain *E. coli* ET12567 (triple defective in DNA methylation, Cm^R) carrying the non-transmissible plasmid pUZ8002 (containing the transfer functions, Kan^R) was used. It is important to use strain ET12567 in order to bypass the methyl-sensing restriction system of some *Streptomyces* strains, like *S. coelicolor*.

The *oriT*-containing vector to be transferred into *Streptomyces* was first introduced into *E. coli* ET12567/pUZ8002 by electroporation (high competence is required when methylated plasmids are introduced into methylation-deficient strains, therefore, electroporation is preferred to the less efficient $CaCl_2$-mediated transformation). Selection was with kanamycin (50 µg/ml), chloramphenicol (25 µg/ml), and the antibiotic for the incoming vector. A colony was inoculated into 5 ml LB medium containing the antibiotics mentioned above and grown overnight at 37 ºC. The overnight culture was diluted 1:100 in LB medium plus antibiotics and grown at 37 ºC to an OD_{600} of approximately 0.6 (about 4 h). The cells were harvest by centrifugation and washed twice with an equal volume of LB medium (to remove antibiotics) and resuspended in 0.1 volume of LB medium.

Different media (MS, MS supplemented with $MgCl_2$, YMG) and quality of the receptor strain (spores, heat-shocked spores, mycelium from a TSB/YEME culture) were tested. The optimal conditions were as follows.

For *S. spheroides*: A spore suspension was thawed on ice and an aliquot corresponding approximately 10^8 spores was harvest by centrifugation. The supernatant was discarded and 0.5 ml of the *E. coli* suspension described above were mixed gently with the spore pellet. Note that the heat shock treatment described by (Kieser *et al.*, 2000) was omitted, which gave the best results.

For *S. roseochromogenes*: Fresh mycelium from 50 ml TSB/YEME 1:1 liquid culture was used instead of spores. After centrifugation, the mycelium was washed with 10.3% sucrose solution and resuspended in 5 ml 2×YT broth (Sambrook and Russell, 2001). 0.5 ml of the *Streptomyces* suspension was gently mixed with 0.5 ml of the *E. coli* suspension.

Each mixture was spread on two MS plates (supplemented with 10 mM $MgCl_2$ for *S. spheroides*, and without $MgCl_2$ for *S. roseochromogenes*), and incubated at 30 ºC for about 18 h, then overlaid with 1 ml water containing 0.5 mg nalidixic acid and an appropriate amount of the respective antibiotic for the incoming vector (e.g. 1.25 mg apramycin for cosmids H10, Z3 and E1). Incubation at 30 °C was continued for about a week to allow outgrowth of the exconjugants. Potential exconjugants (resistant colonies) were picked off to selective media containing nalidixic acid (25 µg/ml) and the antibiotic for the incoming vector, and analysed by Southern blotting.

II.7.10. DNA sequencing and computer-assisted sequence analysis

Double-stranded sequencing of recombinant plasmids was done by the dideoxynucleotide chain termination method on a LI-COR automatic sequencer (MWG-Biotech AG, Ebersberg, Germany).

The DNASIS software package (Version 2.1, Hitachi Software Engineering, San Bruno, CA, USA) was used for sequence analysis. Amino acid sequence homology searches were performed by using the BLAST program (Version 2.0) available on the web at www.ncbi.nim.nih.gov/BLAST/. The secondary structure of NovG was predicted using the PHD (Profile network prediction Heidelberg) method (Rost, 1996), available on the web at http://www.embl-heidelberg.de/predictprotein.

II.8. Methods of biochemistry and biology

II.8.1. Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The discontinuous SDS-PAGE was carried out according to the method of Laemmli (1970). Probes were mixed with sample buffer in ration 1:1 to 1:4 and incubated for 5 min in boiling water. 4% and 12% Polyacrylamide gel were used as stocking and resolving gel, respectively. Gel electrophoresis was carried out with working voltage of 200 V using the Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad, München, Germany). Protein bands were stained with Coomassie Brilliant Blue G-250 (s. Table II.13): 1-5 min in Fixing buffer, 15 min in Coomassie Brilliant Blue G-250 solution, and 90 min in Bleaching solution. To determine protein sizes, the Low Molecular Weight Calibration Kit for SDS gel electrophoresis (Amersham Biosciences, Freiburg, Germany) was used.

II.8.2. Overexpression and purification of recombinant protein from *E. coli*

Overexpression and purification of 6×His-tagged NovG protein from *E. coli* were carried out as described by the user manual of Qiagen (Qiagen, Hilden, Germany), “a handbook for high level expression and purification of 6×His-tagged proteins”. Different conditions for cultivation and purification were tested (e.g. different cultivation temperatures, amount of IPTG used for induction, concentration of imidazol in the washing buffer, elution; see also Results). The preparative method described bellow yielded the best results.

II.8.2.1. Cultivation

Five litres of LB medium containing 50 µg carbenicillin/ml and 35 µg chloramphenicol/ml were inoculated with *E. coli* BL21(DE3)pLysS/pAE-G5 cells; sufficient inoculum was used to reach an initial optical density of 0.1 at 600 nm (OD_{600}). Cells were then cultured at 30 ºC. When the OD_{600} reached a value of 0.6, IPTG was added to a final concentration of 0.5 mM. After further growth for 1.5 h at 30 ºC, cells were harvested by centrifugation and frozen at -70 ºC.

II.8.2.2. Preparation of cell-free extract

All the following steps were carried out at 4 ºC. After thawing on ice, cells (16 g) were suspended in 20 ml ice cold lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole, 2 mg lysozyme/ml) and incubated on ice for 30 min. The cell suspension was sonicated for 7 × 30 s with 30 s intervals between each sonication treatment (Branson Sonifier 250). Subsequently, cellular debris was removed by centrifugation (17,500×*g* for 30 min).

II.8.2.3. Purification by nickel affinity chromatography

Histidine fusion protein was purified from the soluble cell extract by metal affinity chromatography using Ni-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, Hilden, Germany) according to the manufacturer´s instructions. 3 ml of Ni-NTA-agarose slurry (50% (w/v) Ni-NTA agarose resin suspension in 30% (v/v) ethanol, precharged with Ni^{2+}) were added to 3 ml lysis buffer and stirred gently for about 15 min. The cell free extract was added to the Ni-NTA-agarose mixture described above and stirred gently for 60 min. 10 ml of washing buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole) were added and the protein-Ni-NTA-agarose mixture was harvested by centrifugation (5,000×*g* for 10 min at 4 ºC) and washed twice with 30 ml washing buffer (5,000×*g* for 10 min at 4 ºC) to remove unbound proteins. The pellet was suspended in washing buffer and loaded into a column. After further washing with 3 × 4 ml washing buffer containing 50 mM imidazole, unspecifically bound proteins were eluted using a stepwise imidazole gradient: 2 × 1 ml 100 mM, 1 × 1 ml 150 mM, and 1 × 1 ml 200 mM imidazole in washing buffer. The NovG fusion protein was subsequently eluted with 4 ml of elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole). 10 µl-aliquots were analysed by SDS-PAGE. 1 ml

Eluate fractions containing the NovG fusion protein (38.1 kDa) were applied onto a NAP 10 column (Amersham Biosciences) and eluted with 1.2 ml storage buffer (25 mM Tris-HCl (pH 7.5), 10% (w/v) glycerol, 2 mM DTT). Aliquots of NovG fusion protein in storage buffer can be shock frozen in liquid nitrogen and stored at -70 °C.

II.8.3. Gel mobility-shift assays

II.8.3.1. Preparation of 3´-end DIG-labeled DNA fragments

The following DNA fragments were used in gel mobility-shift assays:

(**1**) the 325 bp *Ava*I-fragment obtained from pMS32 (position 4 463 to 4 787 in AF170880, i.e. upstream of *novE*); (**2**) the 341 bp *Pvu*I-*Sal*I-fragment obtained from pMS32 (position 4 231 to 4 571 in AF170880, i.e. upstream of *novE*); (**3**) the 272 bp PCR product obtained using pMS63 as template and the primer pair Pnov$F_f (5´-AGG ACC ACT GGC TCG ATT TCG-3´) and Pnov$F_r (5´-GTC ACG CGC GAA GCC GTG AG-3´) (position 5 238 to 5 509 in AF170880, i.e. upstream of *novF*); (**4**) the 265 bp PCR product obtained using pMS33 as template and the primer pair Pnov$G_f (5´-GAG CTG GCC CGC CTC TTC GA-3´) and Pnov$G_r (5´-ACT TAA TGG TCT GGT CCG GAT CG-3´) (position 6 451 to 6 715 in AF170880, i.e. upstream of *novG*); (**5**) the 180 bp *Van*91I-*Hind*III-fragment obtained from pMS63 (position 7 725 to 7 904 in AF170880, i.e. upstream of *novH*); (**6**) the 209 bp *Sal*I-*Eco*RI-fragment obtained from pMS33 (position 7 613 to 7 821 in AF170880, i.e. upstream of *novH*); (**7**) the 244 bp *Bam*HI-fragment obtained from pMS61 (position 17 264 to 17 507 in AF170880, i.e. upstream of *novO*); (**8**) the 252 bp PCR product obtained using pMS61 as template and the primer pair Pnov$O_f (5´-TGT ACG AGC TGC TCA CCC ACG-3´) and Pnov$O_r (5´-TGA ATT GAG CCT ACA CGG ACA C-3´) (position 17 101 to 17 352 in AF170880, i.e. upstream of *novO*); (**9**) the 231 bp PCR product obtained using pMS62 as template and the primer pair Pgyr$_f (5´-GCG CAG AGG TGC TCT CGT TCC-3´) and Pgyr$_r (5´-TGC GGG TGT CGT AAG AAG TCA C-3´) (position 168 to 398 in AF205854, i.e. upstream of $gyrB^R$); (**10**) the 277 bp *Sma*I-fragment obtained from pMS62 (position 25 608 to 25 617 in AF170880 and 4 to 270 in AF205854, i.e. upstream of $gyrB^R$); (**11**) the 235 bp *Not*I-*Mlu*I-fragment obtained from pAE9 (position 12 654 to 12 888 in AF329398, i.e. upstream

of *cloY*); (**12**) the 255 bp PCR product obtained using cosmid K1F2 as template and the primer pair Pclo$H_1f (5´-GAA CGG CTC CTA TCT GGT CC-3´) and Pclo$H_1r (5´-CGG CCT TCG AAC AAC CTT CG-3´) (position 12 946 to 13 200 in AF329398, i.e. upstream of *cloH*); and (**13**) the 260 bp PCR product obtained using cosmid K1F2 as template and the primer pair Pclo$H_2f (5´-TGG GTG GCG AGT AGC ATC TG-3´) and Pclo$H_2r (5´-CTT AAG TCT CCA TGC CAT TGG-3´) (position 13 122 to 13 381 in AF329398, i.e. upstream of *cloH*).

PCR products were obtained using the Expand High Fidelity PCR System (Table II.20). Plasmids and cosmids used are described in Tables II.14 and II.15.

After purification by NuSieve GTG-agarose (FMC BioProducts) gel electrophoresis, DNA fragments were 3´-end labeled with DIG-11-ddUTP using the DIG Gel Shift Kit, 2nd Generation (Roche) according to the manufacturer's instructions.

II.8.3.2. Gel mobility-shift assay

Gel mobility-shift assays were performed using the DIG Gel Shift Kit, 2nd Generation (Roche) according to the manufacturer's instructions.

The DNA-binding reaction conditions were adapted from (Retzlaff and Distler, 1995). It was carried out at 25 ºC in 20 µl of 12.5 mM Tris-HCl (pH 7.5), 5% (w/v) glycerol, 62.5 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 50 ng poly [d(I-C)] μl^{-1}, 5 ng poly L-lysine μl^{-1}.

In general, about 4 ng of DIG-labeled DNA fragment and approximately 0.5 µg of purified His_6 tagged NovG were used for each assay.

For testing the specificity of binding, competitor plasmid-DNA or the respective empty vector were added in approximately 125-fold molar excess in comparison to the labeled fragment.

After 15 min of incubation, the reaction mixture was applied to a pre-run (20 min at 35 V) 6% (w/v) native polyacrylamide gel (85 × 75 × 0.75 mm) with 0.5×TBE as running buffer (Table II.14). The gel was run at 35 V for about 3 h, and transferred to a positively charged Hybond-N+ nylon membrane (Amersham) by contact blotting.

Crosslinking and detection were carried out following the manufacturer's instructions of the DIG Gel Shift kit.

II.8.4. Bioassay with *Bacillus subtilis*

Antibacterial activity was determined by a disc diffusion assay using *Bacillus subtilis* ATCC 14893 as the indicator strain. Different amounts of aminocoumarin antibiotics dissolved in 5-20 µl of methanol were applied to filter paper disks (Ø 6 mm; MN 440 B blotting paper, Macherey-Nagel), air dried under the clean bench and placed on the top of nutrient agar plates, which were seeded with 2×10^5 *B. subtilis* spores per ml of agar medium. Plates were cultured overnight at 37 °C, and the diameters of growth inhibition zones measured. Novobiocin (Fluka) and clorobiocin (Aventis) were used as reference substances.

II.9. Construction of deletion mutants of *S. roseochromogenes* and *S. spheroides*

II.9.1. Inactivation of *clo-hal* in *S. roseochromogenes*

clo-hal was inactivated using the PCR targeting system (Gust *et al.*, 2003), which takes advantage of the λ-Red recombination functions (*gam*, *bet*, *exo*) to promote gene replacement. An *aac(3)IV* (=apramycin resistance gene)*/oriT* cassette for replacement of *clo-hal* was generated by PCR (see Table II.22) using the primer pair P1-clo-hal (5´- ATT GGC GAT TTA TCG TCA GCG GTT GGA GGA AGT AGC GTG ATT CCG GGG ATC CGT CGA CC -3´) and P2-clo-hal (5´- AGC CTT CGG GCG AGA AGT CCT CGT CAC GTA CCG CGC TTA TGT AGG CTG GAG CTG CTT C-3´). Underlined letters represent 39 nt homologous extensions to the DNA regions immediately upstream and downstream of *clo-hal* including the putative start and stop codons of *clo-hal*, respectively. This cassette was introduced into *E. coli* BW25113/pIJ790, containing cosmid D1A8 (SuperCos1-based, kanamycin-resistant) which included the entire biosynthetic gene cluster of clorobiocin. The gene replacement was confirmed by restriction analysis and PCR using test primers TP1-

clo-hal (5´- GGA ACG GAA GCT TGG CTA TC-3´) and TP2-clo-hal (5´- GAA GTG CGA CAG GAT CTG GA-3´).

The mutated cosmid (termed D1A8-h-773, = H10) was introduced into *Streptomyces roseochromogenes* by conjugation from *E. coli* ET12567 carrying the non-transmissible pUZ8002 (see II.7.9.2). Apramycin-resistant, kanamycin-sensitive colonies were identified by replica plating and characterised by PCR (using *Taq* polymerase and the test primers mentioned above) and Southern blot analysis.

II.9.2. Inactivation of *cloZ* in *S. roseochromogenes*

cloZ was inactivated as described for *clo-hal*. The primer pair used for amplification of the *aac(3)IV/oriT* cassette was: P1-cloZ (5´-GGC CTG TCA GGC GTT AAC GGC GTT GAC TCG TCG ATG ATG ATT CCG GGG ATC CGT CGA CC-3´), P2-cloZ (5´-CCG GCG TAA CCC TCC GCG GTC GCC AGT CGC GCT GCG TCA TGT AGG CTG GAG CTG CTT C-3´).

The mutated cosmid was termed D1A8-Z-773 (= Z3).

Test primers were: TP1-cloZ (5´-GAT CCT GAA TTG GCC ATC AAC-3´), TP2-cloZ (5´-ATG CGT CAG ACG AGA ACT CAC-3´).

II.9.3. Inactivation of *novE* in *S. spheroides*

Within cosmid 10-9C (SuperCos1-based) containing the biosynthetic gene cluster of novobiocin, *novE* was inactivated as described for *clo-hal*. The primer pair used for amplification of the *aac(3)IV/oriT* cassette was: P1-novE (5´-GCC GGT CCG CTT GTC CCG AGG GGA AGA GAG GCA TCG TGA TTC CGG GGA TCC GTC GAC C-3´); P2-novE (5´-GCC GTG AGG CCG CGA AAT GGA TCG GAG TGC GTC CGG TCA TGT AGG CTG GAG CTG CTT C-3´).

The mutated cosmid was termed 10-9C-E-773 (= E1).

Test primers were: TP1-novE (5´-CCG TCA ACA AGT GAA CCC AA-3´) and TP2-novE (5´-GTC CAT GAG GTA GGA GTC GA-3´).

II.10. Heterologous expression of the novobiocin and clorobiocin biosynthetic gene clusters

Plasmid pIJ787 was constructed by Dr. Bertolt Gust (John Innes Centre, Norwich) using standard cloning techniques and λ-Red-mediated recombination. pIJ787 is a SuperCos1 derivative, in which the ampicillin resistance gene (*bla*) was replaced by a cassette containing the integrase gene (*int*) and attachment site (*attP*) of phage ϕC31 as well as a resistance marker (*tet*, tetracycline resistance gene).

The *Dra*I-*Bsa*I-fragment of pIJ787, containing the integrase cassette and flanked by about 100 bp *bla* sequence on one site and about 300 bp *bla* sequence on the other side was used to replace the respective *bla* gene in the SuperCos1 backbone of cosmids 10-9C and D1A8, via λ-Red-mediated recombination (Datsenko and Wanner, 2000; Gust *et al.*, 2003), generating nov-BG1 and clo-BG1, respectively (by Dr. Bertolt Gust).

The modified cosmids nov-BG1 and clo-BG1, still carrying the kanamycin resistance gene *neo*, were then introduced into *S. coelicolor* M512 and *S. lividans* TK24 via PEG-mediated protoplast transformation (see II.7.9.1). Kanamycin resistant clones were checked for site specific integration into the genome by Southern blot analysis.

II.10.1. Protocol for single or multiple deletions within the cosmids

The apramycin resistance cassette (approximately 1 kb) was excised from pUG019 by digestion with *Eco*RI and *Hind*III and amplified by PCR using the forward primer 5´-$(N)_{39}$ ATT CCG GGG ATC TCT AGA TCT-3´ and the reverse primer 5´-$(N)_{39}$ ACT AGT CTG GAG CTG CTT C-3´. $(N)_{39}$ represents 39 nt extensions for λ-Red-mediated recombination, homologous to the regions upstream and downstream of the DNA fragment to be deleted; underlined are the *Xba*I and *Spe*I restriction sites. Amplification was performed using the Expand High Fidelity PCR system as described at II.7.6.2. The PCR product was used for gene replacement in cosmids via λ-Red-mediated recombination, according to the instructions of the REDIRECT© technology kit (Gust *et al.*, 2003). The PCR product was introduced into the cells by electroporation. Cosmids which had integrated the cassette were isolated and analyzed by restriction enzyme digestion. For excision of the resistance cassette,

cosmid DNA was isolated from *E. coli* ET12567, digested with *Xba*I and *Spe*I and 100 ng DNA were religated overnight at 4 °C. $CaCl_2$-competent *E. coli* XL1 Blue MRF' cells were transformed with the ligation reaction. Apramycin-sensitive kanamycin-resistant clones were analyzed by restriction enzyme digestion and gel electrophoresis. Correct excision of the cassette was indicated by the absence of *Xba*I and *Spe*I sites, as well as by the absence of a *Bgl*II site internal to the cassette. For subsequent gene deletions, the identical procedure was used.

II.10.2. Removal of non-essential DNA regions from the cosmid inserts

Using the procedure described above (II.10.1), cosmids **nov-AE6** and **nov-AE4** were generated (see Fig. III.6). First, the region downstream of $gyrB^R$ was removed from nov-BG1, using primers PnovgyrB_f (5´-GGT TCC TCC AGC GTG GCC ACG ACC ATG ACC GGG AGG TCG ATT CCG GGG ATC TCT AGA TC-3´) and PT3SC1_r (5´-GTC TTC AAG AAT TCG CGG CCG CAA TTA ACC CTC ACT AAA ACT AGT CTG GAG CTG CTT C-3´) generating cosmid nov-AE2. Underlined letters represent the 39 nt extensions homologous to the region downstream of $gyrB^R$ and to the T3 promoter of SuperCos1, respectively. Subsequently, *ORF21* was deleted from nov-AE2 using primers PT7SC1_f (5´-ACA TGA GAA TTC GCG GCC GCA TAA TAC GAC TCA CTA TAG ATT CCG GGG ATC TCT AGA TC-3´), and Pnov20_r (5´-CTT CCC GAG GTT CAA TTC CGC CGC GCA CGT CAG CTC CTC ACT AGT CTG GAG CTG CTT C-3´) generating cosmid nov-AE6. Underlined are sequences homologous to the T7 promoter region of SuperCos1 and to the region downstream of *ORF20*, respectively. Alternatively, the entire region upstream of *novE* was deleted from nov-AE2, using primers PT7SC1_f (see above), and PnovE_r (5´-GCT GGA ATG CGC GGC TGC CGT CGC CGG GAC GGT CCC GGC ACT AGT CTG GAG CTG CTT C-3´) generating cosmid nov-AE4. Underlined is the sequence homologous to the region directly downstream of *novD*.

clo-AE2, containing genes from *cloE* to $gyrB^R$, was generated from clo-BG1 in the same way using primers Pcloorf9_f (5´-TAG TAT GGC GAA ATT GGG TGA TCT GCT TGC CGC CGT CGA ATT CCG GGG ATC TCT AGA TC-3´) and PT3SC1_r (see above).

See Table II.15 for details about the insert's sequence of nov-AE4, nov-AE6 and clo-AE2.

II.10.3. Inactivation of *novO* in cosmid nov-BG1, and heterologous expression of the *novO*⁻ cosmid

In cosmid nov-BG1, *novO* was replaced by the apramycin resistance (*aac(3)IV*) cassette from pUG019 as described at II.10.1. The cassette for replacement of *novO* was generated by PCR using the primer pair P1-NovO (5´-AGA TCA GCT CAC TGA CCC AAC ACG AGG GGC ATC GAG ATG ATT CCG GGG ATC **TCT AGA** TC-3´) and P2-NovO (5´-CGG GTC CAG GCG CTC TGT TCG GGA CAA TTC CGC CGC TCA **ACT AGT** CTG GAG CTG CTT C-3´). Underlined letters represent 39 nt homologous extensions to the DNA regions immediately upstream and downstream of *novO*, respectively, including the putative start and stop codons of *novO*; the *Xba*I and *Spe*I restriction sites are presented in bold letters.

The generated Δ*novO* cosmid nov-AE8, carrying the kanamycin resistance gene *neo*, was introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation. Kanamycin resistant clones were checked for specific genomic integration of cosmid nov-AE8 into the ϕC31 attachment site by Southern blot analysis.

II.10.4. Inactivation of *novG* in cosmid nov-BG1, and heterologous expression of the *novG*⁻ cosmid

In cosmid nov-BG1, *novG* was replaced by the apramycin resistance (*aac(3)IV*) cassette from pUG019 as described at II.10.1. The cassette for replacement of *novG* was generated by PCR using the primer pair P1-novG (5´-GAT CCG GAC CAG ACC ATT AAG TCC TAT GGG GGT TAC ATG ATT CCG GGG ATC **TCT AGA** TC-3´) and P2-novG (5´-CAA CCG AAT GAT TCG AGC AGT TGA ACG TCA GGC GGT GTC **ACT AGT** CTG GAG CTG CTT C-3´). Underlined letters represent 39 nt homologous extensions to the DNA regions immediately upstream and downstream of *novG*, including the putative translational start and stop codons of *novG*, respectively; the *Xba*I and *Spe*I restriction sites are presented in bold letters.

The generated Δ*novG* cosmid nov-AE10, carrying the kanamycin resistance gene *neo*, was introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation. Kanamycin resistant clones were checked for specific genomic

integration of cosmid nov-AE10 into the ϕC31 attachment site by Southern blot analysis.

II.11. Analysis and isolation of secondary metabolites

II.11.1. HPLC analysis

II.11.1.1. Analysis of clorobiocin and derivatives

Constructed mutants, transformants and the wild-type strain of *S. roseochromogenes*, and *S. lividans* or *S. coelicolor* carrying the clorobiocin cluster were cultured in production medium as described at II.6.2.2. 1-5 ml bacterial culture were acidified with HCl to pH 3 and extracted twice with an equal volume of ethyl acetate. After evaporation of the solvent, the residue was redissolved in 0.25-1 ml ethanol. After centrifugation, 10 to 100 µl of the clear supernatant was analysed by HPLC with a Multosphere RP18-5 column (250×4 mm; 5 µm; C+S Chromatographie Service, Düren, Germany) at flow rate of 1 ml/min, using a linear gradient from 40 to 100% of solvent B in 25 min (solvent A, MeOH : H_2O : HCOOH 50:49:1; solvent B, MeOH : HCOOH 99:1) with detection at 340 nm. Authentic clorobiocin (Aventis) was used as standard.

II.11.1.2. Analysis of novobiocin and derivatives

Constructed mutants, transformants and the wild-type strain of *S. spheroides*, and *S. lividans* or *S. coelicolor* carrying the novobiocin cluster were cultured in CDM medium as described at II.6.2.2. After centrifugation, the clear supernatant was analysed by HPLC with a Multosphere RP18-5 column (250 x 4 mm; 5 µm; C+S Chromatographie Service, Düren, Germany) with a linear gradient from 60 to 100% methanol in 1% aqueous formic acid and detection at 305 nm. Authentic novobiocin (Fluka) was used as standard.

II.11.2. Preparative isolation of aminocoumarins

II.11.2.1. Column chromatography using Sephadex® LH-20

Column chromatography was used to pre-purify the culture extract. Crude culture extract (max. 3 ml) was loaded onto a glass column (100×2.6 cm) with Sephadex LH-20 (Amersham Biosciences, Freiburg, Germany). The column was eluted with degased methanol with a flow rate of 1 ml/min. 15 ml eluates were collected by a fraction collector and controlled by HPLC. Fractions containing the expected compounds were pooled and the solvent removed in vacuum. The residue was dissolved in methanol and used for further purification by HPLC.

II.11.2.2. Preparative HPLC

Preparative HPLC was carried out on a Multosphere 120 RP 18-5 column (5 µm, 250×20 mm; C+S Chromatographie Service, Düren Germany) with a flow rate of 3 ml. Solvents were as described at II.11.1. Concentrated culture extract or LH-20 fractions were injected and analysed. The expected eluates after HPLC analysis were collected and the solvent was evaporated in vacuum. The residues were dried over phosphorus pentoxide and subjected to MS and NMR (^{1}H-NMR and ^{13}C-NMR) analysis.

II.11.3. Spectroscopic methods for structural elucidation

II.11.3.1. Negative-ion FAB mass spectroscopy

Negative-ion fast atom bombardment (FAB) mass spectra were recorded on a TSQ70 spectrometer (Finnigan, Bremen, Germany) using diethanolamine as matrix.

Negative-ion FAB MS data (*m/z* (relative intensity in %)) were as follows:

Clorobiocin: 697 (24, $[M-H]^-$, ^{37}Cl), 696 (28, $[M-H]^-$, ^{13}C), 695 (80, $[M-H]^-$, ^{35}Cl), 661 (17, $[M-HCl]^-$), 588 (7, $[M-C_6H_7ON$, = M-pyrrol unit$]^-$), 507 (23), 473 (9), 413.5 (38), 379 (32), 283 (31), 226 (100).

Novclobiocin 101: 661 (50, $[M-H]^-$), 473 (20), 380 (15), 339 (38), 283 (37), 209 (100).

Novclobiocin 102: 675 (72, $[M-H]^-$), 661 (6, $[M-CH_4]^-$), 568 (7, $[M-C_6H_7ON$, = M-pyrrol unit$]^-$), 487 (28, $[M-C_{12}H_{14}O_2$, = M-ring A$]^-$), 447 (22), 394 (24), 271 (50), 251 (70), 205 (100).

Novobiocin: 611 (8, $[M-H]^-$), 568 (7, $[M-CONH]^-$), 395 (7), 378 (6), 281 (10), 255 (24), 209 (100).

Novclobiocic acid 106: 380 (18, $[M-H]^-$), 283 (10), 255 (22), 209 (100).

Novclobiocin 117: 597 (7, $[M-H]^-$), 380 (4), 314 (6), 283 (10), 255 (13), 209 (100).

Novclobiocin 114: 633 (5, $[M-H]^-$, ^{37}Cl), 632 (5, $[M-H]^-$, ^{13}C), 631 (15, $[M-H]^-$, ^{35}Cl), 588 (5, $[M-CONH]^-$), 554 (4, $[M-CH_2ONCl]^-$), 380 (7), 255 (46), 209 (100).

Novclobiocin 119: 633 (10, $[M-H]^-$, ^{37}Cl), 632 (11, $[M-H]^-$, ^{13}C), 631 (32, $[M-H]^-$, ^{35}Cl), 588 (12, $[M-CONH]^-$), 554 (6, $[M-CH_2ONCl]^-$), 400 (25), 255 (68), 209 (100).

II.11.3.2. Nuclear magnetic resonance (NMR)

^1H NMR and ^{13}C NMR spectra were measured on an AC 250, on an AMX 400, or on an AMX 600 spectrometer (250, 400, and 600 MHz, respectively; Bruker, Karlsruhe, Germany), using CD_3OD as solvent.

For ^1H and ^{13}C NMR data of clorobiocin, novclobiocin 101 and novclobiocin 102, see Table III.1 and III.2, respectively. For ^1H NMR data of novobiocin, novclobiocic acid 106, novclobiocin 117, 114 and 119, see Table III.4.

In case of strains harboring nov-BG1, nov-AE6 and nov-AE4 (comprising the novobiocin cluster), the isolated compound showed a molecular ion $[M-H]^-$ at *m/z* 611 (novobiocin = $C_{31}H_{36}N_2O_{11}$; molecular weight, 612). The isolated substance gave the following ^1H NMR signals: δ_{ppm} 7.81 (1H, d, *J*=8.9 Hz, H-5´); 7.75 (1H, br (=broad) s, H-2); 7.72 (1H, br d, *J*=8.3 Hz, H-6); 7.23 (1H, br d, *J*=8.9 Hz, H-6´); 6.84 (1H, d, *J*=8.3 Hz, H-5); 5.57 (1H, br s, H-1´´); 5.34 (1H, mt, *J*=7 Hz, H-8, overlapped with H-3´´, *J* not exactly determinable); 5.31 (1H, dd, *J*=3, 10 Hz, H-3´´, overlapped with H-8, *J* not exactly determinable); 4.23 (1H, br s, H-2´´); 3.57 (1H, d, *J*=9.9 Hz, H-4´´); 3.55 (3H, s, H-8´´); 3.34 (2H, d, *J*=7.3 Hz, H-7); 2.31 (3H, s, H-11´); 1.75 (3H, s, H-11);

1.74 (3H, s, H-10); 1.34 (3H, s, H-7´´); 1.14 (3H, s, H-6´´). These signals corresponded to those obtained from authentic novobiocin.

Strains harboring clo-BG1 and clo-AE2 (comprising the clorobiocin cluster), showed four peaks in the HPLC analysis (Fig. III.8D). The first peak (1), with the shortest retention time, showed a molecular ion $[M-H]^-$ at *m/z* 661 and the following isotopic pattern: mass (intensity): 661 (100.0%), 662 (39.9%), 663 (11.9%). Furthermore, this compound gave the following ^{1}H NMR signals: δ_{ppm} 7.93 (1H, d, *J*=9.3 Hz, H-5´); 7.77 (1H, d, *J*=2.0 Hz, H-2); 7.72 (1H, br d, *J*=8.5 Hz, H-6); 7.06 (2H, complex signal, H-6´ and H-8´); 6.90 (1H, d, *J*=3.8 Hz, H-3´´´); 6.83 (1H, d, *J*=8.3 Hz, H-5); 5.94 (1H, d, *J*=3.8 Hz, H-4´´´); 5.61 (1H, d, *J*=2.6 Hz, H-1´´); 5.59 (1H, dd, *J*=3.3, 9.9 Hz, H-3´´, overlapped with H-1´´); 5.35 (1H, mt, *J* not determinable, H-8); 4.23 (1H, t, *J*=2.7 Hz, H-2´´); 3.69 (1H, d, *J*=9.8 Hz, H-4´´); 3.51 (3H, s, H-8´´); 3.34 (2H, d, *J* not determinable, overlapped with solvent signal, H-7); 2.29 (3H, s, H-6´´´); 1.74 (6H, br s, H-11 and H-10); 1.37 (3H, s, H-7´´); 1.20 (3H, s, H-6´´). The MS and ^{1}H NMR data corresponded to those obtained for C-8´-deschloro-clorobiocin (=novclobiocin 101, Table III.1). The second peak (2) showed a molecular ion $[M-H]^-$ at *m/z* 695 and the typical isotopic pattern caused by the chlorine isotopes ^{35}Cl and ^{37}Cl: mass (intensity): 695 (100.0%), 696 (36.1%), 697 (39.5%), 698 (14.6%). This compound gave the following ^{1}H NMR signals: δ_{ppm} 7.91 (1H, d, *J*=9.0 Hz, H-5´); 7.76 (1H, br s, H-2); 7.73 (1H, dd, *J*=2.4, 10 Hz, H-6, overlapped with H-2, *J* not exactly determinable); 7.35 (1H, d, *J*=9.1 Hz, H-6´); 6.90 (1H, d, *J*=3.7 Hz, H-3´´´); 6.85 (1H, d, *J*=8.3 Hz, H-5); 5.94 (1H, br d, *J*=3.7 Hz, H-4´´´); 5.72 (1H, d, *J*=3.3 Hz, H-1´´); 5.70 (1H, dd, *J*=3.2, 7.0 Hz, H-3´´, overlapped with H-1´´, *J* not exactly determinable); 5.35 (1H, mt, *J* not determinable, H-8); 4.32 (1H, t, *J*=2.8 Hz, H-2´´); 3.72 (1H, d, *J*=10.2 Hz, H-4´´); 3.52 (3H, s, H-8´´); 3.35 (2H, d, *J*=6.8 Hz, H-7); 2.29 (3H, s, H-6´´´); 1.75 (3H, s, H-11); 1.74 (3H, s, H-10); 1.35 (3H, s, H-7´´); 1.18 (3H, s, H-6´´). The MS and ^{1}H NMR data corresponded to those obtained for clorobiocin (Table III.1). The third peak (3) had the same mass as the first one, molecular ion $[M-H]^-$ at *m/z* 661, and showed the same isotopic pattern (mass (intensity): 661 (100.0%), 662 (30.2%), 663 (17.5%)), indicating absence of a chlorine atom. The fourth peak (4) had the same mass as clorobiocin, molecular ion $[M-H]^-$ at *m/z* 695, and the isotopic pattern caused by the chlorine isotopes ^{35}Cl and ^{37}Cl: mass (intensity): 695 (100.0%), 696 (37.6%), 697 (36.0%), 698 (14.6%). Substances (3) and (4) are therefore likely to represent

structural isomers of (1) and (2) which carry the acyl group at 2-OH rather than 3-OH of the deoxysugar, as identified previously (Galm *et al.*, 2004a).

III. RESULTS

III.1. Identification of the gene responsible for the halogenation reaction in clorobiocin biosynthesis

The characteristic aminocoumarin moiety of the aminocoumarin antibiotics is substituted at position 8 in novobiocin and coumermycin A_1 with a methyl group and in clorobiocin with a chlorine atom (Fig. I.1). This structural difference is perfectly reflected in the organization of the gene clusters: the novobiocin and coumermycin A_1 clusters contain a C-methyltransferase gene, i.e. *novO* and *couO*, respectively, whereas the clorobiocin cluster contains the gene *clo-hal*, which shows sequence similarity to the recently discovered class of $FADH_2$-dependent halogenases (van Pée, 2001), at the corresponding position (Fig. I.3). In addition, the clorobiocin cluster also contains the ORF *cloZ*, which shows no sequence similarity to known genes. For all genes of the clorobiocin cluster, with the exception of *clo-hal* and *cloZ*, homologues exist in the novobiocin and/or coumermycin cluster. Since the halogen atom represents the only structural characteristic of clorobiocin absent from both novobiocin and coumermycin, it can be questioned whether *cloZ*, together with *clo-hal*, may play a role in the halogenation of clorobiocin.

III.1.1. Sequence analysis of *clo-hal* and *cloZ*

The predicted gene product of *clo-hal* comprises 525 amino acids and resembles $FADH_2$-dependent halogenases which use phenols or pyrrole derivatives as substrates, such as BhaA of the balhimycin gene cluster (Pelzer *et al.*, 1999; Puk *et al.*, 2002), ComH of the complestatin gene cluster (Chiu *et al.*, 2001), and PltA of the pyoluteorin gene cluster (Nowak-Thompson *et al.*, 1999). The sequence identity of Clo-hal to those three proteins is 36%, 35% and 31%, respectively.

The predicted gene product of *cloZ* comprises 254 amino acids and shows no homology to known proteins.

III.1.2. Inactivation of *clo-hal* and *cloZ* in *S. roseochromogenes*

It was first attempted to inactivate *clo-hal* and *cloZ* by conventional methods reported for *in frame* gene deletion (Δ) in *Streptomyces* (Kieser *et al.*, 2000). For this purpose, a Δ*clo-hal* and a Δ*cloZ* allele was constructed by cloning two non-contiguous fragments of about 1 kb, carrying the 5´ and 3´ ends of the target gene, in a suitable vector (non-replicative in *Streptomyces*), which finally led to plasmids pAE-ha3 ($Thio^R$) and pAE-Z3 ($Thio^R$) (s. Table II.15 and Figs. III.1 and III.2). These plasmids were then independently transformed into *S. roseochromogenes* by PEG-mediated protoplast transformation. Selection for thiostrepton allowed the isolation of mutants in which a single crossover event had occurred, due to homologous recombination between one fragment of the plasmid and the respective DNA region of the *S. roseochromogenes* genome, as shown by Southern blot analysis (Figs. III.1 and III.2). Note that homologous recombination may have occurred upstream or downstream of *clo-hal* and *cloZ*, respectively, since the used probes do not distinguish between these two crossover events.

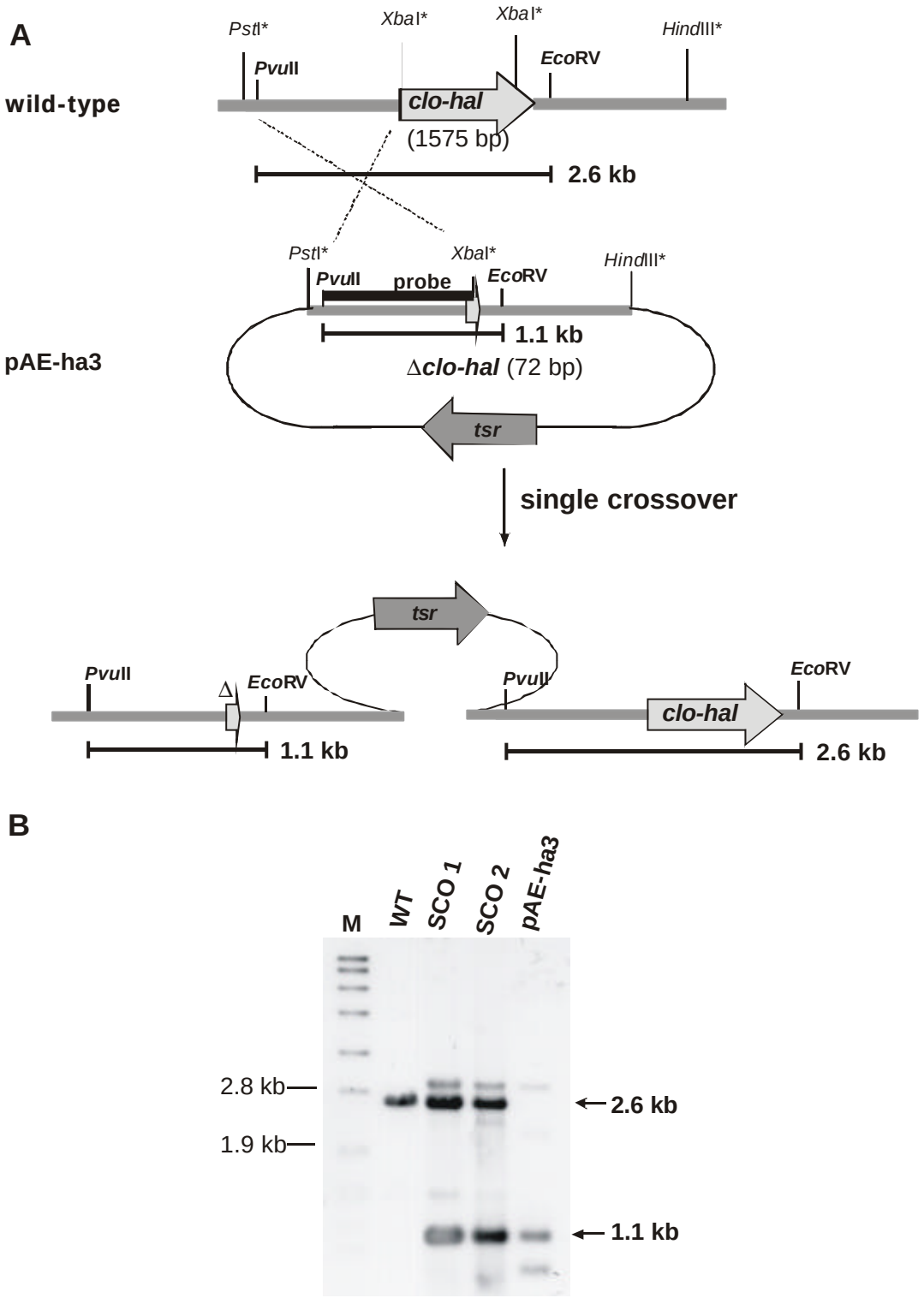


Fig. III.1: Single crossover mutants of *clo-hal* in *S. roseochromogenes*.

(**A**) Schematic presentation of a single crossover event. The DNA fragment used as probe is indicated as a black bar. *tsr*: thiostrepton resistance gene. Restriction sites marked with an asterisk are not presented in the original sequence, but only in pAE-ha3 (introduced by PCR). Out of scale. (**B**) Southern blot analysis of wild-type (WT) and *clo-hal* single crossover mutants (SCO) of *S. roseochromogenes*. Genomic and plasmid DNA were digested with *Pvu*II and *Eco*RV. M: DIG-labeled DNA Molecular Weight Marker VII (Roche).

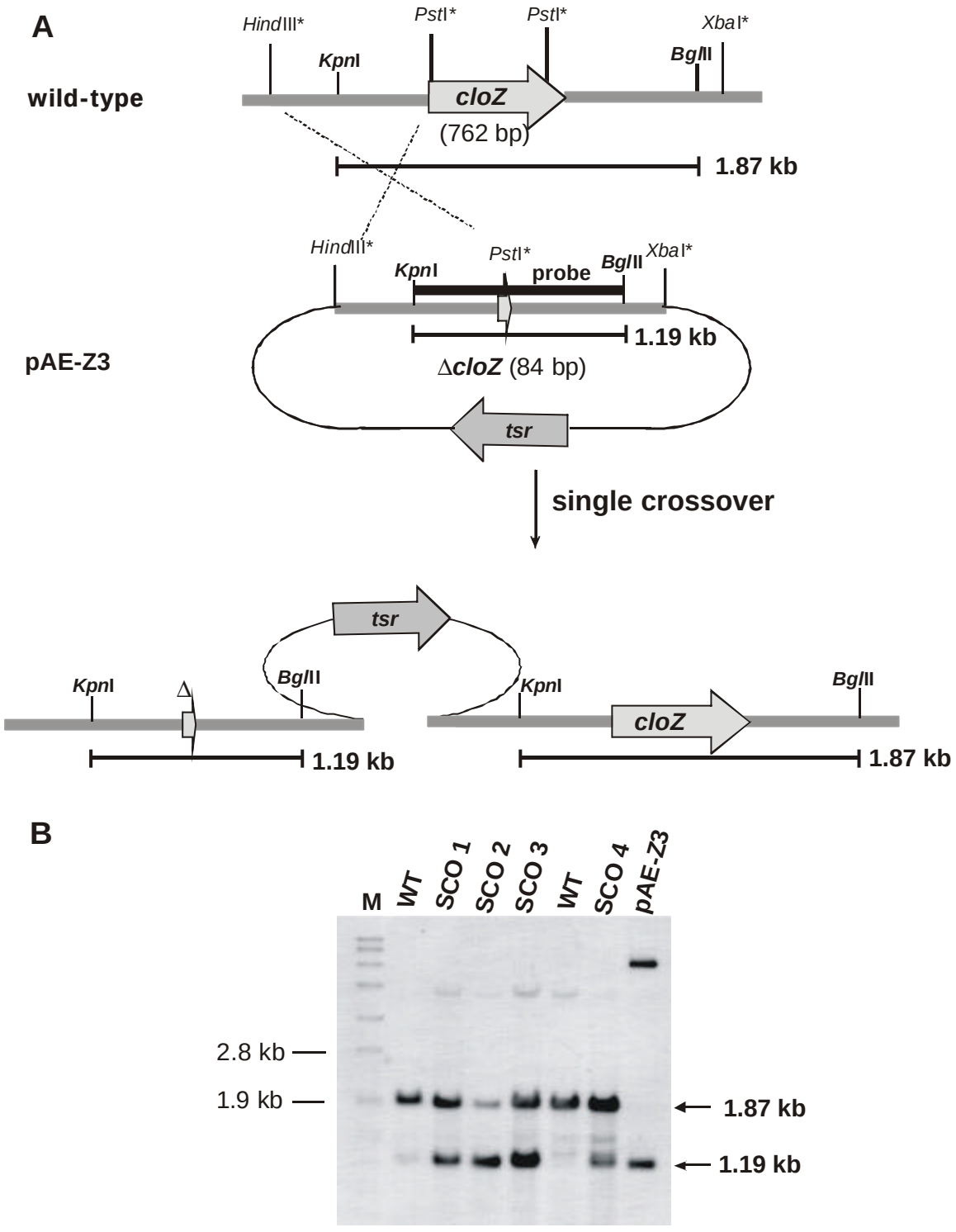


Fig. III.2: Single crossover mutants of *cloZ* in *S. roseochromogenes*.

(**A**) Schematic presentation of a single crossover event. The DNA fragment used as probe is indicated as a black bar. *tsr*: thiostrepton resistance gene. Restriction sites marked with an asterisk are not presented in the original sequence, but only in pAE-Z3 (introduced by PCR). Out of scale. (**B**) Southern blot analysis of wild-type (WT) and *cloZ* single crossover mutants (SCO) of *S. roseochromogenes*. Genomic and plasmid DNA were digested with *Kpn*I and *Bgl*II. M: DIG-labeled DNA Molecular Weight Marker VII (Roche).

Single crossover mutants were then cultured without addition of antibiotics to allow selection for another crossover event in which the vector was lost (thiostrepton-sensitive mutants). These second crossover events may either lead to a reversion to the wild-type or to the desired gene replacement mutant (Kieser *et al.*, 2000). To select for double crossover mutants, it is necessary to grow the single crossover mutants non-selectively for several generations, and to screen single colonies (often thousands) by replica plating (sporulation is here desirable). It is therefore a very time-consuming method, especially for *S. roseochromogenes*, which sporulates (if at all) only very slowly (Mancy *et al.*, 1974).

During the selection for double crossover events, we became acquainted with a new approach, the PCR targeting system (Gust *et al.*, 2003), which allows gene inactivation experiments to be carried out much more simply and quickly than by previous methods (Kieser *et al.*, 2000). Datsenko and Wanner (2000) have developed a rapid method to disrupt chromosomal genes in *Escherichia coli* by replacement with a selectable marker. The marker is generated by PCR, using primers with 36-50 nt extensions which are homologous to the targeted gene. Recombination of these short homologous sequences with chromosomal DNA is promoted by the λ-Red functions (*gam*, *bet*, *exo*). This strategy was adapted for use in *Streptomyces coelicolor* by (Gust *et al.*, 2003).

We have therefore abandoned the experiments described above and decided to use this PCR targeting system to inactivate *clo-hal* and *cloZ* in *S. roseochromogenes*.

Within cosmid D1A8, which contained the biosynthetic gene cluster of clorobiocin in the SuperCos1 vector (carrying a kanamycin resistance gene), *clo-hal* was replaced by an apramycin resistance/*oriT* cassette using λ-Red-mediated recombination in *E. coli* (see Methods). The modified cosmid (named D1A8-h-773, = H10) was introduced into *S. roseochromogenes* by conjugation from *E. coli*. Apramycin-resitant, kanamycin-sensitive colonies, resulting from double crossover events, were selected. From the 17 obtained ex-conjugants, 15 were Apramycin-resitant, kanamycin-sensitive, representing therefore potential double crossover mutants. Southern blot analysis of three candidates confirmed that in these mutants *clo-hal* was replaced by the apramycin resistance/*oriT* cassette (Fig. III.3A and B).

cloZ was inactivated in the same way, and the correct genotype of the resulting double crossover mutants was confirmed by Southern blot analysis (Fig. III.3C and D).

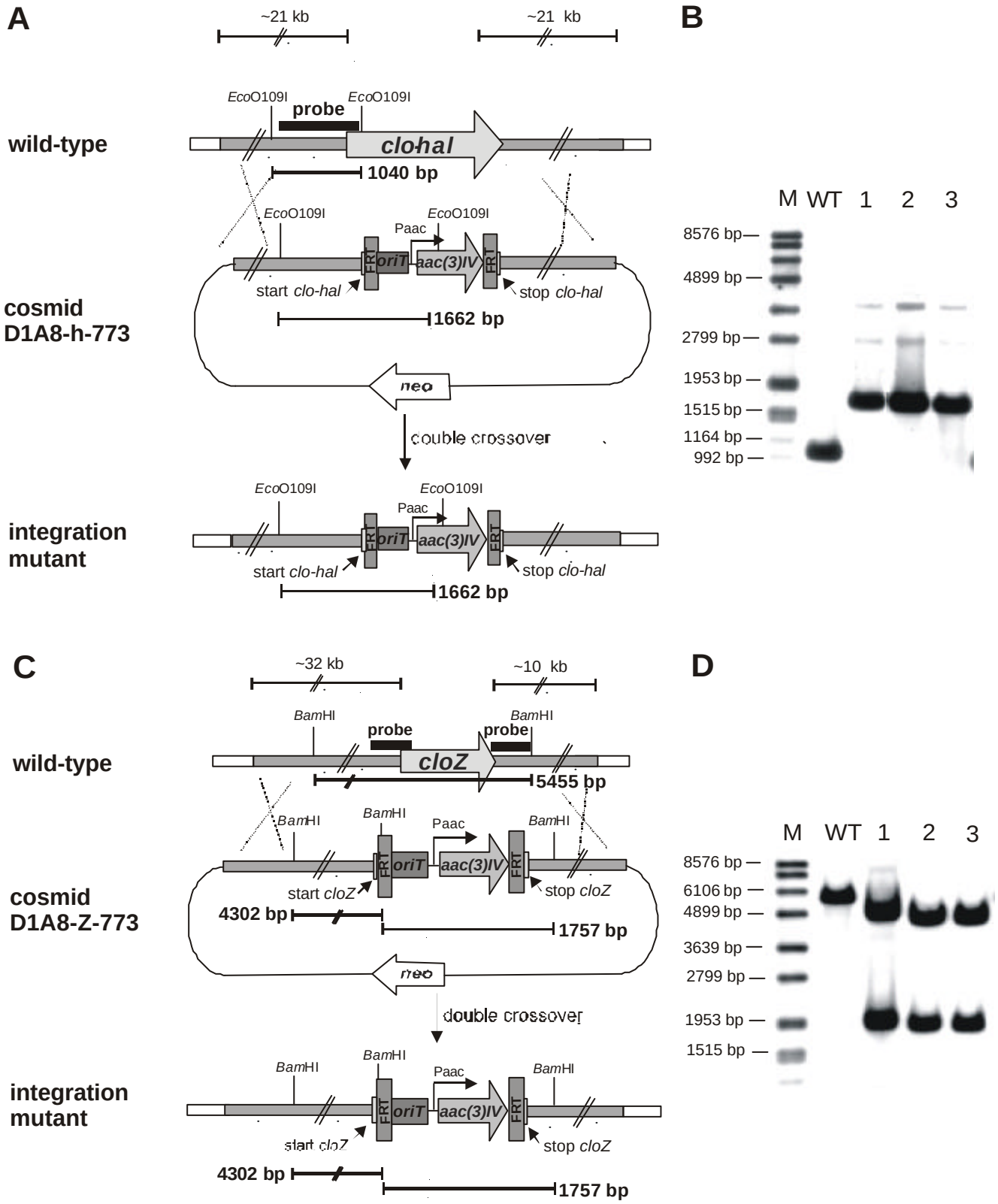


Fig. III.3: Inactivation of *clo-hal* and *cloZ* in *S. roseochromogenes*. See next page for details.

(**A**) Schematic presentation of the *clo-hal* inactivation experiment. The DNA fragment used as probe is indicated as a black bar. *aac(3)IV*: apramycin resistance gene, Paac: promoter of the apramycin resistance gene, FRT: FLP recognition target, *oriT*: origin of transfer from RK2, *neo*: kanamycin resistance gene. Out of scale. (**B**) Southern blot analysis of wild-type and *clo-hal*$^-$ mutants. Genomic DNA was restricted by *Eco*0109I. M: DIG-labeled DNA Molecular Weight Marker VII (Roche); WT: *S. roseochromogenes* var. *oscitans* wild-type; 1-3: *clo-hal*$^-$ mutants (strains AE h2, AE h10, and AE h11). (**C**) Schematic presentation of the *cloZ* inactivation experiment. The DNA fragment used as probe is indicated as a black bar. Out of scale. (**D**) Southern blot analysis of wild-type and *cloZ*$^-$ mutants. Genomic DNA was restricted by *Bam*H1. M: DIG-labeled DNA Molecular Weight Marker VII (Roche); WT: *S. roseochromogenes* var. *oscitans* wild-type; 1-3: *cloZ*$^-$ mutants (strains AE-Z4, AE-Z25, and AE-Z40).

With some modifications of the conjugation procedure (see Methods), the PCR targeting system could therefore be used successfully in *S. roseochromogenes*, allowing gene inactivation experiments to be indeed carried out much more simply and quickly than by previous methods (Kieser *et al.*, 2000).

III.1.3. Characterization of secondary metabolites

Three independent *clo-hal*$^-$ mutants (AE-h2, AE-h10, and AE-h11) and three independent *cloZ*$^-$ mutants (AE-Z4, AE-Z25, and AE-Z40) as well as the wild-type were cultured in clorobiocin production medium (see Methods). After extraction of the cultures with ethyl acetate, secondary metabolites were analysed by HPLC in comparison with clorobiocin standard.

The production of clorobiocin was abolished in all *clo-hal*$^-$ mutants (Fig. III.4B). These mutants produced, instead, a new substance with a shorter retention time than clorobiocin. This compound was isolated on a preparative scale. Negative-ion FAB MS analysis showed a molecular ion $[M-H]^-$ at *m/z* 661, consistent with the loss of a chlorine atom in comparison to clorobiocin ($[M-H]^-$ at *m/z* 695). Clorobiocin shows the typical isotopic pattern caused by the chlorine isotopes ^{35}Cl and ^{37}Cl (mass [intensity]: 695 [100.0%], 696 [32.6%], 697 [31.8%], 698 [11.1%]), whereas the negative-ion FAB MS of the new substance did not show this pattern, indicating the absence of

chlorine (mass [intensity]: 661 [100.0%], 662 [25.9%], 663 [9.5%]). ^{1}H NMR (Table III.1) and ^{13}C NMR (Table III.2) unequivocally confirmed that the new substance was a clorobiocin derivative containing an hydrogen instead of a chlorine atom at C-8´ of the aminocoumarin moiety. In the ^{1}H NMR spectrum of the new compound, the signal at 7.33 ppm corresponding to H-6´ of clorobiocin had disappeared. Instead, a signal at 7.06 ppm for two protons was observed as a broad singlet, which could be assigned to H-6´ and H-8´ by correlated spectroscopy (COSY). The coincidence of the signals of H-6´ and H-8´ as a broad singlet is in accordance with the spectrum reported from a naturally occurring novobiocin derivative lacking the 8´-methyl group (Sasaki *et al.*, 2001). In the ^{13}C NMR spectrum, the signal corresponding to C-8´ was found at 104.0 ppm (instead of 110.7 ppm as for clorobiocin), and the signal corresponding to C-6´, at 115.0 ppm (clorobiocin: 112.5 ppm). This is also in accordance with literature data (Sasaki *et al.*, 2001).

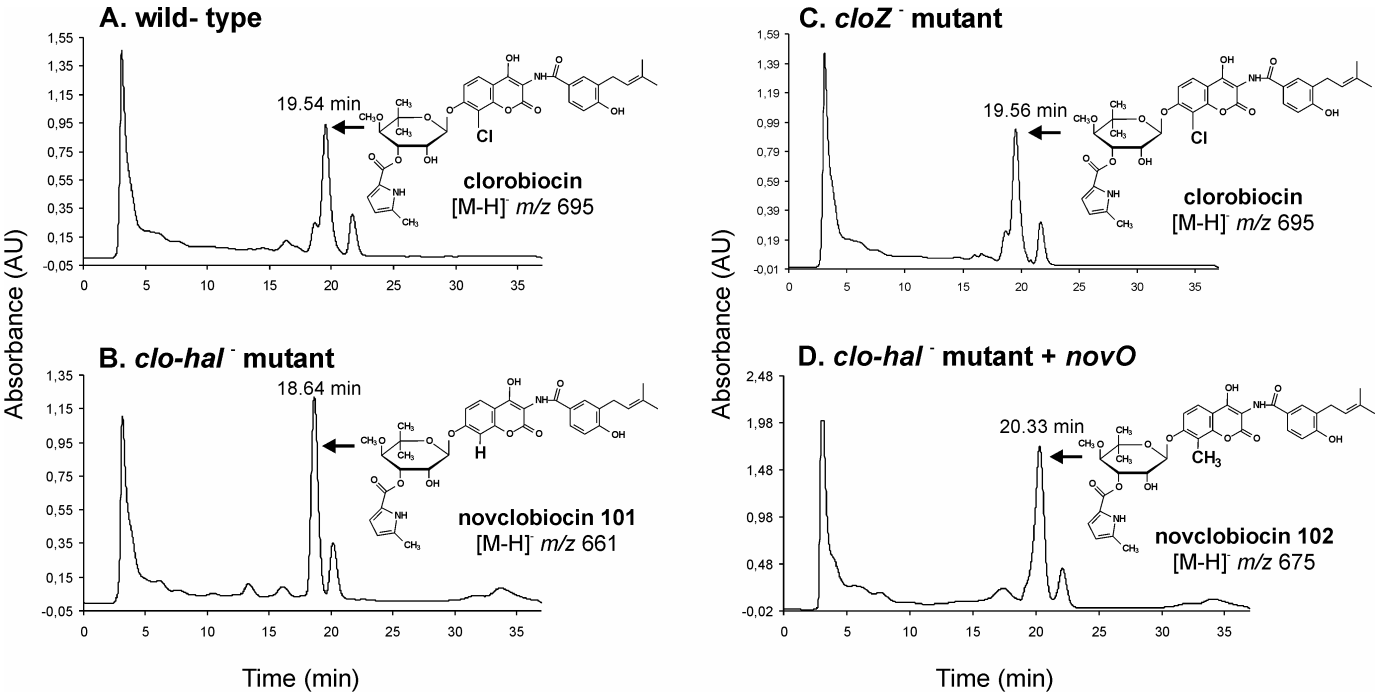
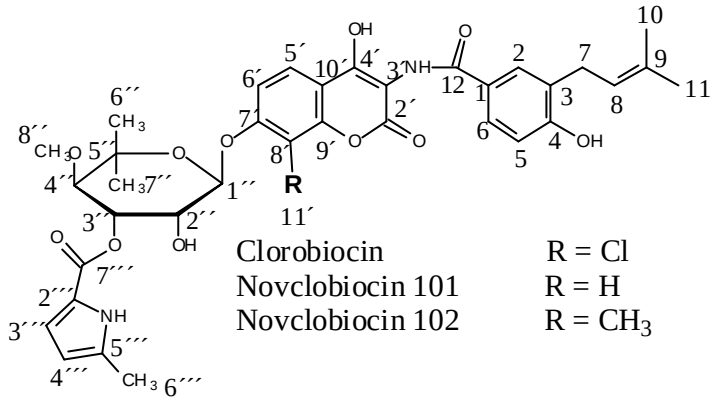


Fig. III.4: HPLC analysis of secondary metabolites.
(**A**) *S. roseochromogenes* var. *oscitans* wild-type. (**B**) *clo-hal*$^{-}$ mutant. (**C**) *cloZ*$^{-}$ mutant. (**D**) *clo-hal*$^{-}$ mutant transformed with the *novO* expression construct (pTLO5).

The new compound was named novclobiocin 101. The *clo-hal*$^{-}$ mutants produced 35-45 µg novclobiocin 101 per ml medium, which exceeded the clorobiocin production of the wild-type (25 µg/ml).

TABLE III.1: ^{1}H NMR data of clorobiocin, novclobiocin 101, and novclobiocin 102 in *d*$_4$-methanol



	Compound		
	Clorobiocin	Novclobiocin 101	Novclobiocin 102
Position	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)
2-H	7.76 d (2.5)	7.77 d (2.1)	7.76 br s
5-H	6.84 d (8.4)	6.84 d (8.4)	6.84 d (8.3)
6-H	7.72 dd (8.4; 2.5)	7.72 br d (8.0)	7.72 br d (8.5)
7-H_2	3.34 d (7.1)	3.34 d (7.0)	3.34 d (7.2)
8-H	5.35 br[a] t (7.1)	5.35 br t (7.3)	5.35 br t (7.2)
10-H_3	1.74 s	1.74 s	1.74 s
11-H_3	1.75 s	1.75 s	1.75 s
5´-H	7.90 d (9.2)	7.92 d (9.4)	7.82 d (8.8)
6´-H	7.33 d (9.2)	7.06[b]	7.24 d (8.7)
8´-H	-	7.06[b]	-
11´-CH_3	-	-	2.34 s
1´´-H	5.73 d (1.8)	5.63 d (2.0)	5.63 d (1.6)
2´´-H	4.34 t (2.7)	4.24 t (2.6)	4.29 br s
3´´-H	5.71 dd (10.3; 2.9)	5.59 dd (9.8; 3.2)	5.67 dd (9.9; 3.1)
4´´-H	3.72 d (10.3)	3.70 d (9.8)	3.71 d (9.9)
6´´-H_3	1.18 s	1.20 s	1.18 s
7´´-H_3	1.35 s	1.37 s	1.36 s
8´´-OCH_3	3.52 s	3.51 s	3.52 s
3´´´-H	6.90 d (3.6)	6.90 d (3.6)	6.90 d (3.6)
4´´´-H	5.94 br d (3.6)	5.94 d (3.6)	5.94 br d (3.4)
6´´´-H_3	2.29 s	2.29 s	2.29 s

δ is given in ppm. The solvent signal (3.30 ppm) was used as reference. Spectra were obtained at 400 MHz (clorobiocin and novclobiocin 102) or at 600 MHz (novclobiocin 101).

[a]br = broad signal.

[b]Complex, overlapping signals; *J* not determinable.

TABLE III.2: ^{13}C NMR data of clorobiocin, novclobiocin 101, and novclobiocin 102 in d_4-methanol at 100 MHz

	Compound		
	Clorobiocin	Novclobiocin 101	Novclobiocin 102
Position	δ (ppm)	δ (ppm)	δ (ppm)
1	124.2	124.3	124.3
2	130.9	130.8	130.8
3	129.9	129.8	129.8
4	161.0	160.9	160.9
5	115.6	115.6	115.6
6	128.5	128.5	128.4
7	29.2	29.2	29.2
8	123.2	123.2	123.2
9	133.8	133.8	133.8
10	17.9	17.9	17.9
11	26.0	26.0	26.0
12	170.0	169.8	169.7
2´	162.4[a]	163.3[a]	163.5[a]
3´	103.7	103.4	103.3
4´	157.8[a]	158.7[a]	158.8[a]
5´	123.9	126.4	123.2
6´	112.5	115.0	111.7
7´	161.8[a]	161.3[a]	158.8[a]
8´	110.7	104.0	114.9
9´	156.5	154.1	151.7
10´	113.3	112.4	112.4
11´	---	---	8.7
1´´	100.4	100.1	100.0
2´´	71.0	70.9	71.0
3´´	71.6	71.6	71.8
4´´	82.7	82.7	82.7
5´´	80.5	80.15	80.1
6´´	22.9	23.4	23.2
7´´	29.3	29.0	29.2
8´´	62.1	62.0	62.0
2´´´	121.8	121.8	121.8
3´´´	118.33	118.4	118.4
4´´´	109.8	109.8	109.8
5´´´	136.3	136.4	136.3
6´´´	12.9	12.9	12.9
7´´´	161.8[a]	161.9[a]	161.9[a]

The signal of the solvent (49.0 ppm) was used as reference. Assignments were made with the help of ^{1}H, ^{13}C COSY and of the litreature (Chadwick, 1990; Sasaki *et al.*, 2001).

[a]Signal of carbon 2´, 4´, 7´, and 7´´´ showed similar chemical shift (157.8-163.5 ppm), and their assignment may be interchanged.

In contrast to the *clo-hal*⁻ mutants, all *cloZ*⁻ mutants produced clorobiocin (Fig. III.4C). The production level was similar to that of the wild-type strain (25 µg/ml). The identity of the clorobiocin produced by the *cloZ*⁻ mutants was confirmed by negative-ion FAB MS (molecular ion $[M-H]^-$ at *m/z* 695) and ^1H NMR analysis (the spectrum obtained was identical to that of authentic clorobiocin, Table III.1).

Although the presence of *cloZ* within the clorobiocin cluster is suggestive of a function related to the formation of this antibiotic, the above experiments show that *cloZ* is clearly not essential for clorobiocin biosynthesis under the present culture conditions, and apparently not involved in the halogenation reaction.

III.1.4. Complementation of the *clo-hal*⁻ mutation

To prove that only the inactivation of *clo-hal* was responsible for the loss of the chlorine atom, the *clo-hal*⁻ mutant was complemented by expression of an intact copy of *clo-hal* under the control of the constitutive *ermE** promoter, using the expression vector pUWL201 (plasmid pAE-ha7, Table II.15). HPLC analysis showed a chromatogram identical to that of the wild-type, i.e. clorobiocin production could be fully restored by expression of *clo-hal*. The authenticity of the obtained peak was confirmed by negative-ion FAB MS analysis (mass [intensity]: 695 [100.0%], 696 [36.5%], 697 [30.2%], 698 [10.5%]).

III.2. Generation of a hybrid antibiotic by metabolic engineering

III.2.1. Expression of *novO* in the *clo-hal*⁻ mutant

novO encodes a putative methyltransferase (Steffensky *et al.*, 2000b). A very similar gene, *couO*, in the coumermycin A_1 biosynthetic cluster (Wang *et al.*, 2000) (Fig. I.3) has been experimentally confirmed to determine the methylation of C-8´ of the aminocoumarin ring (Li *et al.*, 2002). NovO shows 84% identity to CouO, and both proteins are of equal size (230 aa). The conserved motif III [LL(R/K)PGG(R/I/L)(L/I)(L/F/I/V)(I/L)] for S-adenosyl-methionine-dependent

methyltransferases (Kagan and Clarke, 1994) is found in both gene products from amino acid 135 to 144 (CouO: LVKPGGAILN, NovO: LAKPGGAVLN).

In order to produce a hybrid antibiotic, the putative methyltransferase gene *novO* was expressed in the *clo-hal*$^-$ mutant. For this purpose, plasmid pTLO5 – which was previously constructed by T. Luft by cloning *novO* into the replicative expression vector pUWL201 (Table II.14), placing it under the control of the constitutive *ermE** promoter – was introduced into the *clo-hal*$^-$ mutant by protoplast transformation. As control, the *clo-hal*$^-$ mutant was transformed with the empty vector pUWL201.

Culture extracts of the two strains were analysed by HPLC. In each case, three independent transformants were examined. While transformants containing the empty vector still produced novclobiocin 101 (data not shown), transformants containing the *novO* construct showed instead a new compound (Fig. III.4D) with a longer retention time than novclobiocin 101. Negative-ion FAB MS analysis of the isolated substance showed a molecular ion $[M-H]^-$ at *m/z* 675, corresponding to the addition of a methyl group to the molecule of novclobiocin 101. ^{1}H NMR (Table III.1) and ^{13}C NMR (Table III.2) analysis unequivocally confirmed that this substance was a clorobiocin derivative in which the chlorine atom at C-8´ of the aminocoumarin ring is replaced with a methyl group. In comparison to the ^{1}H NMR spectrum of clorobiocin (Table III.1), an additional singlet at 2.34 ppm corresponding to three protons was detected, which could be assigned to 8´-CH_3, based on literature data (Sasaki *et al.*, 2001). In comparison to novclobiocin 101, the signal of H-8´ had disappeared, and the signal of H-6´ was found as doublet at 7.24 ppm, as reported in the literature (Sasaki *et al.*, 2001). In the ^{13}C NMR spectrum (Table III.2) a new signal at 8.7 ppm was observed, which could be assigned to C-11´, using ^{1}H, ^{13}C correlated spectroscopy (COSY). This was also in accordance with the literature (Sasaki *et al.*, 2001).

The new compound was named novclobiocin 102. It was produced in an amount of 28-58 µg per ml medium (data of three independent transformants), exceeding the amount of clorobiocin produced in the wild-type (25 µg/ml).

In the wild-type and in all mutant strains, an additional peak with a slightly longer retention time than the respective main product was observed (Fig. III.4A-D). Negative-ion FAB MS analysis of these minor peaks showed the same molecular ion

as the corresponding main product. These compounds are likely to represent isomers of the main products, possibly carrying the pyrrole carboxylic acid moiety in position 2 instead of position 3 of the deoxysugar (Galm *et al.*, 2004a; Westrich *et al.*, 2003).

III.2.2. Antibiotic activity of clorobiocin and derivatives against *B. subtilis*

Authentic clorobiocin (Aventis), novclobiocin 101 (from the *clo-hal*$^-$ mutant) and novclobiocin 102 (from the *clo-hal*$^-$ mutant transformed with *novO*) were assayed for antibiotic activity against *Bacillus subtilis* (Fig. III.5). Clorobiocin showed the highest antibacterial activity, followed by novclobiocin 102 (approximately half as active as clorobiocin) and novclobiocin 101 (approximately eight times less active than clorobiocin). The results show that a substitution at C-8´ of the aminocoumarin unit strongly enhances the activity of the aminocoumarin antibiotics against this test organism. A chlorine atom at this position appears to be superior to a methyl group, although the difference is moderate.

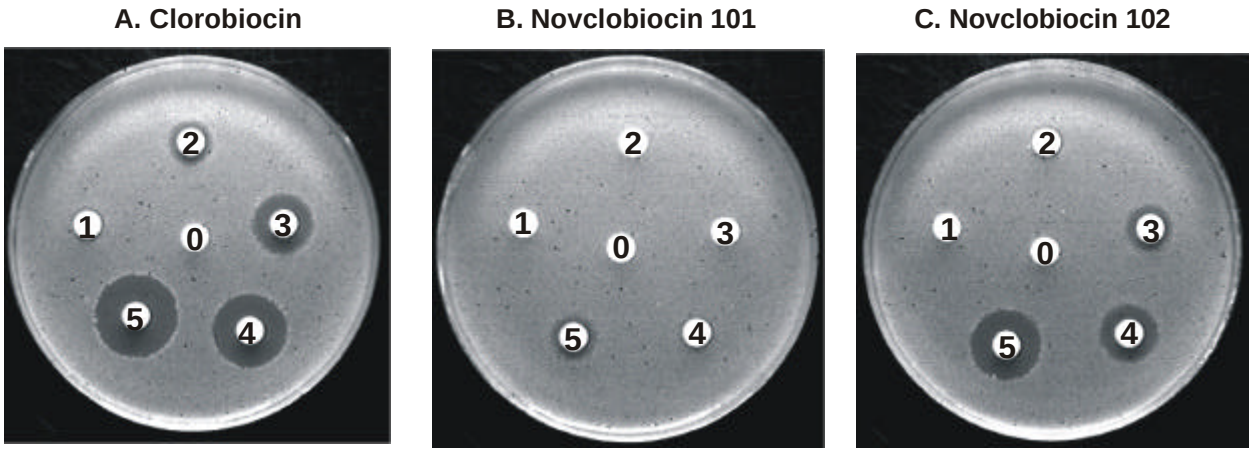


Fig. III.5: Antibacterial activity of clorobiocin and derivatives.
(**A**) clorobiocin, (**B**) novclobiocin 101, (**C**) novclobiocin 102. Bioassay with *Bacillus subtilis*. 0: methanol, 1-5: 0.5 µg, 1 µg, 2 µg, 4 µg, and 8 µg of the respective substance. For structures see Table III.1.

III.3. Heterologous expression of the novobiocin and clorobiocin biosynthetic gene clusters

III.3.1. Integration into the genome of host strains and analysis of secondary metabolites

Cosmids containing the entire biosynthetic gene clusters of novobiocin and clorobiocin have been obtained previously (Pojer *et al.*, 2002; Steffensky *et al.*, 2000b), employing the widely used cosmid vector, SuperCos1 (Stratagene), which contains an ampicillin (*bla*) and a neomycin/kanamycin (*neo*) resistance gene.

The *bla* gene within the SuperCos1 backbone of cosmids 10-9C and D1A8 (containing the novobiocin and clorobiocin cluster, respectively) was replaced with a cassette containing the integrase gene (*int*) and the attachment site (*attP*) of phage ϕC31, as well as a selectable marker (tetracycline resistance), using λ-Red-mediated recombination in *E. coli*. This one-step procedure yielded the desired modified cosmids termed nov-BG1 and clo-BG1, respectively (constructed by Dr. Bertolt Gust, John Innes Centre, Norwich, UK; presently at Prof. Dr. Heide's department).

Cosmids nov-BG1 and clo-BG1 were introduced into *S. coelicolor* M512 and into *S. lividans* TK24 by protoplast transformation. Selection for kanamycin resistance resulted in the desired integration mutants (approximately 10^3 mutants per microgram cosmid DNA for *S. coelicolor* and 10^5 mutants for *S. lividans*). Southern blot analysis showed that the entire cosmids had integrated site-specifically into the *attB* site of the chromosome in both *Streptomyces* strains (Figs. III.6C and III.7C; only results for *S. coelicolor* strains are shown).

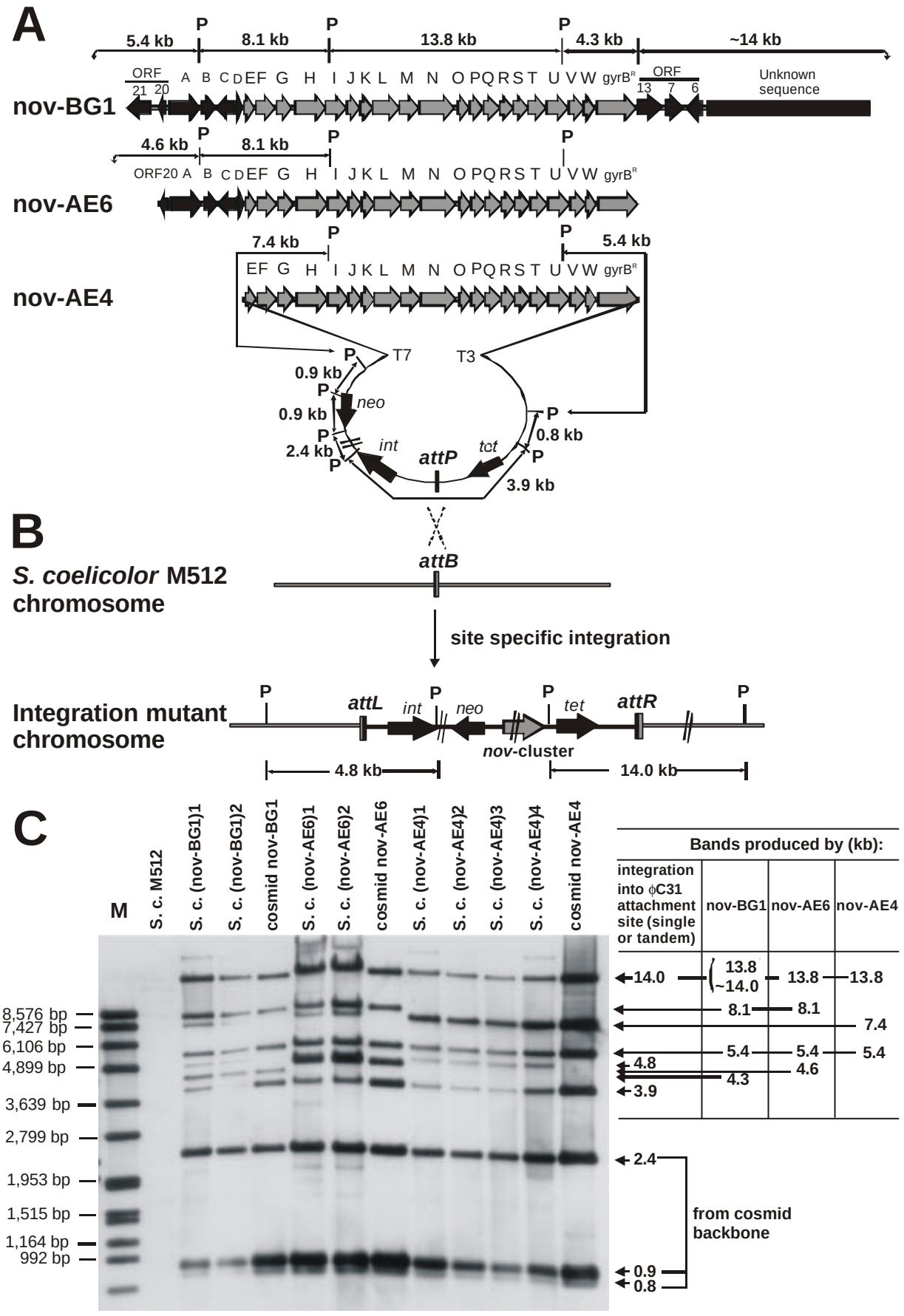


Fig. III.6: Cosmid constructs containing the novobiocin biosynthetic gene cluster and their integration into the *S. coelicolor* chromosome. See details on next page.

(**A**) Cosmid constructs nov-BG1, nov-AE6 and nov-AE4. P = *Pst*I restriction site. T3, T7 = T3 and T7 promoter of the SuperCos1 vector. *tet* = tetracycline resistance gene; *neo* = neomycin/kanamycin resistance gene; *int*, *attP* = integrase gene and attachment site of phage ϕC31. Fragment sizes resulting from digestion with *Pst*I are indicated. Cosmid backbone out of scale. (**B**) Schematic presentation of site specific integration of constructs nov-BG1, nov-AE6 or nov-AE4. The integrase, *int*, derived from *Streptomyces* phage ϕC31 catalyses integration via recombination between *attP* (from phage or vector) and *attB* (from *Streptomyces* genome) sites generating the hybrid sites *attL* and *attR* (Thorpe *et al.*, 2000). Junction fragments which prove specific integration into the ϕC31 attachment site of the *S. coelicolor* genome are indicated. Out of scale. (**C**) Southern blot analysis of *S. coelicolor* (= S. c.) M512 parental strain, of *S. coelicolor* M512 integration mutants harboring nov-BG1, nov-AE6 or nov-AE4, and of the respective cosmid constructs. M = DIG-labeled DNA Molecular Weight Marker VII (Roche). Genomic and cosmid DNA were digested with *Pst*I. The DIG-labeled cosmid nov-BG1 was used as probe. The 14.0 kb band resulting from site specific integration overlaps with the 13.8 kb band from the cosmid inserts.

Integration mutants and parental host strains were cultured in production media. The analysis of secondary metabolites by HPLC (Fig. III.8) showed that, in contrast to the untransformed host strains, the integration mutants accumulated novobiocin and clorobiocin, respectively. The identity of these substances was confirmed, after preparative isolation, by negative-ion FAB MS and ^{1}H NMR analysis (see Methods). *S. coelicolor* strains expressing the clorobiocin cluster additionally accumulated three clorobiocin analogs (Fig. III.8D), identical to those observed in the natural clorobiocin producer *S. roseochromogenes* (see Methods).

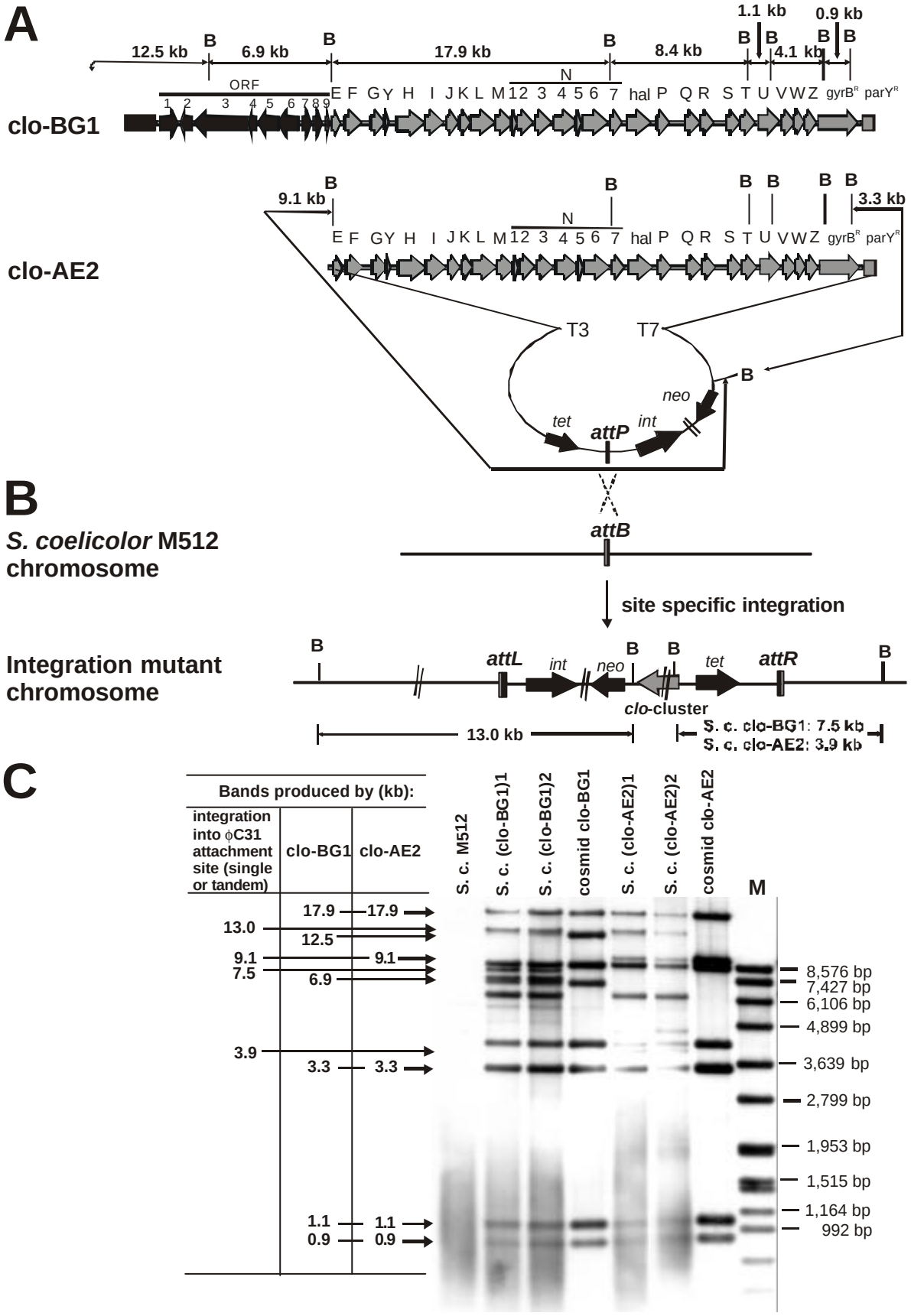


Fig. III.7: Cosmid constructs containing the clorobiocin biosynthetic gene cluster and their integration into the *S. coelicolor* chromosome. See details on next page.

(**A**) Cosmid constructs clo-BG1 and clo-AE2. B = *Bgl*II restriction site. T3, T7 = T3 and T7 promoter of the SuperCos1 vector. Fragment sizes resulting from digestion with *Bgl*II are indicated. Cosmid backbone out of scale. (**B**) Schematic presentation of site specific integration of constructs clo-BG1 and clo-AE2. See Fig. III.6 for details of the integration mechanism. (**C**) Southern blot analysis of *S. coelicolor* (= S. c.) M512 parental strain, of *S. coelicolor* M512 integration mutants harboring clo-BG1 or clo-AE2, and of the respective cosmid constructs. M = DIG-labeled DNA Molecular Weight Marker VII (Roche). Genomic and cosmid DNA were digested with *Bgl*II. The DIG-labeled cosmid clo-BG1 was used as probe.

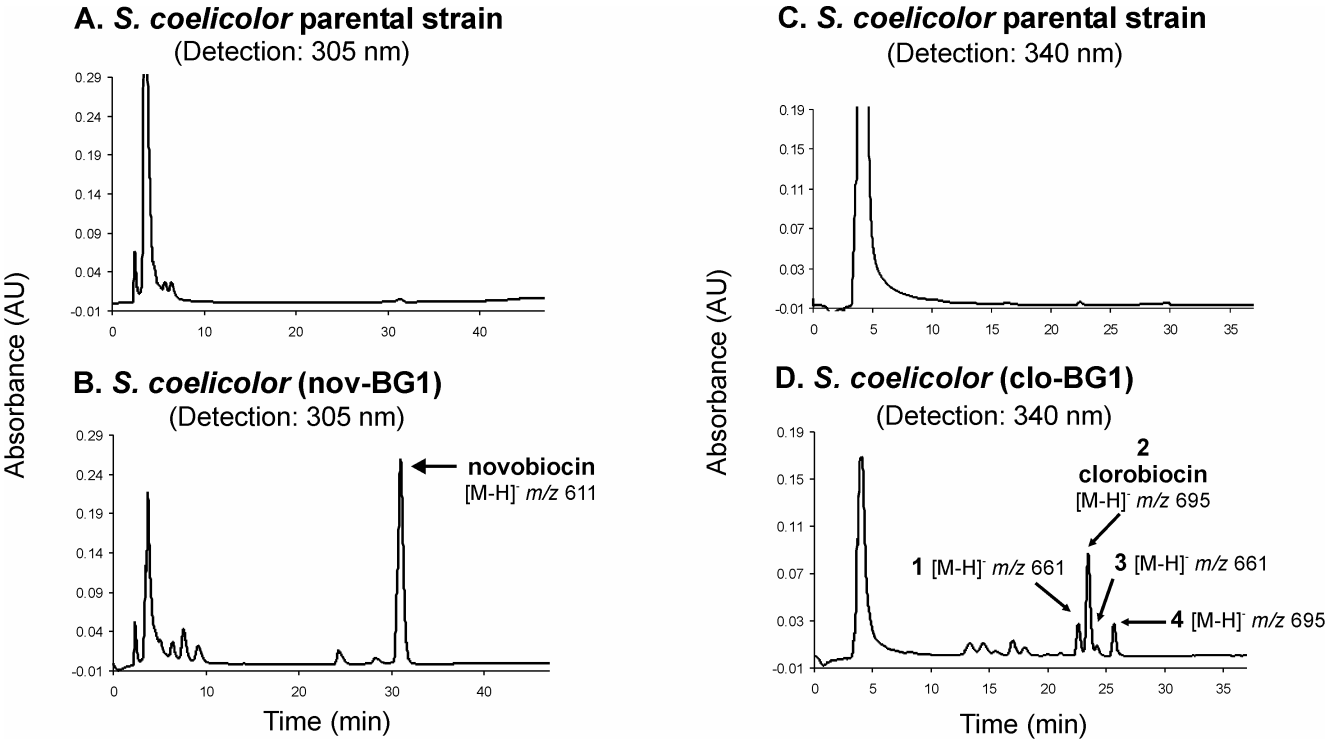


Fig. III.8: HPLC analysis of secondary metabolites.

(**A**) *S. coelicolor* M512 parental strain (detection at 305 nm). (**B**) *S. coelicolor* harboring nov-BG1 (**C**) *S. coelicolor* M512 parental strain (detection at 340 nm). (**D**) *S. coelicolor* harboring clo-BG1.

The productivity of the integration mutants of *S. coelicolor* was comparable to that of the original producers *S. spheroides* and *S. roseochromogenes* (Table III.3). *S. lividans* as host was much less productive. Therefore, *S. coelicolor* was used in the subsequent experiments.

TABLE III.3: Novobiocin and clorobiocin production by *S. coelicolor* and *S. lividans* strains

Strain	Novobiocin[a] (mg/l)	Clorobiocin[a, b] (mg/l)
Parental strains		
S. spheroides	35 (30-40)	---
S. roseochromogenes	---	25 (20-30)
S. coelicolor M512	---	---
S. lividans TK24	---	---
Strains carrying full length cosmids		
S. coelicolor (nov-BG1)	31 (20-42)	---
S. lividans (nov-BG1)	<1	---
S. coelicolor (clo-BG1)	---	26 (18-34)
S. lividans (clo-BG1)	---	5 (4-6)
Strains carrying shortened cosmids		
S. coelicolor (nov-AE6)	24 (23-25)	---
S. coelicolor (nov-AE4)	1.5 (0.8-2.2)	---
S. coelicolor (clo-AE2)	---	14 (8-19)

[a]mean values (range) from at least two independent experiments.
[b]total amount of clorobiocin and the three major analogs (see Fig. III.8D).

III.3.2. Removal of non-essential DNA regions from the cosmid inserts

III.3.2.1. Orientation of inserts in cosmids nov-BG1 and clo-BG1

Removal of putative non-essential DNA regions in the SuperCos1-derived cosmids nov-BG1 and clo-BG1 was carried out using gene deletion via PCR targeting (Gust *et al.*, 2003). For this purpose, the sequence of the inserts' extremities or the orientation of the inserts must be known. Therefore, cosmids nov-BG1 and clo-BG1 were short-sequenced using T3 and/or T7 primers. The sequencing results allowed the following conclusions about the orientation of the inserts:

- nov-BG1: (T7) *ORF21* incomplete (682 bp of 1 485 bp, start at position 4 927 of AY227005), *ORF20*, *novA* to *ORF6*, unknown sequence (T3). See Fig. III.6.
- clo-BG1: (T3) unknown sequence (the sequence data with T3 primer showed no homology to the known sequence of the clorobiocin cluster), *ORF1* to *gyrB*^{R}, *parY*^{R} incomplete (123 bp of 2.1 kb, till position 2 238 in AY136281) (T7)

(the sequence data with T7 were identical to the reverse-complementary sequence of AY136281, position 2 238 – 1 968). See Fig. III.7.

III.3.2.2. Removal of non-essential DNA regions

The apramycin resistance cassette from the previously described plasmid pIJ773 of the REDIRECT© technology kit for PCR targeting (Gust *et al.*, 2003) is flanked by FLP recognition targets (FRT). After gene replacement, this cassette can be conveniently removed by action of FLP recombinase, leading to the excision of the DNA region in between the FRT sites and leaving an 81 bp "scar" sequence. However, the presence of this scar sequence makes further knock-outs in the same cosmid difficult, because it represents a functional FRT site as well as a target for λ-Red-mediated recombination.

To overcome this problem, Ute Galm (Pharmazeutische Biologie, Universität Tübingen) has generated an apramycin resistance cassette by PCR using primers with either an *Xba*I or a *Spe*I recognition site between the apramycin resistance marker and the 39 bp flanking sequence for λ-Red-mediated recombination, leading to plasmid pUG019 (Table II.14). *Xba*I and *Spe*I sites are rare in the GC-rich *Streptomyces* genome. After gene replacement, this cassette can be removed by digestion with *Xba*I and *Spe*I and religation of the resulting compatible ends (Fig. III.9). This procedure leaves a minimal *in frame* "scar" of 18 nucleotides, which does not interfere with further gene deletions or replacements.

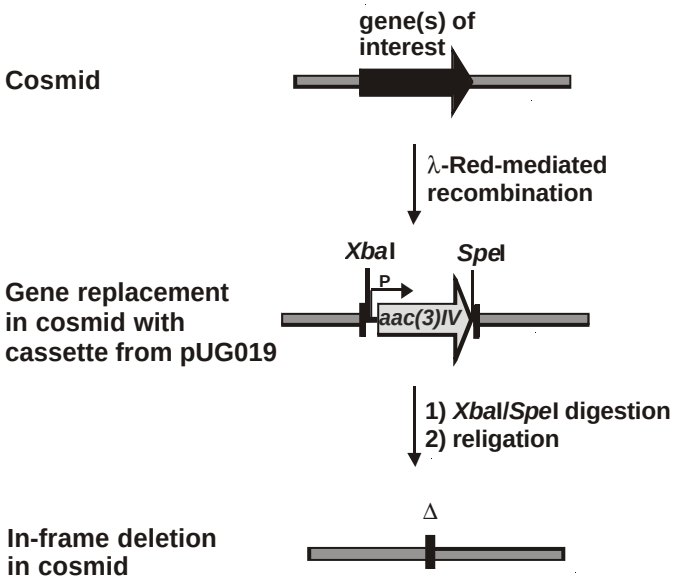


Figure III.9 Gene deletion using the apramycin resistance cassette from pUG019 containing flanking *Xba*I and *Spe*I recognition sites. *aac(3)IV* = apramycin resistance gene; P = promoter of the apramycin resistance gene.

The biosynthetic gene cluster of clorobiocin is flanked on one side by the two resistance genes *gyrB*^{R} and *parY*^{R} (Fig. I.3). Of the latter 2.1 kb gene, only 123 bp are contained in cosmid clo-BG1 (Fig. III.7A, right-hand side of the insert). On the opposite end, the gene *cloE* may represent the border of the cluster (Eustáquio *et al.*, 2003b; Schmutz *et al.*, 2003a) (Fig. I.3 and Fig. III.7A).

In order to prove that the genes upstream of *cloE* are indeed non-essential for clorobiocin biosynthesis, these were deleted from cosmid clo-BG1 using the cassette from pUG019 (see also Methods). The insert of the resulting cosmid, clo-AE2, starts 148 bp upstream of the start codon of *cloE* (Fig. III.7).

This cosmid was transformed into *S. coelicolor* M512, and integration mutants were selected. Site specific integration of the entire cosmid was confirmed by Southern blot analysis (Fig. III.7C, lanes 5 and 6). When these mutants were cultured in production medium, HPLC analysis clearly showed that clorobiocin was still produced in significant amounts (Table III.3), proving that the 34 kb sequence from *cloE* to *gyrB*^{R} contains all genes required for clorobiocin biosynthesis.

A similar experiment was performed with the novobiocin cluster, contained in cosmid nov-BG1. It has been suggested previously that *novE* and *gyrB*^{R} (Fig. III.6A) may delineate the left and right border of this cluster, respectively (Eustáquio *et al.*, 2003b; Schmutz *et al.*, 2003a). However, *novA* which is located outside of this region shows sequence similarity to ABC transporters and has been suggested to be involved in novobiocin transport (Méndez and Salas, 2001; Schmutz *et al.*, 2003a). Therefore, two different shortened cosmids were produced: nov-AE4, containing only the genes from *novE* to *gyrB*^{R}; and nov-AE6, containing additionally the genes *novABCD* and *ORF20* (Fig. III.6A). *ORF20* shows sequence similarity to transcriptional regulators of the MarR-type (MarR is a transcriptional repressor of the mar, multiple antibiotic resistance, operon of *E. coli*), and may encode a regulator of *novA*, as suggested by sequence comparison with database entries.

Cosmids nov-AE4 and nov-AE6 were transformed into *S. coelicolor* M512, and site specific integration of the entire cosmids was confirmed by Southern blot analysis (Fig. III.6). Cultivation in production medium and HPLC analysis revealed that integration mutants containing the larger cosmid, i.e. nov-AE6, produced still two thirds of the novobiocin amount found in the parental strain *S. spheroides*. In contrast,

mutants containing the smaller cosmid nov-AE4 produced only 6% of that amount (Table III.3). This proves that the DNA region from *novE* to *gyrB*^{R} contains all genes which are essential for novobiocin biosynthesis, but also that the DNA region from *ORF20* to *novD* contains elements which are required for a high productivity of novobiocin.

III.4. Production of 8´-halogenated and 8´-unsubstituted novobiocin derivatives in genetically engineered *S. coelicolor* strains

III.4.1. Heterologous expression of a modified novobiocin cluster (*novO*$^{-}$)

Since heterologous expression of the intact novobiocin biosynthetic gene cluster was successful, the generation of a modified (8´-unsubstituted) antibiotic by inactivation of the methyltransferase gene *novO* in cosmid nov-BG1 and introduction of this modified cosmid into the genome of *S. coelicolor* was attempted.

Therefore, *novO* was replaced by the apramycin resistance cassette from pUG019 (Fig. III.9) via λ-Red-mediated recombination (Gust *et al.*, 2003). The cassette was then removed by digestion with *Xba*I and *Spe*I and religation. As explained above, this procedure leaves a minimal *in frame* “scar” of 18 nucleotides inside the targeted sequence. The modified cosmid (named nov-AE8) was then introduced into *S. coelicolor* by transformation. Southern blot analysis confirmed the integration into the genome, and the deletion of *novO* was clearly shown by the shortening of the relevant *Bgl*II restriction fragment (Fig. III.10).

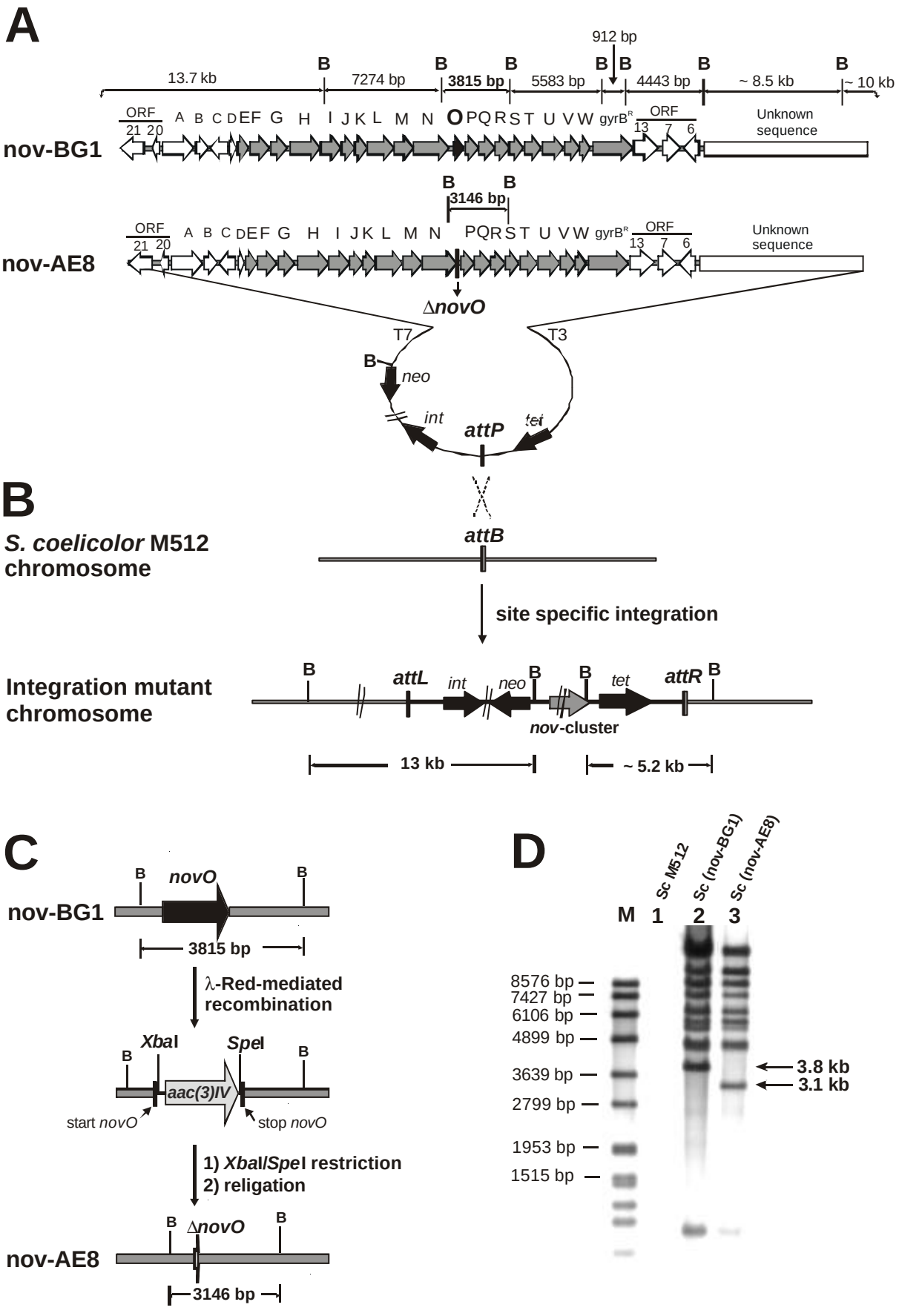


Fig. III.10: Inactivation of *novO*. See next page for details.

(**A**) Schematic presentation of cosmids nov-BG1, containing the intact novobiocin biosynthetic gene cluster, and nov-AE8 in which *novO* was deleted. The novobiocin cluster is presented in grey, the flanking regions in white, and *novO* in black. B = *Bgl*II restriction site. Cosmid backbone out of scale. (**B**) Schematic presentation of site specific integration. Junction fragments which prove specific integration into the ϕC31 attachment site of the *S. coelicolor* genome are indicated. Out of scale. (**C**) Schematic presentation of the *novO* inactivation. *novO* (693 bp) was first replaced by an apramycin resistance (*aac(3)IV*) cassette. Afterwards, the cassette was excised by digestion with *Xba*I and *Spe*I and religated, leaving an *in frame* "scar" of 18 nucleotides between the start and stop codons of *novO*. Out of scale. (**D**) Southern blot analysis of *S. coelicolor* M512 (lane 1), and M512-derived integration mutants bearing cosmid nov-BG1 (lane 2), or the *novO*$^-$ cosmid nov-AE8 (lane 3). M = DIG-labeled DNA Molecular Weight Marker VII (Roche). Genomic DNA was digested with *Bgl*II. The DIG-labeled cosmid nov-BG1 was used as probe. The *novO* inactivation is confirmed by the 3.1 kb band (lane 3) instead of a 3.8 kb band (lane 2); otherwise the band pattern should be identical. See Fig. III.6 for further details.

The analysis of secondary metabolites by HPLC (Fig. III.11C) showed complete absence of novobiocin. Instead, the novobiocin derivative novclobiocin 117 accumulated, carrying a hydrogen instead of a methyl group at C-8´. The structure was confirmed by negative-ion FAB MS (molecular ion $[M-H]^-$ at *m/z* 597 corresponding to the loss of a methyl group in comparison to novobiocin) and by ^1H NMR analysis (Table III.4) of the isolated compound, which clearly showed the absence of the signal for 8´-CH_3. These spectroscopic data are in accordance with the litreature (Sasaki *et al.*, 2001; Xu *et al.*, 2004). The *novO* mutant also produced a minor product (= novclobiocic acid 106) with shorter retention time than novclobiocin 117. This compound lacked the entire deoxysugar moiety, as shown by a molecular ion $[M-H]^-$ at *m/z* 380 in MS analysis and by the absence of sugar protons in ^1H NMR analysis (Table III.4). In addition, a third compound was detected with a slightly higher retention time than novclobiocin 117. This substance showed a molecular ion $[M-H]^-$ at *m/z* 597, i.e. identical to novclobiocin 117. As explained below, this compound is likely to represent an isomer of novclobiocin 117, carrying the carbamoyl moiety in position 2 instead of 3 of the deoxysugar. The production of such 2´´-acylated isomers has previously been observed in strains of *S.*

roseochromogenes, the clorobiocin producer (Galm *et al.*, 2004a; Westrich *et al.*, 2003).

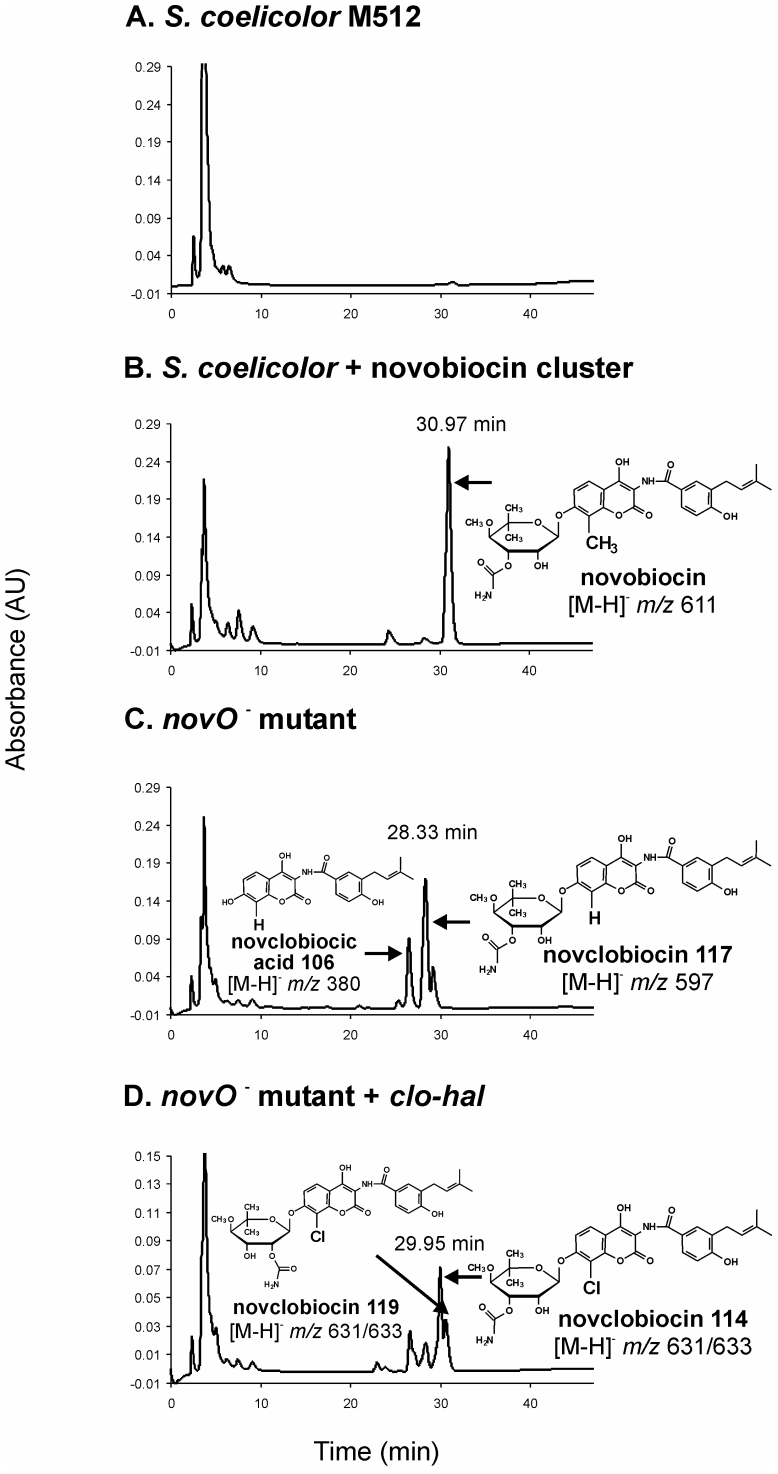
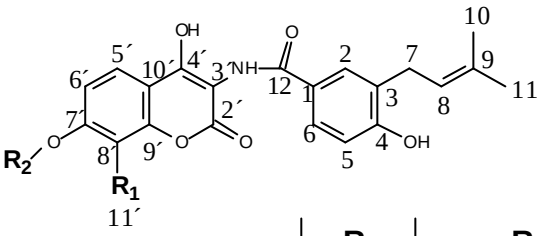
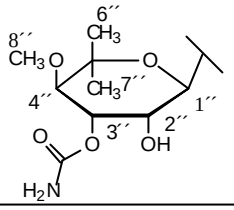
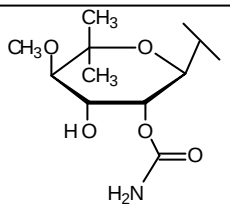


Fig. III.11: HPLC analysis of secondary metabolites.

(**A**) *S. coelicolor* M512 parental strain. (**B**) *S. coelicolor* (nov-BG1), carrying the novobiocin biosynthetic gene cluster. (**C**) *S. coelicolor* (nov-AE8), *novO*$^{-}$ mutant. (**D**) *S. coelicolor* (nov-AE8)/pAE-ha7, *novO*$^{-}$ mutant transformed with the *clo-hal* expression construct (pAE-ha7). Detection at 305 nm.

TABLE III.4: ^{1}H NMR data of novobiocin, novclobiocic acid 106, novclobiocin 117, novclobiocin 114, and novclobiocin 119 in *d_4*-methanol.



	R_1	R_2
Novobiocin	CH_3	
Novclobiocin 117	H	
Novclobiocin 114	Cl	
Novclobiocic acid 106	H	H
Novclobiocin 119	Cl	

	Compound				
	Novobiocin	Novclobiocic acid 106	Novclobiocin 117	Novclobiocin 114	Novclobiocin 119
Position	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)	δ, Multiplicity (*J*/Hz)
2-H	7.75 br[a] s	7.76 br s	7.76 br s	7.75 br s	7.76 br s
5-H	6.84 d (8.3)	6.83 d (8.4)	6.82 d (8.2)	6.84 d (8.3)	6.82 d (8.3)
6-H	7.72 br d (8.3)	7.72 br d (8.4)	7.72 br d (8.2)	7.72 br d (8.3)	7.72 br d (8.3)
7-H_2	3.34 d (7.3)	3.34 d (7.5)	3.33[c]	3.33 d (7.1)	3.33 d (6.9)
8-H	5.34 mt (7)[b]	5.35 br t (7.5)	5.35 mt (7)[d]	5.35 mt (7)[b]	5.35 br t (7)[d]
10-H_3	1.74 s	1.74 br s	1.74 br s	1.74 s	1.74 br s
11-H_3	1.75 s	1.74 br s	1.74 br s	1.75 s	1.74 br s
5´-H	7.81 d (8.9)	7.81 br d (8.0)	7.91 br d (7.5)	7.88 d (8.9)	7.88 d (8.8)
6´-H	7.23 br d (8.9)	6.78 br d (8.3)	7.00 br d (7.6)[b]	7.29 d (8.9)	7.26 d (9.1)
8´-H	-	6.68 s	7.00 s	-	-
11´-H_3	2.31 s	-	-	-	-
1´´-H	5.57 br s	-	5.55 d (2.3)	5.67 br s	5.74 br s
2´´-H	4.23 t[c]	-	4.18 t (2.8)	4.28 t[c]	5.10 t[c]
3´´-H	5.31 dd (3; 10)[b]	-	5.25 dd (3.1; 9.6)	5.36 dd (2.8; 10.0)[b]	4.35 dd (3.4; 9.9)
4´´-H	3.57 d (9.9)	-	3.54 d (10.2)	3.58 d (10.0)	3.40 d (9.9)
6´´-H_3	1.14 s	-	1.17 s	1.15 s	1.14 s
7´´-H_3	1.34 s	-	1.35 s	1.32 s	1.32 s
8´´-H_3	3.55 s	-	3.53 s	3.55 s	3.60 s

δ is given in ppm. The solvent signal (3.30 ppm) was used as reference. Spectra were obtained at 400 MHz.

[a]br = broad signal.

[b]Complex, overlapping signals; *J* not exactly determinable.

[c]*J* not determinable.

[d]Complex signal; *J* not exactly determinable.

The *S. coelicolor* strain harboring the *novO*-defective cluster produced 30 mg/l novclobiocin 117 and 10 mg/l novclobiocic acid 106. Its productivity was therefore similar to that of the *S. coelicolor* strains carrying the intact novobiocin biosynthetic gene cluster (31 mg/l on average).

III.4.2. Complementation of the *novO* mutation

To prove that only the inactivation of *novO* was responsible for the loss of the methyl group, we complemented the mutant by expressing an intact copy of *novO*. For this purpose, plasmid pTLO5 was used, a derivative of the replicative vector pUWL201, in which *novO* was placed under the control of the constitutive *ermE** promoter (Table II.14). After pTLO5 was used to transform the *novO* mutant, HPLC analysis showed that the novobiocin production was restored to about 80% of the productivity observed before *novO* inactivation (data not shown). The identity of novobiocin was confirmed by negative-ion FAB MS analysis (molecular ion $[M-H]^-$ at *m/z* 611).

III.4.3. Production of the hybrid antibiotic novclobiocin 114

In order to obtain the desired novobiocin analog carrying a chlorine atom instead of a methyl group at C-8´, the halogenase gene *clo-hal* from the clorobiocin biosynthetic gene cluster was expressed in the *S. coelicolor* strain harboring the *novO*-defective cluster, using the replicative plasmid pAE-ha7 in which *clo-hal* was under the control of the constitutive *ermE** promoter (Table II.15). As shown by HPLC (Fig. III.11D), the resulting *clo-hal* transformants produced a substance which was absent from transformants carrying the empty replicative vector pUWL201 (data not shown). Negative-ion FAB MS of the isolated compound showed a molecular ion $[M-H]^-$ at *m/z* 631, corresponding to the substitution of a hydrogen by a chlorine atom in the molecule of novclobiocin 117. The typical isotopic pattern caused by the chlorine isotopes ^{35}Cl and ^{37}Cl was clearly visible (mass [intensity]: 631 [100.0%], 632 [30.6%], 633 [31.9%], 634 [11.2%]). ^1H NMR analysis (Table III.4) unequivocally confirmed that the chlorine atom was attached at C-8´ of the aminocoumarin core: the signal at 7.00 ppm corresponding to the two protons H-6´ and H-8´ of novclobiocin 117 had disappeared, and instead a signal at 7.27 ppm (doublet, 1H) was observed, which could be assigned to H-6´. In comparison, the H-6´ signal of novobiocin was seen as doublet (1H) at 7.23 ppm (Table III.4). As observed previously (Table III.1 and (Xu *et*

al., 2004)), the chlorine atom at C-8´ in clorobiocin causes a slightly different magnetic shift of the H-6´ signal than a methyl group at the same position. The accumulated substance was therefore unequivocally identified as novclobiocin 114 (Fig. III.11D). The three independent *clo-hal* transformants tested produced on average 14 mg novclobiocin 114 per litre medium. The minor peak with slightly longer retention time represents the isomer of novclobiocin 114, i.e. novclobiocin 119, which carries the carbamoyl moiety in position 2 instead of 3 of the deoxysugar. In the negative-ion FAB MS, it showed the same molecular ion $[M-H]^-$ at *m/z* 631 and similar isotopic pattern (mass [intensity]: 631 [100.0%], 632 [34.7%], 633 [32.1%], 634 [12.5%]) as novclobiocin 114. Furthermore, in the ^{1}H NMR analysis (Table III.4), the signal of H-2´´ was seen at 5.10 ppm instead of 4.28 ppm and the signal of H-3´´ at 4.35 instead of 5.36 ppm. Therefore, the chemical shift of the sugar protons demonstrates the presence of the carbamoyl moiety in position 2 instead of 3, in accordance with published data on novobiocin and isonovobiocin (Crow *et al.*, 1999).

The minor peaks with shorter retention times than novclobiocin 114 (Fig. III.11D) represent remaining non-halogenated compounds, i.e. novclobiocin 117 and novclobiocic acid 106.

III.4.4. Antibacterial activity of novobiocin and derivatives

The obtained novclobiocins (i.e. novclobiocin 114, 119, 117, and novclobiocic acid 106; see Table III.4 for structures) were assayed for antibiotic activity against *Bacillus subtilis* in comparison to authentic novobiocin (Fig. III.12). In accordance with results obtained previously (see III.2.2 and (Galm *et al.*, 2004b; Xu *et al.*, 2004)), substitution at C-8´ with either $-CH_3$ or –Cl was important for biological activity: novclobiocin 117 (8´-H) showed only 4% of the activity of novobiocin, while novclobiocin 114 (8´-Cl) was half as active as novobiocin (8´-CH_3). Further investigations of the structure-activity relationships of these compounds are in progress.

As reported for clorobiocin derivatives (Galm *et al.*, 2004b), the transfer of the acyl group from 3´´-OH to 2´´-OH resulted in a reduction but not in a complete loss of activity: novclobiocin 119 still showed approximately 20% of the activity of novclobiocin 114. In contrast, the removal of the deoxysugar moiety and the 8´-substituent (= novclobiocic acid 106) resulted in 99% loss of activity.

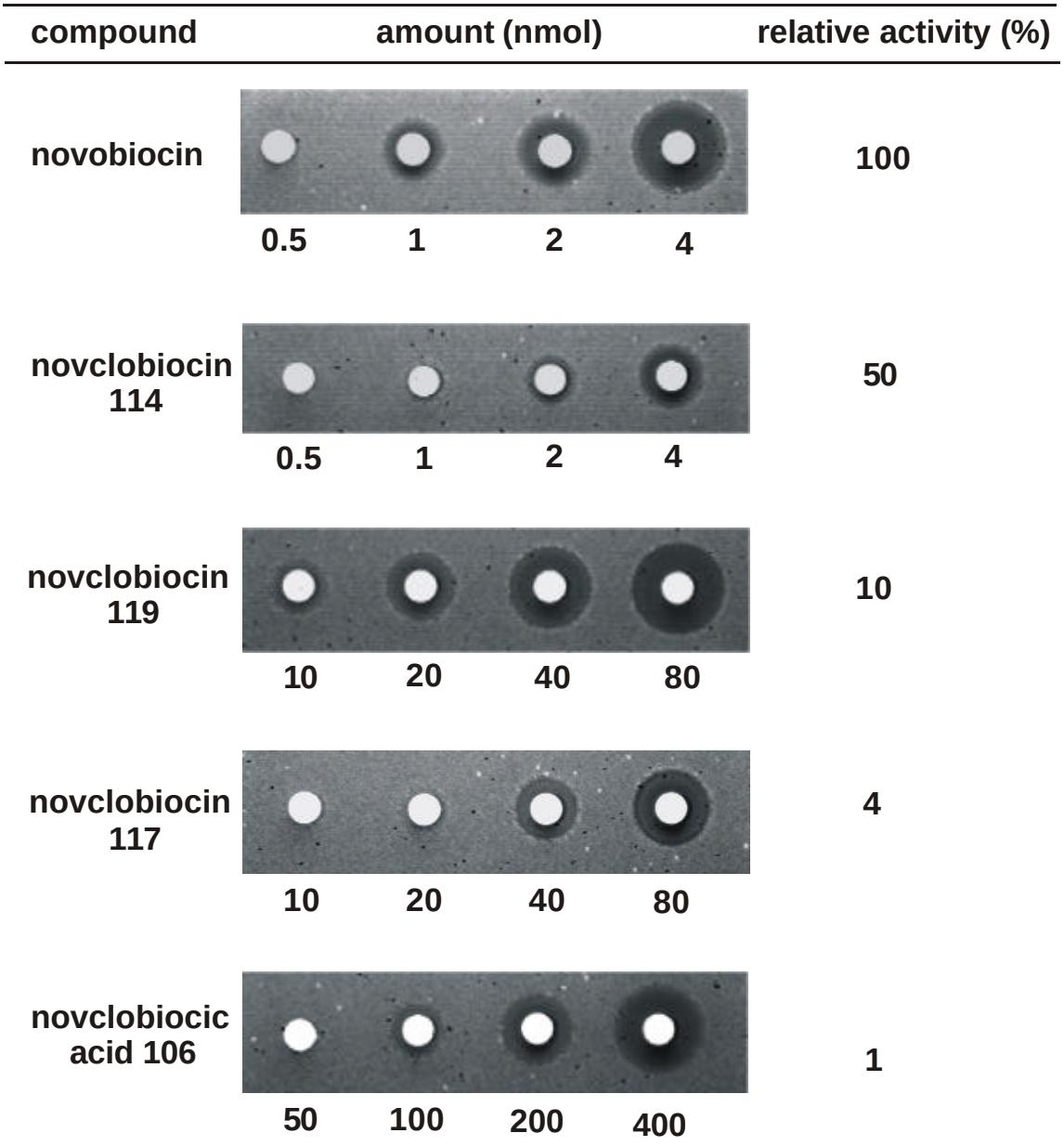


Fig. III.12: Antibacterial activity of novobiocin and derivatives.
Bioassay against *Bacillus subtilis*. Activities are expressed relative to novobiocin (100%). For structures see Table III.4.

III.5. Are the halogenases of clorobiocin and balhimycin biosynthesis interchangeable?

The glycopeptide antibiotic balhimycin (Fig. III.13) and the aminocoumarin antibiotic clorobiocin both contain 3-chloro-*b*-hydroxy-tyrosyl-derived moieties. In both compounds, the biosynthesis of these moieties starts from L-tyrosine, which forms a

thioester bond with an activating enzyme, i.e. BpsD or CloH, respectively. Subsequently, the cytochrome P_{450} enzymes OxyD or CloI, respectively, introduce the *b*-hydroxy group (Fig. III.13) (Chen and Walsh, 2001; Süssmuth and Wohlleben, 2004). The predicted protein sequences of CloH and CloI show clear sequence similarity to BpsD and OxyD. The introduction of the chlorine atom into position 3 of the aromatic nucleus of the *b*-hydroxy-tyrosyl-derived moieties is catalyzed by enzymes with sequence similarity to $FADH_2$-dependent halogenases (van Pée, 2001). The predicted amino acid sequence of BhaA of the balhimycin cluster (Puk *et al.*, 2002) shows 36% identity to Clo-Hal of the clorobiocin cluster (Pojer *et al.*, 2002). In the biosynthesis of both antibiotics, it is unknown which exact intermediate serves as substrate for the halogenation.

In order to verify whether *bhaA* could functionally replace *clo-hal*, we placed *bhaA* under the control of the constitutive *ermE** promoter using the same expression vector, pUWL201, as employed for the experiments with *clo-hal* described above. The resulting plasmid was termed pAE-B3. pAE-B3 was constructed by cloning a *Bam*HI-*Xba*I fragment of plasmid pSETbhaA, containing the *bhaA* gene and its natural ribosome binding site (Puk *et al.*, 2002) into the same sites of pUWL201. pSETbhaA has been successfully used previously for complementation of a *bhaA*$^-$ mutant of *Amycolatopsis balhimycina* (Puk *et al.*, 2002).

pAE-B3 was introduced by protoplast transformation into the *S. coelicolor* strain containing the *novO* defective novobiocin cluster. In three independent transformants, the presence of the intact plasmid was confirmed by plasmid isolation and restriction analysis. However, HPLC analysis of the three transformants showed no changes in comparison to the untransformed control, or to controls transformed with the empty vector pUWL201. The detected compounds still showed the same molecular ions in the negative-ion FAB MS analysis as those seen in the *S. coelicolor* strain with the *novO*$^-$ cluster ($[M-H]^-$ at *m/z* 597, corresponding to novclobiocin 117; and $[M-H]^-$ at *m/z* 380, corresponding to novclobiocic acid 106). Therefore, no chlorinated products could be observed after expression of *bhaA* in the *novO*$^-$ mutant.

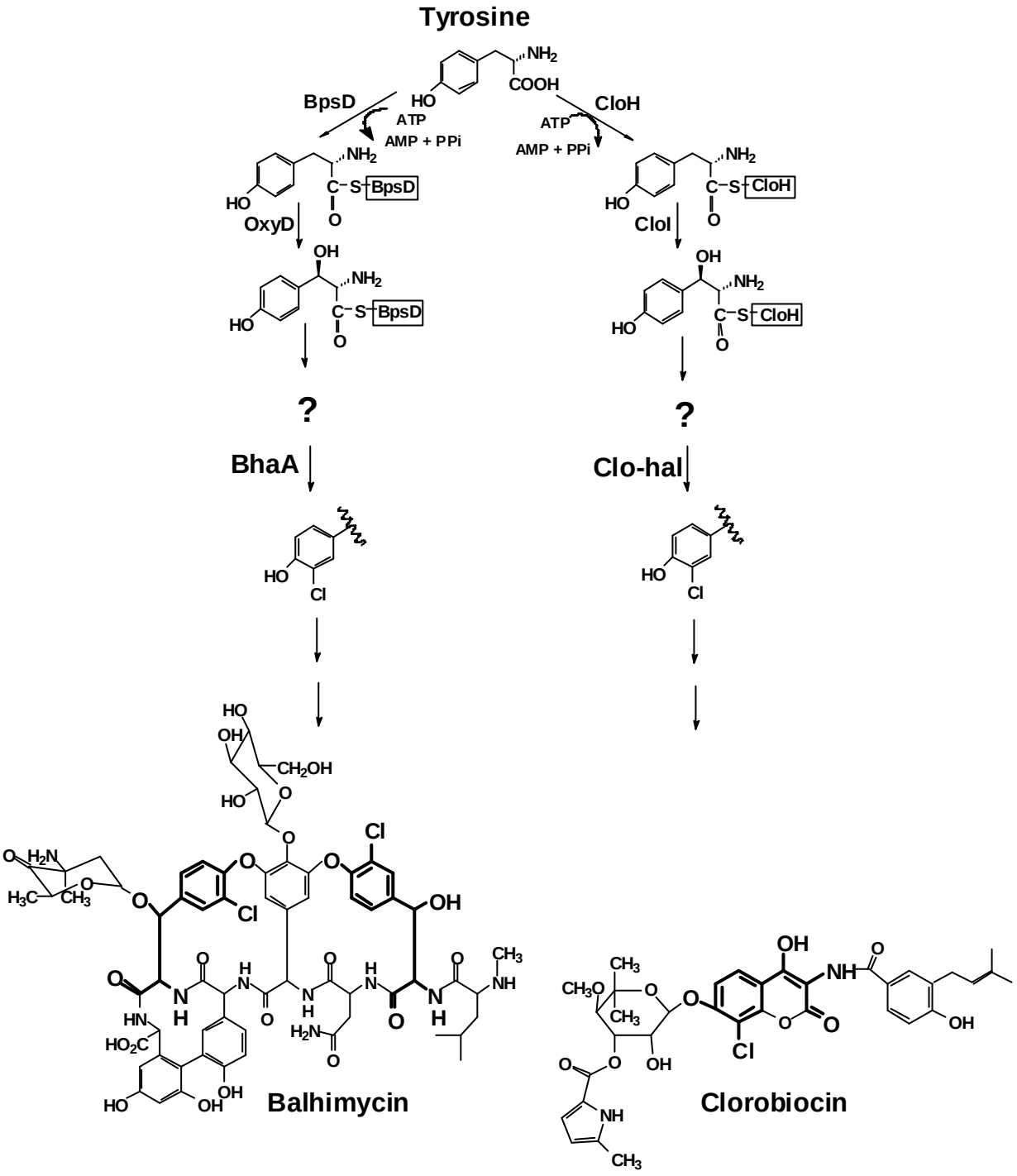


Fig. III.13: Biosynthesis of the 3-chloro-*b*-hydroxy-tyrosyl moieties of clorobiocin and balhimycin.

In both pathways, it is unknown at which exact step during the biosynthesis the halogenation takes place (see text). The 3-chloro-*b*-hydroxy-tyrosyl-derived moieties of clorobiocin and balhimycin are emphasized by larger font size.

In order to confirm this result, the *bhaA* expression plasmid pAE-B3 was also introduced into the *clo-hal*$^-$ mutant of the clorobiocin producer *S. roseochromogenes* (see III.1.2). Again, *bhaA* was unable to complement the mutant. HPLC analysis of three independent transformants, as well as FAB MS analysis of the isolated compounds, showed only the same metabolites as detected in the *clo-hal*$^-$ mutants prior to transformation. The dominant product was a clorobiocin derivative with a hydrogen instead of a chlorine atom at C-8´ ([M-H]$^-$ at *m/z* 661). If, however, the *clo-hal*$^-$ mutant was transformed with plasmid pAE-ha7, which contains *clo-hal* rather than *bhaA* and is otherwise identical to pAE-B3, clorobiocin production was readily restored. The resulting chromatogram corresponded to that of the wild-type *S. roseochromogenes*, and the identity of clorobiocin was confirmed by negative-ion FAB MS analysis (mass [intensity]: 695 [100.0%, [M-H]$^-$], 696 [36.5%], 697 [30.2%], 698 [10.5%]). These experiments provide clear evidence that *clo-hal* cannot be functionally replaced by *bhaA*.

III.6. Regulation of novobiocin production

III.6.1. NovG, a positive regulator of novobiocin biosynthesis

III.6.1.1. Sequence analysis of *novG*

novG codes for a protein which comprises 318 amino acids and shows 41% identity on the amino acid level to StrR, a pathway-specific transcriptional activator of streptomycin biosynthesis in *Streptomyces griseus* and *S. glaucescens* (Retzlaff and Distler, 1995; Thamm and Distler, 1997).

The predicted amino acid sequence of NovG shows a putative helix-turn-helix (HTH) motif in the central region of the protein, which is typical for a family of bacterial and phage DNA-binding proteins (Pabo and Sauer, 1992). This motif is also found in StrR (Retzlaff and Distler, 1995; Thamm and Distler, 1997). Prediction of the secondary structure of NovG using the PHD (Profile network prediction Heidelberg) method confirmed the presence of an HTH motif (Fig. III.14).

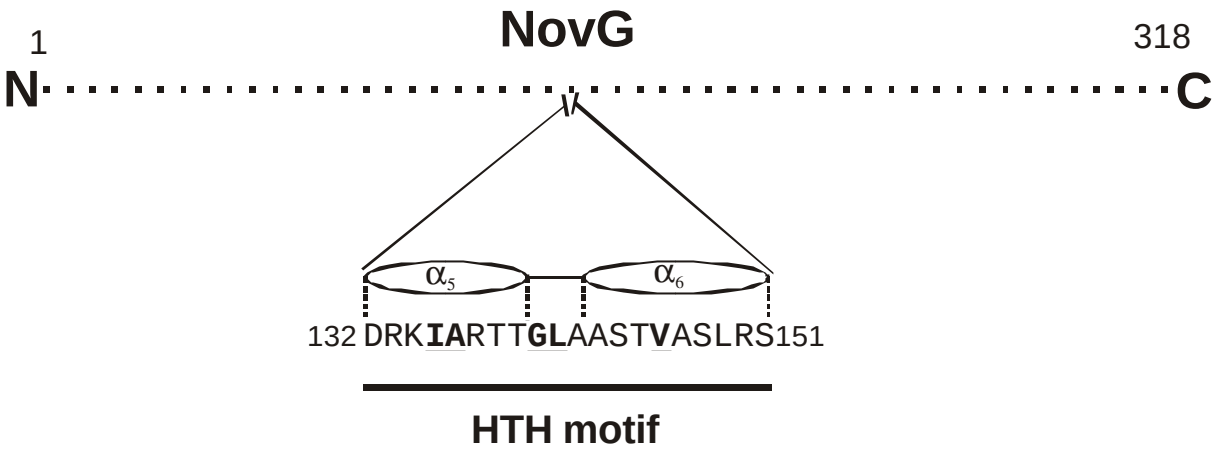


Fig. III.14: Putative helix-turn-helix (HTH) motif of NovG.

The most highly conserved residues in the HTH motif are underlined and include a glycine (G) in the turn and several hydrophobic residues (I, A, L, V) (Pabo and Sauer, 1992). The secondary structure was predicted using the PHD (Profile network prediction Heidelberg) method (Rost, 1996) (available on the web at http://www.embl-heidelberg.de/predictprotein).

novG contains the rare TTA leucine codon (as codon 182) which indicates its dependence of *bldA*, the structural gene for $tRNA^{UUA}$. This may represent a further indication for a regulatory role of NovG in novobiocin biosynthesis, since most of the known TTA-containing genes specify regulatory or resistance proteins associated with biosynthetic gene clusters for antibiotics (Leskiw *et al.*, 1991).

Orthologues of *novG* are found in the biosynthetic gene clusters of the aminocoumarin antibiotics clorobiocin and coumermycin (Pojer *et al.*, 2002). They are termed *cloG* and *couG* and code both for proteins of 319 amino acids, which share on average 78% sequence identity with *novG*. Just as *novG*, both show the putative HTH motif in their central region. *cloG* contains three TTA codons (as codon 28, 83, and 149), whereas *couG* contains none.

III.6.1.2. Inactivation of *novG*

In order to investigate the function of *novG*, the effects of its inactivation and its overexpression were first examined. Using the method described above to circumvent genetic manipulation in the novobiocin producer *S. spheroides*, a Δ*novG* strain was generated by deletion of *novG* in cosmid nov-BG1 and introduction of this modified cosmid into the genome of *S. coelicolor* (Fig. III.15).

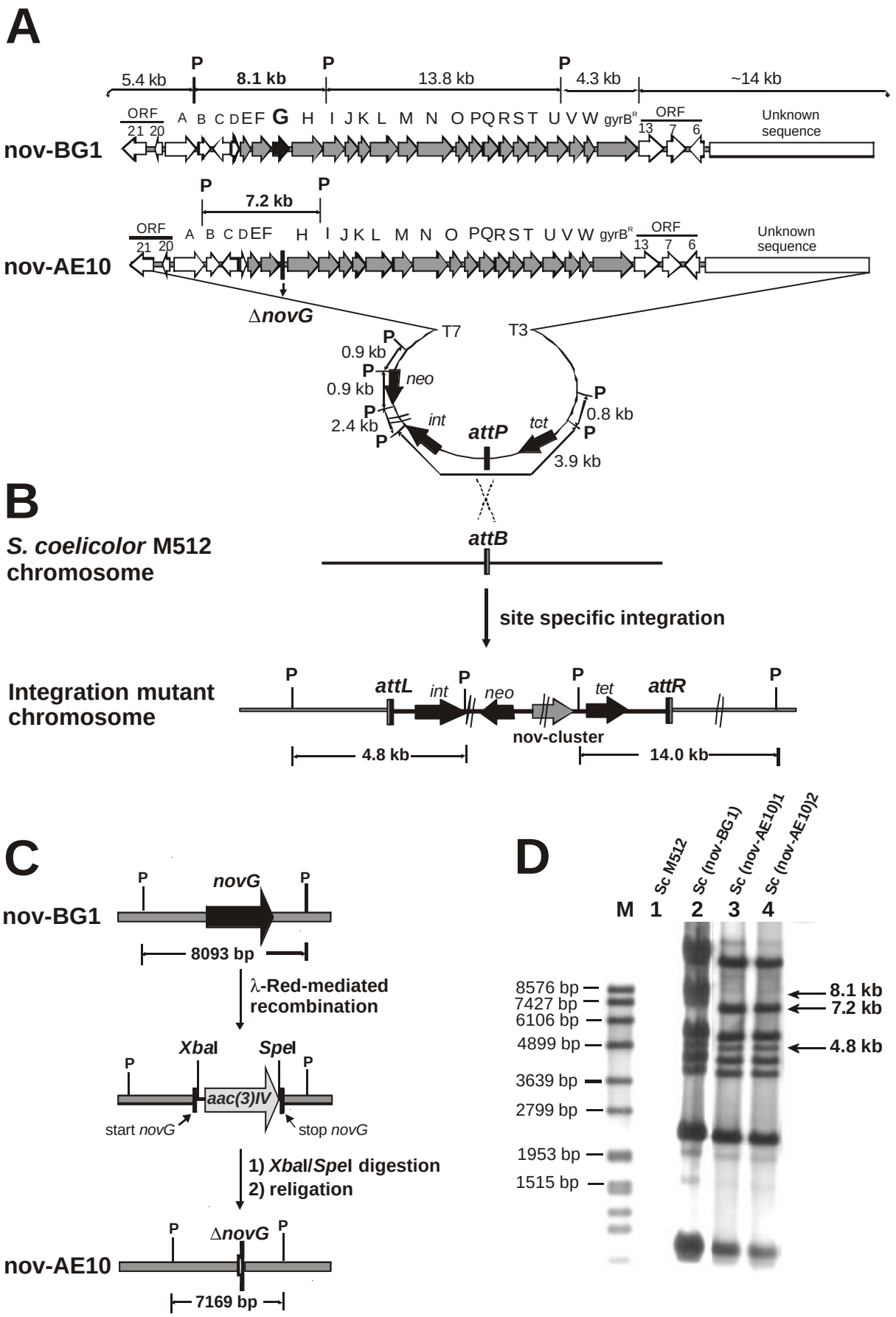


Fig. III.15: Inactivation of *novG*. See next page for details.

(**A**) Schematic presentation of cosmids nov-BG1, containing the intact novobiocin biosynthetic gene cluster, and nov-AE10 in which *novG* was deleted. The novobiocin cluster is presented in grey, the flanking regions in white, and *novG* in black. P = *Pst*I restriction site. For further details see Fig. III.6. Cosmid backbone out of scale. (**B**) Schematic presentation of site specific integration. Junction fragments which prove specific integration into the ϕC31 attachment site of the *S. coelicolor* genome are indicated. For details about the integration system see Fig. III.6. Out of scale. (**C**) Schematic presentation of the *novG* inactivation. *novG* (957 bp) was first replaced by an apramycin resistance (*aac(3)IV*) cassette. Afterwards, the cassette was excised by digestion with *Xba*I and *Spe*I and religated, leaving an *in frame* "scar" of 18 nucleotides between the start and stop codons of *novG*. Out of scale. (**D**) Southern blot analysis of *S. coelicolor* M512 (lane 1), and M512-derived integration mutants bearing cosmid nov-BG1 (lane 2), or the *novG*$^-$ cosmid nov-AE10 (lane 3 and 4). M = DIG-labeled DNA Molecular Weight Marker VII (Roche). Genomic DNA was digested with *Pst*I. The DIG-labeled cosmid nov-BG1 was used as probe. The *novG* inactivation is confirmed by the 7.2 kb band (lane 3 and 4) instead of an 8.1 kb band (lane 2); otherwise the band pattern should be identical.

For this purpose, *novG* was replaced by the apramycin resistance cassette from pUG019. The cassette was then removed by digestion with *Xba*I and *Spe*I, and subsequent religation of the outer ends lead to excision of the cassette (Fig. III.15C). The modified cosmid (named nov-AE10) was then introduced into *S. coelicolor* by protoplast transformation. Southern blot analysis confirmed the site specific integration into the genome, and the deletion of *novG* was clearly shown by the smaller size of the relevant *Pst*I restriction fragment in comparison to nov-BG1 strains (Fig. III.15D, lanes 2, 3 and 4).

Analysis of secondary metabolites by HPLC showed that the resulting Δ*novG* strains still produced novobiocin, which was identified by negative-ion FAB MS analysis in comparison to an authentic standard (molecular ion $[M-H]^-$ at *m/z* 611; novobiocin, $C_{31}H_{36}N_2O_{11}$, has a molecular weight of 612). However, the amount of novobiocin produced by the Δ*novG* mutants was reduced by 98% in comparison to *S. coelicolor* strains carrying the intact novobiocin cluster (Table III.5).

TABLE III.5: Effect of *novG* inactivation or expression on novobiocin production by recombinant *S. coelicolor* strains carrying the novobiocin cluster

Strain	**Expression plasmid**	**Novobiocin production[a]**	
		mg/l	**(%)**
S. coelicolor M512	---	0	0
S. coelicolor(nov-BG1), complete cluster	pWHM3 (empty vector)	20	100
S. coelicolor(nov-AE10), Δ*novG*	pWHM3 (empty vector)	0.4	2
S. coelicolor(nov-AE10), Δ*novG*	pAE-G2_1 (*novG* with 100 bp upstream sequence, opposite orientation to *lacZa*)	0.4	2
S. coelicolor(nov-AE10), Δ*novG*	pAE-G2_2 (*novG* with 100 bp upstream sequence)	3	15
S. coelicolor(nov-AE10), Δ*novG*	pAE8 (*novG* with 336 bp upstream sequence)	16	79
S. coelicolor(nov-BG1), complete cluster	pAE-G2_2 (*novG* with 100 bp upstream sequence)	38	190
S. coelicolor(nov-BG1), complete cluster	pAE8 (*novG* with 336 bp upstream sequence)	54	270

[a]Values are means from two to four independent mutants with two determinations each.

III.6.1.3. Complementation of the *novG* mutation

To prove that indeed the inactivation of *novG* was responsible for the low productivity of the Δ*novG* strains, we attempted to complement one of these strains by expressing *novG* under control of its own promotor. For this purpose, we used a derivative of the promoterless, replicative shuttle vector pWHM3 (Vara *et al.*, 1989), into which *novG* and 336 bp of the upstream DNA region (from the putative translational start point) was cloned, resulting in pAE8.

Transformants of the Δ*novG* strain carrying pAE8 produced on average 79% of the novobiocin amount accumulated by *S. coelicolor* strains bearing the intact novobiocin cluster (Table III.5). Therefore, complementation was successful, establishing that the low productivity of Δ*novG* strains was due to lack of *novG* and not to possible polar effects of the deletion on downstream genes.

Notably, a pWHM3 construct containing *novG* with only 100 bp of the upstream DNA region (termed pAE-G2_2) was much less effective in restoration of novobiocin

biosynthesis (Table III.5). If the orientation of its insert was reversed (i.e. placed against the *lacZα* orientation), the resulting plasmid pAE-G2_1 was completely unable to restore novobiocin production. Apparently, these constructs did not contain a functional promoter upstream of *novG*, and the low increase of productivity observed in pAE-G2_2 transformants may be due to a slight activity of the *lacZα* promoter in the *Streptomyces* host.

III.6.1.4. Overexpression of *novG* in *S. coelicolor*(nov-BG1) leads to overproduction of novobiocin

The results presented above indicate that NovG could act as a positive regulator in novobiocin biosynthesis. It has been reported that overexpression of pathway-specific activators can lead to overproduction of the respective antibiotic (Gramajo *et al.*, 1993; Stutzman-Engwall *et al.*, 1992). Therefore, the multicopy plasmids pAE-G2_2 and pAE8 were introduced into *S. coelicolor*(nov-BG1) by protoplast transformation. As presented in Table III.5, pAE-G2_2 led to 1.9-fold and pAE8 to 2.7-fold increase in novobiocin biosynthesis in comparison to strains carrying only the empty vector pWHM3.

III.6.1.5. Overexpression and purification of NovG as a His_6 fusion protein

For further investigation of the function of *novG*, its gene product was expressed as an N-terminal His_6 fusion protein in *E. coli* (see Methods). When cultivating at 30 ºC and inducing with 1 mM IPTG for 5 h, most of the protein was insoluble. The amount of soluble protein did not improve significantly when the growth temperature was reduced to 15 ºC and the IPTG concentration to 0.25 mM. However, useful amounts of soluble protein could be obtained reproducibly when the induction period was shortened to only 1.5 h, using 0.5 mM IPTG at 30 ºC (see Methods). The His_6 tagged NovG protein was purified from the soluble fraction by nickel affinity chromatography. SDS-PAGE analysis showed a band of about 40 kDa (calculated mass 38.1 kDa) in the eluate (Fig. III.16). The protein yield of purified NovG was about 15 µg/g cells (fresh weight), determined by SDS-PAGE.

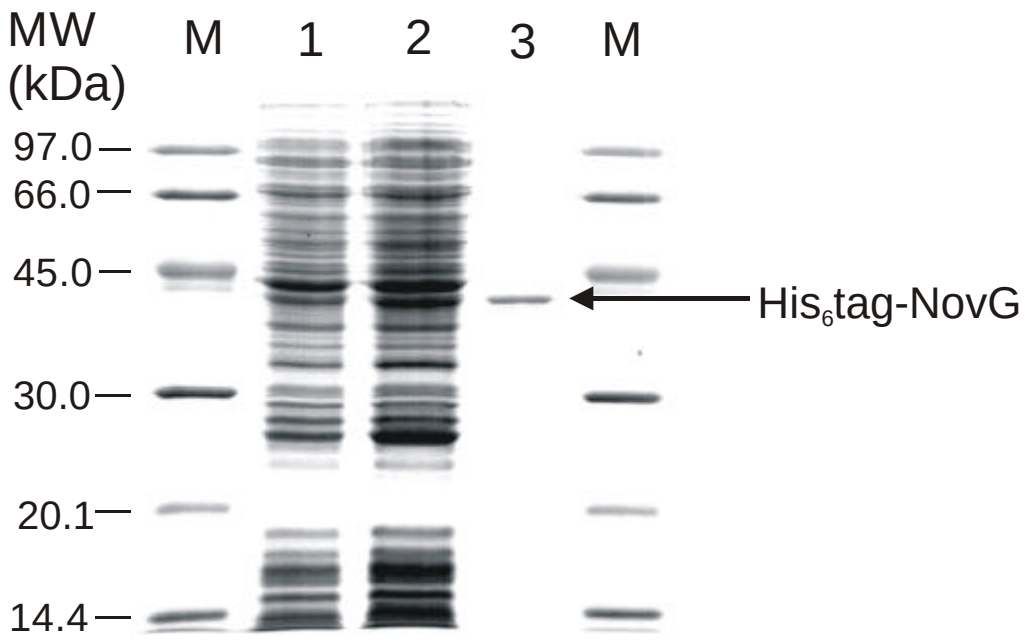


Fig. III.16: Purification of NovG after overexpression as a fusion protein with a N-terminal His_6 tag in *E. coli* BL21(DE3)pLysS.

SDS/12% PAGE. Lane 1, soluble protein before induction; lane 2, soluble protein after induction with 0.5 mM IPTG for 1.5 h; lane 3, eluate from nickel affinity chromatography; M, molecular weight (MW) marker. The molecular weights (kDa) of the marker proteins are indicated.

III.6.1.6. DNA-binding activity

NovG may act as a positive regulator of novobiocin biosynthesis by binding at or near the promoter region of biosynthetic genes and thereby activating their transcription (see Introduction). In order to verify whether NovG has indeed DNA-binding activity, gel mobility-shift assays were carried out using the purified His_6 tagged NovG and selected DNA fragments from the novobiocin biosynthetic gene cluster.

In the novobiocin cluster, the intergenic regions between most genes are very short, and often the coding regions even overlap indicating a possible translational coupling (Steffensky *et al.*, 2000b). Only six of the intergenic regions are larger than 70 bp (Fig. III.17A), and those we chosen for gel mobility-shift experiments, which are based on the differential mobility of free DNA and DNA-protein complexes in a gel matrix. As shown in Fig. III.17B, also DNA fragments extending into the coding sequences upstream of these intergenic regions were investigated. The NovG fusion protein

showed very clear binding activity for one of the two fragments obtained from the *novG-novH* intergenic region (Fig. III.17), but not for any other of the investigated ten DNA fragments.

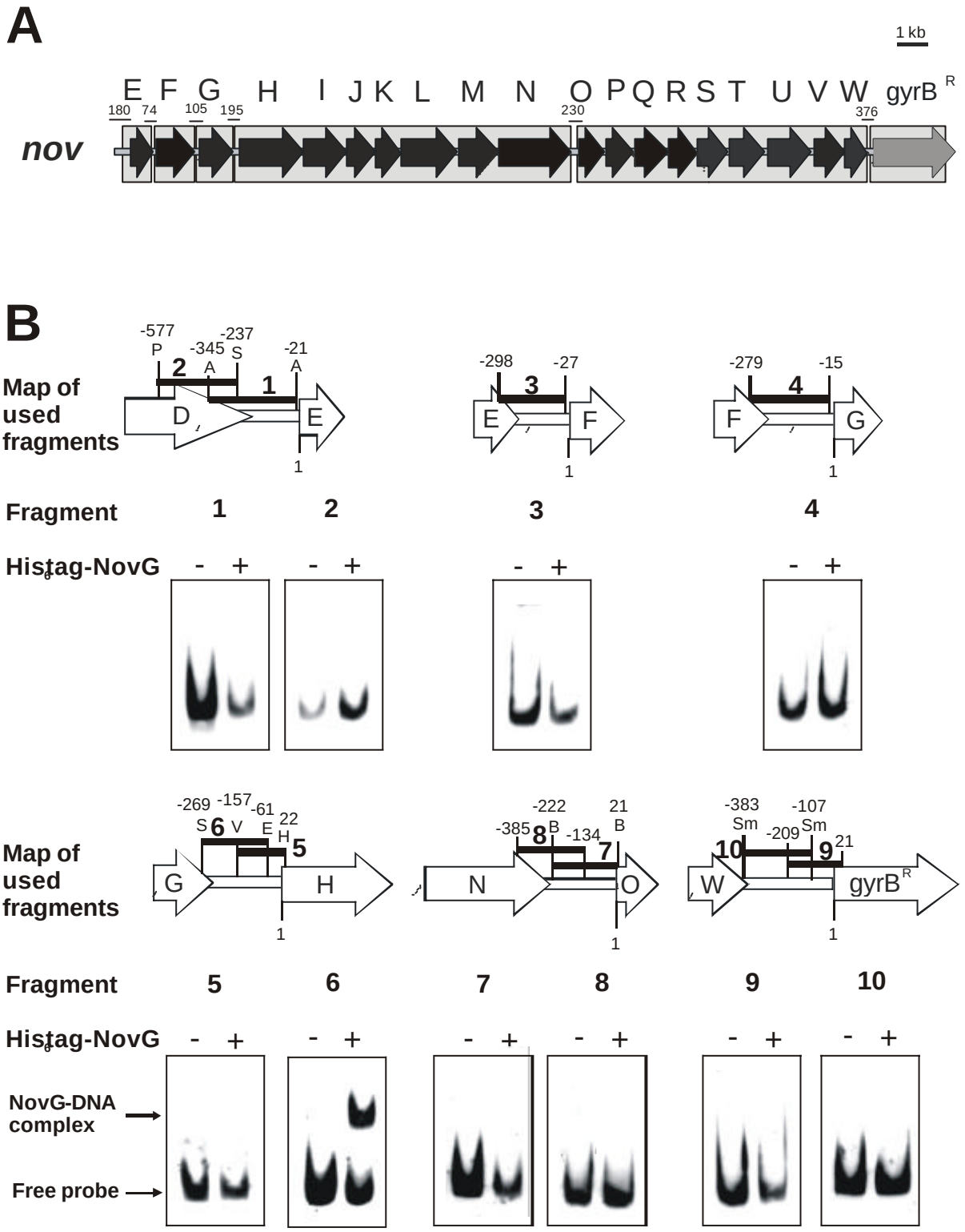


Fig. III.17: Gel mobility-shift assays with DNA fragments from the novobiocin biosynthetic gene cluster. See next page for details.

(**A**) Schematic presentation of the novobiocin biosynthetic gene cluster. All intergenic regions larger than 70 bp are shown and their exact size is given. (**B**) Autoradiogram of gel mobility-shift assays using purified His_6 tagged NovG. The DNA-binding activity was analysed as described in the Methods section, using about 4 ng of the indicated DIG-end-labelled fragment and no protein (-) or 0.5 µg of purified His_6 tagged NovG protein (+). A map showing the location of the DNA fragments used is indicated above each autoradiogram (out of scale, the intergenic regions are oversized in comparison to the ORFs). The fragments were obtained by digestion of appropriate plasmids or by PCR amplification (see Methods). For fragments obtained by digestion, restriction sites are indicated (P = *Pvu*I, S = *Sal*I, A = *Ava*I, V = *Van*91I, E = *Eco*RI, H = *Hind*III, B = *Bam*HI, Sm = *Sma*I).

The novobiocin and the clorobiocin clusters show very high similarity between each other. For most of the *nov* genes, orthologues exist in the clorobiocin cluster, which show 75-80% sequence identity to the genes of the novobiocin cluster on the amino acid level (Pojer *et al.*, 2002). This is also the case for both *novG* and *novH*. However, in the novobiocin cluster the gene *novH* (coding for a biosynthetic enzyme (Chen and Walsh, 2001)) is situated directly downstream of the regulatory gene *novG*, whereas in the clorobiocin cluster their orthologues *cloG* and *cloH* are separated by a small open reading frame of unknown function, i.e. *cloY*. As shown in Fig. III.18, we tested whether NovG could also bind to the DNA regions upstream of *cloY* and/or *cloH*. Very clearly, NovG did bind to the DNA region upstream of *cloY*, but not to those upstream of *cloH*.

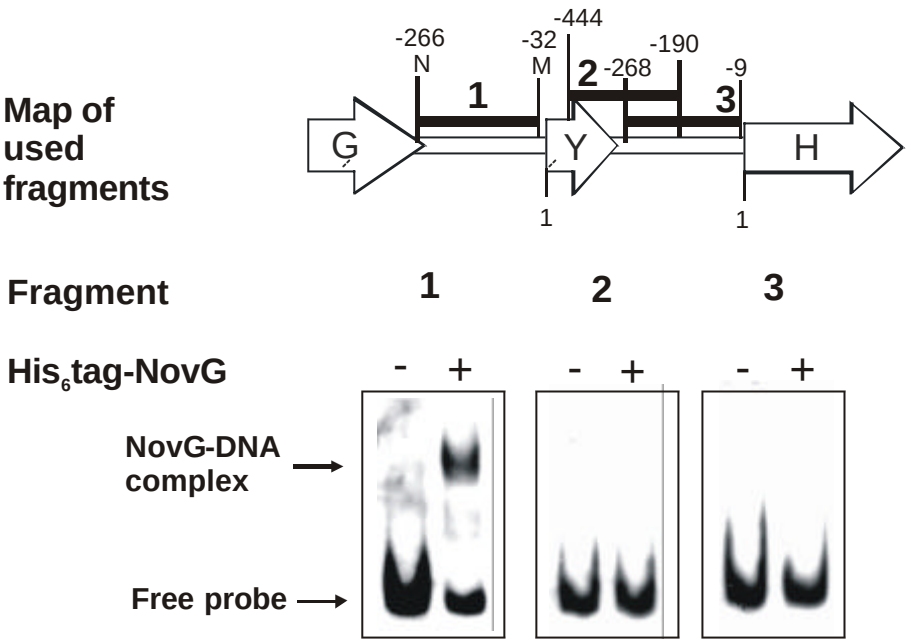


Fig. III.18: Gel mobility-shift assays with DNA fragments from the clorobiocin biosynthetic gene cluster.

The fragments were obtained by digestion of pAE9 (fragment 1) or by PCR amplification from cosmid K1F2 (fragments 2 and 3, see Methods). N = *Not*I, M = *Mlu*I. See legend of Fig. III.17 for further explanations.

III.6.1.7. NovG binds specifically to the *novG-novH* and *cloG-cloY* intergenic regions

To provide proof that binding of NovG to the *novG-novH* and *cloG-cloY* intergenic regions is specific, a gel mobility-shift assay was carried out in which a competitor plasmid containing the respective binding site was added in approximately 125-fold molar excess. As negative control, the same amount of empty vector was used. As shown in Fig. III.19, the presence of the specific competitor plasmid indeed strongly reduced the intensity of the band caused by the NovG-DNA complex, while the empty vector caused no significant effect.

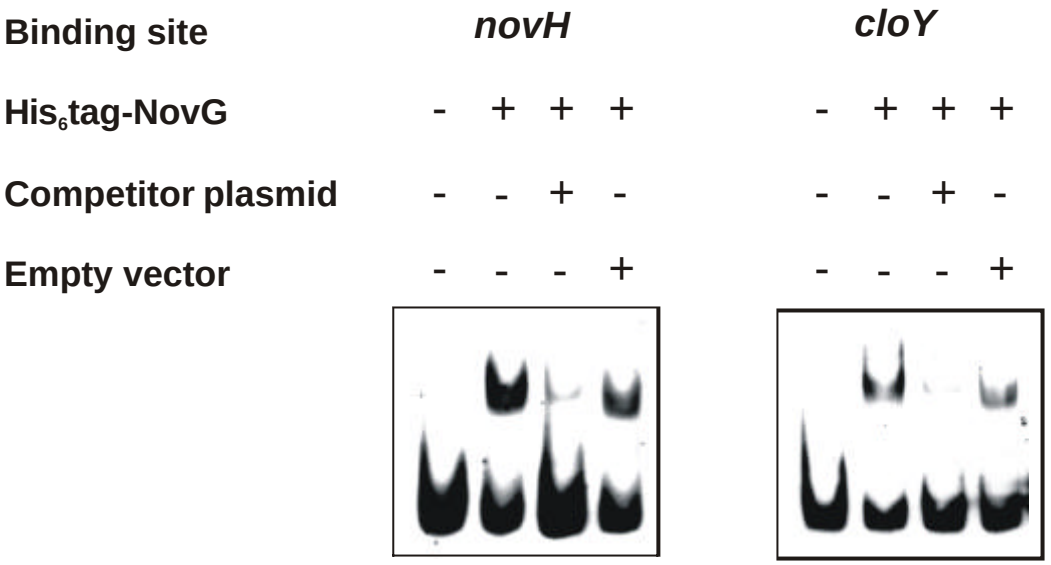


Fig. III.19: Specifity of His_6 tagged NovG binding to DNA.

Approximately 4 ng of DIG-end-labelled fragment (*novH*: fragment 6 of Fig. III.17, *cloY*: fragment 1 of Fig. III.18) and no protein (-) or 0.5 µg purified His_6 tagged NovG protein (+) were used in each lane for a standard DNA-binding assay (see Methods). When indicated (+), an additional 125-fold molar excess of specific competitor plasmid DNA (pAE7 or pAE9, respectively) or of the non-specific empty vector-DNA (pBluescript SK(-) or pSPORT1, respectively) was added.

III.6.1.8. *In silico* analysis of the NovG binding DNA fragments

When the DNA sequences of the *novG-novH* and *cloG-cloY* intergenic regions were compared, similar palindromic structures could be found in both fragments (Fig. III.20). Notably, similar inverted repeats were identified as the binding site for StrR by DNase I footprinting assay (Retzlaff and Distler, 1995). The putative NovG-binding sites and the StrR-binding sites all possess a conserved palindromic structure with the consensus sequence GTTCRACTG$(N)_{11}$CRGTYGAAC.

putative NovG binding site (*novH*) CT**GAC**GTTCAACTG**CTCGAA**T**CAT**T**C**GGTTGAACA**CCA

putative NovG binding site (*cloY*) GG**GA**T**GTTCAACTG**CTCGAA**C**CAT**A**C**GGTTGAACA**TGG

StrR binding site (consensus sequence) **GTTC**G**ACTG**NNNNNNNNNNN**C**A**GT**C**GAAC**

Consensus sequence **GTTCRACTGNNNNNNNNNNNCRGTYGAAC**

Fig. III.20: Sequences of putative NovG-binding sites of *S. spheroides* (upstream of *novH*) and *S. roseochromogenes* (upstream of *cloY*) in comparison to the consensus sequence of StrR-binding sites of *S. griseus* and *S. glaucescens* (see (Retzlaff and Distler, 1995)). Nucleotides conserved in at least two of the sequences are written in bold. Nucleotides conserved in all three sequences are underlined. The inverted repeats are indicated with arrows. A putative consensus sequence for NovG- and StrR-binding sites is indicated.

III.6.2. Inactivation of *novE* in *S. spheroides*

novE, *cloE* and *couE* show, on average, 82% sequence identity with each other on the amino acid level. The predicted gene product of *novE* comprises 217 amino acids and shows 45% identity to the gene product of *lmbU* of the lincomycin biosynthetic gene cluster. Lincomycin, like the aminocoumarin moiety of novobiocin, is formed from tyrosine, but the reaction sequence is unknown. Peschke *et al.* (1995) speculated that *lmbU* may encode a regulatory protein, but a catalytic function for LmbU could not be excluded.

novE contains the rare TTA leucine codon (as codon 189) which may represent an indication for a regulatory role of NovE in novobiocin biosynthesis, since most of the known TTA-containing genes specify regulatory or resistance proteins associated with biosynthetic gene clusters for antibiotics (Leskiw *et al.*, 1991).

In order to gain more insights into the role of *novE* in novobiocin biosynthesis, it was inactivated in *S. spheroides* NCIMB 11891. Within cosmid 10-9C, which contained the biosynthetic gene cluster of novobiocin in the SuperCos1 vector (Kan^R), *novE* was replaced by an apramycin resistance/*oriT* cassette using the PCR targeting system (Gust *et al.*, 2003). The modified cosmid (named 10-9C-E-773, = E1) was then introduced into *S. spheroides* by conjugation. Apramycin-resistant, kanamycin-sensitive colonies, resulting from double crossover events, were selected. Southern

blot analysis confirmed that in these mutants *novE* was replaced by the Apra^R/*oriT* cassette (Fig. III.21).

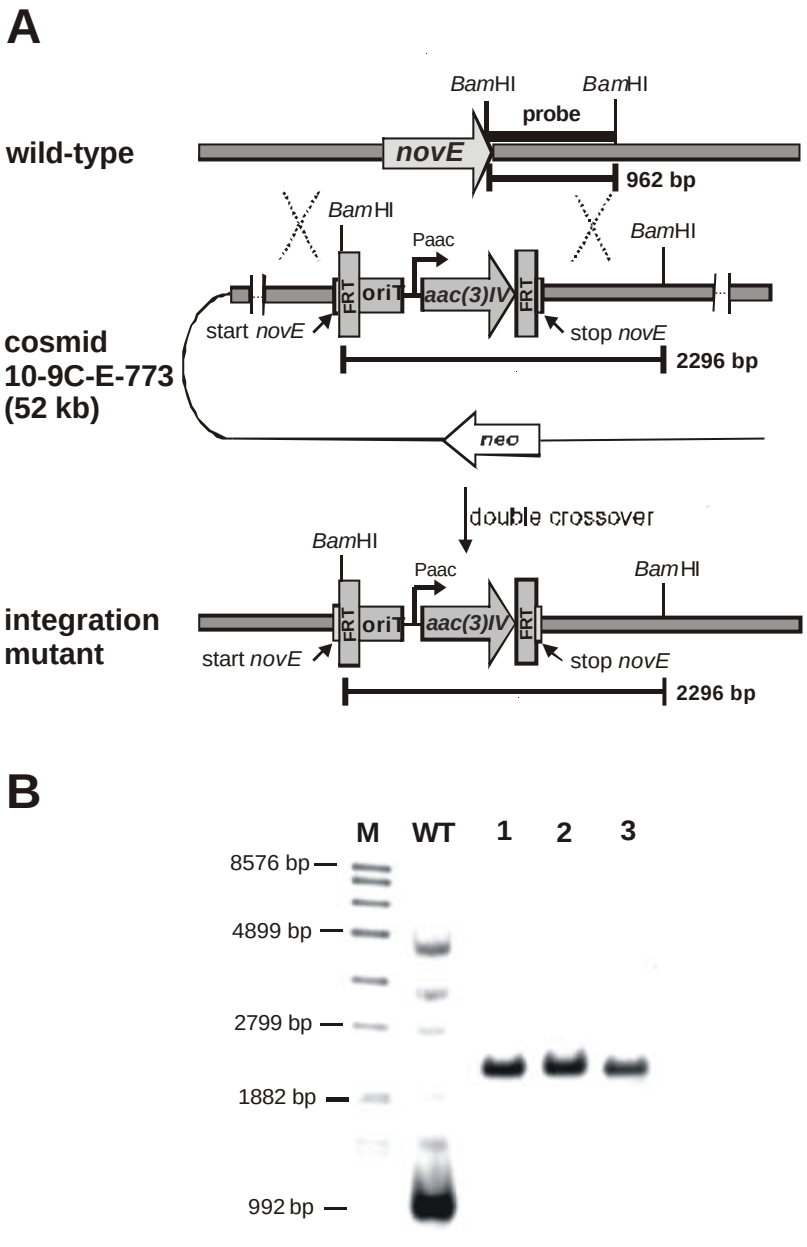


Fig. III.21: Inactivation of *novE* of the novobiocin biosynthetic gene cluster.
(**A**) Schematic presentation of the gene inactivation experiment. The DNA fragment used as probe is indicated as a black bar. *aac(3)IV*: apramycin resistance gene; Paac: promoter of the apramycin resistance gene; FRT: FLP recognition target; *oriT*: origin of transfer from RK2; *neo*: kanamycin resistance gene. (**B**) Southern blot analysis of wild-type and mutants. Genomic DNA was restricted by *Bam*HI. M: DIG-labeled DNA Molecular Weight Marker VII (Roche); WT: *S. spheroides* wild-type; 1-3: *novE* mutants (strains *S. spheroides* AE-E2, AE-E3, and AE-E4).

The secondary product formation of three independent *novE* mutants (named AE-E2, AE-E3, and AE-E4) and the wild-type strain of *S. spheroides* were analysed by HPLC. All three mutants, as well as the wild-type strain, produced novobiocin, which was identified by ^{1}H NMR and negative-ion FAB MS analysis in comparison to an authentic standard. However, the level of production in the mutants was significantly lower than in the wild-type (average 1.5 mg/l in the mutants, compared to 37 mg/l in the wild-type). This finding rules out the possibility that NovE has an essential catalytic function. Rather, NovE may act as a positive regulator in novobiocin biosynthesis.

IV. DISCUSSION

IV.1. Clorobiocin biosynthesis in *S. roseochromogenes*: Identification of the halogenase and generation of structural analogues

The results presented in this thesis demonstrate that the gene *clo-hal* is responsible for halogenation of position 8 of the aminocoumarin ring of clorobiocin, while *novO* is responsible for methylation of the corresponding position of novobiocin. *cloZ* is not involved in the halogenation, nor is it essential for clorobiocin biosynthesis under the present culture conditions.

The *clo-hal* gene product is responsible for the introduction of the halogen atom of clorobiocin. The mechanism of halogenation reactions, which are involved in the biosynthesis of at least 3000 natural halometabolites (Gribble, 1998), is not yet completely understood. Clo-hal shows sequence similarity to the recently discovered class of $FADH_2$-dependent halogenases (van Pée, 2001), such as are involved in the biosynthesis of chlortetracycline (Dairi *et al.*, 1995), the glycopeptide antibiotic balhimycin (Puk *et al.*, 2002), and pyoluteorin (Nowak-Thompson *et al.*, 1999). It has been speculated that the reactions catalysed by these enzymes may proceed via an epoxide intermediate (van Pée, 2001). *In vitro*, these enzymes require the presence of an unspecific flavin reductase generating $FADH_2$. This reductase appears not to be encoded within the gene clusters of the above mentioned secondary metabolites, similar to the finding for the clorobiocin cluster (Pojer *et al.*, 2002). The functional identification of *clo-hal* reinforces the importance of this new class of halogenases for natural product biosynthesis.

Moreover, expression of *novO* from the novobiocin cluster in the *clo-hal*$^-$ mutant led to the very efficient formation of an 8´-methylated compound, i.e. novclobiocin 102. Together with the previously published inactivation of the very similar gene *couO* from the coumermycin cluster (Li *et al.*, 2002), this result provides conclusive evidence that NovO and CouO catalyse the C-methylation reaction in the biosynthesis of the aminocoumarin rings of novobiocin and coumermycin.

It is not clear at present at which step of aminocoumarin biosynthesis the methylation or halogenation reactions takes place. For novobiocin biosynthesis, Chen and Walsh (Chen and Walsh, 2001) provided evidence that the methylation occurs after activation of tyrosine, which is the first step in aminocoumarin formation (Fig. I.4), and an inactivation experiment by Steffensky *et al.* (Steffensky *et al.*, 2000b) demonstrated that methylation takes place before glycosylation of novobiocic acid. In any case, for the assembly of the entire novobiocin or clorobiocin molecule, several enzymatic steps are required to take place after the methylation or halogenation, respectively (Fig. I.4). It is remarkable that the yields of novclobiocin 101 (8´-H), produced by the *clo-hal*$^-$ mutant, and 102 (8´-CH_3), produced by the *clo-hal*$^-$ mutant expressing *novO*, were at least as high as that of clorobiocin (8´-Cl) in the wild-type. This indicates a low specificity of the subsequent biosynthetic enzymes for the substituent at the 8´ position, a very useful feature for the development of new aminocoumarin antibiotics by genetic approaches.

In the present study, this allowed the production of the two clorobiocin analogues mentioned above, and a first comparison of these compounds with clorobiocin regarding their antibacterial activity (see IV.4).

Moreover, with some modifications of the conjugation procedure (see Methods), the PCR targeting system could be used successfully in *S. roseochromogenes*, allowing gene inactivation experiments to be carried out much more simply and quickly than by previous methods (Kieser *et al.*, 2000). The modified cosmid can be introduced in *Streptomyces* by conjugation from *E. coli*, which represents usually a more efficient method than transformation, since restriction barriers may be reduced by the transfer of single-stranded concatemers of DNA (Kieser *et al.*, 2000). Furthermore, by using the cosmid as vector, the flanking DNA region of the inactivated allele is much bigger than 1 kb (e.g. for *clo-hal* about 20 kb on either side). Since the frequency of homologous recombination is proportional to the lengths of the DNA fragments used (Kieser *et al.*, 2000), the frequency of desired double crossover events is also proportionally higher. Indeed, by omitting the selection for the vector backbone (in this case, by overlaying the conjugation plates only with apramycin), colonies carrying the desired double crossover events grew already on these plates, making time-consuming non-selective growth and screenings unnecessary.

IV.2. Heterologous expression of the novobiocin and clorobiocin gene clusters

The expression of biosynthetic genes in heterologous hosts which are more amenable for genetic manipulation can be used to circumvent the challenge associated with developing suitable protocols for the genetic manipulation of natural producers (Eppelmann *et al.*, 2001; Sánchez *et al.*, 2002; Tang *et al.*, 2000). Furthermore, the successful heterologous expression of a biosynthetic cluster proves that all genes essential for antibiotic biosynthesis are included in that cluster. In this study, the clusters for novobiocin and clorobiocin were expressed in *S. coelicolor* and *S. lividans*, phylogenetically very near species which have been extensively studied and for which well established genetic tools are available (Kieser *et al.*, 2000).

The complete genome sequence of strain M145 of *S. coelicolor* has been published (Bentley *et al.*, 2002). *S. coelicolor* M145 is able to produce three antibiotics, i.e. prodiginines, actinorhodin and calcium-dependent antibiotic (CDA). To avoid interference in the chemical and biological analysis of antibiotic formation, the novobiocin and clorobiocin biosynthetic gene clusters were expressed in *S. coelicolor* M512, which is derived from M145 but which is unable to produce prodiginines and actinorhodin, since the activator genes *redD* and *actII*ORF4 were deleted in this strain (Floriano and Bibb, 1996). For the heterologous expression of clorobiocin, a prodiginine-deficient strain offers the additional advantage that any competition for proline or proline derivatives, which are precursors of both prodiginines and clorobiocin (Thomas *et al.*, 2002), is avoided.

Historically, *S. lividans* has been distinguished phenotypically from *S. coelicolor* by the inability of the former to normally produce actinorhodin, which lends the characteristic blue color of *S. coelicolor* colonies responsible for its name. Yet, a functional biosynthetic actinorhodin gene cluster is present in *S. lividans*, suggesting different regulation of the onset of secondary metabolism in the two species (Kim *et al.*, 2001; Shima *et al.*, 1996). Kim *et al.* (2001) reported that actinorhodin biosynthesis in *S. lividans* is repressed by glucose in the media and that a change of the carbon source (glycerol instead of glucose) promotes production of the antibiotic. Furthermore, a strain of *S. lividans*, TK24 (*str-6*, SLP1$^-$, SLP2$^-$), was found to produce actinorhodin under “normal” culture conditions (which are described for *S. coelicolor*),

while the parental strain TK21 (SLP1$^-$, SLP2$^-$) and the wild-type isolate, strain 66, do not. It has been suggested that the streptomycin-resistant mutation *str-6* (representing a point mutation in the *rpsL* gene encoding ribosomal protein S12, changing Lys-88 to Glu) may be responsible for induction of antibiotic synthesis (Shima *et al.*, 1996). Shima *et al.* (1996) proposed that this mutation leads to a change in the ribosomal structure which may give rise to initiation of the onset of secondary metabolism (by an unknown mechanism).

Therefore, to test *S. lividans* as host, strain TK24 was chosen. However, *S. coelicolor* M512 showed at least five times higher productivity than *S. lividans* TK24 (Table III.3), under the present culture conditions, and was therefore used for subsequent experiments.

Since heterologous expression of cosmid clo-AE2 (Fig. III.7) led to significant production of clorobiocin, it could be proven, for the first time, that the DNA region from *cloE* to *gyrB*^{R} contains all genes necessary for clorobiocin production. This finding is of special interest since no candidate gene has been identified which may code for the enzyme responsible for the introduction of the ring oxygen of the coumarin moiety of the aminocoumarin antibiotics (see also Introduction) (Chen and Walsh, 2001; Eustáquio *et al.*, 2003b; Holzenkämpfer and Zeeck, 2002). Unless this oxygenation is carried out by primary metabolic enzymes of *S. coelicolor* (a rather unlikely scenario), this catalytic function will have to be identified in one of the proteins encoded in cosmid clo-AE2.

The principal resistance gene of the novobiocin producer *S. spheroides* is *gyrB*^{R}, encoding for an aminocoumarin-resistant gyrase B subunit (Schmutz *et al.*, 2003a; Schmutz *et al.*, 2004; Thiara and Cundliffe, 1988). Since this gene is contained within the novobiocin cluster (Fig. III.6), the *S. coelicolor* mutants generated in this study were able to tolerate the accumulation of the antibiotic.

The clorobiocin producer *S. roseochromogenes* contains, besides *gyrB*^{R}, an additional resistance gene, *parY*^{R}, which encodes an aminocoumarin-resistant topoisomerase IV subunit (Schmutz *et al.*, 2003a; Schmutz *et al.*, 2004). Only 123 bp of the 2.1 kb *parY*^{R} gene were contained in the cosmids clo-BG1 and clo-AE2 (Fig. III.7). Nevertheless, clorobiocin was produced in good yield by the mutants expressing these cosmids, consistent with the previous finding that *gyrB*^{R} alone is

sufficient to provide resistance against aminocoumarin antibiotics (Schmutz *et al.*, 2003a).

Vectors containing the integrase gene and the *attP* site of ϕC31 can integrate not only as a single copy, but also in tandem (Combes *et al.*, 2002). Tandem integration of cosmids was observed in approximately 90% of all integration mutants obtained in this study, as identified in Southern blot analysis by a hybridizing fragment containing the unchanged *attP* site (e.g. 3.9 kb in Fig. III.6C). This 3.9 kb band may in principle also indicate the presence of the cosmid as covalently closed circular DNA. However, since the SuperCos1-derived cosmids are non-replicative in *Streptomyces*, their presence after extended sub-culturing is unlikely. Mutants with tandem integration showed somewhat higher antibiotic production than mutants with single copy integration, but the difference, and the number of available strains, was not large enough to draw unequivocal conclusions.

In contrast, the 16-fold higher productivity of strains bearing cosmid nov-AE6, compared to strains harboring nov-AE4 (Table III.3 and Fig. III.6) was observed in more than ten independent mutants each and clearly proves that the DNA sequence from *ORF20* to *novD* contains elements required for a high novobiocin production. The gene *novA* which codes for an ABC transporter may be responsible for this effect. Alternatively, two other hypotheses could explain this finding. First, genes between *ORF20* and *novD* could have a regulatory function. Second, even though the used cosmid contained all intergenic region between *novD* and *novE* (180 bp), it is possible that it does not include all necessary regulatory regions for the normal transcription of *novE*, which likely codes for a positive regulator in novobiocin biosynthesis (see IV.6.2), leading to the drop in novobiocin production. Further experiments are necessary to identify which part of the sequence between *ORF20* and *novD* is responsible for the observed effect.

IV.3. Production of 8´-halogenated and 8´-unsubstituted novobiocin derivatives in genetically engineered *S. coelicolor* strains

In this study, a strategy was developed for the production of a hybrid antibiotic, novclobiocin 114, which carries a chlorine atom instead of a methyl group at position

8´ of the aminocoumarin unit of novobiocin. This compound could not be produced by gene inactivation and heterologous gene expression experiments in the novobiocin producer *S. spheroides*, because of difficulties in the genetic manipulation of this strain (Pojer and Heide, unpublished results). Yet, through heterologous expression of the entire novobiocin biosynthetic gene cluster in *S. coelicolor*, novobiocin and, after appropriate modification of the cluster, both the 8´-H compound (novclobiocin 117) and the 8´-Cl compound (novclobiocin 114) were readily obtained from the resulting strains.

An accumulation of truncated intermediates, lacking the deoxysugar moiety, was observed in mutants carrying modified biosynthetic pathways, i.e. in the *novO*$^-$ mutant (Fig. III.11C), and in the *novO*$^-$ mutant complemented with *clo-hal* (Fig. III.11D). This may indicate that the remaining biosynthetic machinery does not accept the modified substrates as efficiently as the genuine intermediates. Notably, this was not the case for modified clorobiocin pathways (see III.1 and III.2).

The development of suitable protocols for the genetic manipulation of *Streptomyces* strains which produce useful antibiotics is time-consuming and often, e.g. for the novobiocin producer *S. spheroides*, gives unsatisfactory results. This problem presents a severe limitation for basic research on microbial secondary metabolism and for drug discovery programs from streptomycetes.

The modification of cosmids in *E. coli* followed by heterologous expression of the modified cosmid in the well-studied *S. coelicolor* offers an alternative approach of unprecedented speed and versatility in comparison to the manipulation of natural producers. It may also allow the exploitation of biosynthetic gene clusters from organisms which are difficult to cultivate, using heterologous expression in culturable hosts.

IV.4. Structure-activity relationships

Structurally, clorobiocin differs from novobiocin in the substitution at C-8´ of the aminocoumarin ring and at the C-3´´ of the deoxysugar moiety (Fig. I.5). Clorobiocin has been reported to show a six times higher antibacterial activity than novobiocin *in*

vitro (Hooper *et al.*, 1982), and to bind more efficiently to isolated gyrase (Lafitte *et al.*, 2002; Lewis *et al.*, 1996a; Tsai *et al.*, 1997). Most authors have attributed the higher activity of clorobiocin primarily to the pyrrole moiety at C-3´´ (Berger and Batcho, 1978; Tsai *et al.*, 1997). Interestingly, however, clorobiocic acid (Fig. I.4), but not novobiocic acid, was found to inhibit both DNA synthesis *in vivo* and gyrase activity *in vitro* (Althaus *et al.*, 1988; Reusser and Dolak, 1986), suggesting that the chlorine atom makes an important contribution to the biological activity of this molecule.

The substances produced in this study (novobiocin and clorobiocin analogs with –CH_3, –H, or –Cl at C-8´) will allow a detailed investigation of the structure-activity relationships of such aminocoumarin compounds, using both antibacterial assays and *in vitro* investigations with purified gyrase. In a first comparison using a bioassay with *Bacillus subtilis* (Fig. III.5), clorobiocin (8´-Cl) was twice as active as novclobiocin 102 (8´-CH_3), and eight times more active than novclobiocin 101 (8´-H); whereas Fig. III.12 shows that novobiocin (8´-CH_3) was twice as active as novclobiocin 114 (8´-Cl), and 25 times more active than novclobiocin 117 (8´-H). Therefore, in both cases, substitution at C-8´ with either –Cl or –CH_3 was important for biological activity; –CH_3 or –Cl leading to comparable activity, and –H causing eight- to 25-fold loss in activity. Notably, the difference between substitution with –CH_3 or –Cl, though moderate, depended upon the compound being a novobiocin or a clorobiocin derivative, i.e. depended upon the substitution at 3´´-OH of the deoxysugar. Further experiments are necessary to identify the cause for this observation.

As reported for clorobiocin derivatives (Galm *et al.*, 2004b), the transfer of the acyl group from 3´´-OH to 2´´-OH resulted in a reduction but not in a complete loss of activity: novclobiocin 119 still showed approximately 20% of the activity of novclobiocin 114. In contrast, the removal of the deoxysugar moiety and the 8´-substituent (= novclobiocic acid 106) resulted in 99% loss of activity. These results are in accordance with X-ray data, which showed that the carbamoyl group of novobiocin forms important hydrogen bonds with *E. coli* GyrB (directly with Ala47 and Asp73 and through ordered water molecules to different residues) (Lewis *et al.*, 1996a). Lewis *et al.* (1996a) speculated that the reduced affinity of isonovobiocin (in which the carbamoyl group is presented at 2´´-OH instead of 3´´-OH of the deoxysugar) to gyrase is probably a consequence of steric clashes. In addition to further hydrogen bonding network (involving the 2´´-OH and the 4´´-OCH_3), there are

a number of hydrophobic contacts between gyrase and the deoxysugar, which explains the importance of this moiety for activity. Yet, the substituted deoxysugar alone has shown no antibacterial or anti-gyrase activity (Althaus *et al.*, 1988), showing also the relevance of the aminocoumarin unit (which forms two hydrogen bonds with Arg136) (Lewis *et al.*, 1996a) and of the 3-dimethyl-allyl-4-hydroxybenzoate moiety (Galm *et al.*, 2004b) for activity.

IV.5. Are the halogenases of clorobiocin and balhimycin biosynthesis interchangeable?

The aminocoumarin antibiotic clorobiocin and the glycopeptide antibiotic balhimycin both contain 3-chloro-*b*-hydroxy-tyrosyl-derived moieties, and the biosynthesis of these moieties show striking similarities (see Fig. III.13). It is not clear at present at which step of clorobiocin biosynthesis the halogenation reaction takes place. In novobiocin biosynthesis, the methylation at C-8´ (corresponding to the chlorination at C-8´ in clorobiocin biosynthesis) occurs only after activation of tyrosine (Fig. III.13) (Chen and Walsh, 2001), and before glycosylation of novobiocic acid (Steffensky *et al.*, 2000b). Possible substrates of Clo-hal include therefore *b*-hydroxy-tyrosyl-CloH, 3-amino-4,7-dihydroxycoumarin, and the amide of this aminocoumarin unit with 3-dimethylallyl-4-hydroxybenzoic acid.

Likewise, the substrate of the halogenase BhaA of balhimycin biosynthesis is still elusive. Possible candidates are tyrosine, *b*-hydroxytyrosine in either free or enzyme-bound form, or substrates derived from later stages of peptide assembly (Süssmuth and Wohlleben, 2004).

The present study clearly showed that BhaA was unable to functionally replace Clo-hal. The structural genes for both halogenases have been cloned into the same expression vector, which contains the constitutive promoter *ermE**. Furthermore, the identical *bhaA* gene under control of this promoter has been used successfully for complementation of a *bhaA*-defective mutant (Puk *et al.*, 2002). However, only expression of *clo-hal* resulted in the formation of halogenated aminocoumarins in this thesis.

To date, three different halogenating enzymes are known: haloperoxidases, perhydrolases and $FADH_2$-dependent halogenases. Clo-hal and BhaA shows sequence similarity to the recently discovered class of $FADH_2$-dependent halogenases, which in contrast to haloperoxidases and perhydrolases show substrate specificity and regioselectivity. The mechanism of halogenation catalyzed by $FADH_2$-dependent halogenases is not yet completely understood (see also IV.1). *In vitro*, these enzymes require the presence of an unspecific flavin reductase generating $FADH_2$ (van Pée, 2001). Recently, the co-crystallization of tryptophan 7-halogenase with tryptophan and FAD was described, representing an important step towards understanding the reaction mechanism of such enzymes (Dong *et al.*, 2004).

Therefore, the inability of BhaA to functionally replace Clo-hal is unlikely to result from the specificity of the flavin reductase. Rather, the results presented here suggest that the genuine substrates for the halogenation reactions catalyzed by BhaA and Clo-hal are different. This is in accordance with recent evidence showing that in balhimycin biosynthesis halogenation apparently takes place during the formation of the oligopeptide backbone (Puk *et al.*, 2004).

IV.6. Regulation of novobiocin biosynthesis

IV.6.1. NovG, a positive regulator of novobiocin biosynthesis

The present work provides experimental proof for the function of NovG as a DNA-binding protein which acts as a positive regulator of novobiocin biosynthesis. Inactivation of *novG* led to a 98% reduction of the novobiocin productivity of the heterologous producer strain. Since Δ*novG* strains still produced some novobiocin, a catalytic role for *novG* can be ruled out. By complementation with *novG* under control of its own promoter, the novobiocin production could be restored nearly to the same level as observed before *novG* inactivation, indicating that the observed phenotype of Δ*novG* strains was indeed caused by lack of *novG*.

Expression of *novG* from a multicopy plasmid in a *S. coelicolor* strain carrying the intact novobiocin cluster led to almost three-fold overproduction of the antibiotic, suggesting that novobiocin biosynthesis in the investigated heterologous expression

host is limited by the availability of enough of the activator protein, as has been shown for other pathway-specific activators in the respective natural antibiotic producers (Gramajo *et al.*, 1993; Stutzman-Engwall *et al.*, 1992).

Furthermore, it was demonstrated that His_6 tagged NovG protein binds specifically to the *novG-novH* intergenic region and, in so doing, probably activates the transcription of *novH*, just as described for StrR which activates transcription of streptomycin biosynthetic genes by binding to their promoter regions (Retzlaff and Distler, 1995). The putative binding site of NovG is located directly downstream of the translational stop codon of *novG*, i.e. between positions -165 and -194 upstream of the putative translational start codon of *novH*. Since nickel affinity purified protein, generated in *E. coli*, was used for the DNA-binding assays, it is very likely that NovG can bind to DNA with no further macromolecular factor involved. However, our results do not completely rule out the possibility that other proteins may be required for activating the transcription of *novH*.

The novobiocin and the clorobiocin clusters show very high similarity between each other. Indeed, NovG could also bind to a DNA region of the clorobiocin cluster, i.e. the *cloG-cloY* intergenic region; the putative binding site is located between positions -160 and -189 upstream of the putative translational start of *cloY*. The function of the small ORF *cloY* remains to be elucidated. Genes with obvious similarity to *cloY* have been found in many other clusters, such as those for coumermycin A_1 (Wang *et al.*, 2000), teicoplanin (Sosio *et al.*, 2004), complestatin (Chiu *et al.*, 2001), CDA (Hojati *et al.*, 2002), and balhimycin (Pelzer *et al.*, 1999), but not in the novobiocin cluster.

The *in silico* analysis of the DNA fragments from the novobiocin and the clorobiocin cluster which bind NovG showed the presence of a perfectly conserved 9 bp inverted repeat, separated by a somewhat less conserved (two mismatches) 11 bp spacer sequence (Fig. III.20). Notably, the previously identified StrR binding sites in *S. griseus* and *S. glaucescens* contain a similar palindromic structure, i.e. conserved inverted repeats of 9 bp each separated by a non-conserved 11 bp spacer (Retzlaff and Distler, 1995). The same putative NovG binding site, with exactly the same inverted repeat and spacer sequences as found upstream of *cloY*, is also present in the coumermycin A_1 cluster, in between of the genes *couG* and *couY*. The strict similarity of the putative NovG/CloG/CouG binding sites in the novobiocin, clorobiocin

and coumermycin A_1 clusters further indicates a common evolutionary origin for these clusters (Eustáquio *et al.*, 2003b).

The consensus sequence GTTCRACTG$(N)_{11}$CRGTYGAAC could be found nowhere else in the gene clusters of novobiocin or clorobiocin except in the mentioned regions upstream of *novH* and *cloY*, respectively. This is in accordance with the experimental results of the gel shift assays with the novobiocin cluster depicted in Fig. III.17. In contrast, four StrR binding sites had been identified in the streptomycin biosynthetic gene cluster in *Streptomyces griseus*, and three in the 5´-OH-streptomycin cluster in *S. glaucescens* (Retzlaff and Distler, 1995). Comparatively, it cannot be completely excluded that NovG may also bind to additional sequences which are different from the motif shown above. However, the possibility should also be considered that only a single transcription unit is expressed under control of *novG*. In the novobiocin and clorobiocin clusters, all genes are orientated in the same direction, and may in principle be transcribed as a single mRNA from *novH* to *novW* or from *cloY* to *cloZ*, respectively. In contrast, previous results suggest that the resistance gene $gyrB^R$, at the right border of the cluster depicted in Fig. III.17, is under control of its own promoter and may be regulated by changes in DNA superhelical density (Thiara and Cundliffe, 1989). NovG was not required for expression of $gyrB^R$ in *S. lividans* (Thiara and Cundliffe, 1988), and correspondingly binding of NovG to the promoter sequence of $gyrB^R$ was not observed in the gel shift assays.

Analysis of different gene clusters encoding biosynthetic pathways for antibiotics has revealed the existence of distinct families of pathway-specific regulatory proteins. One of these families comprises the *strR*-like genes and includes, besides *novG*, *cloG* and *couG*, also *dbv4* of the glycopeptide antibiotic A40926 (Sosio *et al.*, 2003), *bbr* of the balhimycin (Pelzer *et al.*, 1999), and *tcp28* of the teicoplanin (Sosio *et al.*, 2004) cluster. Another family comprises the SARPs, i.e. ActII-ORF4 and DnrI (see Introduction) and SnoA of the nogalamycin, RedD of the undecylprodigiosin, and CcaR of the cephamycin cluster, respectively (Wietzorrek and Bibb, 1997). However, no genes with sequence similarity to this family have been found in the gene clusters of the aminocoumarin antibiotics.

An understanding of the complex regulatory mechanisms that determine the onset of antibiotic biosynthesis in actinomycetes is only just beginning to emerge (Wietzorrek and Bibb, 1997). Activation of any particular pathway of any particular organism might be expected to require its own combination of signals (Chater and Bibb, 1997). Therefore, analysis of regulatory genes from different species is crucial to build a comprehensive picture of these processes.

The aminocoumarin antibiotics may provide useful model systems for such studies, due to their very stringent genetic organization (Pojer *et al.*, 2002) and due to the availability of detailed data on the function of most genes contained therein. The fact that they can be successfully expressed in *Streptomyces coelicolor*, the genome sequence of which is available (Bentley *et al.*, 2002), makes them even more attractive for investigating the regulation cascades for improved antibiotic production in streptomycetes.

IV.6.2. Inactivation of *novE* in *S. spheroides*

In this study, it was shown that *novE*$^-$ mutants of *S. spheroides* are still able to produce novobiocin, although in significantly lower amounts in comparison to the wild-type.

This finding rules out the possibility that NovE has an essential catalytic function. Rather, NovE may act as a positive regulator in novobiocin biosynthesis. This hypothesis is supported by the presence of a TTA codon in *novE*. Many of the known TTA-containing genes specify regulatory or resistance proteins associated with biosynthetic gene clusters for antibiotics (Leskiw *et al.*, 1991).

Since *novE* was inactivated by replacement with a resistance cassette, it cannot be excluded that the observed down-regulation of novobiocin biosynthesis was due to polar effects, i.e. the transcription of downstream genes could be affected by the presence of the resistance marker. However, such effects were not observed during inactivation of *clo-hal* and *cloZ* using the same cassette. Yet, complementation experiments are necessary to confirm that the observed phenotype is only dependent on the absence of *novE*.

Indeed, it was attempted to complement the *novE*$^-$ mutant by expressing an intact copy of *novE* using plasmid pAE-E4 (a multi-copy plasmid, see Table II.15). Such experiments were however unsuccessful, due to difficulties in the genetic manipulation of *S. spheroides*, i.e. the presence of the plasmid in potential transformants could not be demonstrated.

If *S. spheroides* is so difficult to manipulate, how could a *novE*$^-$ mutant be at all constructed? There are several answers: (1) the PCR targeting method developed by Gust *et al.* (2003) allows gene inactivation experiments to be carried out much more simply and efficiently than by previous methods; (2) the modified cosmid can be introduced in *Streptomyces* by conjugation from *E. coli*, which represents usually a more efficient method than transformation, since restriction barriers may be reduced by the transfer of single-stranded concatemers of DNA (Kieser *et al.*, 2000); (3) yet the efficiency for *S. spheroides* was very low and poorly reproducible (only six ex-conjugants were obtained in one of several experiments).

Further experiments are necessary to determine a possible role for *novE* in the regulation of novobiocin biosynthesis. The successful heterologous expression of the novobiocin cluster in *S. coelicolor* reported here offers a much simpler and easier way for functional investigation of biosynthetic genes and may help reaching this goal.

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