Biosynthesis of aminocoumarin antibiotics in *Streptomyces*: Investigations on the regulation of novobiocin production

Biosynthese von Aminocoumarin-Antibiotika in *Streptomyces*: Untersuchungen zur Regulation der Novobiocinproduktion

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CONTENTS

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ABBREVIATIONS

°C	degree celsius
µ	micro
aa	amino acids
aac(3)IV	apramycin resistance gene from pIJ773
aacC4	apramycin resistance gene from Ω (omega) interposon
Amp	ampicillin
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
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
IPTG	isopropyl-*β*-thiogalactoside
k	kilo
KAc	potassium acetate
Kan	kanamycin
kb	kilo base pairs
kDa	kilo dalton
l	litre
lacZα	gene portion for α-complementation of *β*-galactosidase
M	molar
m	milli
Mb	mega base pairs
min	minute
MW	molecular weight
n	nano
NaOAc	sodium acetate
NaOH	sodium hydroxide
neo	neomycin/kanamycin resistance gene

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
PEG	polyethylene glycol
^{R}	resistant
RBS	ribosome binding site
Ring A	3-dimethylallyl-4-hydroxybenzoic acid
RNA	ribonucleic acid
RNase	ribonuclease
RP	reverse phase
rpm	rotation per minute
RT	Reverse transcriptase
s	second
s.	see
S.	*Streptomyces*
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-*β*-D-galactopyranoside

SUMMARY

The aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 are produced in different *Streptomyces* strains and are potent inhibitors of DNA gyrase. Cloning and sequencing of the corresponding biosynthetic gene clusters allowed detailed investigations of their biosynthetic pathways as well as the generation of novel antibiotics by metabolic engineering, chemo-enzymatic synthesis and precursor-directed biosynthesis. On the other hand, only limited knowledge is available about the regulation of the biosynthesis of the aminocoumarin antibiotics.

The biosynthetic gene cluster of novobiocin, clorobiocin and coumermycin A_1 each contains two putative regulatory genes with high similarity in between the clusters, i.e. *novG/cloG/couG* and *novE/cloE/couE*. The function of NovG as a DNA binding protein and positive regulator of novobiocin biosynthesis has been established previously. In the first part of this thesis, functional proof for the role of *novE* as a positive regulator of novobiocin biosynthesis is provided. Overexpression of *novE*, using a replicative shuttle vector in *S. coelicolor* strains carrying the intact novobiocin cluster has been shown to lead to almost two-fold overproduction of novobiocin, suggesting that novobiocin production is limited by the availability of NovE protein. In contrast, a *novE*-defective mutant, generated by an in-frame deletion in this study, produced only 0.7 % of the novobiocin amount formed by an *S. coelicolor* strain harboring the intact novobiocin cluster. Novobiocin production in this Δ*novE* mutant could be restored by introduction of an intact copy of *novE*, but also by overexpression of the regulatory gene *novG*.

NovE was expressed in *E. coli* and purified. However, in contrast to NovG, no DNA binding properties could be shown for NovE. The following RT-PCR experiments showed that at least some *novG* transcription can occur in the absence of NovE, and that *novE* transcription can occur in the absence of NovG. Correspondingly, overexpression of *novG* under control of its own promoter stimulated novobiocin production even in a *novE*-defective mutant.

Another part of this thesis focuses on the determination of promoter regions within the novobiocin biosynthetic gene cluster. For this purpose Ω (omega) interposons, i.e.

DNA fragments containing an antibiotic resistance marker flanked by short inverted repeats containing termination signals for transcription, were introduced into genes downstream of putative promoter regions, i.e. into *novE*, *novF*, *novG*, *novH*, *novO*, *novP*, *novQ* and *novS*, resulting in termination of mRNA synthesis at the place of insertion. Transcription is re-initiated at the next active promoter-sequence downstream of the Ω insertion. RT-PCR analysis of the generated mutants showed that the novobiocin biosynthetic gene cluster contains, in addition to previously identified promoter regions upstream of *novE* and $gyrB^R$, six further promoter regions situated upstream of *novF*, *novG*, *novH*, *novO*, *novP* and *novQ*.

In order to confirm the importance of the promoter regions identified upstream of *novO*, *novP* and *novQ*, quantitative RT-PCR experiments were carried out to quantify transcription of *novH*, *novP* and *novQ* in the Ω*novH* mutant in comparison to *S.coelicolor* strains containing the intact novobiocin cluster. The results of these investigations clearly showed that the Ω insertion into *novH* resulted not only in an almost complete loss of *novH* transcription (< 1 %), but additionally in a very strong reduction of transcription of *novP* and *novQ* (<3 %). This finding strongly suggests that transcription of *novO*, *novP* and *novQ* (and of the genes located downstream thereof) is mainly controlled by the *novH* promoter initiating a large transcript of at least 18 kb, i.e. from *novH* to *novW*. Furthermore, quantitative RT-PCR was used for investigations of the interplay of the two positive regulators *novE* and *novG*, as well as on their influence on the novobiocin biosynthetic genes. These investigations showed that both *novE* and *novG* act as transcriptional activators of the genes of novobiocin biosynthesis by initiating transcription from the *novH*-promoter. *novE* and *novG* act in a cascade-like reaction mechanism, i.e. *novE* positively regulates transcription of *novG* and NovG regulates transcription of all genes from the *novH* promoter by binding to a well-defined inverted repeat sequence in the intergenic region between *novG* and *novH*.

Based on the results presented above, the final part of this thesis deals with the uncoupling of novobiocin production from its natural regulation cascade by replacing the entire *novEFG*-region, including the promoter region upstream of *novH*, by a strong inducible promoter. For this purpose, the tetracycline-controllable promoter 830 (tcp830) was used. It has been shown previously, by using *luxAB* genes expressing luciferase as a reporter system, that induction factors of up to 270 could

be obtained for tcp830 by induction with anhydrotetracycline. HPLC analysis of novobiocin production in the resulting mutants showed that an induced tcp830 promoter is sufficient to cause transcription of the genes from *novH* to *novW*, i.e. of a polycistronic mRNA of >18.000 nt, resulting in a two-fold overproduction of novobiocin in comparison to strains containing the unmodified novobiocin gene cluster. Therefore, regulation of novobiocin production by tcp830 has been confirmed as a strategy to uncouple novobiocin production from its natural regulation and notably as a further, newly discovered tool to enhance antibiotic production.

ZUSAMMENFASSUNG

Die Aminocoumarin-Antibiotika Novobiocin, Clorobiocin und Coumermycin A_1 werden von unterschiedlichen *Streptomyces*-Stämmen gebildet und sind potente Gyrasehemmstoffe. Klonierung und Sequenzierung der Biosynthesegencluster dieser Antibiotika ermöglichten detaillierte Untersuchungen der Biosynthesewege und die Herstellung neuer Antibiotika durch metabolisches Engineering, chemo-enzymatische Synthese und Vorstufen-gerichtete Biosynthese. Das Wissen über die Regulation der Aminocoumarin-Biosynthese ist jedoch begrenzt.

Die Biosynthese-Gencluster von Novobiocin, Clorobiocin und Coumermycin A_1 enthalten jeweils zwei putative Regulatorgene, *novG/cloG/couG* und *novE/cloE/couE,* mit starker Ähnlichkeit zwischen diesen Clustern. Die Funktion von NovG als DNA-bindendes Protein und positiver Regulator in der Novobiocinproduktion wurde bereits vor Beginn dieser Arbeit nachgewiesen. Im ersten Teil dieser Arbeit wurde der Funktionsnachweis für *novE* als positiver Regulator der Novobiocinproduktion erbracht. Die Überexpression von *novE* mit Hilfe eines replikativen Shuttle-Vektors in *Streptomyces* coelicolor-Stämmen mit intaktem Novobiocin-Biosynthesegencluster führte zu einer nahezu zweifachen Überproduktion von Novobiocin, was darauf hindeutet, dass die Novobiocinproduktion durch die Verfügbarkeit von NovE-Protein begrenzt wird. Im Gegensatz dazu produzierte die in dieser Arbeit durch „in-frame“ Deletion hergestellte *novE*-Defektmutante lediglich 0.7% der Novobiocinmenge, die von *Streptomyces coelicolor* Stämmen mit intaktem Novobiocincluster produziert wird. Die Novobiocinproduktion in dieser *novE*-Defektmutante konnte nicht nur durch das Einbringen einer intakten Kopie von *novE*, sondern auch durch Überexpression von *novG* wiederhergestellt werden.

NovE wurde in *Escherichia coli* exprimiert und aufgereinigt. Im Gegensatz zu Experimenten mit NovG konnte für NovE jedoch keine DNA-bindende Eigenschaft nachgewiesen werden. Die folgenden RT-PCR Untersuchungen zeigten, dass zumindestens etwas *novG*-Transkription in Abwesenheit von NovE, und *novE*-Transkription in Abwesenheit von NovG stattfindet. In Übereinstimmung damit stimulierte die Überexpression von *novG* unter Kontrolle des eigenen Promotors die Novobiocinproduktion auch in der *novE*-Defektmutante.

Der folgende Teil dieser Arbeit zielte auf die Identifizierung der Promotorregionen innerhalb des Novobiocin-Biosynthesegenclusters. Zu diesem Zweck wurden Ω-(omega) Interposons, das heißt DNA-Fragmente, die einen Antibiotika-Resistenzmarker beidseitig flankiert durch Sequenzen, die Terminationssignale für die Transkription beinhalten, in Gene downstream von putativen Promotorregionen eingebracht, das heißt in *novE*, *novF*, *novG*, *novH*, *novO*, *novP*, *novQ* und *novS*. Dies führte zu einer Termination der mRNA-Synthese. Die Wiederaufnahme der Transkription erfolgt von der nächsten aktiven Promotor-Sequenz downstream der Ω-Insertion. RT-PCR Untersuchungen der generierten Mutanten zeigten, dass im Novobiocin-Biosynthesegencluster, zusätzlich zu den bereits identifizierten Promotorregionen upstream von *novE* und $gyrB^R$, sechs weitere Promotorregionen enthalten sind, die sich upstream von *novF*, *novG*, *novH*, *novO*, *novP* und *novQ* befinden.

Zur Bestätigung der Rolle der Promotorregionen upstream von *novO*, *novP* und *novQ*, wurden quantitative RT-PCR (qRT-PCR) Experimente durchgeführt um die Transkription von *novH*, *novP* und *novQ* in der Ω*novH*-Mutanten im Vergleich zu *Streptomyces* coelicolor-Stämmen mit intaktem Novobiocincluster zu quantifizieren. Die Ergebnisse dieser Untersuchungen zeigten eindeutig, dass die Ω-Insertion in *novH* nicht nur zu einem nahezu vollständigen Ausfall der *novH*-Transkription (< 1%), sondern auch zu einer sehr starken Reduktion der Transkription von *novP* und *novQ* (<3 %) führt. Diese Ergebnisse machen wahrscheinlich, dass die Transkription von *novO*, *novP* und *novQ* sowie der downstream davon liegenden Gene hauptsächlich über den *novH*-Promotor kontrolliert wird, von dem ein mindestens 18 kb großes Transkript, das heißt von *novH* bis *novW*, gebildet wird. Außerdem wurde mittels qRT-PCR das Zusammenspiel der beiden positiven Regulatoren *novE* und *novG*, sowie deren Einfluss auf die Novobiocin-Biosynthesegene untersucht. In diesen Untersuchungen wurden *novE* und *novG* als Transkriptionsaktivatoren der Novobiocinproduktion bestätigt, welche in einer Regulationskaskade die Transkription vom *novH*-Promotor aus initiieren. Offenbar aktiviert *novE* die Transkription von *novG* und NovG reguliert über den *novH*-Promotor die Transkription aller Gene durch Bindung an eine definierte Sequenz im intergenischen Bereich zwischen *novG* und *novH*.

Basierend auf den oben präsentierten Ergebnissen beschäftigte sich der letzte Teil dieser Arbeit mit der Entkopplung der Novobiocinproduktion von deren natürlichem Regelkreis durch Austausch der gesamten *novEFG*-Region, einschließlich der Promotorregion upstream von *novH*, gegen einen starken Promotor. Hierzu wurde der Tetracyclin-induzierbare Promotor 830 (tcp830) verwendet, für den mit den Luciferase-kodierenden Genen *luxAB* als Reporter-System Induktionsfaktoren von bis zu 270 beschrieben wurden. Die HPLC-Analyse der Novobiocinproduktion in den generierten Mutanten zeigte, dass der tcp830-Promotor erfolgreich eingesetzt werden kann, um eine 18 kb umfassende Region von *novH* bis *novW* zu transkribieren, was zu einer zweifachen Überproduktion von Novobiocin führte. Die Regulation der Novobiocinproduktion über tcp830 wurde dadurch nicht nur als eine Strategie zur Entkopplung der Novobiocinproduktion von deren natürlichem Regulationskreis, sondern auch als ein weiteres, neue entdecktes Werkzeug zur Erhöhung der Antibiotikaproduktion bestätigt.

I. INTRODUCTION

I.1. *Streptomyces* – the largest antibiotic-producing genus

Streptomyces spp. are Gram-positive, filamentous, soil bacteria and ubiquitous in nature. They belong to the order Actinomycetales. Remarkable features of *Streptomyces* are the high G+C ratio of their DNA (> 70%) and their large chromosome, which consists of 8-10 Mb (Bentley *et al.*, 2002; Bibb *et al.*, 1984; Ikeda & Nakagawa, 2003).

Furthermore, they are remarkable in terms of their morphological and metabolic differentiation phenomena (Hopwood, 1999). During their complex life cycle, *Streptomyces* differentiate into at least three distinct cell types: substrate hyphae, aerial hyphae and spores. Germination of spores is the first step in the development of *Streptomyces*, giving rise to a basal or substrate mycelium, which develops in close contact with the nutritive substrate. In response to poorly identified signals emanating from the environment and from cellular metabolism, the basal mycelium develop aerial hyphae, which ramify and differentiate into spores (Chater, 1998; Chater & Horinouchi, 2003). The passage from one mycelial state to the other is characterised by a pause in growth and requires the expression of numerous genes specific to the aerial mycelium.

This morphological diffentiation is accompanied by metabolic differentiation. Generally, *Streptomyces* spp. produce a large number of very diverse secondary metabolites during the later stages of this development. Genes responsible for the synthesis of specific secondary metabolism products are clustered on the chromosome. These clusters may contain all biosynthetic, regulatory and resistance genes required for production of antibiotics. Notably, over two thirds of the clinically useful antibiotics of natural origin are produced by Streptomycetes (Kieser *et al.*, 2000). Till now, over 3000 biological active compounds have been isolated from Streptomycetes (including important antibiotics like tetracyclins, vancomycin and erythromycin) (Watve *et al.*, 2001). Further bioactive compounds produced by streptomycetes are used as antitumoural, antifungal and antiviral agents or as herbicides.

I.2. Regulation and overproduction of antibiotics production in *Streptomyces*

Antibiotics represent the industrially most important group of secondary metabolites. Since two thirds of the clinically useful antibiotics of natural origin are produced by *Streptomyces* (Kieser *et al.*, 2000) it is of major interest to understand how these filamentous soil bacteria synthesize antibiotics, to allow manipulation of biosynthetic pathways for production of novel compounds with improved properties. Further investigations focus on the identification and characterisation of genes involved in the regulation of antibiotic biosynthesis, and the use of the resulting knowledge to enhance secondary metabolite production.

Biosynthesis of secondary metabolites in Streptomycetes is a complex process involving different mechanisms of regulation. Two phylogenetically distant species have received especially extensive attention so far: *S. coelicolor* A3(2) because of its early development to a thoroughly studied genetic system which allowed the analysis of the interrelation between morphological differentiation and secondary metabolite formation; and *S. griseus* because it provided the first well-studied bacterial example of extracellular signalling by a hormone-like lactone, the γ-butyrolactone A-factor (Chater & Horinouchi, 2003). In both organisms, antibiotic production is affected by pleiotropic regulatory genes that influence the expression of pathway-specific regulatory genes which are clustered together with the structural genes of the biosynthetic enzymes.

In *S. griseus*, transcription of streptomycin biosynthetic genes is activated by StrR, a pathway-specific regulatory protein which binds to DNA loci containing an inverted repeat with the consensus sequence GTTCGActG$(N)_{11}$CagTcGAAc. These loci are situated upstream of the respective promoter regions (Retzlaff & Distler, 1995; Tomono *et al.*, 2005). Four StrR binding sites have been identified in the streptomycin biosynthetic gene cluster in *S. griseus* and three in *S. glaucescens*. StrR contains a putative helix-turn-helix (HTH) motif (Retzlaff & Distler, 1995) which is typical for a family of bacterial and phage DNA-binding proteins (Pabo & Sauer, 1992).

ActII-ORF4 from *S. coelicolor* (Arias *et al.*, 1999; Wietzorrek & Bibb, 1997) and *DnrI* from *S. peucetius* (Sheldon *et al.*, 2002; Wietzorrek & Bibb, 1997) belong to another family of regulatory proteins (SARPs). Their predicted secondary structure is an OmpR-like DNA-binding domain, and this structure is different from the typical HTH motif (Sheldon *et al.*, 2002; Wietzorrek & Bibb, 1997). These proteins act as transcriptional activators of target genes by binding to DNA loci that contain direct heptameric repeats with the consensus sequence TCGAGCG/C. These loci are situated close to the transcriptional start sites (Arias *et al.*, 1999; Wietzorrek & Bibb, 1997).

Another extensively studied system is the regulation of tylosin biosynthesis in *Streptomyces fradiae*. Tylosin biosynthesis is activated by two positive regulatory genes *tylS* and *tylR*, which act in a cascade-like reaction mechanism, and their overexpression could be used to enhance tylosin production (Bate *et al.*, 2006; Bibb, 2005).

In other *Streptomyces* strains further types of pathway-specific regulatory genes have been found, like *smrR* in *S. ambofaciens* which encoded protein shows no significant sequence similarity to any other known regulatory protein; the negative regulator *mmyR* of the methylenomycin cluster in *S. coelicolor*; and *dnrN* of the daunorubicin cluster in *S. peucetius* as well as *redZ* of the undecylprodigiosin cluster in *S. coelicolor*, both representing response regulatory genes of two-component systems. However, in some pathways no regulatory gene has been found, i.e. in erythromycin pathway (Rawlings, 2001).

Large-scale industrial fermentation requires strains producing high amounts of compound to ensure cost effective production, which has been traditionally obtained by strain improvements, i.e. expression of the biosynthetic gene cluster in a heterologous host or in an industrially optimized strain. Increased secondary metabolite production has additionally been obtained by i) metabolic engineering redirecting primary metabolic fluxes by introduction of genetic modifications through recombinant DNA technology, in a manner to support high secondary metabolite productivities (Adrio & Demain, 2006; Nielsen, 1998), ii) engineering antibiotic resistance, i.e. actinorhodin and undecylprodigiosin in *S. coelicolor* and *S. lividans* by introducing point mutations in the *rspL* gene, encoding the ribosomal protein S12,

which confers resistance to streptomycin (Okamoto-Hosoya *et al.*, 2003; Shima *et al.*, 1996), iii) deregulation of the expression of secondary metabolite pathways, by overexpressing pathway-specific positive regulators, i.e. actinorhodin and undecylprodigiosin in *S. coelicolor* by *actII-orf4* and *redD* (Fernández-Moreno *et al.*, 1991; Narva & Feitelson, 1990), undecylprodigiosin in *S.lividans* and *S.parvulus* by *redD* (Malpartida *et al.*, 1990), nikkomycin in *S.ansochromogenes* by *sanG* (Liu *et al.*, 2005) and clavulanic acid in *S.clavuligerus* by *ccaR* (Hung *et al.*, 2007; Pérez-Llarena *et al.*, 1997) or vice versa, by inactivation of pathway repressors, i.e. chromomycin in *S.griseus* subsp. *griseus* by inactivation of the transcriptional repressor *cmmRII (Menendez et al., 2007)*. Subsequently, the exchange of regulatory genes and promoter regions with ermEp*, representing a constitutive promoter, has been reported not only to abolish secondary metabolite production from its origin regulation circuit, but also to result in enhanced production titers, i.e. the replacement of four regulatory genes including the promoter region P_J in the jadomycin gene cluster by ermEp* resulted in increased jadomycin B production in *Streptomyces venezuelae* (Zheng *et al.*, 2004).

I.3. Aminocoumarin antibiotics

The closely related aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 are produced by different *Streptomyces* strains. Novobiocin is produced by *S. spheroides* NCIMB 11891 (Berger *et al.*, 1978) and *S. niveus* (Smith *et al.*, 1956). However, Lanoot *et al.* have proposed that these two strains are synonyms of *S. caeruleus* LMG 19399T (Lanoot *et al.*, 2002). 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 produced by *S. rishiriensis*, *S. hazeliensis* var. *hazeliensis*, *S. spinichromogenes* and *S. spinicoumarensis* (Berger *et al.*, 1978; Kawaguchi *et al.*, 1965). Simocyclinone D8 (Schimana *et al.*, 2000) and rubradirin (Sohng *et al.*, 1997) are two further aminocoumarins found in nature, which are also produced by different *Streptomyces* strains.

I.3.1. Chemical structure

Novobiocin, Clorobiocin and Coumermycin A_1 contain three structural moieties: a 3-amino-4,7-dihydroxycoumarin moiety (named Ring B), a deoxysugar moiety (named Ring C) and an acyl component (Fig. I.1). Ring B is linked to the acyl component via an amide bond, and to Ring C via a glycoside bond. Clorobiocin and novobiocin share the same 3-dimethylallyl-4-hydroxybenzoyl moiety (named Ring A) as acyl component. Clorobiocin differs from novobiocin at two positions: novobiocin bears a carbamoyl group at position 3 of the noviose moiety, while clorobiocin contains a 5-methylpyrrole-2-carboxylic acid, and clorobiocin, as indicated by its name, carries a chlorine atom at position 8 of Ring B, while novobiocin carries a methyl group. Coumermycin A_1 contains two noviosyl aminocoumarin moieties, and carries a different acyl component in comparison to novobiocin and clorobiocin, i.e. a 3-methyl-pyrrole-2,4-dicarboxylic acid. Coumermycin A_1 contains, similar to novobiocin, a methyl group at position 8 of Ring B and contains, similar to clorobiocin, the same 5-methylpyrrole-2-carboxylic acid at position 3 of the noviose moiety (Fig. I.1).

Simocyclinone D8 and rubradirin contain the same aminocoumarin ring as the three closely related aminocoumarin antibiotics described above. Furthermore, simocyclinone D8 carries a chlorine atom at position 8 of its aminocoumarin ring just as clorobiocin. In contrast to novobiocin, clorobiocin and coumermycin A1, simocyclinone D8 and rubradirin do not carry a noviose moiety, and their acyl components are large and complicated structures (Fig. I.1).

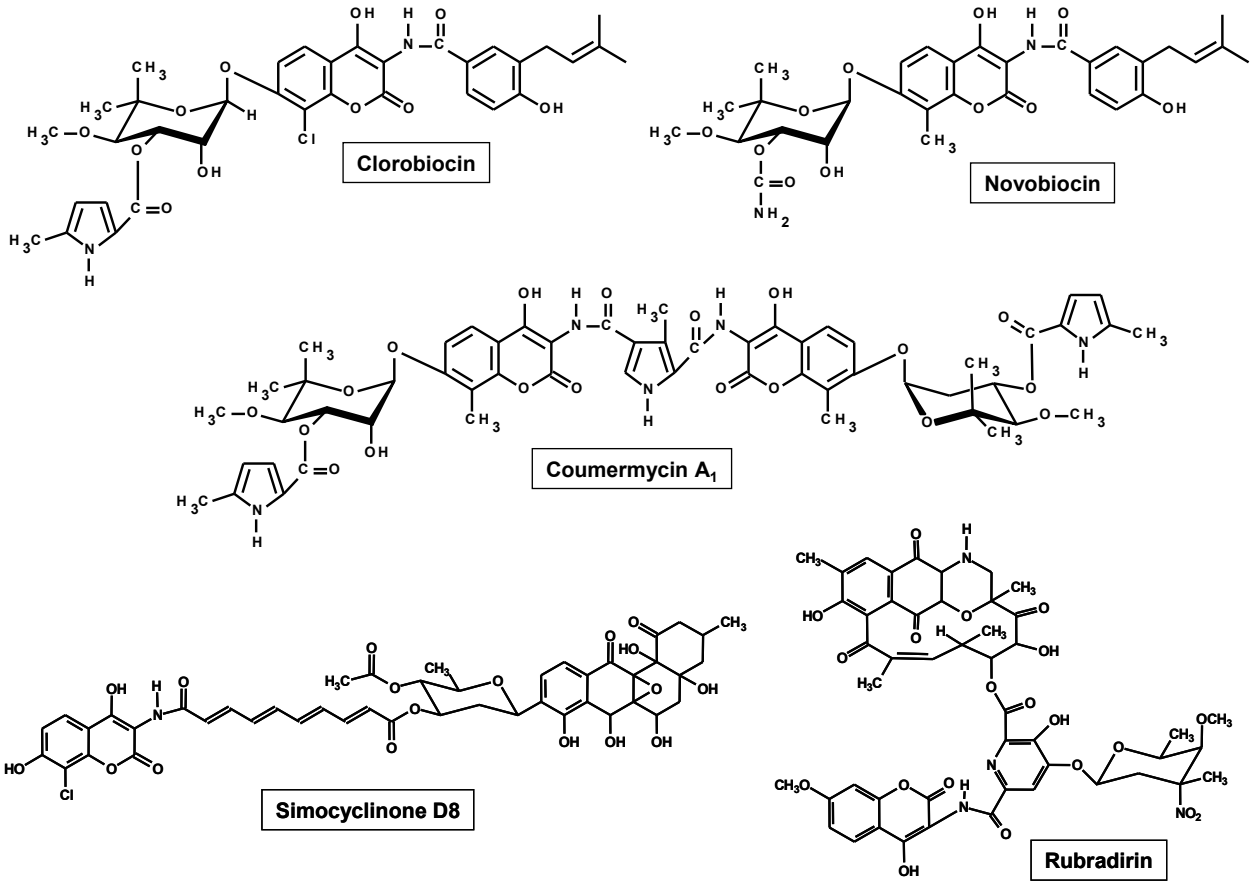

Fig. I.1: Structure of the aminocoumarin antibiotics.

I.3.2. Mechanism of action and clinical application

Novobiocin, Clorobiocin and Coumermycin A_1 are potent inhibitors of bacterial DNA gyrase. The affinity of these drugs to bacterial gyrase is extremely high. The equilibrium dissociation constants (KDs) are in the 10 nM range, i.e. two orders of magnitude lower in comparison to modern fluoroquinolones. Gyrase belong to type II topoisomerase, which are present in all cell types. DNA topoisomerases are essential for cell viability and involved in important DNA-processes, i.e. DNA transcription, recombination and replication. The presence of DNA-gyrase in all bacteria, but not in eukaryotes makes it to a good target for antibacterial agents. With regard to its function DNA-Gyrase is responsible for the ATP-dependent introduction of negative supercoils into double-stranded DNA. It consists of two subunits: GyrA and GyrB, which together build up the active enzyme complex, an A_2B_2 heterodimer. DNA

gyrase has already been isolated from many different bacteria, but so far the one by *Escherichia coli* represents the best characterised. GyrA, a protein of 97 kDa, includes an N-terminal domain involved in breakage-resealing reactions and a C-terminal domain, involved in the DNA-protein interaction. GyrB, a protein of 90 kDa, also includes a N-terminal and a C-terminal domain. While the N-terminus (43 kDa) catalyses ATP hydrolysis, the C-terminus interacts with GyrA and DNA (Maxwell, 1997; Maxwell, 1999).

All three aminocoumarin antibiotics compete with ATP for binding to the B subunit of DNA gyrase and inhibit the ATP-dependent DNA supercoiling catalysed by gyrase (Maxwell, 1999; Maxwell & Lawson, 2003). In comparison, fluorquinolones inhibit gyrase-catalysed supercoiling by disrupting the DNA breakage-reunion reaction on GyrA (Jacoby, 2005). X-ray crystallographic studies with the 24 kDa N-terminal subdomain of *Escherichia coli* GyrB showed that both, the aminocoumarin and the substituted deoxysugar moieties of these substances are essential for their binding to the B-subunit of Gyrase. In these studies the resulting complexes between novobiocin (Lewis *et al.*, 1996) and clorobiocin (Tsai *et al.*, 1997) and the protein involve hydrophobic interactions and a network of hydrogen bonds. Especially hydrogen bonds between Arg136 and the aminocoumarin ring, Asp73 and the 3-acyl group on the deoxysugar, and Asn46 and the 2-hydroxyl group on the deoxysugar represent key bonds. 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.

Further studies showed that Coumermycin A_1 stabilize a dimer form of the 43 kDa N-terminal fragment of GyrB. This is consistent with the pseudo-dimeric structure of coumermycin (Fig. I.1)(Maxwell & Lawson, 2003).

Topoisomerase IV (topo IV), another bacterial type II topoisomerase has been proposed of being a further target of novobiocin (Hardy & Cozzarelli, 2003). Topoisomerase IV has a structure similar to bacterial DNA-gyrase: two ParC and two ParE subunits building up a C_2E_2-complex. ParC is responsible for DNA breakage and reunion, and ParE contains the ATP binding-site. Mutational experiments in the ParE subunit led to the conclusion, that novobiocin inhibits topo IV by the same mechanism as DNA gyrase is being inhibited.

Aminocoumarin antibiotics show a strong activity against gram-positive bacteria, including methicillin- and vancomycin-resistant *Staphylococcus* strains. Novobiocin (Albamycin®, Pharmacia & Upjohn) was licensed as drug 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, clinical use of these antibiotics remains restricted, 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) (Maxwell, 1993). In comparison, clorobiocin and coumermycin have not been used clinically. Therefore, the generation of new, structurally modified aminocoumarin antibiotics, and of course the test whether they may be able to overcome limitations of the known compounds, is still of major interest (Maxwell & Lawson, 2003).

Further studies showed that aminocoumarins act synergistically with antitumour drugs (Lorico *et al.*, 1992; Rappa *et al.*, 1992; Rappa *et al.*, 2000a; Rappa *et al.*, 2000b). Additionally, interactions with eukaryotic heat shock protein 90 (Hsp90) have been described (Burlison & Blagg, 2006; Burlison *et al.*, 2006; Huang & Blagg, 2007).

I.3.3. Structure-activity relationships

In vitro investigations on the inhibitory activity of aminocoumarin antibiotics and their analogues towards *E. coli* DNA gyrase as well as investigations on their antibacterial activity against *E. coli* cells showed, that the aminocoumarin moiety linked to the substituted deoxysugar is important and that lack of the pyrrole or carbamoyl substituent resulted in loss of inhibitory activity (Hooper *et al.*, 1982). Furthermore, novenamine, i.e. aminocoumarin moiety plus substituted deoxysugar has been described to represent the minimal structural entity of novobiocin to obtain interaction with DNA gyrase (Reusser & Dolak, 1986). This finding is in accordance with X-ray data presented above. The aminocoumarin and deoxysugar moiety alone did not show any activity in antibacterial and anti-gyrase activities (Althaus *et al.*, 1988; Hooper *et al.*, 1982; Reusser & Dolak, 1986).

Coumermycin A_1 was the most active compound (Hooper *et al.*, 1982). As mentioned above, it has been speculated that this effect may be caused by its pseudo-dimeric

structure (Maxwell & Lawson, 2003). In comparison to many aryl and alkyl substituents tested, the prenylated benzoic acid moiety of novobiocin and clorobiocin, i.e. Ring A, was the most effective (Galm *et al.*, 2004; Hooper *et al.*, 1982). Ring A was supposed to be unimportant in DNA gyrase interactions and to only facilitate the absorption of aminocoumarin compounds (Lewis *et al.*, 1996), which, however, was disproved (Freitag *et al.*, 2004; Galm *et al.*, 2004; 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.*, 1996; Tsai *et al.*, 1997). Most authors have attributed the higher activity of clorobiocin primarily to the pyrrole moiety at C-3 of the deoxysugar moiety (Berger *et al.*, 1978; Tsai *et al.*, 1997). Interestingly, however, clorobiocin acid, the aglycon of clorobiocin, but not novobiocin acid, was found to inhibit both DNA synthesis *in vivo* and gyrase activity *in vitro* (Althaus et al., 1988; Reusser & Dolak, 1986), suggesting that the chlorine atom makes an important contribution to the biological activity of this molecule.

I.3.4. Biosynthetic gene clusters

Structural differences and similarities between novobiocin, clorobiocin and coumermycin A_1 have been found to be remarkably well reflected by differences and similarities in the organization of the respective biosynthetic gene clusters (Pojer *et al.*, 2002) (Fig. I.2).

In addition to candidate genes for the biosynthetic reactions, in all three clusters a gene for aminocoumarin-resistant *gyrB* subunit was found downstream of the biosynthetic genes for the deoxysugar. The clorobiocin and coumermycin A_1 clusters contain a second aminocoumarin-resistant *gyrB* homologue, i.e. *parY*^{R} (Fig. I.2).

The genes located upstream of *nov/clo/couE* and downstream of the resistance genes are completely different for each cluster (Eustáquio *et al.*, 2003; Schmutz *et al.*, 2003). These genes may be involved in the primary metabolism, indicating that *nov/clo/couE* and *gyrB*^{R}*/parY*^{R} may represent the left and the right borders of these clusters respectively (Eustáquio *et al.*, 2003; Schmutz *et al.*, 2003).

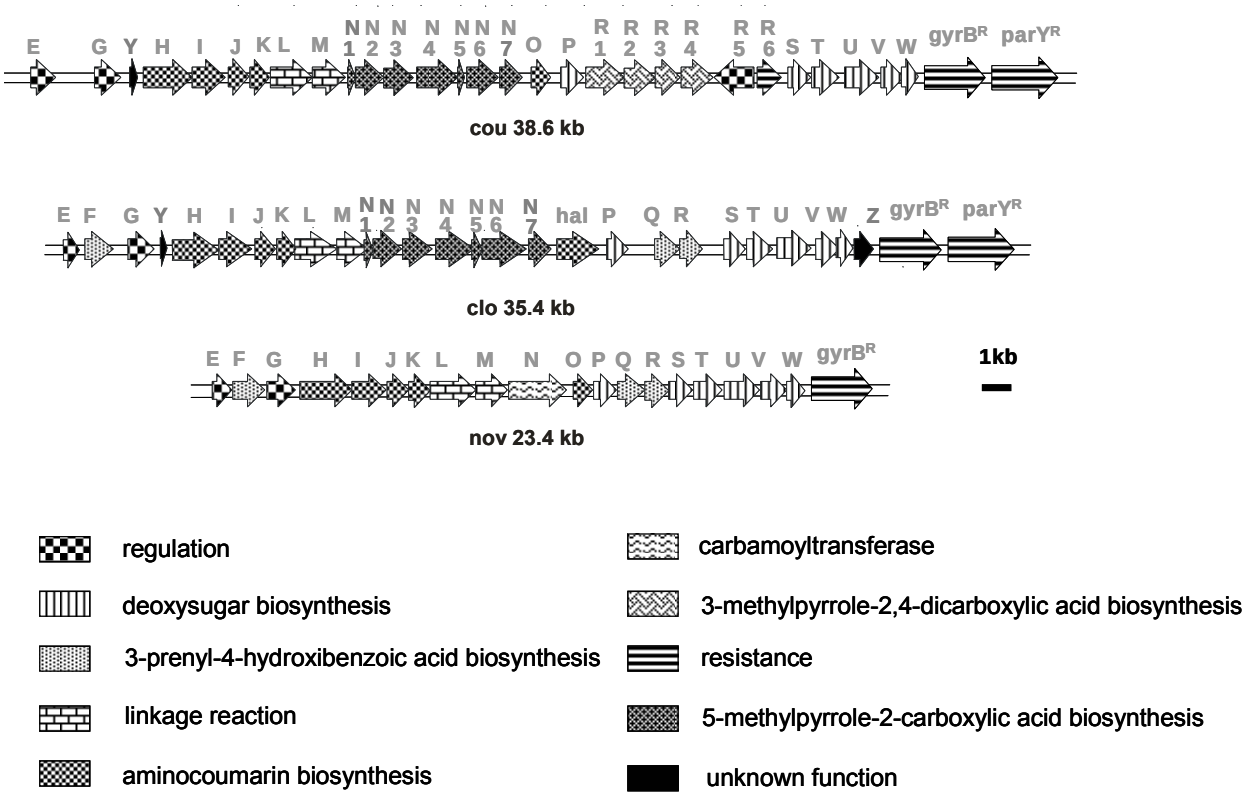

Fig. I.2: The biosynthetic gene clusters of novobiocin (*nov*), clorobiocin (*clo*) and coumermycin A_1 (*cou*). Accession numbers: novobiocin cluster AF170880, clorobiocin cluster AF329398, coumermycin cluster AF235050 (Li & Heide, 2004).

I.3.4.1. Novobiocin biosynthesis

The novobiocin biosynthetic gene cluster spans 23.4 kb and comprises 20 coding sequences (Li & Heide, 2004; Li *et al.*, 2006; Li & Heide, 2006). The genes *novHIJKLM* are responsible for the synthesis and the linkage of the aminocoumarin moiety. *novQR* are responsible for the generation of the prenylated 4-hydroxybenzoate moiety, and *novSTUVW* for the generation of the deoxysugar. *novN*, *novO* and *novP* encode enzymes involved in tailoring reactions, i.e. the carbamoylation and methylation of the novobiocin skeleton. *novF* is probably responsible for the availability of 4-hydroxyphenylpyruvate, a precursor of both aromatic moieties of novobiocin.

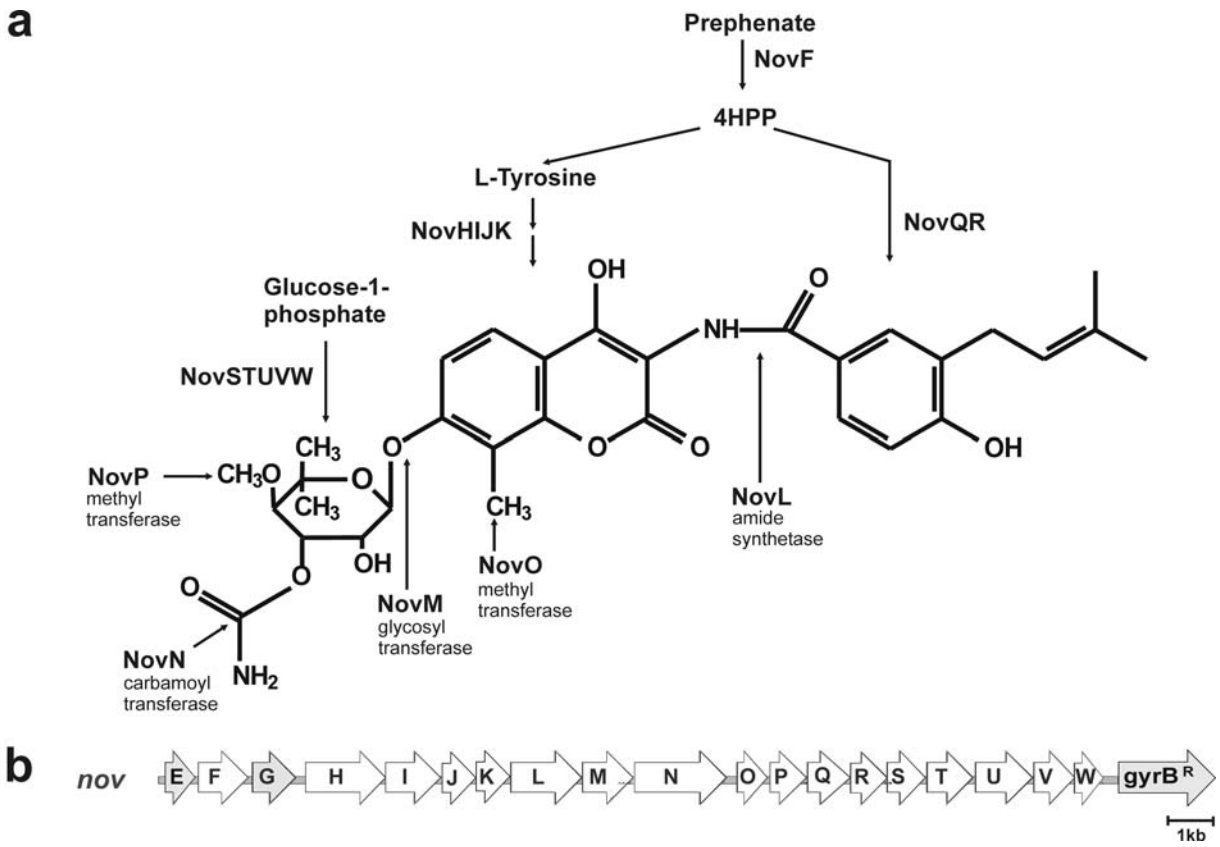

Fig. I.3: (a) Structure of novobiocin, and function of the gene products of *novHIJKLMNOPQRSTUVW* in novobiocin biosynthesis; 4 HPP, 4-hydroxyphenylpyruvate (Li & Heide, 2004). **(b)** Novobiocin biosynthetic gene cluster.

I.3.5. Resistance genes

Biosynthesis of antibiotics with high biological activity requires mechanisms to protect the producer against the inhibitory effect of these compounds. It has been shown that the resistance mechanism of the novobiocin producer *S. spheroides* is based on the *de novo* synthesis of the amincoumarin-resistant GyrB subunit (Thiara & Cundliffe, 1988). This is obtained by the replacement of the aminocoumarin-sensitive $GyrB^S$ subunit in the active heterodimer of bacterial DNA-gyrase by $GyrB^R$-protein. Therefore, the respective *gyrB*^{R}-gene was identified in all three clusters, as expected, since the producers must obviously protect their gyrases from the inhibitory effect of aminocoumarins during antibiotic production. In addition to *gyrB*^{R}, the coumermycin A_1 and clorobiocin, but not the novobiocin cluster contain a further gene, i.e. *parY*^{R}, with high sequence similarity to the B-subunit of type II topoisomerases. Heterologous expression of *gyrB*^{R} and *parY*^{R} in *S. lividans* TK24 resulted in similar levels of resistance against novobiocin and coumermycin A_1 (Schmutz *et al.*, 2003).

Furthermore, it has been shown that $GyrB^R$ leads to resistance against novobiocin and coumermycin A_1. Heterologous expression and purification of $GyrB^R$ and $ParY^R$ of the coumermycin A_1 gene cluster and the corresponding topoisomerase subunits GyrA and ParX of *Streptomyces coelicolor* showed, that ATP-dependent DNA supercoiling is catalysed by the *in vitro* complex of GyrA and $GyrB^R$. For the complex of ParX and $ParY^R$ an ATP-dependent function of decatenation and relaxation of DNA was demonstrated. This function was usually assigned to the topoisomerase IV (Schmutz *et al.*, 2004). This finding strongly supports the hypothesis, that topo IV may represent a further target for aminocoumarins.

I.3.6. Regulation of aminocoumarin-antibiotic production

Cloning and sequencing of the biosynthetic gene clusters of the aminocoumarin antibiotics (Pojer *et al.*, 2002; Steffensky *et al.*, 2000; Wang *et al.*, 2000) allowed detailed investigations of the biosynthetic pathways (Li & Heide, 2004) as well as the generation of novel antibiotics by metabolic engineering, chemo-enzymatic synthesis and precursor-directed biosynthesis (Li & Heide, 2005).

On the other hand, only limited knowledge is available on how aminocoumarin antibiotic production is regulated. The novobiocin biosynthetic gene cluster contains two putative regulatory genes, *novE* and *novG*. Close orthologues of both genes are found in the gene clusters of clorobiocin and coumermycin A_1.

NovG shows 41 % identitiy at the amino acid level to StrR, the pathway-specific transcriptional activator of streptomycin biosynthesis in *Streptomyces glaucescens* and *S. griseus* (Retzlaff & Distler, 1995; Thamm & Distler, 1997; Tomono et al., 2005). The predicted amino acid sequence of NovG shows a putative HTH motif in the central region of the protein, which is typical for a family of bacterial and phage DNA-binding proteins (Pabo & Sauer, 1992). This motif is also found in StrR (Retzlaff & Distler, 1995; Thamm & Distler, 1997).

Since the natural novobiocin producer *Streptomyces spheroides* is difficult to manipulate genetically (Hussain & Ritchie, 1991), the biosynthetic gene cluster of novobiocin was expressed in *S. coelicolor* M512 (Eustáquio *et al.*, 2005a) which genome sequence (of the wild-type strain *S.coelicolor*A3(2)) is available (Bentley *et*

al., 2002). In comparison to the natural producer strain the heterologous host showed equal novobiocin production titers (Eustáquio *et al.*, 2005a). However, engineering of the novobiocin pathway is much easier in the heterologous host than in the wild-type strain.

Inactivation of *novG* led to a 98 % reduction of the novobiocin productivity of the heterologous producer strain (Eustáquio *et al.*, 2005b). Due to the fact that Δ*novG* strains still produced some novobiocin, an essential catalytic role for *novG* could be ruled out. This finding also indicated a low level expression of the biosynthetic genes in absence of NovG. Introduction of *novG* with its own putative promoter (i.e. 336 bp upstream of the start codon of *novG*) in the expression vector pWHM3 into the Δ*novG* strain led to restoration of novobiocin production nearly to the same level (80%) as observed before *novG* inactivation, indicating that the observed phenotype of Δ*novG* strains was indeed caused by the lack of *novG* (Eustáquio *et al.*, 2005b).

In comparison, expression of *novG* from a multicopy plasmid in a *S. coelicolor* M512 strain carrying the intact novobiocin cluster resulted in a 2.7-fold overproduction of novobiocin, suggesting that novobiocin biosynthesis in the heterologous expression host is limited by availability of the activator protein (Eustáquio *et al.*, 2005b).

As mentioned above, NovG shows sequence similarity to StrR a positive regulator of streptomycin biosynthesis. StrR binds to DNA loci containing an inverted repeat with the consensus sequence GTTCGActG$(N)_{11}$CagTcGAAc. These loci are situated upstream of the respective promoter regions (Retzlaff & Distler, 1995).

EMSA (electrophoretic mobility shift assays), in which selected DNA fragments of the novobiocin biosynthetic gene cluster were used, demonstrated that His_6-tagged NovG protein binds specifically to the *novG-novH* intergenic region (Eustáquio *et al.*, 2005b). Therefore, it has been speculated that this binding may activate transcription of *novH*, just as described for StrR, which activates transcription of streptomycin biosynthetic genes by binding to their promoter regions (Retzlaff & Distler, 1995), (Tomono *et al.*, 2005). The putative binding-site of NovG is located directly downstream of the translational stop codon of *novG*, i.e. between -165 and -194 bp upstream of the putative translational start codon of *novH*. Due to the fact that EMSA was carried out with nickel affinity purified protein, generated in *E. coli*, it is very likely that NovG binds to DNA with no further macromolecular factor involved. However,

the possibility that other proteins may be required for activating the transcription of *novH* may not completely be ruled out.

As mentioned above, the novobiocin and clorobiocin gene clusters are highly similar to each other on amino acid level. It was demonstrated that 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 codon of *cloY* (Eustáquio *et al.*, 2005b).

The *in silico* analysis of the DNA fragments from the novobiocin and clorobiocin clusters which bind NovG showed the presence of a conserved 9 bp inverted repeat , separated by a less-conserved (two missmatches) 11 bp spacer sequence (Eustáquio *et al.*, 2005b). 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 & 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, between genes *couG* and *couY*. The close 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.*, 2003).

The consensus sequence GTTCRACTG$(N)_{11}$CRGTYGAAC or similar motifs were not found anywhere else in the gene clusters of novobiocin and clorobiocin, except in the mentioned regions upstream of *novH* and *cloY*, respectively. In contrast, four StrR binding sites have been identified in the streptomycin biosynthetic gene cluster in *S. griseus* and three in the 5´-OH-streptomycin cluster in *S. glaucescens* (Retzlaff & Distler, 1995).

The predicted gene product of *novE* comprises 217 amino acids and contains the rare TTA-leucine codon (as codon 189) which suggests its dependence on *bldA*, the structural gene for $tRNA^{UUA}$. This may indicate 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 addition to that, NovE shows 45 % identity at the

amino acid level to LmbU in the lincomycin biosynthetic gene cluster. It has been speculated that *lmbU* may code for a regulatory protein (Peschke *et al.*, 1995).
Orthologues of *novE* and *lmbU* include *cloE* and *couE* from the clorobiocin and coumermycin gene clusters, *rubC4* from the gene cluster of the aminocoumarin antibiotic rubradirin (GenBank AJ871581) and five further genes deposited in the database. All of these genes have been found in actinobacteria. However, their function is yet unknown.

Heterologous expression experiments showed that the expression of the complete novobiocin cluster, i.e. nov-BG1, including several kb of DNA upstream of the start codon of *novE* (Fig. I.4), in the heterologous host *Streptomyces coelicolor* M512 resulted in a high production of novobiocin. In contrast, the expression of a similar construct, i.e. novAE4, including only 180 bp upstream of the *novE* start codon (Fig. I.4) had given only 5 % of the production achieved with nov-BG1 (Eustáquio *et al.*, 2005a). Subsequently, the expression of a construct which contained 484 bp upstream of the *novE* start codon (Fig. I.4), i.e. nov-AE12, resulted in 12 times higher production levels in comparison with nov-AE4 (Dangel *et al.*, 2008). The additional DNA region contained in nov-AE12 but not in nov-AE4 comprises only the 3´-end of the structural gene *novD*, without a start codon. Therefore, this effect cannot be attributed to the expression of a functional NovD protein. This finding may represent an indication that the promoter region of *novE* extends more than 180 bp upstream of the *novE* start codon, and that expression of *novE* is important for novobiocin biosynthesis.

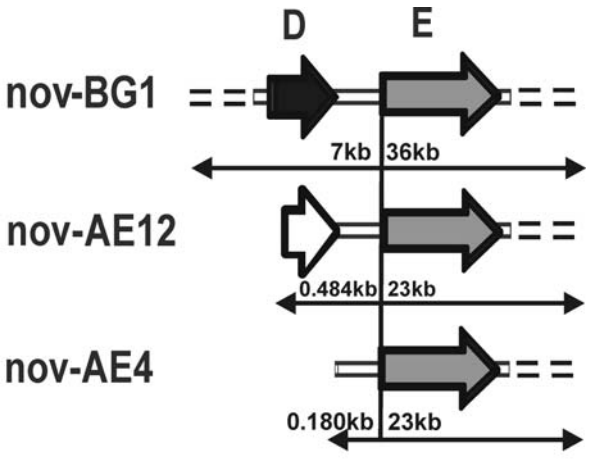

Fig. I.4: Schematic comparison of the inserts of cosmids nov-BG1, nov-AE12 and nov-AE4.

In the course of investigations on the genes at the borders of the novobiocin, clorobiocin and coumermycin gene clusters, a replacement of *novE* with an apramycin resistance gene has been carried out in the novobiocin producer *Streptomyces spheroides* (syn. *Streptomyces caeruleus*) (Eustáquio *et al.*, 2003). This resulted in a reduction of the novobiocin production by 96 % but not in a complete abolishment. This result indicated that *novE* may act as a positive regulator of novobiocin biosynthesis. However, downstream effects of the introduced apramycin resistance cassette can not be excluded.

I.4. Aims of this study

The first aim of this study was to supply functional evidence for the regulatory role of *novE* in novobiocin biosynthesis. For this reason the following experiments have been carried out:

- Inactivation of *novE* in cosmid nov-BG1 and heterologous expression of the resulting Δ*novE*-cosmid in *Streptomyces coelicolor* M512.
- Complementation of the Δ*novE*-mutation by introducing an intact copy of *novE* on a multicopy number plasmid (pWHM3-based) into *Streptomyces coelicolor* M512 (Δ*novE*).
- Overexpression of an intact copy of *novE* on a multicopy number plasmid (pWHM3-based) in *Streptomyces coelicolor* M512 (nov-BG1).
- Introduction of the positive regulator *novG* under control of the constitutive *ermE** promoter (pUWL-based) into *Streptomyces coelicolor* M512 (Δ*novE*).
- HPLC analysis of secondary metabolite production in the generated mutants.

The second aim of this study was to show whether *novE* and *novG* act in a cascade-like or a parallel mechanism. Therefore, the following experiments have been carried out:

- Introduction of an intact copy of *novG* under control of its own putative promoter on a multicopy number plasmid (pWHM3-based) into *Streptomyces coelicolor* M512 (Δ*novE*).
- Introduction of an intact copy of *novE* under control of its own putative promoter on a multicopy number plasmid (pWHM3-based) into *Streptomyces coelicolor* M512 (Δ*novG*).
- Establishment of methods for RNA isolation from *Streptomyces* and for RT-PCR.
- RT-PCR analysis for investigations on *novG* transcription in *Streptomyces coelicolor* M512 (Δ*novE*) and *novE* transcription in *Streptomyces coelicolor* M512 (Δ*novG*).

Further aims were the determination of promoter regions and investigations on the transcriptional regulation of the novobiocin biosynthetic gene cluster. Investigations on the determination of promoter regions within the novobiocin biosynthetic gene

cluster were carried out in cooperation with Johannes Härle as diploma student and included the following experiments:

- Introduction of Ω (omega) interposons downstream of putative promoter regions into cosmid nov-BG1, resulting in interruption of transcription up to the next active promoter region.
- Heterologous expression of the modified constructs in *Streptomyces coelicolor* M512.
- RT-PCR analysis of the generated mutants.

Investigations on the transcriptional regulation required the establishment of quantitative RT-PCR. Quantitative RT-PCR experiments were carried out for contribution of the promoter regions within the region from *novH* to *novW* and for investigations on the interplay of *novE* and *novG* as well as on the influence of *novE*- and *novG*-inactivation on the transcription of novobiocin biosynthetic genes and the resistance gene $gyrB^R$.

Subsequently, the final aim of this study was the generation of *Streptomyces coelicolor* M512 strains with improved novobiocin production, independent from its origin regulation cascade. Therefore the following experiments were carried out:

- Generation of *S. coelicolor* M512 (Δ*novE*Δ*novG*).
- Introduction of tetracycline-controllable-promoters (tcp830) into cosmid nov-BG1 in exchange for the entire *novEFG*-region and/or for the intergenic region between *novP* and *novQ*.
- Heterologous expression of the modified cosmids in *Streptomyces coelicolor* M512.
- HPLC analysis of secondary metabolite production, with and without induction by anhydrotetracycline.

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
Bacto-Difco, Heidelberg, Germany	Agar Casaminoacids Malt extract 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 Methanol
Roth, Karlsruhe, Germany	Anhydro-tetracycline (aTc) 5-Bromo-4-chlor-3-indolyl-β-D-galactopyranoside (X-Gal) Carbenicillin Glacial acetic acid Glass beads Glycine Isopropanol Isopropyl-β-thiogalactoside (IPTG) Maleic acid Na-salicylat Phenol/Chloroform/Isoamylalkohol(25:24:1) Phenol (pH 8.0) Polyethyleneglycol (PEG) 1000 Sodium dodecyl sulphate (SDS)

	Tris-(hydroxymethyl)-aminomethane-maleate (Tris-maleate) N-Tris-(Hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES)
Serva, Heidelberg, Germany	N-Lauroylsarcosine (Na-Salt, 35%)
Sigma-Aldrich, Deisenhofen, Germany	Bromophenol blue Dimethyl formamide (DMF) Dimethyl sulfoxide (DMSO) Ethyleneglycol Kanamycin Polyoxyethylenesorbitan monolaurate (Tween 20) Tetracycline Tris base
Südzucker, Mannheim, Germany	Sucrose

II.2. Materials for Chromatography

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

TABLE II.2: Liquid chromatography media

Supplier	Medium
C+S Chromatographie Service, Düren, Germany	Multosphere® RP 18-5 (commercial colum, 5 µm, 150×4,6 mm

II.3. Enzymes and kits

TABLE II.3: Enzymes and kits

Supplier	Enzymes and kits
Amersham Biosciences, Freiburg, Germany	Restriction endonucleases T4 DNA Ligase
Fermentas	Deoxyribonuclease I (1 U/µl) 10 x buffer with $MgCl_2$ (100 mM Tris-HCl (pH 7.5 at 25 °C), 25 mM $MgCl_2$, 1mM $CaCl_2$) Random hexamer primers (0.2 µg/ml) Revert-Aid™M-MuLV Reverse Transcriptase (200 U/µl) 5 x reaction buffer (250 mM Tris-HCl (pH: 8.3 at 25 °C), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT) RiboLock™ Ribonuclease Inhibitor (20 U/µl)

	10 mM dNTP-mix (10 mM each) 1 kb DNA Ladder
Fluka, Ulm, Germany	Lysozyme (47 000 U/mg) Lysozyme (85 400 U/mg)
Macherey-Nagel, Düren, Germany	Nucleobond® AX100 NucleoSpin® Extract 2 in 1 Nucleo-spin® RNA Clean-up
New England Biolabs, Schwalbach, Germany	Restriction endonucleases T4 DNA Ligase 100 bp DNA Ladder
Plant Bioscience Limited, Norwich, UK	REDIRECT© technology: PCR-targeting system in *Streptomyces coelicolor*
Promega, Madison, WI, USA	pGEM-T Easy® Vector System 1 *Taq* DNA polymerase *Pfu* DNA polymerase
Qiagen, Hilden, Germany	RNase A (100g/ml)
Roche, Mannheim, Germany	DNA Molecular Weight Marker VII, DIG-Labelled Expand High Fidelity PCR System LightCycler® RNA ampflication Kit SYBR Green I
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 room temperature or at 4 °C.

II.4.1.1. Cultivation of *E. coli*

LB (Luria-Bertani) Medium (Sambrook & 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.

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.

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.

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

L-asparagine	0.5 g
K_2HPO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Mannitol	5 g
Agar	10 g
Distilled water	ad 1000ml

Dissolve the ingredients, except agar, in the distilled water, adjust to pH 7.0-7.2 and dispense 200 ml into 300 ml Erlenmeyer flasks each containing 2 g agar. Close the flasks and sterilize by autoclaving.

II.4.1.3. 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.4.1.4. Protoplast transformation of *Streptomyces*

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.

R5 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
Difco Casaminoacids	0.1 g
Trace elements solution	2.0 ml
DifcoYeast extract	5.0 g
TES buffer	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 the following sterile solutions:

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

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 (room temperature 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
Anhydrotetracycline	50	1[b]	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.
[b] anhydrotetracycline was used for induction of tcp830.

II.4.3. Buffers and solutions

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

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 concentration	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
	$K_2SO_4 \cdot$ (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) Trace elements solution K_2SO_4 (140 mM) KH_2PO_4 (40 mM) $MgCl_2 \cdot 6H_2O$ (1 M) $CaCl_2 \cdot 2H_2O$ (5 M) Tris-maleate (0.5 M, pH 8.0) PEG 1000 (50% (w/v) in H_2O); 	1.0 0.03 0.1 0.1 0.1 1.0 1.0 5.0 adjust the volume with distilled water to 10 ml.

II.4.3.6. Solution for isolation of RNA from *Streptomyces*

TABLE II.12: Solution for isolation of RNA from *Streptomyces*

Solution	Components	Final concentration	Preparation
Modified Kirby mix	N-lauroylsarcosine Sodium-salicylate 2 M Tris-HCl pH 8.0 Phenol pH 8.0	1 % (w/v) 6 % (w/v) 20 mM 6 % (v/v)	Dissolve N-lauroylsarcosine, sodium-salicylate in distilled water, add the Tris-HCl buffer and the phenol and add distilled water up to 100 ml. Do not autoclave. Store protected from light at 4°C.

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

II.5.1. Vectors, cosmids and plasmids

TABLE II.13: Vectors, cosmids and plasmids

Name	Description	Source or reference
Vector		
pGEM-T	Linearized vector with T-overhang for direct cloning of PCR fragments with A-overhang, *lacZ*α, ori, f1-origin, Amp^R	Promega

pUWL 201	*E. coli* - *Streptomyces* shuttle vector, *ermE** promoter, Amp^R, $Thio^R$	(Doumith *et al.*, 2000)
pWHM3	*E. coli* - *Streptomyces* shuttle vector, *lacZ*α, Amp^R, $Thio^R$	(Vara *et al.*, 1989)
Cosmid		
nov-BG1	From cosmid 10-9C, *bla* gene replaced by cassette from pIJ787 (*oriT*, *tet*, *attP*, *int* ϕC31), Kan^R.	Bertolt Gust (Eustáquio *et al.*, 2004)
nov-AE10	nov-BG1 (Δ*novG*), Kan^R	(Eustáquio *et al.*, 2005b)
nov-JH4	nov-BG1 (Ω*novH*); Kan^R $Apra^R$	Johannes Härle´s Thesis
Plasmid		
pIJ787	SuperCos1-derivative, *bla* gene replaced by a cassette containing *oriT*, *tet*, *attP*, *int* ϕC31, Kan^R	Bertolt Gust (Eustáquio *et al.*, 2004)
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)
pMS80	Plasmid containing the tetracycline-controllable promoter 830 (tcp830); $Apra^R$	(Rodriguez-Garcia *et al.*, 2005)
pAE8	1.43 kb *Bam*HI-*Eco*RI fragment of pMS33 (*novG*, position 6 393 – 7 821, AF 170880) in the same sites of pWHM3, containing 336 bp before the putative start codon of *novG*; Amp^R, $Thio^R$	(Eustáquio *et al.*, 2005b)
pAE12	1.35 kb *PstI-SphI* fragment of pAE11, cloned into the same sites of pWHM3 (same orientation as *lacZ*), containing *novG* and 336 bp upstream of its start codon (position 6383 - 7687 in AF170880); Amp^R $Thio^R$	Alessandra Eustáquio´s studies

TABLE II.14: Plasmids and cosmids produced in this study

Name	Description
Plasmid	
pVD1	1 kb *BamHI/HindIII* fragment of pAE-G4 into the same sites of pUWL201, cointaining *novG* (position 6720 - 7704 in GenBank entry AF170880); Amp^R, $Thio^R$
pVD4	1.314 kb *PstI/SphI* fragment of pVD7 into the same sites of pWHM3, containing *novE* and 599 bp upstream of its start codon; Amp^R, $Thio^R$

pVD7	PCR fragment comprising *novE* (position 4209 – 5461 in AF170880) in pGEM-T; Amp^R
pVD10	1.31 kb *HindIII-SphI* fragment of PCR product comprising *novE* (position 4209-5461 in AF170880) into the same sites of pAE12
Cosmid	
nov-VD1	From nov-BG1, *novE* replaced by the apramycin resistance cassette from pUG019, Kan^R, $Apra^R$
nov-VD2	From nov-VD1, apramycin resistance cassette excised by expression of FLP-recombinase; Δ*novE* cosmid (Fig. III.1), Kan^R
nov-VD3	From nov-AE10, *novE* replaced by the apramycin resistance cassette from pUG019, Kan^R, $Apra^R$
nov-VD4	From nov-VD3, apramycin resistance cassette excised by expression of FLP-recombinase; Δ*novE*Δ*novG* cosmid (Fig. III.8), Kan^R
nov-VD6	From nov-BG1, region *novD* stop codon to *novH* start codon replaced by apra-tcp830 from pMS80 (Fig. III.8); Kan^R, $Apra^R$
nov-VD7	From nov-VD6, apramycin resistance cassette excised by expression of FLP-recombinase (Fig. III.8); Kan^R
nov-VD8	From nov-BG1, intergenic region *novP_novQ* replaced by apra-tcp830 from pMS80 (Fig. III.8); Kan^R, $Apra^R$
nov-VD9	From nov-VD7, intergenic region *novP_novQ* replaced by apra-tcp830 from pMS80 (Fig. III.8); Kan^R, $Apra^R$

II.5.2. PCR primers used for construction of plasmids

TABLE II.15: Primers used for construction of plasmids

Name	Sequence (5'-3')[a]	Restriction site	Positions (Acession number)	Plasmid
PnovE_f	CCGACGCCTTCACCACCACG	---	4209 - 4228 (AF170880)	pVD7
PnovE_r	GTCAAACGGCCCCGTCCACG	---	5443 - 5462 (AF170880)	
P10_novE_f	AAAAAAAAGCTTCCGACGCCT TCACCACCACG	*Hind*III	4209 - 4228 (AF170880)	pVD10
P10_novE_r	AAAAAAGCATGCGTCAAACGG CCCCGTCCACG	*Sph*I	5443 - 5462 (AF170880)	
[a]Underlines letters represent restriction sites *Hind*III and *Sph*I.				

II.5.3. Primers used for RT-PCR experiments

TABLE II.16: Primers used for RT-PCR experiments

Name	Sequence (5´-3´)	Position	Target gene
P_hrdB_RT_f	TCGGCCAGCACATCCCGTAC	6367668 - 6367687	*hrdB*
P_hrdB_RT_r	TCGGTACCCTCGGGCTCCTC	6368203 - 6368222	
P_novE_RT_f	GACGCGACCCCCGTTGACAG	4880 - 4889	*novE*
P_novE_RT_r	GCCTCCCTCCACCGTTCGAG	5378 - 5397	
P_novG_RT_f	CCAACAGCGGCGATGAGGAG	6734 - 6753	*novG*
P_novG_RT_r	CGGTGGACGACGACCTCAGC	7173 - 7192	
The GenBank accession number for the sequence reported is AF170880 (novobiocin cluster) and SCO5820 (*hrdB*).			

II.5.4. Primers used for qRT-PCR experiments

Table II.17: Primers used for qRT-PCR experiments

Name	Sequence (5´-3´)	Position	Target gene
P_hrdB_qRT_f	TGACGCTGATGGTCAGTGC	6367864 - 6367882	*hrdB*
P_hrdB_qRT_r	GTCGCCTTCCTGCTGGTC	6367969 - 6367987	
P_novE_qRT_f	GATCCCGCGGGACCTCTC	4963 - 4980	*novE*
P_novE_qRT_r	CCAGCCAGTCGCCGATCC	5043 - 5060	
P_novF_qRT_f	GGAATGATCGGCAGATCCAT	5563 - 5582	*novF*
P_novF_qRT_r	CAGTGCGACGGGACTGGT	5628 - 5646	
P_novG_qRT_f	GTCGTGCACCGTGGGACC	6901 – 6918	*novG*
P_novG_qRT_r	ACACATCGGCCGGGGAGC	7001 - 7018	
P_novH_qRT_f	CGAAACGGCTGGCACGAC	8048 – 8065	*novH*
P_novH_qRT_r	GCCACGGCAAGCACGCTG	8138 - 8156	
P_novO_qRT_f	CGTCCTCAACTGCTCCTTCA	17908 – 17927	*novO*
P_novO_qRT_r	GCGTAATGGTGAGCCAGCG	18021 - 18039	
P_novP_qRT_f	GCCGGGACGGTGTACGAG	18301 - 18318	*novP*
P_novP_qRT_r	TGCGTTCGCGGGCCAGTC	18391 - 18408	
P_novQ_qRT_f	GTGTGCTTCTACGCCCTGA	19728 – 19746	*novQ*

P_novQ_qRT_r	CCGAACGACCAGCCGATG	19837 - 19855	
P_gyrB^R_qRT_f	CTCAGAGAACCGCAGTTCG	39918 - 39936	*gyrB^R*
P_gyrB^R_qRT_r	ACCAGTCGGTCAGATGCTC	40002 - 40020	
The GenBank accession number for the sequence reported is AF170880 (novobiocin cluster), SCO5820 (*hrdB*) and AF205854 (*gyrB^R*)			

II.5.5. Bacterial strains

TABLE II.18: 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)
S. coelicolor M512	Δ*redD* Δ*actII-ORF4* SCP1$^-$ SCP2$^-$ (no production of actinorhodin, undecylprodigiosin, and methylenomycin)	(Floriano & Bibb, 1996)
S. coelicolor M512 (nov-BG1)	*S. coelicolor* M512 containing the novobiocin cluster and flanking DNA regions, Kan^R	(Eustáquio *et al.*, 2004)
S. coelicolor M512 (nov-BG1)/pWHM3	Kan^R, Thio^R	This thesis
S. coelicolor M512 (nov-BG1)/pVD4	Kan^R, Thio^R	This thesis
S. coelicolor M512 (nov-AE10)	*S. coelicolor* M512 containing a *novG*-defective novobiocin cluster, Kan^R	(Eustáquio *et al.*, 2005b)
S. coelicolor M512 (nov-AE10)/pWHM3	Kan^R, Thio^R	(Eustáquio *et al.*, 2005b)
S. coelicolor M512 (nov-AE10)/pAE8	Kan^R, Thio^R	(Eustáquio *et al.*, 2005b)
S. coelicolor M512 (nov-AE10)/pVD4	Kan^R, Thio^R	This thesis
S. coelicolor M512 (nov-VD2)	*S. coelicolor* M512 containing a *novE*-defective novobiocin cluster, Kan^R	This thesis
S. coelicolor M512 (nov-VD2)/pWHM3	Kan^R, Thio^R	This thesis
S. coelicolor M512 (nov-VD2)/pVD4	Kan^R, Thio^R	This thesis

S. coelicolor M512 (nov-VD2)/pVD1	Kan^R, $Thio^R$	This thesis
S. coelicolor M512 (nov-VD2)/pAE8	Kan^R, $Thio^R$	This thesis
S. coelicolor M512 (nov-VD4)	*S. coelicolor* M512 containing a *novE,novG*-defective novobiocin cluster, Kan^R	This thesis
S. coelicolor M12 (nov-VD4)/pWHM3	Kan^R, $Thio^R$	This thesis
S. coelicolor M512 (nov-VD4)/pVD10	Kan^R, $Thio^R$	This thesis
S. coelicolor M512 (nov-VD7)	*S. coelicolor* M512 containing nov-BG1 with the region from *novD* stop codon to *novH* start codon replaced by apra-tcp830; Kan^R, $Apra^R$	This thesis
S. coelicolor M512 (nov-VD8)	*S. coelicolor* M512 containing nov-BG1 with the intergenic region *novP_novQ* replaced by apra-tcp830; Kan^R, $Apra^R$	This thesis
S. coelicolor M512 (nov-VD9)	*S. coelicolor* M512 containing nov-VD7 with the intergenic region *novP_novQ* replaced by apra-tcp830; Kan^R, $Apra^R$	This thesis
S. coelicolor M512 (nov-JH4)	*S. coelicolor* M512 containing a Ω*novH* novobiocin cluster; Kan^R; $Apra^R$	Johannes Härle´s Thesis

II.5.6. Probe used in Southern blot analysis

Cosmid nov-BG1 after digestion with *Pst*I and DIG-labelling, was used as probe for Southern blot analysis.

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 & Russell, 2001). 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 coelicolor*

II.6.2.1. General cultivation

Streptomyces coelicolor strains were routinely cultured in liquid YMG medium. These cultures were carried out by inoculation of 10^8 spores into 300 ml baffled Erlenmeyer flasks containing a stainless steel spring and 50 ml YMG medium and cultivated at 200 rpm and 30 °C for 48 hours. For preparation of protoplasts, *S. coelicolor* was cultured in TSB medium containing 0.4% glycine. For isolation of genomic DNA, *S. coelicolor* was cultured in TSB medium. An appropriate concentration of antibiotic(s) was added, if required.

II.6.2.2. Production of secondary metabolites

For production of secondary metabolites *S. coelicolor* strains containing the entire or modified novobiocin biosynthetic gene cluster were cultivated in 300 ml baffled Erlenmeyer flasks or 24-square deepwell plates.

Note: Cultivation of *Streptomyces coelicolor* strains in 24-square deepwell plates (Duetz *et al.*, 2000) resulted in higher reproducibility of cell growth and novobiocin production in comparison to conventional Erlenmeyer flask cultivations. Therefore, cultivation in 24-square deepwell plates are recommended especially for quantitative analysis of secondary metabolite production and (q)RT-PCR experiments.

Cultivation in Erlenmeyer flasks

For production of secondary metabolites, 1ml of the YMG preculture of *S. coelicolor* containing the entire or modified novobiocin gene cluster was inoculated into 300 ml baffled flasks containing a stainless steel spring and 50 ml CDM production medium and cultivated at 30 °C and 200 rpm for 7 days. The cultivation in production medium was carried out without addition of antibiotics.

Cultivation in 24-square deepwell plates

For cultivation in 24-square deepwell plates frozen and homogenized inoculum of *Streptomyces coelicolor* strains were prepared as described in II.6.2.3.. 10^6 CFU of this inoculum was mixed with 40 ml CDM production medium, containing 0.6 % (m/v) siloxylated polyether EO/PO copolymer Q2-5247 (Dow Corning, Auburn, Michigan/USA) and 3 ml of this mixture were placed into each well of the 24-square deepwell plates (Duetz *et al.*, 2000). Cultivation was carried out at 30 °C and 300 rpm for 7 days. This cultivation was carried out without addition of antibiotics.

II.6.2.3. Preparation of homogenized and frozen inoculum

For preparation of frozen inoculum, 50 ml of YMG preculture were centrifuged (2772 x g for 10 min). The cells were resuspended in 10 ml of an aqueous solution of 20 % (w/v) peptone (Bacto® Proteose Peptone Nr. 3, Difco, Sparks, Maryland/USA) and gently homogenized using a potter homogenizer operated manually (B. Braun Biotech, Sartorius AG, Göttingen, Germany). The resulting mixture was devided in aliquots and stored at -70 °C.

II.6.2.4. Preparation of mycelia for storage and 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 coelicolor* strains were spread on MS agar and incubated at 30 °C for about one week. 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.

Note: For selection of *Streptomyces coelicolor* strains with thiostreptone as selection marker, strains were spread on MM agar !

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 & 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 & 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's 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 room temperature 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 (10000×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 room temperature 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 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 room temperature 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 Phenol/Chloroform/Isoamylalcohol (25:24:1). 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 coelicolor*

Genomic DNA was isolated by the Kirby mix procedure (Kieser *et al.*, 2000). 2 ml of a 2-day-old culture in TSB 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 (25:24:1) 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 NaOAc and 300 µl phenol/chloroform/isoamyl alcohol (25:24:1) (1 min vigorous vortexing). The aqueous phase was separated and genomic DNA was precipitated by addition of 0.8-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 by alkaline treatment for ssDNA transformation in *Streptomyces*

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 placed on ice and the reaction was terminated with addition of 2 µl 1 M HCl (Oh & Chater, 1997).

II.7.6. PCR amplification

II.7.6.1. General conditions

PCR amplifications were carried out with the GeneAmp® PCR System 2400 or GeneAmp® PCR-System 9700 (Perkin-Eimer, Weiterstadt, Germany) or iCycler PCR-System (Bio-Rad, Munich, Germany). The amplification conditions for PCR reactions using the Expand High Fidelity PCR system (Roche) are given in Table II.19, and using the GC-Rich PCR system (Roche) in Table II.20.

TABLE II.19: 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.20: 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 pUG019 and the apra-tcp830 cassette from pMS80

The conditions for amplification of the apra-tcp830 from pMS80 (REDIRECT© technology kit for PCR targeting (Gust *et al.*, 2003)) using the Expand High Fidelity PCR system (Roche) are given in Table II.21. Template DNA was prepared by digesting about 10 µg of pMS80 with *Kpn*I and *Sac*II, and by isolating the 1.7 kb cassette fragment from an agarose gel (Gust *et al.*, 2003).

TABLE II.21: Conditions for amplification of the apramycin resistance cassette from pUG019 and apra-tcp830 from pMS80

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 conditions were the same as listed in Table II.21. 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 probe used in this thesis is presented in section II.5.6.

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 room temperature, 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 room temperature. 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 room temperature for 2-5 min protected from light. Excess of solution and air bubbles were removed by wiping the cover sheet with a clean paper towel. 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 room temperature. 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 & Russell, 2001).

II.7.8.1. $CaCl_2$-mediated transformation

Preparation of competent cells: 100 ml LB-medium were inoculated with 1 ml of an overnight culture of *E. coli* and cultivated at 37 °C and 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 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 *lacZ*α-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 β-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 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. PEG-mediated protoplast transformation for introduction of DNA in Streptomyces

Relevant buffers and media are listed in Table II.11 and in section II.4.1.4, respectively.

Preparation of protoplasts from S. coelicolor

Slightly modified from (Kieser *et al.*, 2000). Mycelium from a 40 h old culture (50 ml TSB medium containing 0.4 % 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 R5 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 (R5 agar).

Note: plating of protoplasts was always done by overlaying with R5 soft agar instead of spreading in order to avoid mechanical stress and lysis.

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.

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 (25% (w/v) were immediately added, mixed by pipetting carefully three times and incubated at room temperature for 1 min. Increasing volumes of the resulting suspension (e.g. 10 µl, 100 µl, 200 µl, rest) were mixed with warm R5 soft agar (about 50 °C, 4×3 ml) and plated on four R5 plates. After 16-24 h incubation at 30 °C, the plates were overlaid with 3 ml of R5 soft agar including the required antibiotics for selection of mutants, and incubation was continued for further 3-7 days.

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/.

II.8. RNA methods

II.8.1. RNA isolation, DNase treatment and purification

For RT-PCR experiments *Streptomyces coelicolor* M512 strains were cultivated in 300 ml baffled Erlenmeyer flasks as described above. From each mutant 1 ml of YMG preculture was inoculated into a 300 ml baffled flask containing 50 ml CDM production medium (Kominek, 1972) and cultivated at 30 °C and 200 rpm. After 22, 30, 44, 77 and 94 hours of cultivation the mycelia of 50 ml culture was collected by vaccum filtration, followed by rinsing with 30 ml distilled water.

Note: Cultivation of *Streptomyces coelicolor* M512 strains for RT-PCR experiments were carried out in Erlenmeyer flasks because at this time point the cultivation procedure in 24-square deepwell plates was not established. Everyone working on regulation projects is recommended to carry out cultivation in 24-square deepwell plates (see below) due to its very stable and reproducible cell growth and production!

For qRT-PCR experiments *Streptomyces coelicolor* strains were cultivated in 24-square deepwell plates (Duetz *et al.*, 2000) as described above. From each mutant 2 x 10^6 CFU of frozen inoculum were mixed with 80 ml CDM production medium, containing 0.6 % (m/v) siloxylated polyether EO/PO copolymer Q2-5247 (Dow Corning, Auburn, Michigan/USA) and 3 ml of this mixture were placed into 27 wells of 2 x 24-square deepwell plates. Cultivation was carried out at 30 °C and 300 rpm. After 24, 32, 48, 72 and 96 hours mycelia of 6 to 4 wells were pooled and collected by vacuum filtration, followed by rinsing with 30 ml distilled water. After 168 hours 3 wells were harvested to analyze final novobiocin production.
Then the cells were transferred to a universal plastic tube (50 ml falcon tube) containing appoximately 14 g 3.5 – 4.5 mm diameter glass beads and 15 ml of modified kirby mix Table II.12 and vortexed for 2 min. After that, cell suspension was

sonicated for 6 x 30 s with 20 s intervals between each sonication treatment (Branson sonifier 250). To this cell suspension 1 volume (15 ml) of phenol/chloroform/isoamylalcohol (25:24:1) was added and followed by vortexing for 30 s and centrifugation (5000 x g for 10 min at 4 °C).

Note: Due to the instability of RNA the following steps have to be carried out carefully to avoid contamination with RNAse. Therefore, gloves were always worn to handle the used plastic or glass ware. Plastic, glass ware, water and solutions (except from ethanol, isopropanol, modified kirby mix and phenol/chloroform/isoamylalcohol 25:24:1) were autoclaved twice before use. Furthermore, breezing and talking into probes was avoided.

Clear supernatant was transferred to a clean tube (50 ml falcon tube) and mixed with an equal volume of isopropanol and 0.1 volume 3 M sodium acetate (pH: 5.2) and leaved for 5 min at 20 °C. After centrifugation (5000 x g for 20 min at 4 °C) supernatant was discarded and the pellet was washed with 2 ml ethanol 70 % (v/v). Then pellet was dried and resuspended in 800 µl water. After resuspension DNase treatment was carried out, using Deoxyribonuclease I (1U μl^{-1}), 10 x buffer with $MgCl_2$ (100 mM Tris-HCl (pH 7.5 at 25 °C, 25 mM $MgCl_2$, 1mM $CaCl_2$) and RNase-free water (Fermentas) according to manufacturer´s instructions. After that, RNA was purified with the NucleoSpin® RNA Clean-up Kit (Macherey & Nagel) according to manufacturer´s instruction and eluted from the column with 40 µl water, followed by its quantification by determining the OD at 280 nm. Integrity of RNA was checked by running 1 µg of RNA on a 1.2 % agarose gel. To ensure that RNA is free of DNA, PCR using RNA-probes without previous RT-reaction (PCR-conditions and primers as presented below; concentration tenfold to the concentration that was used in RT-reaction) was carried out.

II.8.2. RT-PCR

After ensuring that RNA is free of DNA the RT-reaction was carried out. Therefore random hexamer primers (0.2 µg μl^{-1}), 5 x reaction buffer (250 mM Tris-HCl (pH: 8.3 at 25 °C), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), RiboLock™ Ribonuclease Inhibitor (20U μl^{-1}), 10 mM dNTP mix (10 mM each), RNase-free water and

RevertAid™M-MuLV Reverse Transcriptase (200U μl^{-1}) (Fermentas) were used. RT-reaction was carried out according to the Fermenta´s "Protocol for First Strand cDNA Synthesis".

After RT-reaction PCR using primer pairs for amplication of *hrdB*, *novE* and *novG* listed in Table II.16 was carried out in 50 µl volume with 2 µl template (cDNA from RT-reaction), 2.5 mM dNTPs each, 50 pmol each primer, 5 % (v/v) DMSO, 10 x buffer (100mM Tris-HCl, pH: 8.8 at 25 °C; 500 mM KCl, 0.8 % (v/v) Nonidet P40 and 15 mM $MgCl_2$) and 1 µl Taq DNA-polymerase (1U μl^{-1}): denaturation at 95 °C for 2 min; 27 cycles with denaturation at 95 °C for 30 s, annealing at 70 °C for 30 s and extension at 72 °C for 50 s, and a final elongation step at 72 °C for 7 min. Finally PCR product was checked by running 22 µl of the PCR-reation with 8 µl loading buffer [50% glycerol, 200mM EDTA, 0.5 % xylen cyanol(Sigma®)] on a 1.2 % agarose gel.

II.8.3. qRT-PCR

LightCycler® quantitative RT-PCR (qRT-PCR) was carried out using the LightCycler® RNA ampflication Kit SYBR Green I (Roche). Master mixes were prepared by following the manufacturer´s instructions for GC-rich templates except from [$MgCl_2$] that was reduced to 6.25 mM for investigations on *novE, novF, novG, novH, novO, novQ* and $gyrB^R$ and 12.5 mM for inverstigations on *hrdB* and *novP*, using the primers listed in Table II.17. After RT for 20 min at 50°C, the following temperature profile was utilized for amplification: denaturation for 1 cycle at 95 °C for 30 s and 45 cycles at 95 °C for 1 s (temperature transition, 20 °C/s), 60 to 55 °C (*novE, novG, novH, novO*), 58 to 53 °C (*hrdB*, *novQ*), 56 to 52 °C (*novF, novP,* $gyrB^R$) (step size, 0.7°C; step delay, 1 cycle) for 10 s (temperature transition, 20 °C/s), and 72 °C for 13 s (temperature transition, 2 °C/s) with stepwise fluorescence acquisition at 60 to 55 °C in single mode. The number of copies of each sample transcript was then determined with the aid of LightCycler® software and normalised to *hrdB*. The specificity of the PCR reaction was verified by ethidium bromide staining on 2% agarose gels.

II.9. Heterologous expression of the modified novobiocin biosynthetic gene clusters

II.9.1. Inactivation of *novE* in cosmids nov-BG1 and nov-AE10, and heterologous expression of the Δ*novE* and Δ*novE*Δ*novG* cosmid

The gene *novE* in cosmid nov-BG1 and cosmid nov-AE10 (Δ*novG* cosmid) (Eustáquio *et al.*, 2005b) was replaced by the apramycin resistance (*aac(3)IV*) cassette from pUG019 (Eustáquio *et al.*, 2004) via λ-Red-mediated recombination (Gust *et al.*, 2003) resulting in cosmid nov-VD1 (from nov-BG1) and nov-VD3 (from nov-AE10). This cassette is flanked by the FRT (FLP recognition target). The cassette for replacement of *novE* was generated by PCR using the primer pair P1_novE (5´-**CGC CGG TCC GCT TGT CCC GAG GGG AAG AGA GGC ATC GTG** ATT CCG GGG ATC CGT CGA CC-3´) and P2_novE (5´-**GCC GTG AGG CCG CGA AAT GGA TCG GAG TGC GTC CGG TCA** TGT AGG CTG GAG CTG CTT C-3´). Bold letters respresent 39 nt homologous extensions to the DNA regions immediately upstream and downstream of *novE*, including the putative translational start and stop codons of *novE*, respectively. The PCR reaction was performed in 50 µl volume with 50 ng template (pUG019 digested with *Eco*RI, *Hind*III and *Dra*I), 0.2 mM dNTPs, 50 pmol each primer, 5 % (v/v) DMSO, using the Expand High Fidelity PCR System (Roche Molecular Biochemicals): denaturation at 94 °C for 2 min, then 10 cycles with denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s, then 15 cycles with annealing at 55 °C, and a final elongation step at 72 °C for 5 min. Subsequently, the cassette was excised by the FLP recombinase, leaving an in-frame “scar” of 81 nucleotides between the start and stop codon of *novE*, resulting in cosmid nov-VD2 (Δ*novE*) and nov-VD4 (Δ*novE*Δ*novG*). *E. coli* XL1 Blue MRF´ cells were analysed using restriction enzymes and gel electrophoresis. The generated Δ*novE* cosmid (nov-VD2) and Δ*novE*Δ*novG* cosmid (nov-VD4), carrying the kanamycin resistance gene *neo*, were then introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation (Kieser et al. 2000). Kanamycin resistant clones were checked for specific genomic integration of cosmid nov-VD2 or cosmid nov-VD4 into the ΦC31 attachment site by Southern blot analysis.

II.9.2. Introduction of tcp830 into cosmid nov-BG1 and heterologous expression of the resulting cosmids

The apramycin-tcp830 cassette (apra-tcp830) from pMS80 (Rodriguez-Garcia *et al.*, 2005) was introduced in nov-BG1 via λ-Red-mediated recombination (Gust *et al.*, 2003), either in exchange with the region from *novD* stop codon to *novH* start codon, resulting in cosmid nov-VD6 or by replacing the intergenic region *novP_novQ*, resulting in cosmid nov-VD8. The apramycin resistance gene (*aac(3)IV*) is flanked by the FRT (FLP recognition target). The required cassettes were generated by PCR using the primer pairs P_*novD*_apra-tcp830 (5´-**AAC CCG GAC CGG TAC GTA CGG CTG AGC TTC CTC GGC TGA** *ACT AGT* GTG TAG GCT GGA GCT GCT TC-3´) and P_*novH*_apra-tcp830 (5´-**CGA CTG ATC AGA AGC TTT GTT CGC ACG TGT GTT GAA CAA** TCT AGA CCT CCG ACG TAC GC -3´) for replacement of the region from *novD* stop codon to *novH* start codon, and P_*novP*_apra-tcp830 (5´-**ATC GAC CGC GAC GGT GTC TAC TGG CAA CGC ACC CGG TAA** *ACT AGT* GTG TAG GCT GGA GCT GCT TC-3´) and P_*novQ*_apra-tcp830 (5´- **TTC GCG GTC GAA TTC TTG ATT CAT CGG GAG TGC GGG CAT** TCT AGA CCT CCG ACG TAC GC-3´) for replacement of the intergenic region *novP_novQ*. Bold letters respresent 39 nt homologous extensions to the DNA regions immediately upstream of *novD* stop codon and downstream of *novH* start codon or upstream of *novP* stop codon and downstream of *novQ* start codon, including the putative translational stop codons of *novD* and *novP* and start codons of *novH* and *novQ*, respectively.

The PCR reaction was performed in 50 µl volume with 50 ng template (pMS80 digested with *KpnI* and *SacII*), 0.2 mM dNTPs, 50 pmol each primer, 5 % (v/v) DMSO, using the Expand High Fidelity PCR System (Roche Molecular Biochemicals): denaturation at 94 °C for 2 min, then 10 cycles with denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s, then 15 cycles with annealing at 55 °C, and a final elongation step at 72 °C for 5 min. Subsequently, nov-VD6 was modified by excision of *aac(3)IV* by the FLP recombinase, leaving an in-frame “scar” of 81 nucleotides between the stop codon of *novD* and tcp830, resulting in cosmid nov-VD7. For construction of nov-VD9, cosmid nov-VD8 was digested with *KpnI* and *SacII* and the obtained 4551 bp restriction fragment comprising the region from 671 bp upstream of *novN* stop codon to 525 bp downstream of *novQ* start codon, including the apra-tcp830 cassette from pMS80 in exchange with the

intergenic region *novP_novQ* was introduced into cosmid nov-VD7 via λ-Red-mediated recombination (Gust *et al.*, 2003). *E. coli* XL1 Blue MRF´ cells were transformed with 100 ng DNA and the obtained clones analysed using restriction enzymes and gel electrophoresis. The generated cosmids nov-VD7, nov-VD8 and nov-VD9, carrying the kanamycin resistance gene *neo*, were then introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation (Kieser *et al.*, 2000). Kanamycin and apramycin resistant (nov-VD8 and nov-VD9) and kanamycin resistant clones (nov-VD7) were checked for specific genomic integration of the respective cosmids into the *Φ*C31 attachment site by Southern blot analysis.

II.10. HPLC Analysis of secondary metabolites

S. coelicolor strains carrying the (modified) novobiocin cluster were cultured in CDM medium as described in section II.6.2.2. After centrifugation, the clear supernatant was analysed by HPLC with a Multosphere RP18-5 column (150 x 4,6 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.

III. RESULTS

III.1. Investigations on the role of *novE* in the regulation of novobiocin biosynthesis and its interplay with *novG*

III.1.1. Inactivation of *novE*

In order to prove that *novE* is important for novobiocin biosynthesis, *novE* was inactivated by an in-frame deletion. For this purpose, *novE* was replaced in cosmid nov-BG1 with an apramycin resistance cassette flanked by FRT (FLP-recognition target) sites via λ RED-mediated recombination (Datsenko & Wanner, 2000; Gust *et al.*, 2003) resulting in nov-VD1 (Fig. III.1c). The cassette was excised using FLP-recombinase, leaving an in-frame "scar" of 81 nucleotides between the start and stop codon of *novE* (Fig. III1c). This modified cosmid (named nov-VD2) was subsequently introduced into *S. coelicolor* M512 by protoplast transformation, and site-specific integration into the genome (Fig. III.1a and b) was confirmed by Southern blot analysis (Fig. III.1d). The deletion of *novE* was clearly shown by the size of the relevant *Pst*I restriction fragments in comparison to nov-BG1 strains (Fig. III.1d, lane 1 and 2).

HPLC analysis showed that the resulting Δ*novE* strains produced novobiocin in a strongly reduced amount (0.7 %) in comparison to *S. coelicolor* strains carrying the intact novobiocin cluster (Table III.1). Subsequently, *novE* together with its own putative promoter, i.e. including 599 bp upstream of the start codon of *novE*, was cloned into the promoterless shuttle vector pWHM3, resulting in pVD4. This plasmid was introduced into the Δ*novE* strain *S. coelicolor* M512 (nov-VD2). Production levels of the resulting transformants reached up to 70 % of those observed in *S. coelicolor* M512 (nov-BG1) which carries the intact *novE* gene. This proved that the very low novobiocin production of *S. coelicolor* M512 (nov-VD2) was indeed due to the inactivation of *novE*.

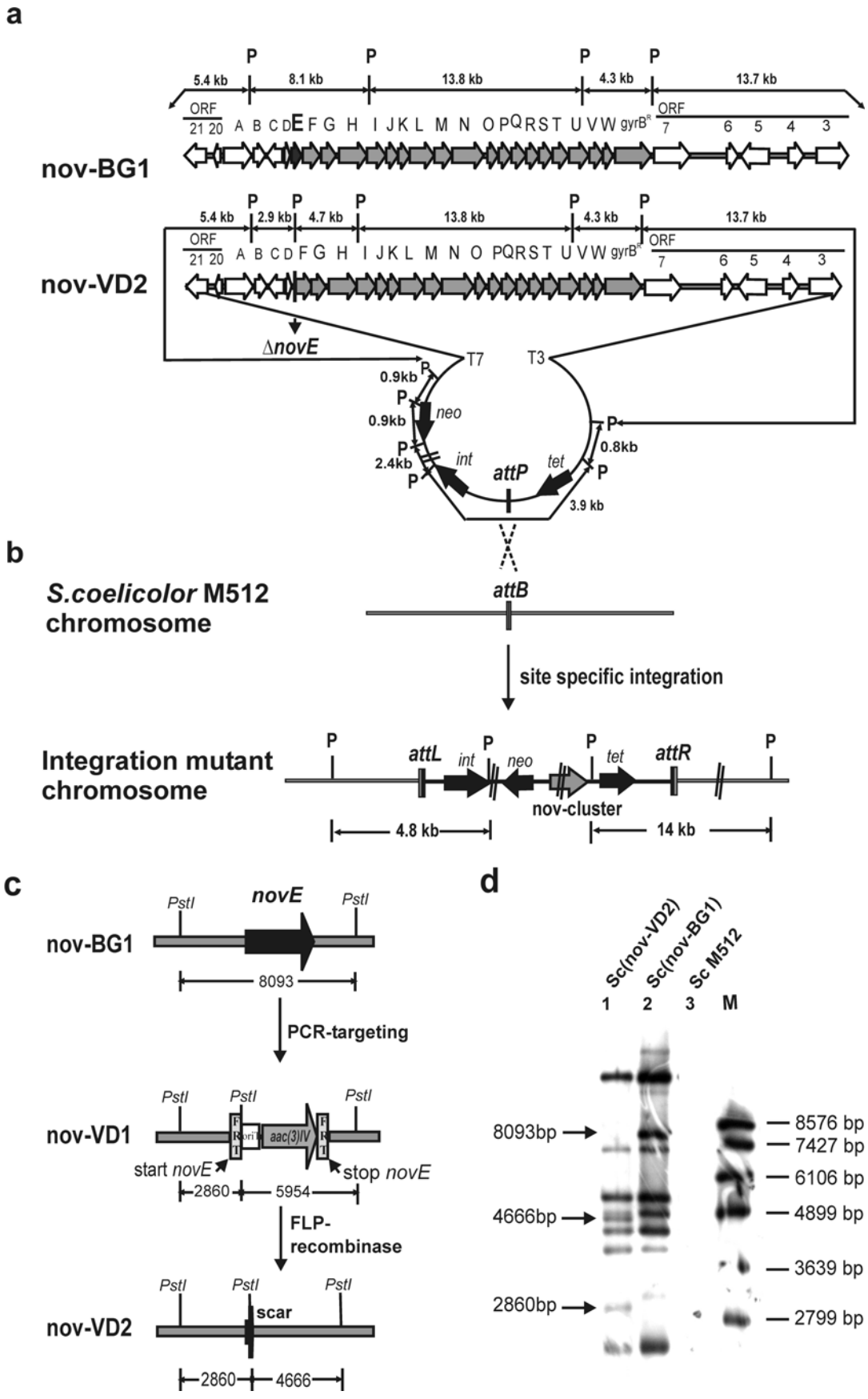

Fig. III.1: Inactivation of *novE*. See next page for details.

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(a) Cosmid constructs nov-BG1 and nov-VD2. P = *PstI* restriction site; T3, T7 = T3 and T7 promoter of the SuperCos1 vector, *tet* = tetracyclin resistance gene; *neo* = neomycin/kanamycin resistance gene; *int*, *attP* = integrase gene and attachment site of ΦC31. Fragment sizes resulting from digestion with *PstI* are indicated. Cosmid backbone out of scale. **(b)** Schematic representation of site specific integration of the cosmids into the genome of *Streptomyces coelicolor* M512. **(c)** Schematic presentation of *novE* replacement and deletion. *novE* (654bp) was first replaced by an apramycin resistance (*aac(3)IV*) cassette. Subsequently the cassette was excised using the FLP recombinase, leaving an in-frame "scar" of 81 nucleotides between the start and stop codons of *novE*. FRT = FLP recognition target. oriT = origin for conjugative transfer. **(d)** Southern blot analysis. M = Molecular Weight Marker; Lane 1: *Streptomyces coelicolor* M512 (nov-VD2); lane 2: *Streptomyces coelicolor* M512 (nov-BG1); lane 3: *Streptomyces coelicolor* M512. Genomic DNA was digested with *PstI.* M = Molecular Weight Marker. The entire cosmid nov-BG1 was used as probe.

Table III.1: Influence of *novE* and *novG* on novobiocin production in the heterologous host *Streptomyces coelicolor* M512.

Strain	Description	Expression plasmid	Novobiocin	
			(mg/l)	(%)
S.coelicolor M512	control	-	0	0
S.coelicolor(nov-BG1)	complete cluster	-	19.3	100
S.coelicolor(nov-BG1)	complete cluster	pWHM3 (empty vector)	20.0	100
S.coelicolor(nov-VD2)	*ΔnovE*	pWHM3 (empty vector)	0.14	0.7
S.coelicolor(nov-BG1)	complete cluster	pVD4 (*novE* under control of its genuine promoter)	38.1	191
S.coelicolor(nov-VD2)	*ΔnovE*	pVD1 (*novG* under control of the constitutive *ermE** promoter)	59.8	299
S.coelicolor(nov-VD2)	*ΔnovE*	pAE8 (*novG* under control of its genuine promoter)	34.6	173
S.coelicolor(nov-AE10)	*ΔnovG*	pWHM3 (empty vector)	0.4	2
S.coelicolor(nov-AE10)	*ΔnovG*	pVD4 (*novE* under control of its genuine promoter)	2.4	12
S.coelicolor(nov-AE10)	*ΔnovG*	pAE8 (*novG* under control of its genuine promoter)	16	80

*Values are means from at least two independent mutants

III.1.2. Overexpression of *novE* in *S. coelicolor* M512 (nov-BG1) results in overproduction of novobiocin

Overexpression of pathway-specific activators can result in overproduction of the respective antibiotic (Stutzman-Engwall *et al.*, 1992). Therefore, the *novE* expression plasmid pVD4 was introduced into *S. coelicolor* M512 (nov-BG1) by protoplast transformation. As presented in Table III.1, transformation with pVD4 led to a 1.9-fold increase in novobiocin production in comparison to *S. coelicolor* M512 (nov-BG1) carrying only the empty vector pWHM3.

III.1.3. Complementation of the *novE* mutation by *novG* under control of the constitutive *ermE** promoter

The results mentioned above are consistent with a role of *novE* as a positive regulator of novobiocin biosynthesis. Previous results (Eustáquio *et al.*, 2005b) had proven that also *novG* acts as a positive regulator of the biosynthesis of this antibiotic. This raised the question whether the two regulators, *novE* and *novG*, act in parallel or in a cascade-like mechanism. In order to investigate the interplay between *novE* and *novG*, a *novG* expression construct was introduced into the Δ*novE* strain.

Therefore, *novG* was placed under control of the constitutive *ermE** promoter in the expression vector pUWL201 (Doumith *et al.*, 2000), resulting in pVD1. This plasmid was transformed into the *ΔnovE* strain *S. coelicolor* M512 (nov-VD2) resulting in a dramatic increase of novobiocin production (Table III.1). The fact that novobiocin production in a *ΔnovE* strain can be restored by overexpression of the regulatory gene *novG* strongly indicates that *novE* has a regulatory rather than catalytic function.

III.1.4. Electrophoretic mobility shift assays (EMSA)

A plausible hypothesis to explain the observation described above would be that *novE* is a positive regulator of *novG*, which in turn is a positive regulator of the expression of the biosynthetic enzymes of novobiocin. NovG has been shown to bind to the DNA region between *novG* and *novH* (Eustáquio *et al.*, 2005b). Using gel-mobility shift assays, Alessandra Eustáquio could readily reproduce this result (Fig.III.2a). Subsequently, the coding sequence of *novE* was amplified by PCR and

cloned into the expression vector pRSET B. Using the resulting plasmid pAE18, NovE was expressed in *E. coli* as soluble, N-terminally His_6 tagged protein and purified by Ni^{2+} affinity chromatography. This protein was used in band shift assays with three different DNA fragments located upstream of the *novG* start codon. However, no mobility shift was observed. Likewise, no binding could be shown with a DNA fragment located upstream of *novF*, and neither with fragments upstream of *novE* which were tested in order to check for a possible autoregulation of *novE* (Fig.III.2b). These results do not support the hypothesis that *novE* acts by direct DNA binding in the *novEFG* region, and this is in accordance with the fact that bioinformatic analysis does not show any DNA binding motif in the predicted amino acid sequence of NovE. The EMSA mentioned above were carried out by Alessandra Eustáquio.

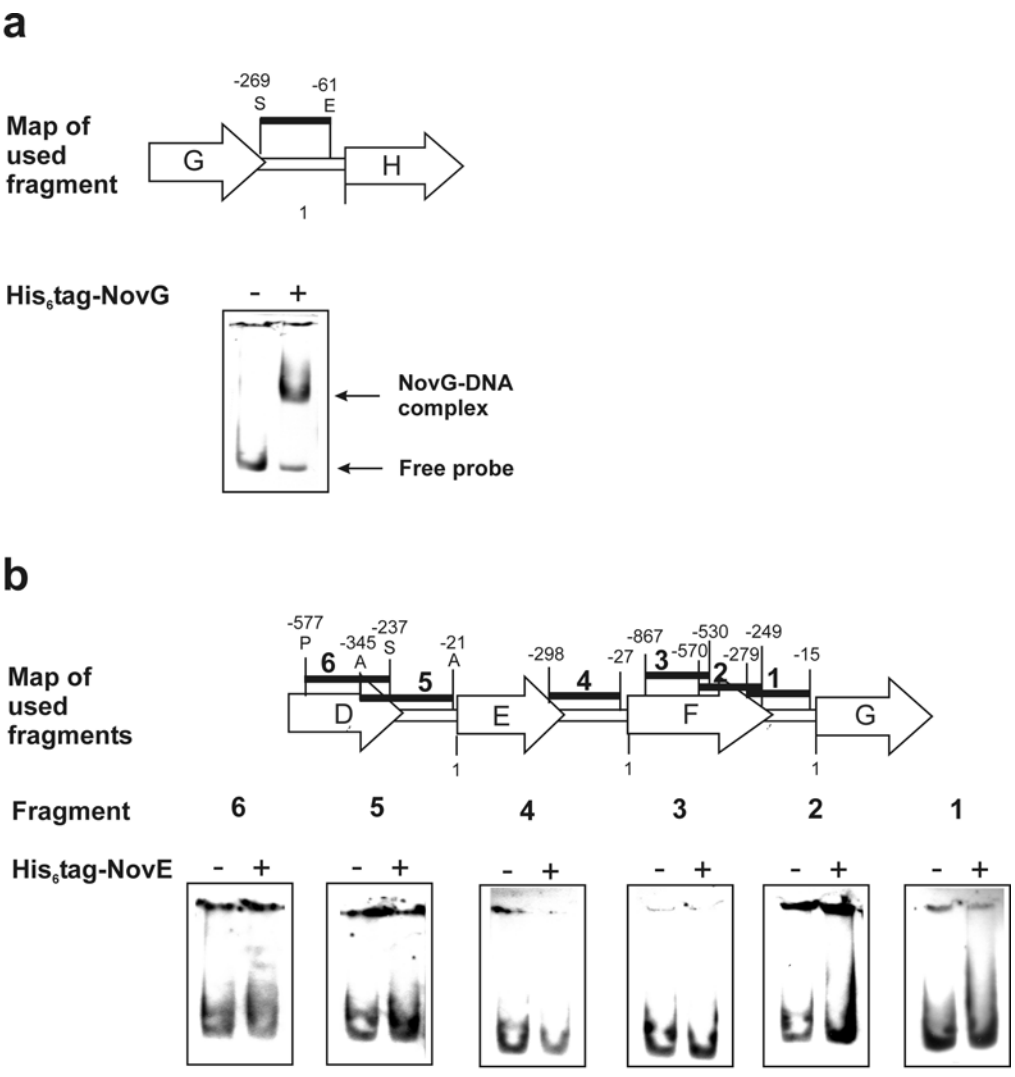

Fig. III.2: Electrophoretic mobility shift assays with fragments from the novobiocin biosynthetic gene cluster. See next page for details.

Autoradiogram of gel mobility-shift assays using **(a)** purified His_6-tagged NovG; **(b)** purified His_6-tagged NovE. Analysis of DNA-binding activity was carried out as described in (Dangel *et al.*, 2008), using approximately 4 ng of the indicated DIG-end-labelled fragment and either no protein (-) or 1.5 µg of purified His_6-tagged NovG or 3 µg of purified His_6-tagged NovE protein (+). Above each autoradiogram a map is given showing the location of the DNA fragments used (out of scale; the intergenic regions are oversized in comparison to the coding sequences). The fragments were obtained by digestion of appropriate plasmids or by PCR amplification. For fragments obtained by digestion, the restriction sites are indicated: E, *EcoR*I; P, *Pvu*I; S, *Sal*I; A, *Ava*I.

III.1.5. Complementation of the *novE* mutation by *novG* under control of its own promoter

The failure to show binding of NovE to the *novG* promoter region questioned the hypothesis that *novE* acts as positive regulator of *novG* transcription. Therefore, plasmid pAE8 (Eustáquio *et al.*, 2005b) which contains the structural gene *novG* together with its genuine promoter region in the promoterless *E. coli/Streptomyces* shuttle vector pWHM3 (Vara *et al.*, 1989) was used to complement the *ΔnovE* strain described above. Indeed, complementation was readily achieved with this construct (Table III.1). This proved that *novG*-expression from its own promoter can occur in the absence of NovE.

III.1.6. Complementation of the *novG* mutation by *novE*

A heterologous expression strain carrying a *novG* deficient novobiocin cluster, i.e. *S. coelicolor* M512 (nov-AE10) (Eustáquio *et al.*, 2005b), produced only 2 % of the novobiocin amount found in the strains carrying the intact cluster (Table III.1). When this strain was transformed with plasmid pVD4, carrying *novE* under control of its own promoter, production levels increased approximately 6-fold (Table III.1), but did not reach the original production levels of the strains carrying the intact cluster. In contrast, complementation of the *novG*-defective strain with an intact copy of *novG* under control of its own promoter (pAE8) restored most of the original production (Table III.1). Therefore, *novG* is able to fully complement a *novE* defect but *novE* can only partly complement a *novG* defect.

III.1.7. RT-PCR experiments

In order to investigate whether *novG* transcription depends on the presence of NovE, and whether *novE* transcription depends on the presence of NovG, RT-PCR experiments were carried out in the strain carrying the complete cluster, as well as in the strains lacking the *novE* or the *novG* gene, respectively (Fig. III.3). The outcome of these experiments showed that at least some *novG* transcription takes place in the absence of NovE, and likewise that *novE* transcription occurs in the absence of NovG.

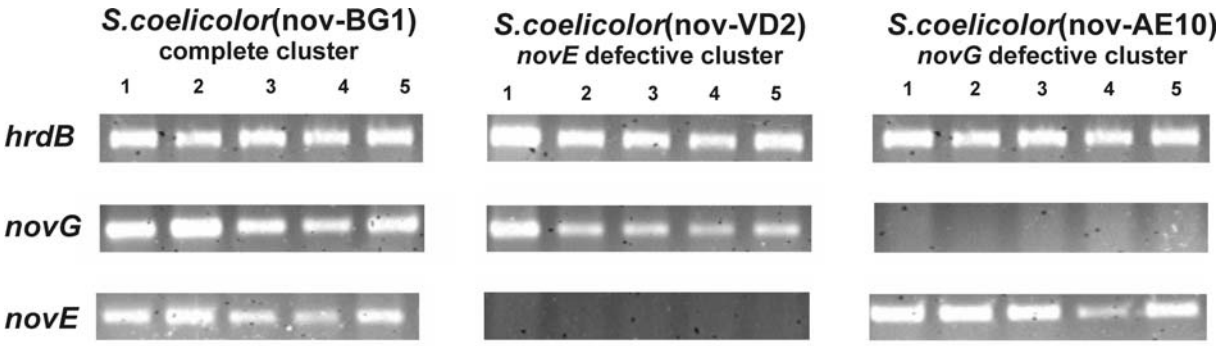

Fig. III.3: RT-PCR analysis. RT-PCR results of *novE* and *novG* transcription in *Streptomyces coelicolor*(nov-BG1) containing the entire novobiocin biosynthetic gene cluster, in the *novE*-defective strain *Streptomyces coelicolor*(nov-VD2) and in the *novG*-defective strain *Streptomyces coelicolor*(nov-AE10) (Eustáquio *et al.*, 2005b)); 1-5 = different times of cultivation (1 = 22 hours, 2 = 30 hours, 3 = 44 hours, 4 = 77 hours, 5 = 94 hours); *hrdB* = house-keeping gene encoding for the sigma factor of the DNA-dependent RNA-polymerase; *novE* and *novG* = putative regulatory genes.

III.2. Investigations on the genetic organization and transcriptional regulation of the novobiocin biosynthetic gene cluster

III.2.1. Sequence analysis of the novobiocin biosynthetic gene cluster

The novobiocin biosynthetic gene cluster spans 23.4 kb and comprises 20 coding sequences (Li & Heide, 2004; Li & Heide, 2006). The genes *novHIJKLM*, separated by very short intergenic regions (<19 bp), are responsible for the synthesis and the linkage of the aminocoumarin moiety (Fig. I.3). *novQR* are responsible for the generation of the prenylated 4-hydroxybenzoate moiety, and *novSTUVW* for the generation of the deoxysugar. The coding sequences for *novQ* and *novR*, as well as of *novS* and of *novV* and *novW*, overlap, suggesting a translational coupling of these genes. *novN*, *novO* and *novP* are responsible for tailoring reactions, i.e. the carbamoylation and methylation of the novobiocin skeleton (Fig. I.3). *novF* is probably responsible for the availability of 4-hydroxyphenylpyruvate, a precursor of both aromatic moieties of novobiocin (Fig. I.3). *gyrB*^{R} codes for a resistance gene, and *novE* and *novG* for putative regulators of novobiocin biosynthesis (see introduction and III.1.). Large intergenic regions, suggestive of the presence of promoters, are found within the cluster, i.e. upstream of *novE* (180 bp intergenic region), *novG* (105 bp), *novH* (195 bp), *novO* (230 bp) and *gyrB*^{R} (376 bp).

For three of these regions, direct or indirect experimental evidence has confirmed their promoter activity, i.e. for the regions upstream *novE* (Dangel *et al.*, 2008), *novH* (Eustáquio *et al.*, 2005b) and *gyrB*^{R} (Thiara & Cundliffe, 1989). In contrast to the streptomycin cluster, which contains four binding-sites of the positive regulator protein StrR, the novobiocin cluster contains only a single binding-site for the StrR-ortholog NovG. This binding-site is situated upstream of *novH* (Eustáquio *et al.*, 2005b). Both, bioinformatic sequence analysis and electrophoretic mobility shift assays confirmed that no NovG binding-site is situated in the large intergenic region upstream of *novO* (Alessandra Eustáquio´s thesis).

Therefore, all 16 genes from *novH* to *novW*, which are arranged in the same orientation and code for all enzymes of novobiocin biosynthesis from 4 HPP (Fig. I.3),

may be transcribed as a single operon under control of the promoter upstream of *novH*. This promoter is probably regulated by the DNA-binding protein NovG.

III.2.2. Insertion of transcriptional terminators into the novobiocin biosynthetic gene cluster

A convenient method for the mapping of transcription units is the use of Ω (omega) interposon, i.e. a DNA fragment containing an antibiotic resistance marker flanked by short inverted repeats which contain termination signals for transcription (Prentki & Krisch, 1984).

In these studies the Ω*aac* cassette (Blondelet-Rouault *et al.*, 1997) was used which contains the apramycin resistance gene *aacC4*, selectable both in *E. coli* and in *Streptomyces*. While previous studies used conventional cloning techniques to introduce their cassette into the transcription unit of interest (Raynal *et al.*, 2006), in these studies the much more versatile λ RED-mediated recombination technique (Gust *et al.*, 2004) was used to insert the 1.8 kb Ω*aac* cassette into the coding sequence of the genes *novE*, *novF*, *novG*, *novH*, *novO* and *novS*. In each case, the cassette was inserted between nucleotides 3 and 7 of the coding sequence of the gene, i.e. replacing the second codon of the coding sequence. For this purpose, the Ω*aac* cassette was amplified with primers containing 39 bp homolog extensions, identical to the sequences upstream and downstream of the second codon of the gene of interest. The PCR products were used for λ RED-mediated recombination in *E. coli*, using cosmid nov-BG1 as target (Eustáquio *et al.*, 2005a). This cosmid contains the complete novobiocin cluster, as well as the integration functions of the phage *ΦC31*. The resulting cosmids with the inserted Ω*aac* cassettes were integrated into the genome of *Streptomyces coelicolor* M512 as described previously (Eustáquio *et al.*, 2005a). Southern blotting confirmed that in all integration mutants the entire cosmid had integrated site-specifically into the *ΦC31* attachment site of the genomic DNA (data presented in Johannes Härle´s diploma thesis).

III.2.3. Identification of promoter regions by reverse transcriptase PCR-analysis of termination mutants

As shown previously, heterologous expression of the intact novobiocin cluster in *S. coelicolor* M512 leads to the production of novobiocin, in amounts similar to those formed by the wild-type novobiocin producer strain (Eustáquio *et al.*, 2005a). Correspondingly, reverse transcriptase PCR (RT-PCR) experiments now revealed the presence of transcripts for all genes of the novobiocin gene cluster when the strain was cultivated in novobiocin production medium (Fig. III.4a). Controls without the preceding reverse transcriptase reaction confirmed that the detected signals were due to cDNA rather than to contamination with genomic DNA.

Insertion of the Ω*aac* cassette into a transcription unit leads to termination of mRNA synthesis, therefore to a lack of RT-PCR signals for the genes downstream of the inserted cassette. Transcription is re-initiated at the next active promoter sequence downstream of the Ω*aac* insertion. As shown in Fig. III.4b, c and d, insertion of the Ω*aac* cassette into *novE*, *novF* and *novG* led, as expected, to a complete abolishment of the transcription of the affected genes. In all three cases, however, transcripts of the adjacent gene, i.e. *novF*, *novG* and *novH*, repectively, were detectable, indicating the presence of promoter regions upstream of these genes. In contrast, insertion of Ω*aac* into *novH* led to a complete abolishment of the transcription of *novHIJKLMN*, indicating that all of these genes form a single transcription unit (Fig. III.4e). Transcripts were detected, however, for *novO* and the genes downstream thereof, indicating the presence of a promoter region upstream of *novO*.

Consequently, three additional mutant strains have been generated, carrying Ω*aac* cassettes in *novO*, *novP* and *novQ* using the same method as described above. In the first two cases, transcripts of the adjacent genes, i.e. *novP* and *novQ* were detected (Fig. III.4f and g), indicating promoter sequences located upstream of these genes. In the strain carrying Ω*aac* cassette in *novQ*, however, transcription of the following genes was completely abolished, indicating that these genes form a transcription unit (Fig. III.4h). This was confirmed by the results from the strain carrying Ω*aac* in *novS*, showing complete abolishment of the transcription of *novSTUVW* (Fig. III.4i).

Therefore, our experiments confirmed the previously supplied indirect evidence that a promoter region is located upstream of *novH*, and additionally showed that promoter regions appear to be localised upstream of *novF*, *novG*, *novO*, *novP* and *novQ*. Experimental evidence for the promoter activity of the DNA region upstream of the putative regulator *novE* and the resistance gene *gyrB*^{R} has been published previously (Dangel *et al.*, 2008; Thiara & Cundliffe, 1989). Therefore, at least eight promoter regions are present within the novobiocin cluster (Fig. III.4k).

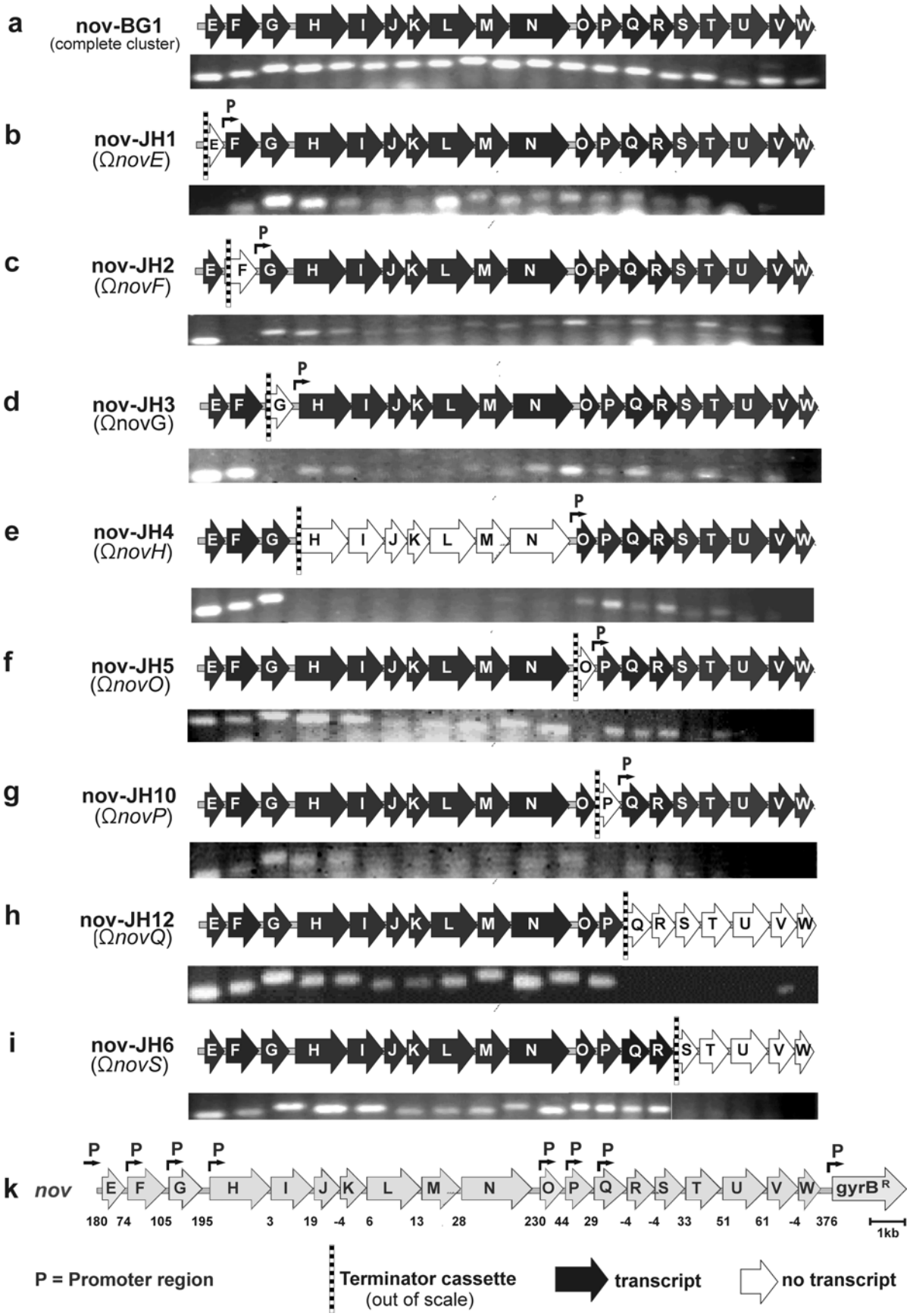

Fig. III.4 See next page for details.

(a-i) Schematic presentation of constructs nov-BG1, nov-JH1 to nov-JH6, nov-JH10 and nov-JH12 including RT-PCR results (the practical part of these investigations was carried out in cooperation with Johannes Härle as diploma student; for details see Johannes Härle´s diploma thesis). **(k)** Transcriptional organisation of the novobiocin biosynthetic gene cluster including *gyrB*^{R}.

III.2.4. Real-time PCR investigations of the transcriptional regulation of the novobiocin cluster by *novE* and *novG*

In order to investigate the influence of *novE* and *novG* on the transcription of the genes of the novobiocin cluster, quantitative RT-PCR (qRT-PCR) experiments using the LightCycler® method were carried out. Suitable primer pairs were chosen for each of the eight genes located downstream of putative promoter sequences within the novobiocin cluster, i.e. *novE*, *novF*, *novG*, *novH*, *novO*, *novP*, *novQ* and *gyrB*^{R}. Reaction conditions, i.e. annealing temperature and $MgCl_2$ concentrations, were optimized for each primer pair until a linear relationship between the logarithm of the mRNA concentration and the cycle number was obtained over a concentration range of at least two orders of magnitude.

The respective reaction conditions are described in the experimental, and the PCR primers are shown in Table II.17. For *novE*, *novG* and *novP* the primers designed for the reverse transcriptase PCR could also be used for qRT-PCR. For *novF*, *novH*, *novO* and *novQ*, however, new primers had to be designed for quantitative mRNA determination. 10 ng of total RNA were used for each LightCycler® reaction. The *hrdB* transcript, coding for the principle sigma-like transcription factor of *Streptomyces coelicolor*, was used as internal standard and the number of transcripts for each sample was normalized to *hrdB*.

qRT-PCR analysis was then carried out for a strain carrying the intact novobiocin cluster, as well as for strains carrying clusters in which either the gene *novE* or the gene *novG* had been inactivated by an in-frame deletion within the coding sequence. The generation of these three strains and the use of *Streptomyces coelicolor* M512 as host for the heterologous expression of the clusters has been described previously (Dangel *et al.*, 2008; Eustáquio *et al.*, 2005a; Eustáquio *et al.*, 2005b).

Cultivation of these three strains in the chemically defined novobiocin production medium was carried out in 24-square deepwell plates (Duetz *et al.*, 2000) which allows much more reproducible cell growth and novobiocin production rates than conventional Erlenmeyer flask cultivations (Stefanie Siebenberg´s thesis).

First, the time course of novobiocin production in this culture system was determined. In accordance with previous results (Stefanie Siebenberg´s thesis) only traces of novobiocin production were detected within the first 48 hours after inoculation, while the highest novobiocin production rate was observed between 72 and 96 hours after inoculation (Fig. III.5a).

In contrast, dry cell weight increases between 24 and 72 hours after inoculation (Stefanie Siebenberg´s thesis). Identical to the observation from the genuine producer strain that novobiocin production starts at the transition from growth phase to stationary phase (Kominek, 1972).

As may be expected, the highest amounts of transcripts for the novobiocin biosynthetic genes *novH*, *novO*, *novP* and *novQ* were detected immediately before the onset of novobiocin production, i.e. 48 hours after inoculation (Fig. III.5, e-h). All transcripts were still clearly detectable after 72 and 96 hours, i.e. well into stationary phase. Expression of all four genes was perfectly synchronous, which is in accordance with the hypothesis stated above that all these genes may be transcribed as a single operon. For the genes coding for enzymes of novobiocin biosynthesis, i.e. *novH* and *novOPQ*, hardly any transcripts were found at 32 hours after inoculation. In contrast, transcripts for the two putative regulators *novE* and *novG* could clearly be detected at this time point, indicating that the expression of the regulatory genes may precede the expression of the biosynthetic enzymes (Fig. III.5,b and d). Maximal transcripts amount of *novE* and *novG* were found 48 hours after inoculation.

The resistance gene $gyrB^R$ showed a low level of transcription even at 24 hours after inoculation. A first maximum of $gyrB^R$ transcripts was detected after 48 hours, i.e. synchronous to the expression of *novH* and *novOPQ*. It is tempting to speculate that $gyrB^R$ may be co-transcribed together with these genes. In contrast to *novH* and *novOPQ*, however, the amount of transcripts for $gyrB^R$ increases again after 72 hours, i.e. when novobiocin concentration is increasing. This is in perfect accordance with

the results of (Thiara & Cundliffe, 1989), who cloned the promoter region of *gyrB*^{R} into a promoter probe vector and showed (by expression in *Streptomyces lividans* TK24) that the promoter was induced by cultivation in the presence of novobiocin. Since cultivation in the presence of ciprofloxacin, a gyrase-inhibitor interacting with the A subunit of this enzyme, had the same inducing effect, Thiara and Cundliffe suggested that the induction is not directly mediated by novobiocin but by the change of superhelical density of chromosomal DNA, caused via the gyrase inhibition excerted by novobiocin or ciprofloxacin.

In contrast to the strain with the intact cluster, the two strains with in-frame deletions in the putative regulators *novE* and *novG* showed only very low novobiocin production (Fig. III.5a). Seven days after inoculation, the strain with the intact cluster had accumulated 45 mg/l novobiocin, whereas the Δ*novE* and Δ*novG* strain had produced less than 0.4 mg/l.

As immediately obvious from Fig. III.5e-h, the amounts of transcripts for the novobiocin biosynthetic genes *novH* and *novOPQ* were dramatically reduced in the Δ*novE* and Δ*novG* strains in comparison to the strain with the intact cluster, providing for the first time, direct evidence that *novE* and *novG* act as transcriptional regulators of novobiocin biosynthesis.

Notably, also *novG* transcription was strongly reduced in the Δ*novE* strain: at 48 hours after inoculation, the amount of transcripts was only 5 % of that observed in the strain with the intact cluster (Fig. III.5d). This suggests that *novG* expression is largely, though not entirely dependent on the presence of *novE*. In contrast, *novE* expression was still high in the Δ*novG* strain (Fig. III.5b), suggesting that *novE* expression is not dependent on *novG*.

The resistance gene *gyrB*^{R} shows low basal transcription during the growth phase of both, the Δ*novE* and the Δ*novG* strain, suggesting a low constitutive expression from the *gyrB*^{R} promoter. An alternative explanation is that the basal transcription rate observed for *gyrB*^{R} results in fact from the highly similar constitutively expressed gene *gyrB* (=SCO3874) of the host strain *S. coelicolor*. This gene has 82% identity with *gyrB*^{R} on the nucleotide level, and the priming-site for the forward and reverse

primer for *gyrB*^{R} can also be found in this gene, although with five and two mismatches, respectively.

The predicted gene product of *novF* shows high sequence similarity to prephenate dehydrogenases and is therefore expected to supply 4-hydroxyphenylpyruvate (4-HPP), the common precursor of both, the prenylated 4-hydroxybenzoate moiety and the aminocoumarin moiety of novobiocin (Fig. I.3). Expression of *novF* is detectable at 32 hours after inoculation (Fig. III.5c), i.e. earlier than the expression of the biosynthetic genes *novH* and *novOPQ*. This may ensure the availability of the precursor 4-HPP just before the novobiocion biosynthesis is initiated. Transcription of *novF* is remarkably similar to that of *novE* in the strain with the intact cluster and in the Δ*novG* strain (Fig. III.5c), which indicates either a close co-regulation or even a co-transcription of both genes.

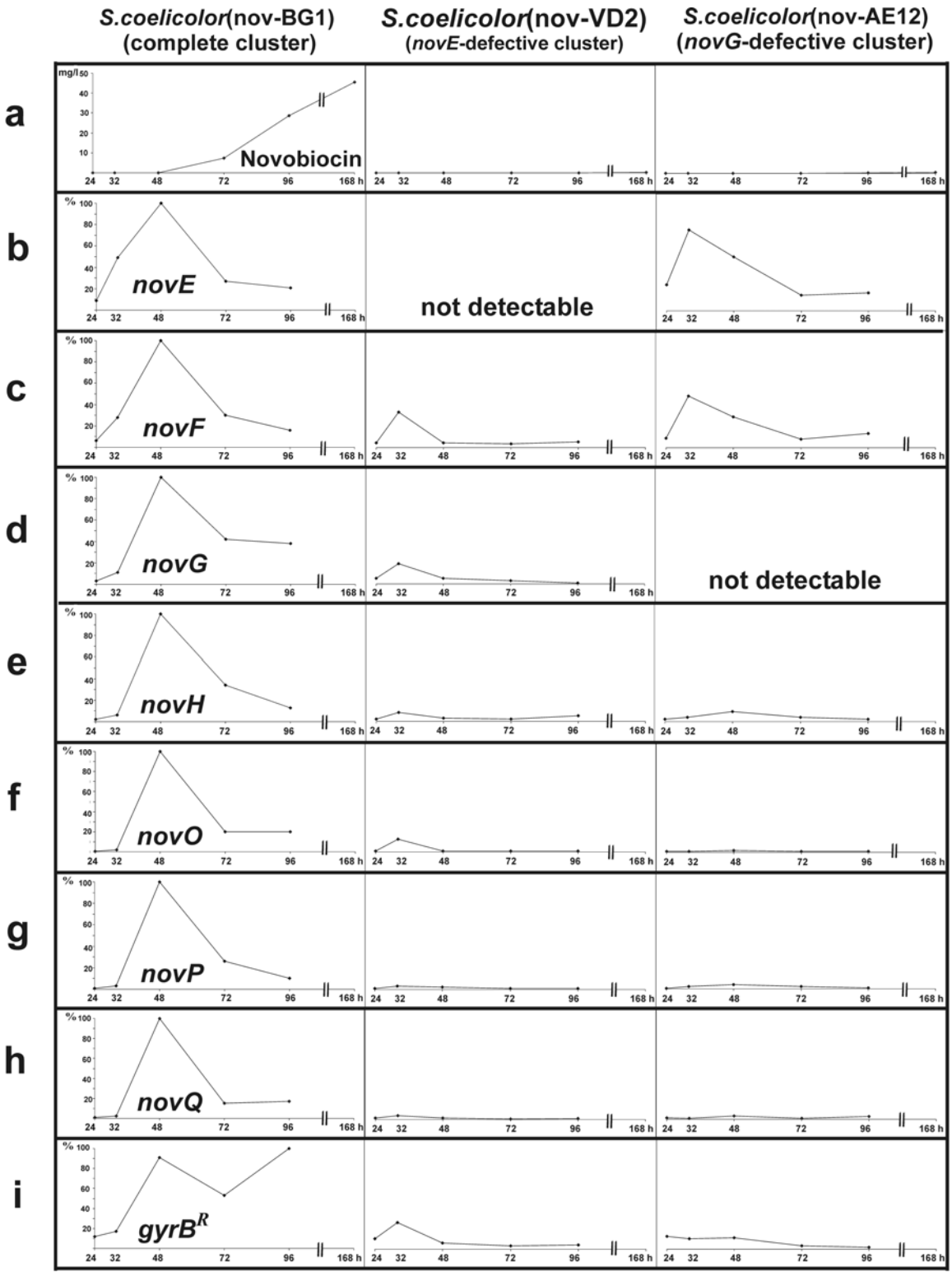

Fig. III.5: (a) Production curves and **(b-i)** results of qRT-PCR experiments for *Streptomyces coelicolor* M512 (nov-BG1) containing the entire novobiocin biosynthetic gene cluster, *Streptomyces coelicolor* M512 (nov-VD2) containing the *novE*-defective cluster (Dangel *et al.*, 2008) and *Streptomyces coelicolor*(nov-AE10) containing the *novG*-defective cluster (Eustáquio *et al.*, 2005b).

III.2.5. Contribution of the promoter regions upstream of *novO*, *novP* and *novQ* to the transcription of the novobiocin cluster

Insertion of the Ω*aac* cassette into *novH* led to the complete termination of the transcription of *novHIJKLMN* (see above). Consistently, the strain carrying this insertion (hereafter called Ω*novH* strain) did not produce any detectable amounts of novobiocin (<0.2 mg/l), while a strain carrying the intact cluster produced 51 mg/l novobiocin. Notably, however, the Ω*novH* strain did also produce detectable amounts of the prenylated 4-hydroxybenzoate moiety (0.3 mg/l). In contrast, a clorobiocin producer strain in which the gene *clol* (orthologues to *novl*) had been inactivated by in-frame deletion produced 3.75 mg/l of the prenylated 4-hydroxybenzoate moiety (Pojer *et al.*, 2003).

This suggested that in the Ω*novH* strain only small amounts of *novQ* and *novR* transcripts (which direct the biosynthesis of prenylated 4-hydroxbenzoate) are formed. This was confirmed by qRT-PCR experiments, composing the expression rate of *novG*, *novH*, *novP* and *novQ* in *S. coelicolor* M512 (nov-BG1) (carrying the intact cluster) and *S. coelicolor* M512 (nov-JH4) (carrying the Ω*novH* cluster) (Fig. III.6). In the latter strain the transcription of *novH* was reduced to <1 % in comparison to the former strain, but also the transcription of *novP* and *novQ* was reduced to 3% in comparison to the strain with the intact cluster (Fig. III.6). This can be explained by the hypothesis that transcription of *novO*, *novP* and *novQ* (and of the genes located downstream thereof) is mainly controlled by the *novH* promoter initiating a large transcript from *novH* to *novW*, while the promoter regions upstream of *novO*, *novP* and *novQ* have only minor relevance for the amount of transcripts formed. This is consistent with the results from bioinformatic analysis which showed no NovG binding-site in the *novOPQ* region.

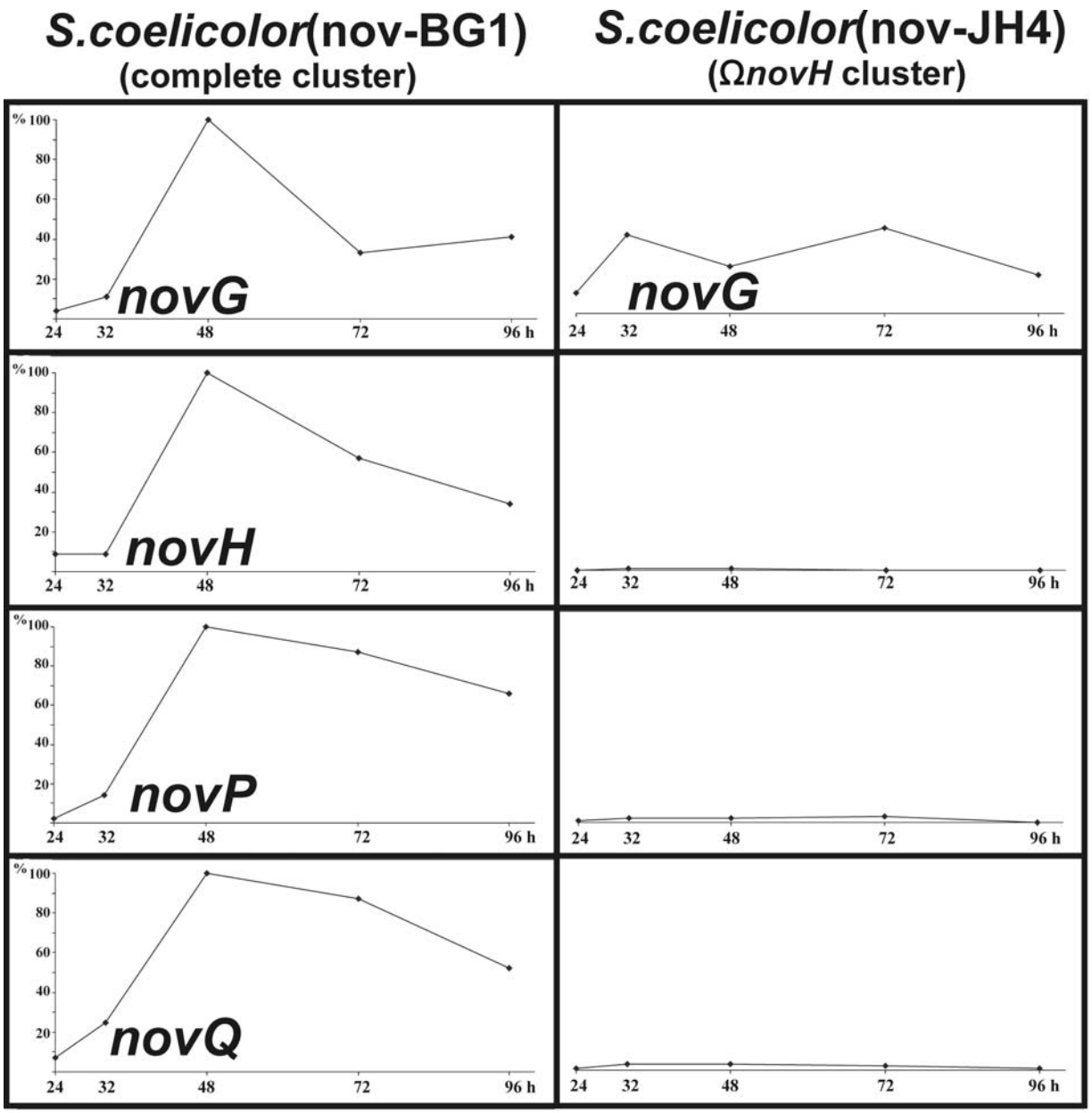

Fig. III.6: Results of qRT-PCR experiments for *Streptomyces coelicolor* M512 (nov-BG1) containing the entire novobiocin biosynthetic gene cluster and *Streptomyces coelicolor* M512 (nov-JH4) containing the Ω*novH*-cluster.

III.2.6. A high novobiocin production is achieved by an optimized *novG* expression vector

The results described above suggest that control of transcription from the *novH* promoter is the central mechanism for the regulation of novobiocin biosynthesis. It is tempting to investigate whether this knowledge can be used to further increase novobiocin yields.

In a previous study (Eustáquio *et al.*, 2005b) novobiocin production could be restored in a *novG*-defective heterologous expression strain by complementation with the multicopy plasmid pAE8, containing an intact copy of *novG*. Notably, in a strain containing an intact novobiocin cluster, i.e. in *S. coelicolor* (nov-BG1), pAE8 caused a 2.7-fold overproduction of novobiocin. In the present study, this result could be reproduced, obtaining 2.9-fold overproduction by pAE8 in comparison to the empty vector control.

NovG has been identified as a DNA binding protein. Both, electrophoretic mobility shift assays and bioinformatic sequence analysis have identified the NovG binding-site, a palindromic structure beginning 2 bp downstream of the *novG* stop codon, i.e. 194 bp upstream of *novH* start codon (Fig. III.7). Due to the recognition that pAE8, the first plasmid for *novG* overexpression, contained 135 bp of the intergenic region downstream of *novG*, i.e. pAE8 included the NovG binding-site (Fig. III.7), it was tempting to speculate that pAE8 may not be an optimal construct for the stimulation of novobiocin production, as the produced NovG protein may bind to the NovG binding-site in pAE8, which is present in many copies in the cell, leaving only a fraction of the produced NovG available for binding to the site in the novobiocin cluster which had been integrated into the genome of the heterologous host. Therefore, a new *novG* expression plasmid was constructed by Alessandra Eustáquio, named pAE12, which contained just 1 bp of the intergenic region downstream of *novG*, and therefore did not contain the NovG binding-site (Fig. III.7). Otherwise, this plasmid was identical to pAE8. Transformation of this plasmid into a heterologous expression strain carrying the intact novobiocin cluster resulted in 8.4-fold overproduction of novobiocin compared to the empty vector control. Therefore, transformation with pAE12 turned out to be the most effective method identified so far in order to increase novobiocin production in a heterologous producer strain.

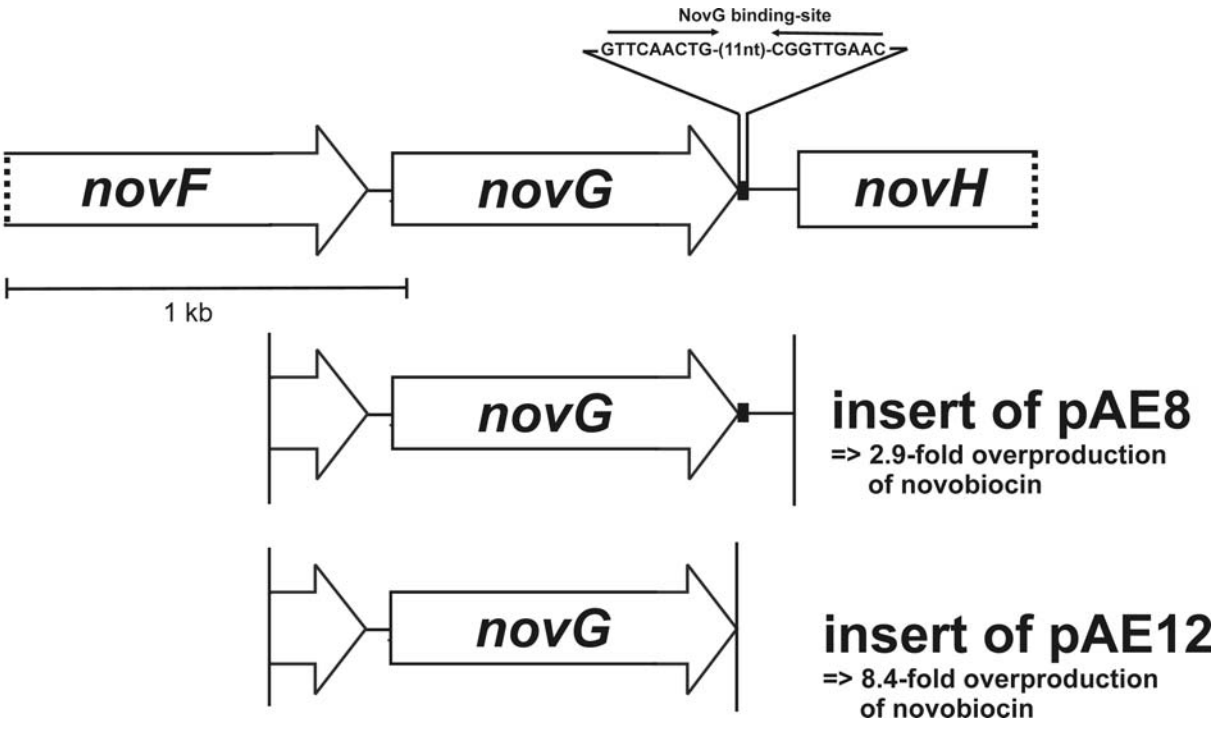

Fig. III.7: Inserts of the *novG* overexpression plasmid pAE8 and pAE12. Both inserts were cloned into the promoterless pWHM3 vector. (Generation of pAE8 and pAE12 were carried out by Alessandra Eustáquio).

III.3. Regulation of novobiocin production by insertion of a tetracycline-controllable promoter 830 (tcp830)

As presented above, initiation of transcription from the promoter situated upstream of *novH*, i.e. in the intergenic region *novG_novH*, is the most important event for the regulation of novobiocin biosynthesis in the heterologous host. In accordance with this finding, the introduction of a strong inducible promoter upstream of *novH* may, in principle, result in high production and, therefore, may represent a promising strategy to uncouple novobiocin production from its natural regulation cascade.

Till now, the thiostrepton inducible promoter *ptipA* (Murakami *et al.*, 1989) represents the most widely used inducible promoter for regulating gene expression in *Streptomyces* spp. providing reliable and controllable gene expression under different circumstances. However, thiostrepton is known to induce a regulon of proteins, and to depend on the presence of an activator, TipAL, and a resistance gene, *tsr* (Ali *et al.*, 2002; Murakami *et al.*, 1989; Weaden & Dyson, 1998). Therefore, the tetracycline controllable promoter 830 (tcp830) represents an alternative valuable inducible promoter due to its activity in a wide range of natural tetracycline resistant *Streptomyces* species (Rodriguez-Garcia *et al.*, 2005). Furthermore, using the *luxAB* genes expressing luciferase as a reporter system, tcp830 has been shown to reach induction factors of up to 270.

The strategy to uncouple novobiocin biosynthesis from its natural regulation cascade, used in this study, was the replacement, not only of the promoter region upstream of *novH* (Fig. III.4k) but additionally of the entire *novEFG* region, by the tetracycline controllable promoter830 (tcp830).

III.3.1. Generation of a *novE-novG*-double defective mutant

Since the strategy to uncouple novobiocin production from its origin regulation circuit used in this study includes the deletion of both pathway-specific positive regulators *novE* and *novG*, a *Streptomyces coelicolor* M512 strain with a Δ*novE*Δ*novG*-cluster was generated in order to show the effect of inactivation of *novE* and *novG* on novobiocin production. Therefore, *novE* was deleted in cosmid nov-AE10 (Δ*novG*),

followed by integration of the resulting Δ*novE*Δ*novG*-cosmid (named nov-VD4) into *S.coelicolor* M512.

For this purpose, *novE* was replaced in cosmid nov-AE10 (Δ*novG*) by an apramycin resistance cassette flanked by FRT (FLP-recognition target) sites via λ RED-mediated recombination (Datsenko & Wanner, 2000; Gust *et al.*, 2003), resulting in nov-VD3. This cassette was excised using FLP-recombinase leaving an in-frame "scar" of 81 nucleotides between the start and stop codon of *novE*. The resulting Δ*novE*Δ*novG*-cosmid (named nov-VD4) (Fig. III.8) was subsequently introduced into *S.coelicolor* M512 by protoplast transformation, and its site-specific integration into the genome was confirmed by Southern blot analysis. HPLC analysis showed that inactivation of both pathway-specific regulators, resulted in an almost loss of novobiocin production, i.e. in non detectabable amounts. Notably, previous investigations showed that *Streptomyces coelicolor* M512 strains containing either a *novE*- or a *novG*-defective novobiocin biosynthetic gene cluster still produced novobiocin in detectable, but dramatic reduced amounts (0.7% - 2%) in comparison to strains harboring the entire cluster (Table III.1).

Subsequently, *novE* together with its own putative promoter, i.e. including 599 bp upstream of the start codon of *novE*, was cloned into pAE12, that contains *novG* together with its own putative promoter, i.e. 336 bp upstream of the start codon of *novG* in the promoterless shuttle vector pWHM3, resulting in pVD10. The introduction of pVD10 into *S.coelicolor* M512 (Δ*novE*Δ*novG*) led to restoration of novobiocin production in the resulting transformants (120 % in comparision to *S. coelicolor* (nov-BG1, containing the entire novobiocin cluster) and therefore confirmed that the almost loss of novobiocin production in *Streptomyces coelicolor* M512 (Δ*novE*Δ*novG*) was indeed caused by inactivation of both positive regulators *novE* and *novG*.

III.3.2. Uncoupling of novobiocin production from its natural regulation cascade

In order to increase novobiocin production the apra-tcp830 cassette from pMS80, containing the tetracycline-controllable promoter 830 (tcp830) and an apramycin resistance gene, flanked by FRT-sites (Rodriguez-Garcia *et al.*, 2005), was

introduced into cosmid nov-BG1 in exchange with the region from *novD* stop codon to *novH* start codon via λ RED-mediated recombination. The resulting cosmid was named nov-VD6 (Fig. III.8). Due to the risk that the effect of tcp830 could not be sufficient to ensure high transcription of all genes from *novH* to *novW*, comprising 18 kb, a second tcp830 cassette was introduced into the novobiocin biosynthetic gene cluster in exchange with the intergenic region *novP_novQ*. For this purpose, the apramycin resistance gene in nov-VD6 was firstly deleted by expression of the FLP-recombinase, leaving a “scar” of 81 nucleotides upstream of tcp830. The resulting cosmid was named nov-VD7 (Fig. III.8). The following attempt to introduce a second apra-tcp830 cassette into nov-VD7 in exchange with the intergenic region *novP_novQ*, by the repeated use of λ RED-mediated recombination did not succeed due to sequence homologies between the newly generated PCR-product and the already existing tcp830 sequence situated upstream of *novH*. Instead, a complete loss of the region from *novH* start codon to *novQ* start codon was observed. For this reason, λ RED-mediated recombination was modified as follows: the first step was the introduction of the apra-tcp830 cassette from pMS80 into cosmid nov-BG1 in exchange with the intergenic region *novP_novQ* via λ RED-mediated recombination, resulting in cosmid nov-VD8 (Fig. III.8). After digestion of nov-VD8 with *KpnI* and *SacII*, the obtained 4551 bp restriction fragment, comprising the region from 671 bp upstream of *novN* stop codon to 525 bp downstream of *novQ* start codon, including the apra-tcp830 cassette within the intergenic region *novP_novQ*, was successfully introduced into cosmid nov-VD7 via λ RED-mediated recombination, resulting in cosmid nov-VD9 (Fig. III.8). No illigitime recombination was observed this time due to the long flanking DNA sequences for homologous recombination by using the 4551 bp restriction fragment instead of the 1596 bp PCR product. Subsequently, the obtained cosmids nov-VD7, nov-VD8 and nov-VD9 were introduced into *Streptomyces coelicolor* M512 by protoplast transformation, followed by confirmation of their site-specific integration into the genome by southern blot analysis.

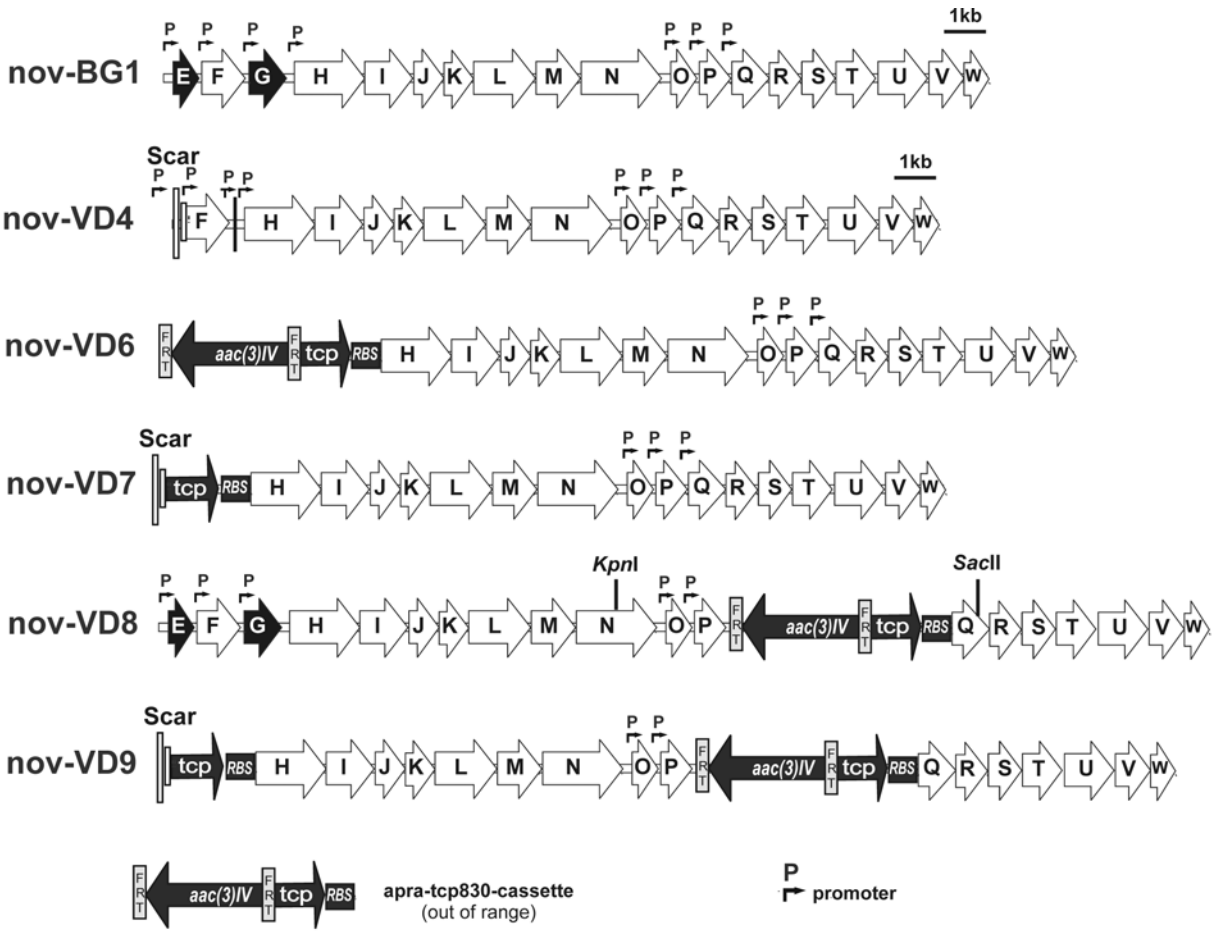

Fig.III.8: Schematic presentation of constructs nov-BG1, nov-VD4, nov-VD6, nov-VD7, nov-VD8 and nov-VD9.

III.3.3. Optimization of induction-conditions for tcp830 towards maximal novobiocin production

Investigations on the determination of optimal aTc (anhydro-Tetracycline)-concentration and time for induction of tcp830 towards maximal novobiocin production were carried out using one transformand of *S.coelicolor* M512 (nov-VD9) and *S.coelicolor* M512 (nov-BG1), harboring the entire novobiocin biosynthetic gene cluster, as a control.

For this purpose, cultivation was carried out in absence and after addition of 0.25, 0.5, 1, 2, 4, 8 or 16 µg/ml aTc at inoculation time. HPLC-analysis confirmed that *S.coelicolor* M512 (nov-VD9) produced novobiocin even in absence of aTc, but only in reduced amounts (24%) in comparison to *S.coelicolor* M512 (nov-BG1) harboring the entire novobiocin biosynthetic gene cluster. In contrast, the induction of tcp830 in

S.coelicolor M512 (nov-VD9) by addition of 0.25 to 2 µg/ml aTc resulted in a dramatic increase of novobicin production (20-fold), representing not only restoration in comparison to *S.coelicolor* M512 (Δ*novE*Δ*novG*), but additionally an up to 4.9-fold overproduction of novobiocin in comparison to *S.coelicolor* M512 (nov-BG1), harboring the entire novobiocin biosynthetic gene cluster under its natural regulation (Fig. III.9a). In contrast, addition of 0.25 to 2 µg/ml aTc did not significantly influenced novobiocin production in *S.coelicolor* M512 (nov-BG1) (Fig. III.9a). Further investigations on dry weight showed, that addition of aTc in concentrations from 0.25 to 2 µg/ml exhibits almost no effect on *S. coelicolor* M512 (nov-BG1) and only small positive effects on *S.coelicolor* M512 (nov-VD9) (Fig. III.9b). In accordance to these results, the addition of 1 µg/ml aTc for induction of tcp830 was determined to achieve maximal novobiocin production.

Subsequently, the optimal time for induction of tcp830 was investigated by addition of 1 µg/ml aTc at inoculation-time and after 6, 12, 24, 36, 48 or 72 hours of cultivation. HPLC-analysis showed that modification of induction-time from inoculation-time did not lead to a further increase of novobiocin production (data not shown). Therefore, addition of 1 µg/ml aTc at inoculation time was used for further investigations.

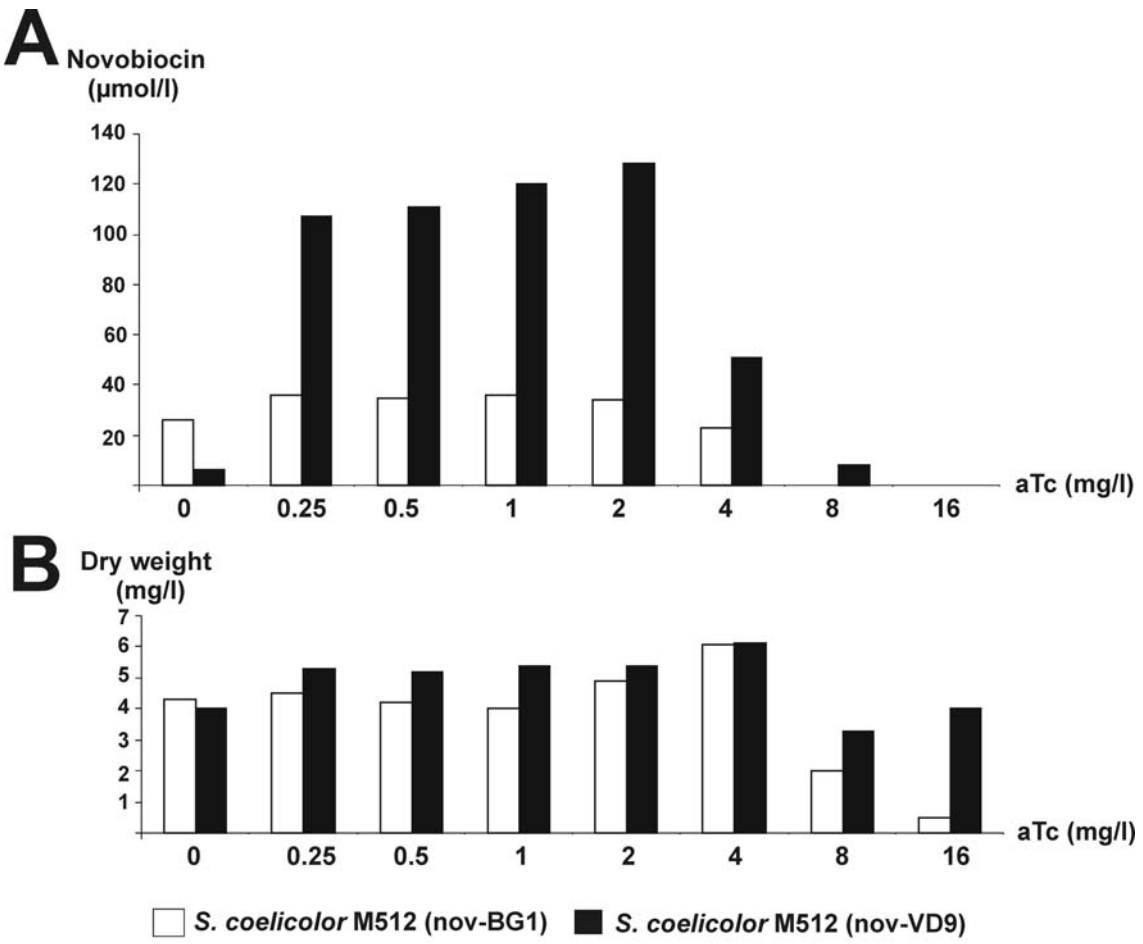

Fig. III.9 (a) Novobiocin production and **(b)** dry weight of *S.coelicolor* M512 (nov-BG1), containing the entire novobiocin cluster and *S.coelicolor* M512 (nov-VD9), containing nov-BG1 with the region from *novD* stop codon to *novH* start codon and *novP* stop codon to *novQ* start codon replaced with (apra-)tcp830 in absence and after addition of 0.25, 0.5, 1, 2, 4, 8 and 16 mg/l aTc at inoculation time.

III.3.4. Quantitative comparison of novobiocin production under natural promotor and inducible promotor control

For quantitative analysis of novobiocin production, three independent transformants of *S.coelicolor* M512 (nov-VD7), *S.coelicolor* M512 (nov-VD8), *S.coelicolor* M512 (nov-VD9) and *S. coeliocolor* M512 (nov-BG1) as a control were cultivated in absence and after addition of 1 µg/ml aTc at inoculation-time. The following HPLC-analysis of secondary metabolite production showed, that all mutants harboring tcp830, produced novobiocin even in absence of aTc, but only in reduced amounts (12 to 48%) in comparison to *S.coelicolor* M512 (nov-BG1) (data not shown).

However, the induction of tcp830 led to a dramatic increase of novobiocin production in all three mutants, resulting in overproduction of novobiocin in comparison to *S.coelicolor* M512 (nov-BG1), i.e. 1.5-fold for *S.coelicolor* M512 (nov-VD8), 1.6-fold for *S.coelicolor* M512 (nov-VD9) and 2-fold for *S.coelicolor* M512 (nov-VD7) (Fig. III.10). In accordance with these results, introduction of tcp830 upstream of *novH*, i.e. in *S.coelicolor* M512 (nov-VD7), has been confirmed of being sufficient not only to fully complement the *novEFG*-deletion, but moreover to overexpress the entire novobiocin biosynthetic gene cluster, resulting in overproduction of novobiocin. Quantitative HPLC-analysis of Ring A formation showed, that *S.coelicolor* M512 (nov-VD7) and *S.coelicolor* M512 (nov-VD9) did not produce any detectable amounts of Ring A. In contrast, the introduction of tcp830 in exchange with the intergenic region *novP_novQ*, i.e. in *S.coelicolor* M512 (nov-VD8), resulted in a 4.2-fold overproduction of Ring A in comparison to *S.coelicolor* M512 (nov-BG1) (Fig. III.10). This observation is in accordance with the localization of tcp830 in cosmid nov-VD8 (Fig. III.8), i.e. upstream of *novQ*, that in addition to *novR* encodes enzymes involved in Ring A formation (Li & Heide, 2004).

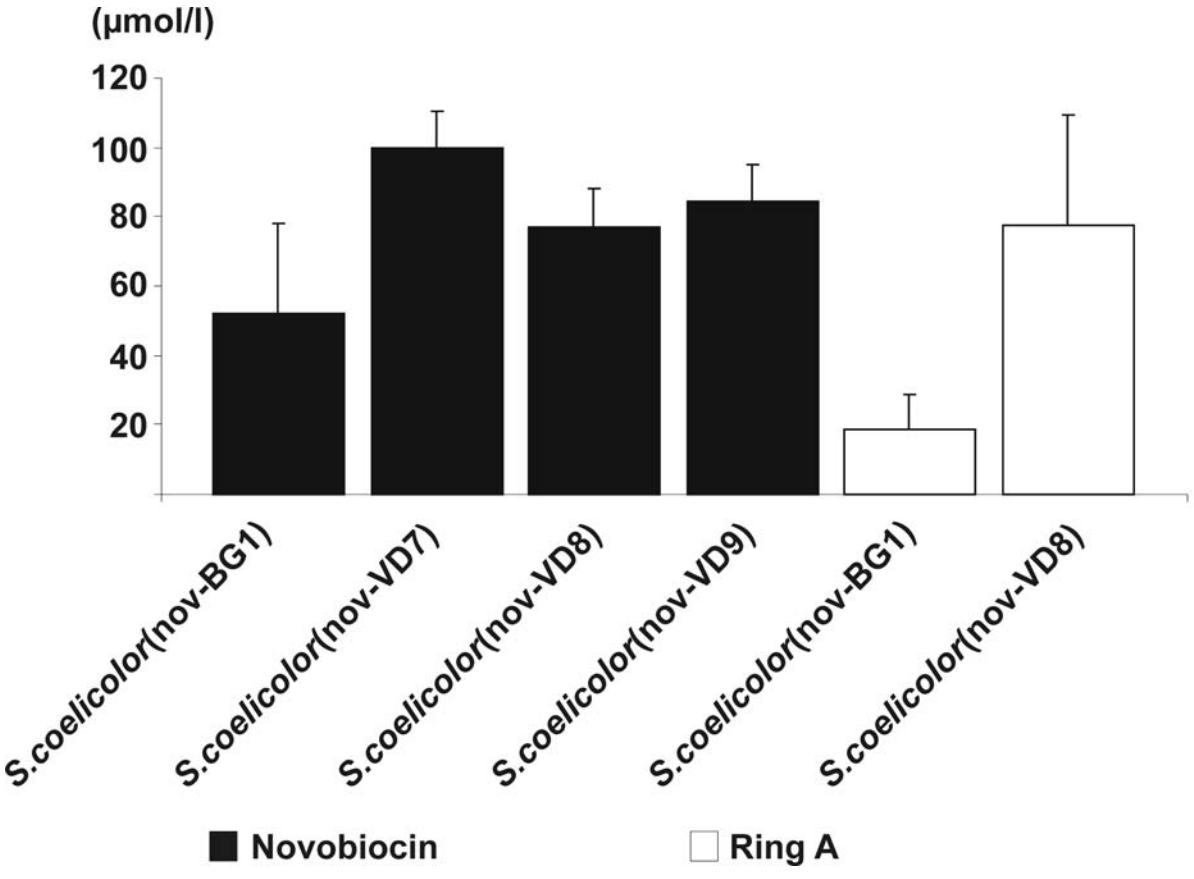

Fig. III.10: See next page for details.

Novobiocin production (black bars) of *S.coelicolor* M512 (nov-BG1) containing the entire novobiocin biosynthetic gene cluster, *S.coelicolor* M512 (nov-VD7) containing nov-BG1 with the region *novD* stop codon to *novH* start codon replaced by tcp830, *S.coelicolor* M512 (nov-VD8) containing nov-BG1 with the region *novP* stop codon to *novQ* start codon replaced by apra-tcp830 and *S.coelicolor* M512 (nov-VD9) containing nov-VD7 with the region *novP* stop codon to *novQ* start codon replaced by apra-tcp830 and Ring A formation (white bars) of *S.coelicolor* M512 (nov-BG1) and *S.coelicolor* M512 (nov-VD8). Values are means of at least three independent mutants and two independent cultivations

IV. DISCUSSION

IV.1. Investigations on the role of *novE* in the regulation of novobiocin biosynthesis and its interplay with *novG*

The biosynthetic gene cluster of the aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A_1 each contain two putative regulatory genes with high similarity within the clusters, i.e. *novG/cloG/couG* and *novE/cloE/couE*. The function of NovG as a DNA binding protein and positive regulator of novobiocin biosynthesis has been established (Eustáquio *et al.*, 2005b). This study investigated the function of *novE*.

The observations that the inactivation of *novE* led to a strong reduction but not to a complete abolishment of novobiocin production, that the overexpression of *novE* led to an increase of novobiocin production and that the *novE* defect could be complemented by an overexpression of the regulatory gene *novG* supported the hypothesis that *novE* had a regulatory rather than a catalytic function.

It was tempting to speculate that NovE may regulate the transcription of *novG*. However, RT-PCR experiments suggested that at least some *novG* transcription can occur in the absence of NovE, and that *novE* transcription can occur in the absence of NovG. Correspondingly, overexpression of *novG* under control of its own promoter stimulated novobiocin production even in a *novE*-defective strain. Vice versa, a *novG* defect strain could be complemented (at least partially) by overexpression of *novE*. These studies were carried out by heterologous expression of the modified cluster in the completely sequenced host *S. coelicolor*. The genome of *S. coelicolor* (Bentley *et al.*, 2002) does not contain any orthologues of *novE* or *novG*, which may functionally replace the experimentally deleted genes within the novobiocin cluster.

Taken together the results of these investigations prove that both *novE* and *novG* act as positive regulators of novobiocin biosynthesis, although the exact mechanism of their interplay was not clearified by these studies. Notably, NovE has been identified as a newly discovered tool to enhance novobiocin production. It remains to be shown whether the nine orthologues of *novE* deposited in the database, among them *lmbU*

from the lincomycin and *rubC4* from the rubradirin biosynthetic gene cluster, can likewise be used to increase the production of the respective secondary metabolites.

IV.2. Genetic organization and transcriptional regulation of the novobiocin biosynthetic gene cluster

In order to investigate whether *novE* and *novG* act as transcriptional activators of novobiocin biosynthetic genes and to investigate the interplay of these two regulators, qRT-PCR experiments have been carried out.

After the establishment of qRT-PCR for investigations on the novobiocin cluster, the following experiments provide the first direct proof that *novE* and *novG* act as transcriptional regulators of novobiocin biosynthesis. For *novG*, this result was expected, as its gene product shows 41 % identity on the amino acid level to *strR*, an established transcriptional regulator of streptomycin biosynthesis (Retzlaff & Distler, 1995; Tomono *et al.*, 2005). In contrast, *novE* has only few orthologues in other secondary metabolic gene clusters and sequence genomes, and no previous evidence existed on the function of these genes.

qRT-PCR experiments, comparing transcription levels in strains containing either an intact or a *novG*-defective novobiocin cluster, showed that effective transcription from the *novH* promoter depended on the presence of *novG* (Fig. III.5e). At least the seven genes *novHIJKLMN* are apparently transcribed as a single operon (Fig. III.4e). In a mutant containing a terminator cassette (Ω*aac*) in the coding sequence of either *novH*, *novO* or *novP*, RT-PCR showed transcripts of *novO*, *novP* and *novQ*, respectively, suggesting the presence of promoters upstream of these genes. However, qRT-PCR with the Ω*novH* mutant proved that transcription of *novQ* was much lower in this mutant than in a strain with the intact cluster, showing that the promoters upstream of *novO*, *novP* and *novQ* contributed only little to the overall transcription of *novQ*. The most plausible explanation of this observation is that the rate of transcription of *novOPQ* is primarily controlled by the *novH* promoter which initiates transcription of a large polycistronic mRNA. Since also *novQRSTUVW* apparently are part of a single operon (Fig. III.4h and i), it appears likely that all of the 16 genes from *novH* to *novW*, which together direct all steps of novobiocin

biosynthesis from glucose-1-phosphate and 4-hydroxyphenylpyruvate or tyrosine (Fig. I.3), are transcribed predominantly in form of a single 18.000 nt transcript. Possibly, even the resistance gene *gyrB*^{R} may be included in this transcript, extending it to 20.000 nt. Transcripts of this rise are not unusual in secondary metabolic gene clusters, as the coding sequences of modular polyketide synthase or nonribosomal peptide synthase genes often span even larger DNA regions. However, other examples of a secondary metabolic gene clusters where 15 or 16 individual genes are transcribed onto a single mRNA have not been described yet.

Whithin the suggested large operon starting with *novH*, RT-PCR had indicated the presence of internal promoters upstream of *novO*, *novP* and *novQ*. Internal promoters within operons have been described previously in *Streptomyces*, e.g. galP2, a low-level constitutive promoter internal to the galactose operon in *Streptomyces lividans* and *Streptomyces coelicolor* A3(2).

A recent study suggested that in *Streptomyces coelicolor* (in contrast to *E. coli* and *Bacillus subtilis*), expression levels of the individual genes of an operon decrease with increasing distance from the transcription start, and suggested that the frequently encountered internal promoters may ensure adequate transcription of the terminal genes of an operon (Laing *et al.*, 2006). Under experimental conditions, however, transcription from the internal promoters upstream of *novO*, *novP* and *novQ* was low, and it cannot be decided whether they have any significant role in the transcription of the novobiocin biosynthetic genes.

Sequence analysis did not show the characteristic sequence of NovG binding-site in the *novOPQ*-region, and EMSA assays did not show binding NovG (or NovE) in the intergenic region upstream of *novO*. This, and the low transcript levels for *novQ* in the Ω*novH* strain suggest, that the weak internal promoters in the *novOPQ* region are not regulated by *novG* or *novE*, and may not be regulated at all.

This study shows that effective transcription of *novG* depends on the presence of *novE* (Fig. III.5d). This suggests a cascade-like regulation mechanism of *novE* and *novG*, i.e. *novE* triggers transcription of *novG*, which in turn triggers transcription of the novobiocin biosynthetic genes. Consistant with this hypothesis, it was shown that novobiocin formation in a *novE*-defective mutant could be restored by an intact copy

of *novG* on a multicopy plasmid, while novobiocin formation in a *novG*-defective mutant remained low even after expression of *novE* from a multicopy plasmid.

Transcription of the novobiocin resistance gene $gyrB^R$ may initially occur as part of the large transcript starting from *novH*, but later on obviously follows a different pattern (Fig. III.5i). Our results may be perfectly explained by the hypothesis of (Thiara & Cundliffe, 1989) that a promoter located upstream of $gyrB^R$ is regulated by the superhelical density of DNA, which in turn is influenced by the accumulation of the gyrase inhibitor novobiocin, acting on the constitutively expressed aminocoumarin-sensitive $gyrB^S$ subunit of gyrase.

Therefore, the results of the study on the genetic organisation and transcriptional regulation of the novobiocin cluster can be summarized in the model depicted in Fig. IV. In this model, *novE* positively regulated transcription of *novG*. Since we could not demonstrate binding of the NovE protein to the DNA region upstream of *novG* (Fig. III.2b), the mechanism of regulation by *novE* is yet unknown. NovE may undergo a modification (e.g. phosphorylation) and bind to another, unknown protein before interacting with the *novG* promoter region, or it may release a repressor from this region, or trigger another event which ultimately induces transcription from the *novG* promoter.

Expression of the *novG*-ortholog *strR* in the streptomycin cluster is triggered by the binding of AdpA to defined binding-sites located in the 406 bp intergenic region upstream of *strR* (Tomono *et al.*, 2005). However, the consensus sequence for the AdpA binding-site is not found in the 105 bp intergenic region upstream of *novG*, and neither in the coding sequence of the preceding gene *novF*. Therefore, regulation of the *novG* expression appears to be different from that of *strR* expression in the streptomycin cluster.

All experiments of this study were carried out in a heterologous producer strain *S. coelicolor* M512 (a derivative of *S. coelicolor* A3(2)) with the novobiocin gene cluster integrated into the *ΦC31* attachment site of the chromosome. Exactly as described for the genuine producer strain (Kominek, 1972) novobiocin production in the CDM medium (see experimental) occured after the growth phase, suggesting that *novE* and *novG* expression may be regulated in a similar timely fashion as in the genuine

novobiocin producer strain. The exact mechanism for this regulation remains to be elucidated in both strains.

Clearly, this study suggests that initiation of transcription from the *novH* promoter is the most important event for the regulation of novobiocin biosynthesis in the heterologous host. Transcription from this promoter is controlled by the DNA-binding protein NovG, which binds to a well-defined inverted repeat sequence in the intergenic region between *novG* and *novH*. (Fig. IV). This knowledge could be utilized to achieve an 8.4-fold overproduction of novobiocin by utilization of an optimized expression vector for *novG*.

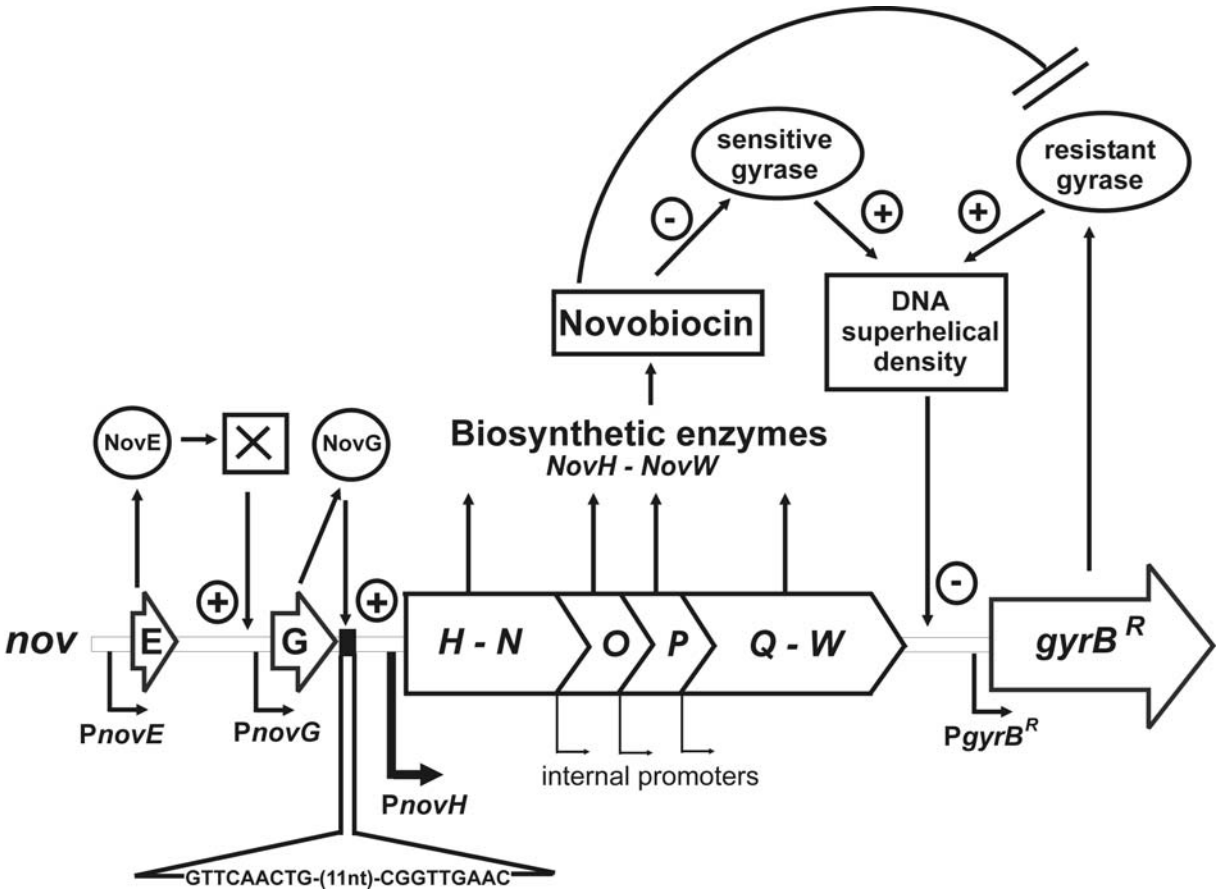

Fig. IV: Model of the genetic organization and transcriptional regulation of the novobiocin biosynthetic gene cluster.

IV.3. Regulation of novobiocin production by tetracycline-controllable promoter 830 (tcp830)

Antibiotic production is regulated by both, pathway-specific and global regulatory genes (Takano, 2006). Since the borders of the novobiocin biosynthetic gene cluster have been identified (Dangel *et al.*, 2008; Eustáquio *et al.*, 2005a) and the functions of most of the genes contained therein has been (Li & Heide, 2004), the presence of further pathway-specific regulators, in addition to *novE* and *novG*, may be excluded. Inactivation of both regulators, i.e. *novE* and *novG*, resulted in an almost loss of novobiocin production.

Strong promoters were commonly used to overexpress single genes, since their use to express entire gene clusters is often limited by the fact that gene clusters consist of multiple transcripts. Recently, the replacement of pathway-specific regulatory genes and native promoter regions within a biosynthetic gene cluster with *ermEp**, has been described as a strategy not only to uncouple secondary metabolite production from its natural regulation cascade, but moreover to result in enhanced antibiotic production, i.e. the replacement of the 3.4 kb regulatory region in the jadomycin biosynthetic gene cluster, including four regulatory genes and the promoter region P_J, by the constitutive promoter *ermEp** resulted in increased jadomycin B production in *Streptomyces venezuelae* (Zheng *et al.*, 2007).

In addition to *ermEp**, representing a constitutive promoter, several inducible promoters are available. Previous investigations confirmed the tetracycline controllable promoter 830 (tcp830) to reach induction factors of up to 270 (Rodriguez-Garcia *et al.*, 2005). In the present study tcp830 has been confirmed of being sufficient to transcribe an entire gene cluster of at least 18 kb, resulting in high antibiotic production. Notably, a recent study (Laing *et al.*, 2006) suggested that in *Streptomyces coelicolor* (in contrast to *E. coli* and *Bacillus subtilis*), expression levels of the individual genes of an operon decreases with increasing distance from the transcription start.

The finding that the replacement of the entire *novEFG*-region, including the promoter region upstream of *novH*, by tcp830, i.e. in *S.coelicolor* M512 (nov-VD7), resulted in 2-fold overproduction of novobiocin is in accordance with the result obtained in this

thesis that initiation of transcription from the *novH* promoter is the most important event for the regulation of novobiocin biosynthesis in the heterologous host.

Moreover, the observed high novobiocin production in *S. coelicolor* M512 (nov-VD7) and *S. coelicolor* M512 (nov-VD9), both lacking the putative prephenate-dehydrogenase *novF*, excludes an essential function for *novF* in novobiocin biosynthesis in the heterologous host. *novF* may be functionally replaced by SCO1761, encoding for a putative prephenate-dehydrogenase with 44% sequence homology on the amino acid level to *novF*.

In conclusion, the control of novobiocin production by use of the inducible promoter tcp830 has been identified as a new tool to enhance antibiotic production.

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