Regulation of antibiotic production in Streptomyces coelicolor

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A bacterial hormone receptor variant and

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Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
vrijdag 7 mei 2010
om 14.45 uur

door

Marco Gottelt

geboren op 7 augustus 1978
te München, Duitsland

Preface

"This one will become a researcher!" This is what my great-grandmother said about me when I was about 5 years old. I remember neither her comment nor what I had picked to pieces just before, but I guess at the latest today one can say that she was right!

I learned a lot on the way towards this booklet, about *Streptomyces*, but mostly about myself. And for both I want to thank a couple of people:

SECONDARY

COELICOLOR

STREPTOMYCES

METABOLITE

SCB BUTYROLACTONES

BIOSYNTHESIS

DNA

GENE

CLUSTER

ANTIBIOTIC

REGULATION

ABCPKK

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General introduction

Submitted for publication together with chapter 5

"γ-Butyrolactones and their role in regulation"
in "Streptomyces Molecular Biology and Biotechnology"
Marco Gottelt and Eriko Takano

The bigger picture: infectious diseases, antibiotic therapy and antibiotic resistance

Infectious diseases

The United Nations' World Health Organisation (WHO) defines infectious diseases as "caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi; the diseases can be spread, directly or indirectly, from one person to another". Infectious diseases were reported responsible for almost one fifth of the total deaths worldwide in 2002. They ranked second in the list of leading causes of death following cardiovascular diseases which, however, can also be caused by infections (World Health Organisation, 1999; World Health Organisation, 2004). And undoubtedly, the impact of infectious diseases would be even more dramatic without the application of antibiotics.

Antibiotics and antibiotic therapy

Antibiotics are the drugs of choice to combat infectious diseases caused by bacteria. The term "antibiotic" (from the Ancient Greek ἀντί – *anti*, "against", and βίος – *bios*, "life") was introduced by Selman Waksman in 1942 to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution (Waksman, 1947). Today, although not clearly indicated by its name, an antibiotic is generally understood as a compound with anti*bacterial* activity (Davey, 2000) and hence to be distinguished from, e.g., antiviral, antifungal, or anticancer drugs.

Pioneering research by Paul Ehrlich in discovering new anti-infectives in the early 20th century paved the way to modern antibiotic therapy (Kaufmann, 2008). A breakthrough – also in public awareness – was the introduction of penicillin which, after being discovered in 1928 by Alexander Fleming, saved the lives of countless soldiers during World War II (Nicolaou *et al.*, 2009). Another milestone was the first successful treatment of tuberculosis (TB) by streptomycin, an antibiotic produced by the soil bacterium *Streptomyces griseus*, in the 1940s (Hopwood, 2007).

Antibiotic resistance

The use of an antibiotic inevitably selects for resistant microbes (Clardy *et al.*, 2006). Every antibiotic introduced in medical treatment has a limited shelf life, the time that a new drug can be effectively used before clinically significant resistance emerges (Clardy *et al.*, 2006; Walsh, 2003). Bacterial resistance to antibiotics is a growing threat to the successful treatment of infectious diseases (Isturiz, 2008). For example, a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) was found to be resistant also against the current "drug of last resort", the oxazolidinon linezolid (Tsiodras *et al.*, 2001). In recent years, multi-drug resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) emerged (Centers for Disease Control and Prevention, 2006), leading to a lengthier and costlier medical treatment, if a cure is possible at all. Once thought to be under control, TB, caused by *Mycobacterium tuberculosis*, has bounced back with a vengeance to kill almost 2 million people each year, often in co-infections with HIV/AIDS (Frieden *et al.*, 2003).

Preventing the development of antibiotic resistance

Antibiotic resistance development can be prevented by limiting the use of antimicrobial agents and by prohibiting the spread of resistant bacteria. Limiting the use of antibiotics is best done by avoiding unnecessary and ineffective use, in particular in animal husbandry (Swedish Ministry of Health and Social Affairs, 2006). In 2003, the EU has banned the non-therapeutic use of antibiotics as growth promotional agents in animal nutrition (European Parliament and Council, 2003). Efficacy of antibiotic therapies could be ensured by the provision of high-quality drugs and sufficient amounts of therapeutic antibiotics for an efficient treatment in poorer regions of the world (World Health Organisation, 1999). In clinical medicine, hygiene and the isolation of patients could help to deescalate the situation (Gaynes *et al.*, 1983; Slaughter *et al.*, 1996).

Furthermore, alternative "non-antibiotic" therapies can reduce the use of antibiotics. Vaccination is an established practice to prevent or combat bacterial infectious diseases. In 1993, 85% of infants in 172 countries were immunized as part of the tuberculosis control programs (Slaughter *et al.*, 1996; World Health Organisation, 1995). On the other hand, antibacterial drugs derived from bacteriocins, proteinaceous bacterial toxins involved in microbial defence systems, can only be

envisaged today and have not yet reached the state of broad clinical applicability (Riley and Wertz, 2002; Sang and Blecha, 2008). Also, bacteriophage therapy will first have to prove its safety and therapeutic use in clinical trials (Mattey and Spencer, 2008). Antisense RNA (asRNA) technology is discussed as an upcoming method to allow a reduction in bacterial pathogenicity and to support the classical antibiotic therapy by the recovery of antibiotic susceptibility (Hebert *et al.*, 2008).

All these alternatives may eventually facilitate the treatment of infectious diseases. However, as most of these approaches are not yet implemented in clinical practice, a continuous supply of structurally novel antibacterial agents with multiple modes of action is needed to combat drug resistance (Pathania and Brown, 2008).

Antibiotic innovation – past, presence and future

During the "golden age" of antibiotic discovery from the late 1940s to the late 1960s, many new antibiotics and other natural products were identified in huge screening programs (Fig. 1) (Hopwood, 2007). Since then, mainly (semi-)synthetic modification of the known natural product scaffolds of β-lactams, macrolides and fluoroquinolones was used to generate new antibacterial drugs. Not until 40 years later, with the approval of the oxazolidinone linezolid in 2000, a new structural class of antibiotic was introduced (Walsh, 2003). Only few large pharmaceutical companies are still active in antibacterial infectious disease research, which overall leaves the clinical pipeline for antibiotics rather empty (Hopwood, 2007). Facing the threat of an increase in resistant pathogens in connection with a decrease in potent drugs and antibiotic diversity, more effort has to be put in the search for new antibiotics again (Newman *et al.*, 2003).

For future antibiotics, new targets such as the non-classical isoprenoid biosynthesis pathway and cell-surface protein sortase enzymes may expand the list of well-established targets (i.e. inhibition of either bacterial cell-wall biosynthesis, bacterial protein synthesis, DNA replication or folate coenzyme biosynthesis). Also, comparative analysis of microbial genome sequencing data will reveal the subset of essential bacterial genes and thus define molecular targets of special interest (Walsh, 2003). Furthermore, new insights into the four "classical" targets allow a

more rational drug design, including efforts to overcome known resistance mechanisms (Clardy *et al.*, 2006).
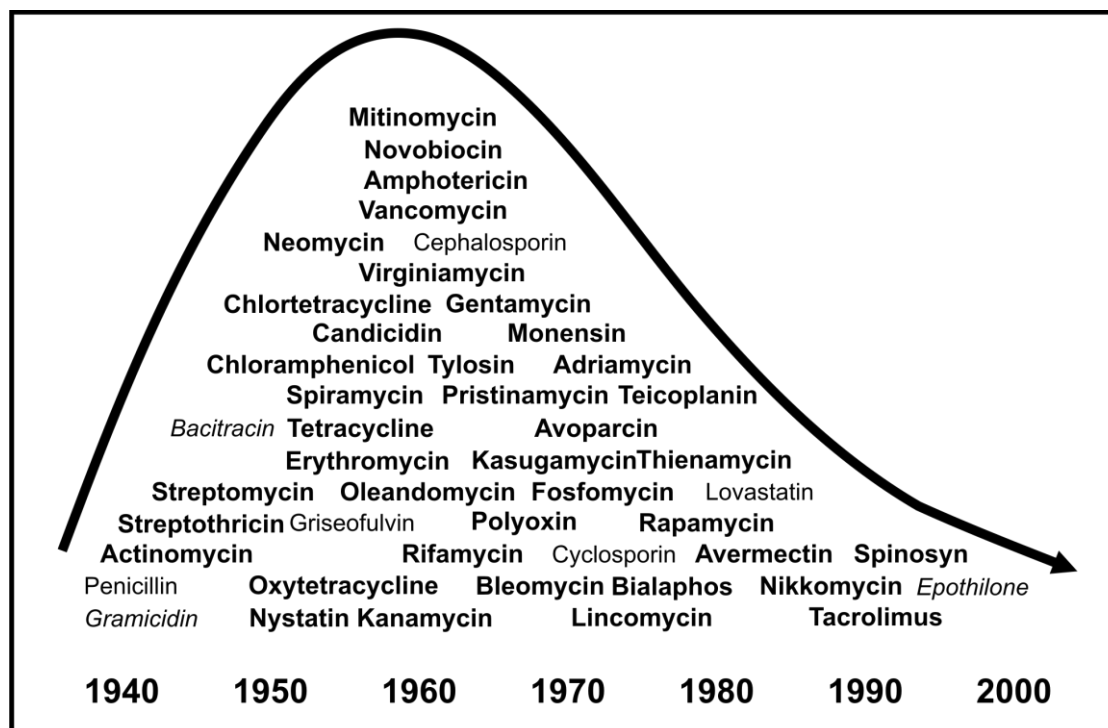
Figure 1 Discovery of important antibiotics and other natural products over the years (adapted from Hopwood, 2007). Bold type indicates actinomycete products; normal type indicates fungal products; italic type indicates products from non-actinomycete bacteria.

New antibiotics – from where?

New antibiotics from synthetic chemistry

Synthetic chemistry produces antibacterial compounds from structures not found in nature, such as the sulfonamide drug family and the fluoroquinolones (Walsh, 2003). It has been argued that combinatorial chemistry will meet its boundaries not by the astronomical number of diverse structures that can be synthesized, but by the limited complexity of the compounds, which is relatively low when compared to natural products. Thus, natural products will continue to be a primary source of new antibiotics for the foreseeable future (Baltz, 2006). However, it is now common sense that both disciplines, combinatorial chemistry and natural product research, will support, fertilize and rely on one another (Bode and Muller, 2005).

New antibiotics from natural products

More than three quarters of the clinically used antibiotics are either natural products or semi-synthetic derivatives of these molecules (Newman *et al.*, 2003). These drugs originally derive mostly from fungi and from the bacterial order Actinomycetales (Fig. 1), especially from the genus *Streptomyces* (Hopwood, 2007). From these, antibiotics are produced as so-called secondary metabolites. In contrast to primary metabolites, secondary metabolites are not directly involved in the normal growth, development, or reproduction of the bacteria, but, nevertheless, can be beneficial under certain circumstances. In the case of antibiotics, for example, one can easily imagine these as "chemical weapons" against competing microorganisms in the struggle for nutrients.

The traditional way to identify antibiotics is to screen extracts prepared from their microbial natural producers against cultures of pathogens (Baltz, 2007). The success of screening programs today is challenged by the rediscovery of known natural products with high frequency and the technical problems associated with purification and structure elucidation of natural products from microbial fermentation (Challis, 2008). Adaptation of the screening conditions, e.g., high-throughput screening against a model organism expressing several antibacterial resistant genes in one strain, would enhance the chances for discovering novel antibiotics and regain effectiveness (Baltz, 2006).

Natural products – still a promising source for novel antibiotics

The continual rediscovery of known natural antibiotics raised the question whether natural products and their producers might soon be exhausted as a source for novel antibacterial agents (Watve *et al.*, 2001). However, even after 70 years of exploitation, the number of bioactive compounds still to be found from natural sources seems to be almost infinite. Estimates based on past success rates in drug discovery and the biodiversity of the producing organisms predict several thousand compounds deriving from the genus *Streptomyces* only, of which not more than 1-3% has been unearthed so far (reviewed in Clardy *et al.*, 2006). Approaches are being developed to culture "uncultivable" antibiotic producers as one way to access the diversity of the yet undiscovered compounds (Joseph *et al.*, 2003; Kaeberlein *et al.*, 2002).

Natural product research in the post-genomic era

Entering the post-genomic era, with currently more than a thousand complete microbial genome sequences available, has revolutionised the field of natural product research (Challis, 2008). Together with the dramatic increase in our understanding of the genetics and enzymology of microbial natural product biosynthesis, and the availability of bioinformatics tools based on this knowledge, it is now possible to identify and analyse groups of genes located in close vicinity (gene clusters) likely to encode natural product biosynthetic pathways in sequenced microbial genomes ("genome mining"; reviewed in Challis, 2008). One example for such antibiotic biosynthesis gene clusters, easily spanning >40 kb, are bacterial Type I polyketide synthase gene clusters (Type I PKSs).

Type I polyketide synthases and combinatorial biosynthesis

Type I polyketide synthases are multifunctional enzymes that are organised into modules, each of which harbours a set of distinct, non-iteratively acting activities (domains) responsible for several biosynthetic steps during the catalysis of one cycle of the polyketide chain elongation. A loading module is followed by several extension modules, eventually releasing a primary PKS product, which then often is further modified by tailoring enzymes encoded in the PKS gene cluster. Typical domains of a Type I PKS module are, for example, acyltransferases (AT), acyl carrier proteins (ACP), ketosynthases (KS), ketoreductases (KR), dehydratases (DH) and a terminal thioesterase function (TE) to release the intermediate compound (Bode and Muller, 2005); also see Fig. 2B). Some domains are highly specific for a distinct substrate. Thus, based on sequence homology with PKSs of which the biosynthetic steps towards the end product are known, probable metabolic starter and extension units, as well as structural properties of the end products of novel polyketide synthase pathways can be predicted (Challis, 2008). The giant Type I PKS "production lines" are involved in the biosynthesis of macrolides, polyethers and polyenes, including several antibiotic scaffolds important in human medicine (e.g. erythromycin and tylosin) (Bode and Muller, 2005).

The modularity and versatility of Type I PKSs made them a major focus of "combinatorial biosynthesis" (reviewed in Bode and Muller, 2005, and Challis, 2008). Combinatorial biosynthesis can be defined as the application of genetic engineering

to modify biosynthetic pathways in order to produce new and altered structures using nature's biosynthetic machinery (Floss, 2006). The genetic and enzymatic structure of Type I PKSs allows "lego-ization", the recombination of modules from several distinct Type I PKS gene clusters in a completely new hybrid arrangement (Sherman, 2005). This approach has led to the production of, for example, several new erythromycin analogues (reviewed in Rodriguez and McDaniel, 2001). Combinatorial biosynthesis is not a recent development in natural product research (Floss, 2006). However, whole-genome analysis dramatically facilitated understanding the structure and function of the huge Type I PKS (Challis, 2008), and makes more and more "Lego brick"-modules available as building blocks for hybrid enzymes (Sherman, 2005).
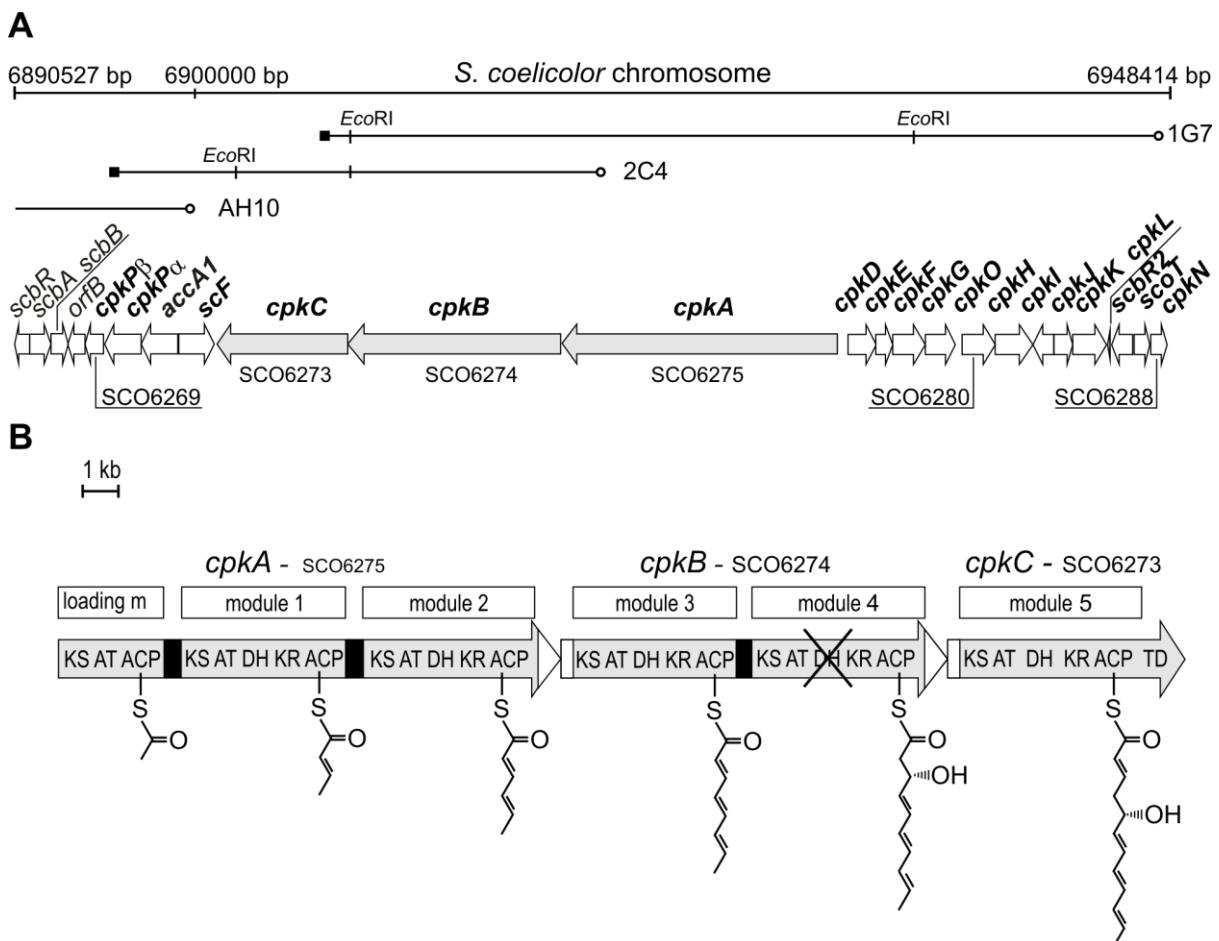
Figure 2 *cpk* gene cluster organisation and PKS domains (adapted from Pawlik *et al.*, 2007). **A** The top line denotes the *S. coelicolor* chromosome. Lines underneath denote the cosmids overlapping the *cpk* gene cluster. ORFs are shown to scale as arrows. Gene names are given above

the arrows with the *cpk* cluster genes in bold type. The SCO numbers and bp numbers correspond to Bentley *et al.* (2002). **B** Modules (boxed on top) and enzymatic domains within the PKS subunits *cpkA*, *cpkB* and *cpkC* are indicated in the grey open arrow. Intermodular linkers are marked with black squares, and docking domains with white spaces. The predicted growing carbon chains are shown and the putative non-active dehydratase domain is crossed out. "Loading m" denotes the loading module. Ketosynthases (KS), acyltransferases (AT), acyl carrier proteins (ACP), dehydratases (DH), ketoreductases (KR), and a terminal reductase domain (TD) are indicated by their abbreviations.

Orphan antibiotic gene clusters and "adoption" strategies

Genome mining of known antibiotic producers revealed the capacity to synthesize many more secondary metabolites than were actually known from these organisms (Bode and Muller, 2005). For example, before the entire genome of the model streptomycete *Streptomyces coelicolor* had been sequenced, despite decades of research, it was known to produce only four antibiotics (Hopwood, 2007). Its genome sequence revealed that it actually possesses more than 20 gene clusters for secondary metabolite biosynthesis (Bentley *et al.*, 2002). This leaves most of these gene clusters without an assigned end product. Many authors described these genetic loci as "cryptic" or "silent" (Gross, 2007). However, these terms are usually associated with a lack of gene expression, which led to a certain terminological confusion. Thus, Gross suggested the term "orphan" biosynthetic gene clusters or pathways to define "biosynthetic loci for which the corresponding metabolite is unknown" (Gross, 2007).

Genomics-guided strategies to identify a metabolite deriving from an orphan gene cluster, and thus "adopt" the orphan cluster, ideally starts with the prediction of the compound's structure, e.g., of a Type I PKS end product (Challis, 2008). Thus, the analytical challenge can be drastically reduced by focusing solely on those metabolites with matching physiochemical properties. If only a precursor or starter unit can be predicted reliably, feeding of the natural producer with adequate isotope-labelled precursors followed by NMR (nuclear magnetic resonance) spectroscopy in the so-called "genomisotopic approach" will allow to identify metabolites containing the labelled molecules. In cases where no sufficient predictions are possible, a combination of gene knockouts or heterologous gene expression with comparative metabolic profiling may provide successful strategies. The first approach involves inactivation of one or more hypothetical, biosynthetic or regulatory, genes of the orphan gene cluster, followed by the comparison of the metabolites produced with

the wild-type and the putative non-producing mutant. In the second strategy, the entire orphan gene cluster is cloned and expressed in a heterologous host. The metabolome profile of the heterologous host harbouring and lacking the biosynthetic gene cluster is then compared. Both methods rely on an appropriate powerful analytical technique such as liquid chromatography-mass spectrometry (LC-MS) for metabolic profiling (reviewed in Gross, 2007, and Challis, 2008).

Streptomyces

Streptomycetes are the predominant source for naturally produced antibiotics (Hopwood, 2007). The genus *Streptomyces* is part of the phylogenetic order Actinomycetales, Gram-positive bacteria with a high G+C content (>55%) ranging from the morphological simple rodlike *Mycobacterium* to the complex filamentous *Micromonospora*. Actinomycetales hence encompasses both *Mycobacterium tuberculosis*, the pathogen causing tuberculosis, and *S. griseus*, the producer of streptomycin, which was the first tuberculosis drug (Hopwood, 2007).

Streptomyces comprises soil-dwelling filamentous bacteria that undergo a remarkable complex life cycle accompanied by a morphological differentiation resembling that of filamentous fungi, making *Streptomyces* a model prokaryote for the study of multicellular differentiation. In solid culture, starting from a germinating spore, septated substrate hyphae are formed, branching and rapidly growing into the medium. From this vegetative mycelium, aerial hyphae emerge subsequently, growing upwards, thereby reusing materials of the partially lysed substrate mycelium. Apical growth of the aerial mycelium stops and septa are formed at regular intervals along the hyphae forming unigenomic compartments that eventually differentiate into a chain of mature spores (reviewed in Horinouchi, 2007).

Streptomyces is characterised by its ability to produce a vast variety of secondary metabolites, including antibiotics and other bioactive substances of high pharmacological and economical interest (Hopwood, 2007). The "physiological differentiation" in secondary metabolism is often correlated with the morphological differentiation in a growth-phase dependent manner, e.g., production of antibiotics coincides with the onset of aerial mycelium formation in agar-grown cultures and with stationary phase in liquid (Champness, 2000). Both processes are simultaneously

controlled by various nutritional factors, such as the carbon-, nitrogen-, and phosphate-sources, and also by trace elements (Horinouchi, 2007).

The model streptomycete *Streptomyces coelicolor*

Streptomyces coelicolor A3(2) has consolidated as the model organism within the streptomycetes (Chater, 2006). It is genetically well characterized and manipulable; the complete genome sequence of *S. coelicolor* strain M145 (a prototrophic derivative of A3(2) lacking the two *S. coelicolor* plasmids SCP1 and SCP2) has been published (Bentley *et al.*, 2002); and an ordered cosmid library (Redenbach *et al.*, 1996), as well as microarrays covering the entire chromosome are available. Also, intensive proteome studies have been carried out (Hesketh *et al.*, 2007).

There are many more A3(2) derived *S. coelicolor* strains besides the sequenced strain M145, some of them auxotrophs after undergoing UV or X-ray mutagenesis (see Weaver *et al.*, 2004 for a linage of *S. coelicolor* strains). Another plasmid-free prototroph is strain M600 that, in contrast to M145, did not undergo any mutagenesis and was shown to possess 1.06 Mb long-terminal inverted repeats (L-TIRs) at the ends of the 8.6 Mb *S. coelicolor* chromosome leading to a duplication of 1005 genes. However, transcript levels for the duplicated genes were similar in M145 and M600 (Weaver *et al.*, 2004).

Streptomyces coelicolor antibiotics

S. coelicolor produces at least four antibiotics: the SCP1 plasmid encoded methylenomycin (Bentley *et al.*, 2004) and the chromosomally encoded calcium dependent antibiotic (CDA) (Hopwood and Wright, 1983; Lakey *et al.*, 1983), the blue-pigmented polyketide (gamma-)actinorhodin (Act) (Bystrykh *et al.*, 1996; Rudd and Hopwood, 1979) and the red-pigmented (undecyl-)prodigiosin (Red) (Feitelson *et al.*, 1985). However, since genome analysis revealed many more secondary metabolite clusters of which 12 were still orphan (Bentley *et al.*, 2002; Challis and Hopwood, 2003), *S. coelicolor* may synthesize more antibiotic compounds than identified to date.

Bacterial communication and "microbial hormones"

Bacteria communicate using chemical signal molecules. As in higher organisms, the information supplied by these molecules synchronises the activities of large groups of cells, and thus allows bacteria to alter behaviour on a population-wide scale. Chemical communication involves producing, releasing, detecting, and responding to small hormone-like molecules termed autoinducers (Waters and Bassler, 2005). In so-called quorum sensing systems the population-density-dependent concentration of such autoinducers affects, for example, bioluminescence in *Vibrio fischeri* or virulence and biofilm formation in *Pseudomonas aeruginosa* (Swift *et al.*, 2001). Two examples of small-molecule autoinducers are acyl homoserine lactones (AHLs) in Gram-negative (Fig. 3) (Fuqua *et al.*, 2001), and modified oligopeptides (Kleerebezem *et al.*, 1997) in Gram-positive bacteria.

C4-homoserine lactone from *Pseudomonas aeruginosa* (exoenzymes, biofilm secretion)

A-factor from *S. griseus* (streptomycin, grixazone and sporulation)

IM-2 from *S. lavendulae* (showdomycin and minimycin)

SCB1

SCB2

SCB3

SCBs from *S. coelicolor* (CPK, Act, Red)

VB-A

VB-B

VB-C

VB-D

VB-E

Virginiae butanolides from *S. virginiae* (virginiamycin)
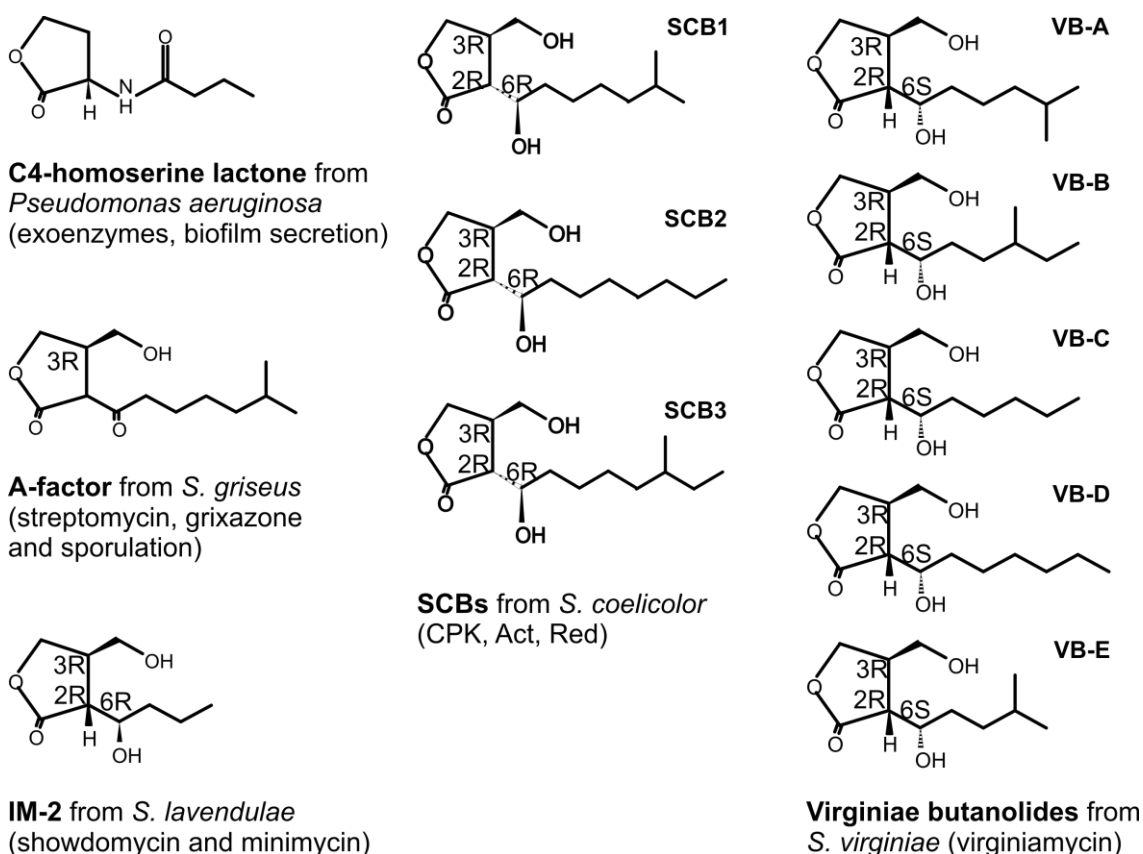
Figure 3 Chemical structures of γ-butyrolactones from *Streptomyces* and C4-homoserine lactone from *Pseudomonas aeruginosa* (adapted from Takano, 2006). The name of the signalling molecule appears in bold type, and the antibiotic that it affects or its other functions are shown in brackets.

γ-Butyrolactone regulatory systems in *Streptomyces*

In actinomycetes, γ-butyrolactones are used as signalling molecules. Diffusible and effective in nanomolar concentrations, they induce and synchronise antibiotic production and, in some cases, also morphological differentiation in the mycelial colonies of streptomycetes (Bibb, 2005; Horinouchi, 2002).

The A-factor system in *Streptomyces griseus*

The best studied *Streptomyces* γ-butyrolactone system is the A-factor system (Fig. 4) regulating streptomycin and grixazone production, as well as sporulation in *S. griseus* (reviewed in Bibb, 2005; Horinouchi, 2007). The γ-butyrolactone A-factor is synthesized by AfsA from a glycol derivative and a β-keto acid (derived from fatty acid biosynthesis) (Ando *et al.*, 1997; Kato *et al.*, 2007). A-factor binds to the cytoplasmic A-factor receptor protein and transcriptional repressor ArpA. ArpA consequently dissociates from the promoter region of *adpA* encoding a pleiotropic transcriptional activator. AdpA binds to the promoter regions of many genes required for morphological development and secondary metabolism. For streptomycin biosynthesis, AdpA activates *strR* encoding the pathway-specific transcriptional activator for the streptomycin biosynthesis gene cluster (Horinouchi, 2007). AdpA was also reported to autorepress its own expression (Kato *et al.*, 2005) and seems to impair A-factor synthesis (Kato *et al.*, 2004). Consistent with the regulatory cascade described, an *afsA* mutant lost A-factor productivity (Hara and Beppu, 1982) and an *arpA* mutant showed precocious antibiotic production (Miyake *et al.*, 1990).

The *Streptomyces coelicolor* butanolide system

In the *S. coelicolor* γ-butyrolactone regulatory system (Fig. 4 and 5) (reviewed in Takano, 2006), *S. coelicolor* butanolides (SCBs) are produced in late transition phase dependent on the AfsA homologue ScbA (Takano *et al.*, 2005). The structures of three distinct SCB compounds, SCB1, 2 and 3 (Fig. 3), have been determined (Takano *et al.*, 2000) and neither was produced in a *scbA* mutant (Takano *et al.*, 2001). The γ-butyrolactones bind to the ArpA receptor homologue ScbR and prevent DNA binding of the transcriptional regulator. In the absence of γ-butyrolactones, ScbR binds to its own promoter, repressing its own expression, and to the promoter of the adjacent and diverging *scbA* gene (Takano *et al.*, 2001). The precise role of

ScbR for *scbA* expression is under investigation (Takano, 2006). Microarray analysis revealed that ScbR directly regulates an orphan Type I PKS gene cluster in close vicinity of *scbAR*, the *cpk* (*c*oelicolor *p*oly*k*etide) gene cluster (Fig. 2) (formally called *kas* gene cluster (Pawlik *et al.*, 2007), via the pathway-specific regulatory gene *cpkO*. In contrast to *scbA* and *scbR*, there are two ScbR binding sites in the *cpkO* promoter region (Fig. 5). Two of the four ScbR target sequences known are completely conserved (Takano *et al.*, 2005). A proposed ScbR binding consensus sequence led to the identification of a single additional site upstream of *orfB*, only two genes away from *scbR*. ScbR binding has been shown experimentally (Takano, unpublished), however, the role of the kinase homologue OrfB has not been determined (Takano, 2006).

Initially, the *S. coelicolor* butanolide system was shown to affect the production of the two pigmented antibiotics Act and Red. A *scbA* mutant overproduces Act and Red, whereas deletion of *scbR* results in delayed Red production. However, the receptor protein and transcriptional regulator ScbR does not bind to the promoter regions of pathway specific regulators for Act and Red production (Takano *et al.*, 2001). Thus, the recently characterised *cpk* gene cluster (Fig. 2) (Pawlik *et al.*, 2007) may be the primary regulatory target of the SCB system, whereas production of Act and Red might be indirectly affected by precursor availability, regulatory cross-talk or by other unknown effects on the expression of the Act and Red antibiotic biosynthesis genes (Fig. 4 and 5) (Bibb, 2005; Takano, 2006).

A-factor system in *S. griseus*   Butanolide system in *S. coelicolor*
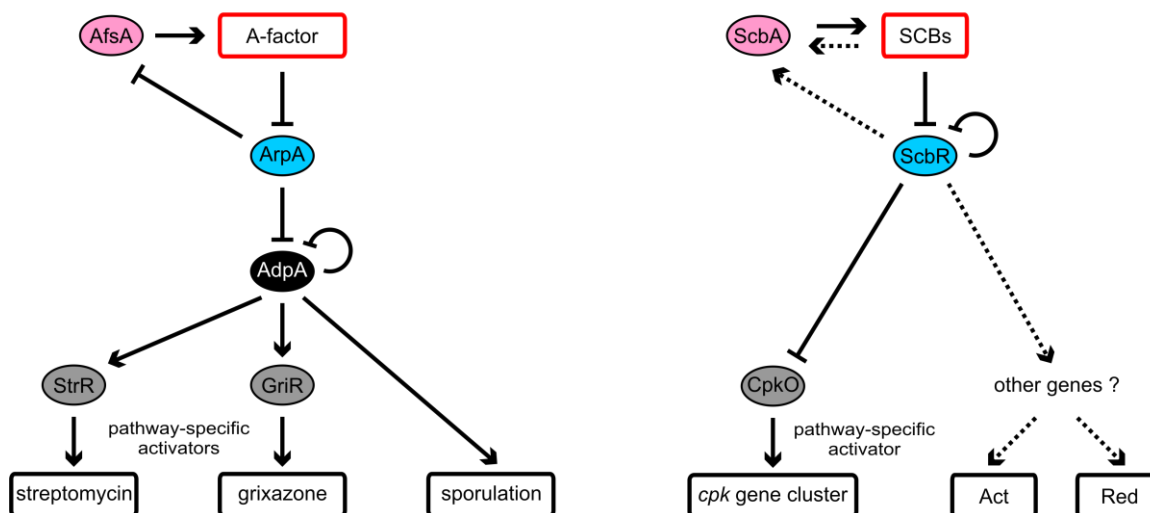
Figure 4 Schematic representations of the A-factor regulatory cascade of *Streptomyces griseus* and the butanolide system of *Streptomyces coelicolor*. Arrows denote activation and lines with a bar denote repression. Dotted lines represent effects currently under investigation. Homologues or homologous functions are indicated by colours. Detailed descriptions are given in the text. For the *S. coelicolor* system, also see Fig. 5.
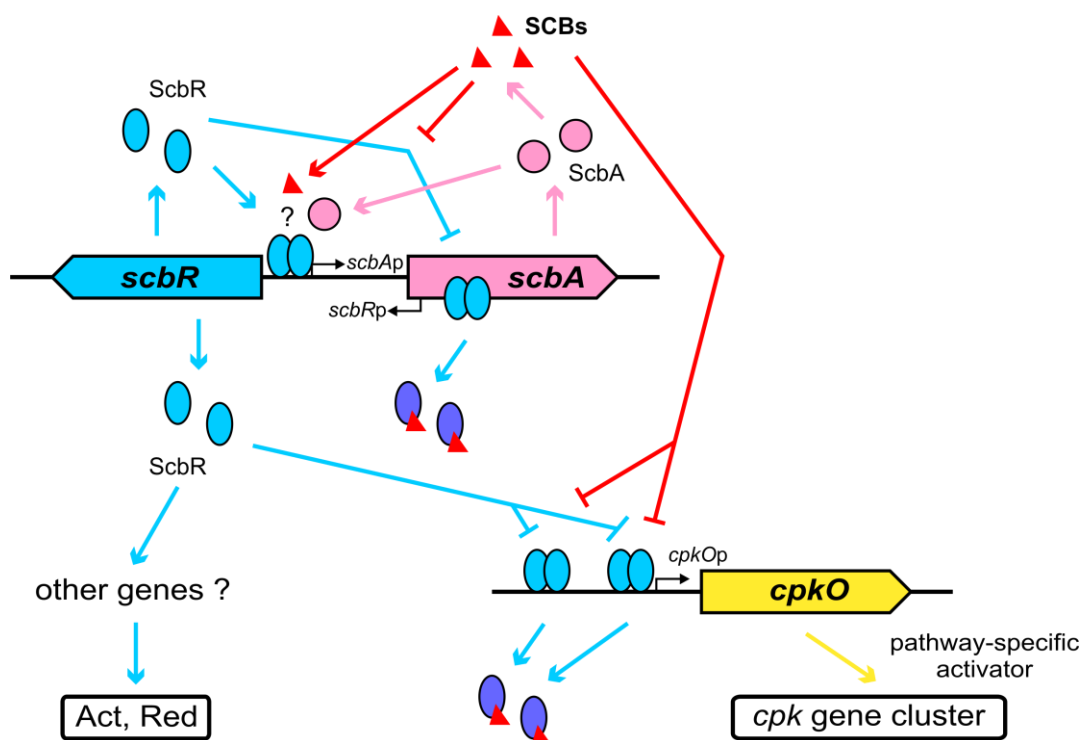
Figure 5 Detailed schematic model of the butanolide system in *Streptomyces coelicolor* (adapted from Takano, 2006). ScbR (light-blue ovals) binds as a dimer to 4 target sites. One

repressor site is in front of its own promoter (*scbR*p) and two other repressor sites are in front of the *cpkO* (pathway-specific activator) promoter (*cpkO*p). In all cases, the γ-butyrolactones (SCBs; red triangles) bind to ScbR (deep-blue ovals with triangle) and relieves the repression. ScbA (pink circles) is involved in production of SCBs and also is required along with SCBs and ScbR for its own expression (*scbA*p, the *scbA* promoter). The precise mechanism remains to be elucidated and is under investigation (shown as '?'). ScbR might regulate other genes which could in turn regulate the production of Act and Red. CpkO actively regulates transcription of the *cpk* gene cluster. Arrows denote activation and lines with a bar denote repression.

γ-Butyrolactone regulatory system diversity in *Streptomyces*

Although both the composition and the molecular mode of action of the key players are studied best with the A-factor system in *S. griseus*, it may not serve as a general model for *Streptomyces* γ-butyrolactones regulatory systems.

Genes involved in the synthesis and binding of the γ-butyrolactones, respectively, are often located in close vicinity of each other and, furthermore, in or close to the target antibiotic biosynthesis gene cluster. ScbA and ScbR in *S. coelicolor* are neighboured and only a few genes upstream from the *cpk* gene cluster (Fig. 2). In contrast, AfsA and ArpA in *S. griseus* are ~100 kb away from each other and ArpA, involved in the regulation of streptomycin production, is not co-located with the cognate gene cluster (Bibb, 2005; Takano, 2006).

AdpA is the central transcriptional activator in *S. griseus*. The *S. coelicolor* butanolide system lacks a counterpart with an analogous function. Nevertheless, we find *adpA* homologues in *S. coelicolor*. With 84% amino acid identity, the one that most closely resembles *adpA* from *S. griseus* is part of the *Bld* cascade related to development and its deletion results in the *bldH* mutant phenotype. Development is suggested not to be influenced by γ-butyrolactones in *S. coelicolor* and, even more important, in contrast to A-factor regulation of *adpA* in *S. griseus*, transcription of *S. coelicolor adpA* is not affected by the SCBs (Fig. 4) (Chater and Horinouchi, 2003).

Differing from A-factor, and similar to the SCBs, most other *Streptomyces* γ-butyrolactones appear to be devoted only to the regulation of secondary metabolism (Bibb, 2005). For example, virginiae butanolides (VBs; Fig. 3) control virginiamycin production in *S. virginiae* (Okamoto *et al.*, 1995), and IM-2 (Fig. 3)

controls showdomycin and minimycin production in *S. lavendulae* (Waki *et al.*, 1997). Furthermore, in contrast to ArpA from *S. griseus*, the γ-butyrolactone receptor proteins of the other three species (ScbR from *S. coelicolor*, BarA from *S. virginiae*, FarA from *S. lavendulae*) all seem to be required for normal production levels of their respective ligands (Kitani *et al.*, 2001, Nakano *et al.*, 1998, Takano *et al.*, 2001, respectively).

All these atypical features of the A-factor system raise doubts about its representativeness for *Streptomyces* γ-butyrolactone regulatory systems. However, despite partial similarities between other γ-butyrolactone regulatory systems, e.g., in *S. coelicolor*, *S. virginiae* and *S. lavendulae*, due to differences concerning other major aspects of the system, there may not be a feasible "general" model for these complex systems above the level of the molecular mode of action of the homologous proteins involved. The more examples are studied, the clearer the diversity and the complexity of *Streptomyces* γ-butyrolactone systems becomes (Chater and Horinouchi, 2003; Takano, 2006).

A yet unidentified γ-butyrolactone is presumed to be involved in the regulation of tylosin production in *S. fradiae*. Besides three other regulatory genes, the unique regulatory cascade contains two γ-butyrolactone receptor homologues, *tylP* and *tylQ*, with TylP regulating *tylQ* transcription (reviewed in Cundliffe, 2008). *S. ambofaciens* (Bunet *et al.*, 2008; Pang *et al.*, 2004) and *S. virginiae* (Matsuno *et al.*, 2004) are other examples of *Streptomycetes* with multiple γ-butyrolactone receptors.

S. coelicolor possesses, in addition to ScbR, at least four other chromosomal ArpA-like receptor proteins: CprA, CprB, SCO6323 and ScbR2 (Takano *et al.*, 2005). Interestingly, ScbR2 is located in the ScbR-controlled *cpk* gene cluster (Pawlik *et al.*, 2007). The detailed functions of CprA, CprB, SCO6323 and ScbR2 are unknown. In particular, binding to a cognate ligand could not be shown for any of them, and no DNA target sequences were identified yet (Takano, 2006). Based on profound amino acid sequence analysis, CprA, CprB and ScbR2, together with, e.g., TylQ from *S. fradiae* and BarB from *S. virginiae*, make up a subfamily within the γ-butyrolactone receptors, which is suggested to comprise "pseudo"-receptors with a function as transcriptional regulators, but without ligand binding ability (Takano *et al.*, 2001).

More work will be needed to resolve the role of this group of regulators in *Streptomyces*.

γ-Butyrolactone biosynthesis

The 2,3-disubstituted lactone ring structure of the γ-butyrolactones resembles that of acyl homoserine lactones (AHLs) from Gram-negative bacteria, except the carbon side chains (Fig. 3). Length, branching and stereochemistry of the fatty acid side chain differ between particular γ-butyrolactones (Fig. 3) (Horinouchi, 2002; Takano, 2006) and seem to determine the highly specific binding of the different γ-butyrolactones to their cognate receptor molecules (Hsiao *et al.*, 2009). Currently, biosynthesis of the small signalling molecules is of strong interest. AfsA was shown to be the key enzyme in A-factor biosynthesis, and the putative biosynthesis pathway in *S. griseus* was recently reported (Ando *et al.*, 1997; Kato *et al.*, 2007). Corresponding functions were shown for BarX, an AfsA homologue in *S. virginiae*, and ScbA from *S. coelicolor* (Hsiao *et al.*, 2007; Lee *et al.*, 2009). In addition to this enzymatic role, ScbA and perhaps BarX may also possess a regulatory function (Hsiao *et al.*, 2007; Kawachi *et al.*, 2000).

Structure-function relationship in γ-butyrolactone receptors

Including ArpA, Horinouchi and co-workers reported 37 ArpA-like γ-butyrolactone receptor homologues in actinomycetes (Nishida *et al.*, 2007). As a general mode of action, the ligand-free γ-butyrolactone receptors bind to specific DNA sequences upstream of their target genes, typically repressing transcription in the absence of the signalling molecules. The γ-butyrolactones inhibit DNA binding of the receptor protein, and the target genes are expressed (reviewed in Takano, 2006).

The cytoplasmic γ-butyrolactone receptors belong to the TetR family of transcriptional repressors (Ramos *et al.*, 2005). Representative for the γ-butyrolactone receptors, the crystal structure of CprB, one of the ArpA homologues in *S. coelicolor* with a yet unknown function, was determined to be a dimer with an Ω shape (Fig. 6) (Natsume *et al.*, 2004). A monomer is composed of two domains, the DNA binding domain, containing a helix-turn-helix (HTH) motif, and a regulatory domain. The latter comprises a dimerization domain and a hydrophobic cavity probably serving as ligand binding pocket (Fig. 6) (Natsume *et al.*, 2004). This

domain structure was confirmed by site-directed mutagenesis yielding a mutant ArpA protein that lacks DNA binding ability but retains γ-butyrolactone binding ability (Val41Ala in the HTH motif) (Sugiyama *et al.*, 1998) or that binds to DNA but does not bind A-factor (Pro115Ser in the regulatory domain) (Onaka *et al.*, 1997). Also tryptophan residue 119 was shown to be essential for ligand binding (Trp119Ala in the ligand binding pocket) (Sugiyama *et al.*, 1998). It is suggested that the binding of γ-butyrolactones induces the relocation of helix α4, which links the binding pocket with the DNA binding domain. As a result of the relocation of the DNA binding domain, the γ-butyrolactone receptor dissociates from its DNA target sequences (Horinouchi, 2007; Natsume *et al.*, 2004). The computer-modelled protein structure shown in Fig. 6 uses crystallographic analysis data of CprB (Natsume *et al.*, 2004) to illustrate the general structure and functional domains of ArpA-like γ-butyrolactone receptors (Horinouchi, 2007).
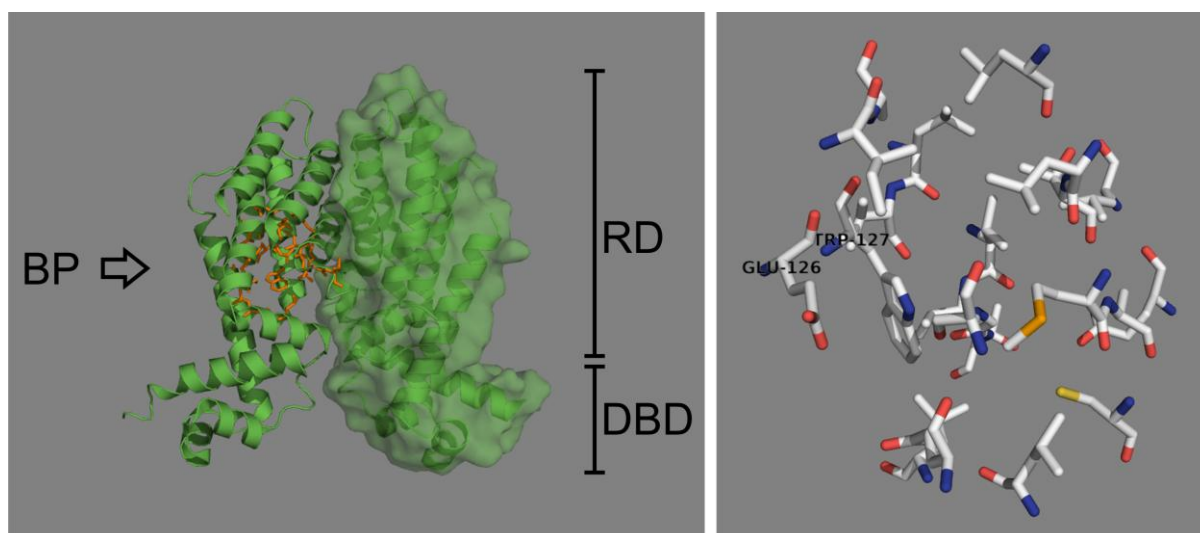
Figure 6 Protein structure of CprB. The first panel shows the overall structure of CprB. The γ-butyrolactone receptor homologue constitutes a dimer, each subunit of which contains an N-terminal DNA binding domain (DBD) with a helix-turn-helix motif, and a C-terminal regulatory domain (RD) including the dimerization domain and the ligand binding pocket (BP). The latter is located in a cavity of each regulatory domain and indicated by an arrow. For one monomer, amino acid residues involved in forming the BP (Natsume *et al.*, 2004) are shown in orange. The second panel shows a close-up of the BP from another angle. Trp127 (corresponding to Trp121 in ScbR) and Glu126 (corresponding to Arg120 in ScbR), that is not part of the BP, are labelled. The structure model was newly created using structure information from the RCSB Protein Data Bank (CprB (form Ia), accession code 1UI5 (Natsume *et al.*, 2004)) and PyMOL (DeLano Scientific) software.

The orphan *cpk* gene cluster in *S. coelicolor*

The *cpk* cluster in *S. coelicolor* had been partially identified previously (Kuczek *et al.*, 1997), but was fully revealed only after analysis of the whole-genome sequence (Fig. 2) (Pawlik *et al.*, 2007). The cluster was shown to be under the control of the *S. coelicolor* butanolide system. The γ-butyrolactone receptor ScbR represses transcription of *cpkO*, a SARP (*Streptomyces* antibiotic regulatory protein) (Wietzorrek and Bibb, 1997) and the pathway-specific activator of the *cpk* gene cluster (Fig. 4 and 5) (Takano *et al.*, 2005). This was determined by microarray analysis and confirmed by further transcription studies. The suggested borders of the cluster are also based on the microarray study. The *cpk* cluster lies in close vicinity to the *scbAR* genes of the SCB system. It spans ~54 kb and contains three large Type I polyketide synthase subunits (*cpkABC*), as well as tailoring and regulatory genes (Fig. 2) (Pawlik *et al.*, 2007).

Among the regulatory genes, besides CpkO, we find another SARP, CpkN. The DNA target sequences for these transcriptional regulators are not identified yet. A third regulatory protein, ScbR2, a ScbR homologue with 32% amino acid sequence identity, was also identified in the *cpk* cluster. Neither binding to a putative (γ-butyrolactone) ligand, nor regulatory activity for a hypothetical target, has been shown with ScbR2 (Pawlik *et al.*, 2007). The three regulatory genes in the γ-butyrolactone-controlled *cpk* cluster add to the complexity of the SCBs-based *S. coelicolor* butanolide regulatory system.

The polyketide synthase subunits CpkA, CpkB and CpkC together comprise a loading module and five extension modules with a unique reductase as a terminal domain instead of a typical thioesterase function (Fig. 2). The intermediate compound produced is predicted to be built from six dicarbon units derived from malonyl-CoA to give a unsaturated 12-carbon chain, without any side chains (Fig. 2) (Pawlik *et al.*, 2007). How tailoring enzymes may further modify this structure is not known. The *cpk* biosynthesis pathway end product has not been identified yet, and thus, the *cpk* gene cluster remains one of the orphan secondary metabolite gene clusters revealed in *S. coelicolor*.

Scope of this thesis

Elucidation of the regulation of secondary metabolite production in *Streptomyces* is fundamental for the discovery of novel natural antibiotics with the potential to be developed into pharmaceutical drugs for the treatment of infectious diseases. The aim of this thesis was to further characterise the regulatory *S. coelicolor* butanolide system and to activate and characterise the orphan *cpk* biosynthetic pathway.

Chapter 2 describes a novel yellow-pigmented secondary metabolite (yCPK). yCPK is conditionally produced in *S. coelicolor* M145 and overproduced in a *scbR2* mutant that is deficient in a γ-butyrolactone receptor homologue located in the *cpk* gene cluster. The yellow compound is shown to be related to the orphan *cpk* cluster. The expression profiles of *scbR2* and other regulatory and biosynthetic genes of the *cpk* cluster were determined, suggesting a role for ScbR2 as transcriptional repressor Together with the activator CpkO, ScbR2 constitutes negative feedback regulation of yCPK production, putatively terminating synthesis of the yellow pigment in M145 after only a short production period.

Chapter 3 describes the discovery of a new antibiotic (abCPK) in *S. coelicolor* under optimised culturing conditions for the production of yCPK. Production of abCPK coincides with that of the yellow pigment, and also abCPK depends on a functional *cpk* gene cluster. yCPK and abCPK, however, are shown to be distinct *cpk* cluster metabolites and to be partially synthesised outside the cell. The physiochemical properties of the new antibiotic were determined after extraction and abCPK is shown to be active against Gram+ and Gram- bacteria.

Chapter 4 describes the identification and *in vitro* characterisation of a second form of the γ-butyrolactone receptor ScbR in the *S. coelicolor* strain M600 (ScbR$_{M600}$). The *scbR*$_{M600}$ gene differs from *scbR* in the sequenced strain M145 (*scbR*$_{M145}$) by a single point mutation (c358a). This mutation leads to an amino acid change (R120S) located in close vicinity to the γ-butyrolactone binding pocket of ScbR. *In vitro* analysis of the ScbR variants, however, revealed no differences in ligand binding abilities, but showed a rapid loss of ScbR$_{M600}$ DNA binding activity.

To study the *in vivo* effect of the amino acid change in ScbR$_{M600}$, a M145 *scbR* mutant was complemented with both ScbR variants with the resulting strains thus

differing only in the single point mutation in $scbR_{M600}$. Compared to $ScbR_{M145}$, DNA binding activity of $ScbR_{M600}$ is also reduced *in vivo*, and expression of target genes involved in antibiotic production and the butanolide system are affected. Consequently, γ-butyrolactone production is slightly diminished. Antibiotic production in the two strains, however, highly resembles each other and is comparable to the parental strain M145.

Chapter 5 summarises and discusses the results of this thesis.

Chapter 2

yCPK

Submitted for publication together with chapter 3

"Deletion of a regulatory gene within the *cpk* gene cluster reveals novel antibacterial activity in *Streptomyces coelicolor* A3(2)"

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Abstract

Genome sequencing of *Streptomyces coelicolor* A3(2) had previously revealed an uncharacterised Type I polyketide synthase gene cluster (*cpk*). Here, we describe a novel, yellow-pigmented secondary metabolite (yCPK) that was discovered by deleting a presumed pathway-specific regulatory gene, *scbR2*, that encodes a member of the γ-butyrolactone receptor family of proteins and which lies in the *cpk* gene cluster. Over-production of yCPK in a *scbR2* deletion mutant, and its absence from a *cpk* deletion mutant, strongly suggest that yCPK is a product of the previously orphan *cpk* gene cluster. Transcriptional analysis suggests that *scbR2* may act in a negative feedback mechanism to eventually limit yCPK biosynthesis. The results described here may represent a novel approach for the discovery of new, biologically active compounds.

Introduction

Streptomycetes are ubiquitous soil-dwelling mycelial bacteria characterised by a complex morphology and the ability to produce a vast variety of secondary metabolites including the majority of antibiotics of natural origin (Weber *et al.*, 2003). Consequently the genus *Streptomyces* is of major interest in the search for new biologically active compounds. The genome sequence of the model organism *Streptomyces coelicolor* A3(2) was recently determined (Bentley *et al.*, 2002), revealing numerous secondary metabolite gene clusters, including those for 11 well-characterised compounds, such as the blue-pigmented polyketide (gamma-)actinorhodin (Bystrykh *et al.*, 1996; Rudd and Hopwood, 1979), the red-pigmented (undecyl-)prodiginines (Feitelson *et al.*, 1985) and the calcium dependent antibiotic (CDA) (Hopwood and Wright, 1983; Lakey *et al.*, 1983), but also 12 "orphan" gene clusters to which no products have been assigned (Challis and Hopwood, 2003).

Orphan gene clusters are found not only in *Streptomyces*, but also in other actinomycetes and fungi with a proven ability to produce biologically active secondary metabolites, and are a promising source of new pharmaceutically useful compounds (Bok *et al.*, 2006; Udwary *et al.*, 2007). With the increasing number of genome sequences, "genome mining" for new secondary metabolite gene clusters

and novel natural products has become feasible. Despite many attempts, the products of orphan gene clusters have not been detected by simply using different growth media (reviewed in Challis, 2008). Consequently, other approaches have been proposed or adopted, including *in silico* analysis and prediction of the products of biosynthetic gene clusters; deletion of biosynthetic genes and identification of the end product by comparative metabolite profiling; heterologous expression of the entire biosynthetic gene cluster (Song *et al.*, 2006); deletion or overexpression of a pleiotropic regulatory gene; and overexpression of a pathway-specific activator (Bergmann *et al.*, 2007) (reviewed in Challis, 2008).

S. coelicolor contains an orphan Type I polyketide synthase (PKS) gene cluster, *cpk* (*coelicolor* polyketide; formerly named *kas*), which was initially detected by hybridisation to an acetyltransferase (AT) gene (Kuczek *et al.*, 1997). However, except for the prediction of a constituent aliphatic 12-carbon chain (Pawlik *et al.*, 2007), which was based on the sequence of the PKS subunits *cpkABC*, nothing was known about the product of the *cpk* cluster, which consequently remained "orphan".

ScbR is a γ-butyrolactone receptor protein of *S. coelicolor* that binds to specific DNA sequences in front of its target genes repressing their transcription (Takano *et al.*, 2001). Binding of SCB1, and potentially other γ-butyrolactones synthesised by ScbA at transition phase (Hsiao *et al.*, 2007; Takano *et al.*, 2001), causes a conformational change in ScbR preventing it binding to DNA. Earlier work had shown that ScbR directly repressed expression of *cpkO*, the pathway-specific transcriptional activator gene of the *cpk* cluster, through two binding sites located in the *cpkO* promoter region (Takano, 2006). In agreement with this, transcription of the *cpk* cluster was increased in a *scbR* mutant (Takano *et al.*, 2001).

There are five *scbR* homologues in *S. coelicolor* (reviewed in Takano, 2006). Interestingly, one of these, *scbR2,* is part of the *cpk* gene cluster (Pawlik *et al.*, 2007) and thus may be involved in its regulation. In this study, *scbR2* was deleted from *S. coelicolor* M145 to assess its role in the regulation of secondary metabolism.

Material and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used are listed in Table 1. *S. coelicolor* was manipulated as previously described (Kieser *et al.*, 2000). *E. coli* strains were grown and transformed as previously described (Sambrook and Russell, 2001).

Difco Nutrient Agar (DNAgar), R2, R2YE (Kieser *et al.*, 2000), and SMMS (Takano *et al.*, 2001) were used to detect antibiotic production. MS agar (Kieser *et al.*, 2000) was used to grow cultures for preparing spore suspensions, and for plating out intergeneric conjugations of *S. coelicolor* with *E. coli* ET12567 containing the RP4 derivative pUZ8002 (Flett *et al.*, 1997; MacNeil *et al.*, 1992). Liquid SMM (Takano *et al.*, 2001) was used to grow cells for the isolation of RNA.

Construction of a *scbR2* disruption mutant

A mutant *scbR2* (SCO6286) allele was constructed in which most of the *scbR2* coding region (amino acids 2-217 out of 224) was deleted and replaced with an apramycin resistance gene using PCR targeting (Gust *et al.*, 2004). Primers mscbR2R and mscbR2F were designed with 5' ends overlapping the 5' and 3' ends of the *scbR2* coding sequence, and 3' (priming) ends to amplify the apramycin resistance disruption cassette of pIJ773 (Gust *et al.*, 2004). The cosmid used for targeting was SCAH10 (Redenbach *et al.*, 1996). Deletions within two independent isolates of *scbR2* were confirmed by Southern hybridization (Kieser *et al.*, 2000) using a 1211 bp PCR-generated probe (primers scbRA2 and scbRFW2, Fig. 1) labelled with digoxygenin (DIG) using Roche's DIG DNA labelling kit. 1385 bp and 892 bp fragments were detected in *Bam*HI + *Xho*I digested genomic DNA of M145 and the *scbR2*::*acc*(3)IV mutant (LW3), respectively, as predicted (Fig. 1, data not shown). Construction of the mutants was also confirmed by PCR using primers scbRA2 and scbRFW3 (Fig. 1) and total DNA isolated from the parental *S. coelicolor* M145 and the two potential mutants as template (Fig. 1, S1). All primers are listed in Table S1. The resulting *scbR2*::*aac*(3)IV mutant strain was designated LW3.

Table 1 Bacterial strains, plasmids and cosmids

Bacterial strains	Description	References
Escherichia coli		
JM101	General cloning strain	(Sambrook and Russell, 2001)
ET12567	Non-methylating strain used for conjugation with *Streptomyces*	(MacNeil *et al.*, 1992)
Streptomyces coelicolor A3(2)		
LW3	M145 *scbR2*::*aac*(3)IV (apramycin resistance gene)	This study
LW6	M145 *cpkO*::*aac*(3)IV	(Takano *et al.*, 2005a)
M145	A3(2) SCP1- SCP2- derivate	(Kieser *et al.*, 2000)
M145Δ*crtEIVB*	M145 Δ*crtEIVB*	(Takano *et al.*, 2005b)
M751	M145 Δ*scbA*	(Takano *et al.*, 2001)
M752	M145 Δ*scbR*	(Takano *et al.*, 2001)
M1142	M145 Δ*act* Δ*red*	Gomez-Escribano (unpublished)
M1146	M145 Δ*act* Δ*red* Δ*cpk* Δ*cda*	Gomez-Escribano (unpublished)
M1148	M145 Δ*act* Δ*red* Δ*cda*	This study
P100	M145 *cpkC*::*aac*(3)IV	(Pawlik *et al.*, 2007)
Plasmids and cosmids		
pBR322	*E. coli* cloning vector	(Sambrook and Russell, 2001)
pIJ12145	Suicide plasmid for substitution of *cdaPSI* and *cdaPSII* with *aac(3)IV*	Gomez-Escribano (unpublished)
pIJ12146	Suicide plasmid for deletion of the rest of the *cda* cluster	Gomez-Escribano (unpublished)
pIJ215	pGEM-T Easy (Promega) harbouring *scbR2*	This study
pIJ216	pIJ82 harbouring *scbR2*	This study
pIJ773	Plasmid harbouring the apramycin resistance gene (*aac*(3)IV) and the RK2 origin of transfer	(Gust *et al.*, 2004)
pIJ82	pSET152 (Bierman *et al.*, 1992) derivative containing the *hyg* gene, replacing a 751 bp *Sac*I fragment containing *aac*(3)IV	(Kieser *et al.*, 2000)
pSET152	*oriT attP int aac(3)*IV	(Kieser *et al.*, 2000)
pUZ8002	RK2 derivative, defective *oriT*, not self-transmissible, but supplies a mobilization function for *oriT*-containing plasmids	(Flett *et al.*, 1997)
SCAH10	Cosmid harbouring *scbR2* (SCO6268)	(Redenbach *et al.*, 1996)
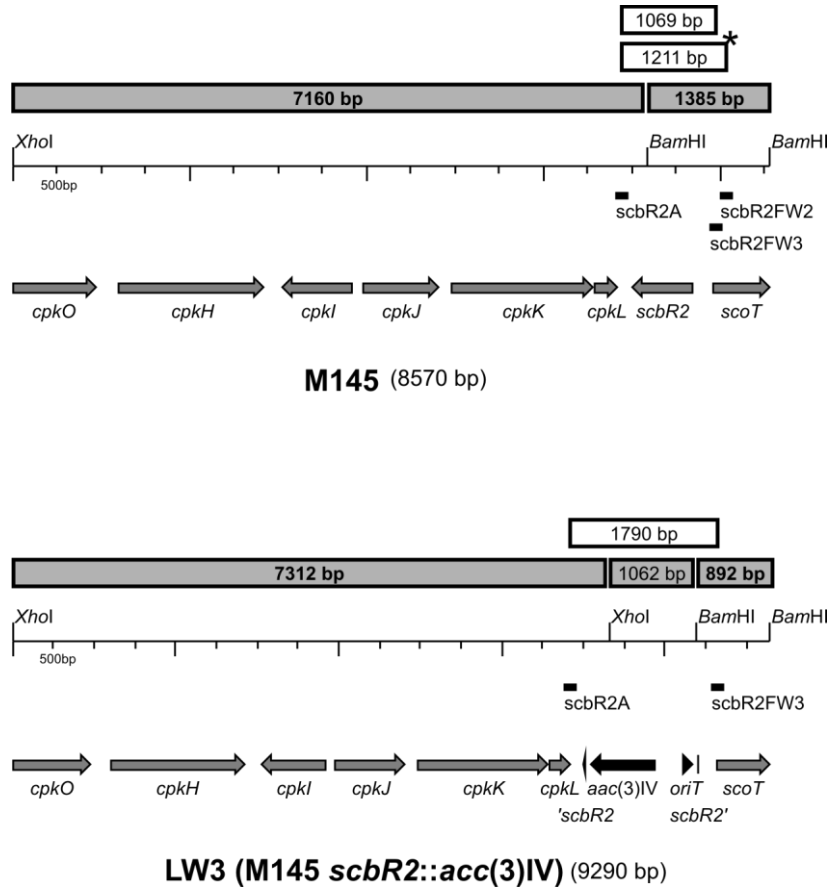
Fig. 1 Genetic organisation of *S. coelicolor* strains M145 and LW3 (M145 *scbR2*::*aac*(3)IV)

Restriction map of the *cpkO* to *scoT* DNA fragment of *S. coelicolor* M145 (8570 bp) and LW3 (9290 bp) with the *aac*(3)IV-*oriT* apramycin resistance cassette replacing most of the *scbR2* coding region in LW3. Genes from M145 and the disruption cassette are represented by grey and black arrows, respectively. Gene names are indicated below; for incomplete gene fragments, the missing part is indicated by an apostrophe at the beginning or the end of the gene name. *Bam*HI and *Xho*I restriction sites are shown, and binding sites for PCR primers scbR2A, scbR2FW3 and scbR2FW2 are indicated by black rectangles. The 1069 and 1790 bp scbR2A + scbR2FW3 PCR fragments from M145 and LW3, respectively, are indicated by a white box on top. With M145, a white box with an asterisk represents the DIG-labelled 1211 bp scbR2A + scbR2FW2 PCR fragment used as Southern probe. Grey boxes indicate the 7160 and 1385 bp, and the 7312, 1062 and 892 bp *Bam*HI + *Xho*I fragments of M145 and LW3, respectively, of which the fragments detected by Southern analysis are labelled in bold. A 500 bp scale bar is also included. Results of the PCR analysis are shown in Fig. S1.

Complementation of the *scbR2* disruption mutant

For complementation of LW3, *scbR2* together with a 227 bp upstream region was amplified from genomic DNA of *S. coelicolor* M145 using primers scbR2A_*Xba*I and scbR2FW3_*Xba*I (Table S1) thereby introducing *Xba*I restriction sites at the ends of the 1087 bp PCR product. The *scbR2* PCR product was introduced into pGEM-T Easy (Promega) to give plasmid pTE215. A 1.1 kb *Xba*I-fragment of pTE215 containing *scbR2* was cloned into pIJ82 (a pSET152 derivative harbouring a hygromycin resistance gene) (Kieser *et al.*, 2000) to give plasmid pTE216. The *scbR2* sequence present in pTE215 and pTE216 was confirmed by DNA sequencing (Seqlab, Göttingen, Germany). The integrative vector pIJ82 and plasmid pTE216 harbouring *scbR2* were introduced into the *S. coelicolor scbR2* mutant strain LW3 (this study) by conjugation using *E. coli* ET12567 containing the RP4 derivative pUZ8002 (Flett *et al.*, 1997;MacNeil *et al.*, 1992). Integration of pTE216 into the chromosome and the *scbR2* genotype of LW3/pTE216 were confirmed by PCR using primers JGattBI-fwd and JGattPint-rev (Hsiao *et al.*, 2007), and primers scbR2A and scbRFW3 (Table S1), respectively (Fig. 1, S1).

Construction of the *act red cda* triple deletion mutant *S. coelicolor* M1148

Most of the gene cluster for CDA biosynthesis (SCO3210-49) was deleted from *S. coelicolor* M1142 (M145 Δ*act* Δ*red*; Gomez-Escribano, unpublished) in a two step process. First, *cdaPSI* and most of *cdaPSII* were replaced with an apramycin resistance cassette by PCR targeting followed by homologous recombination using pIJ12145 (Table 1). The remaining *cda* genes were then removed by homologous recombination with 2 kb flanking segments using pIJ12146 (Table 1). The resulting strain, M1148, was verified by Southern analysis (data not shown).

Assays for secondary metabolite production

For solid cultures, 4 x 10^7 spores of M145, M145Δ*crtEIVB*, LW3, M1148, M1146, P100, LW6, M751 and M752 were streaked out as 2.5 x 2.5 cm^2 patches and incubated for up to 10 days at 30°C in darkness. Plates were scanned from the bottom to determine pigmented secondary metabolite production (Fig. 2, 4).

S1 nuclease protection assay

M145, M751 and M752 were grown in SMM and RNA was isolated as previously described (Takano, 2006). The S1 nuclease protection assay was conducted as

described (Takano *et al.*, 2001). The probe for *scbR2* was made using primers scbR2-REV uniquely labelled at its 5' end with [^{32}P]ATP using T4 polynucleotide kinase and SCBR-FW to generate a 540 bp fragment with *S. coelicolor* M145 total DNA as template. A sequence ladder was generated using the SCBR-REV primer and a Sequenase 7-deaza-GTP sequencing kit (USB). The probe for *cpkJ* was made using primers ksER uniquely labelled at the at its 5' end with [^{32}P]ATP using T4 polynucleotide kinase and ksEF to generate a 502 bp fragment. pBR322 digested with *Msp*I was labelled with [^{32}P]ATP using T4 polynucleotide kinase and used as a marker. A protected 5' *cpkJ* transcript of about 190 nt was detected. All primers are listed in Table S1. The results shown for *hrdB* were previously published (Takano *et al.*, 2005a).

Reverse transcription and quantitative RT-PCR

RNA was isolated as previously described (Takano *et al.*, 2005a) from M145, LW3 (*scbR2*::*aac*(3)IV) and LW6 (*cpkO*::*aac*(3)IV) grown in liquid SMM at three different time points at OD_{450} of: M145: (1) 0.61 (13.3 h), (3a) 1.36 (16.3 h), (5) 1.6 (21.3 h); LW3: (1) 0.46 (17.5 h), (2) 0.89 (21 h), (3) 1.48 (25 h, produced Red); LW6: (2) 0.54 (19 h), (3) 0.72 (21.5 h, produced Red) and (4) 0.90 (25 h). The LW6 samples are identical with those used in (Takano *et al.*, 2005a). DNase treatment was performed on 10 μg of each RNA sample used for the qRT-PCR experiments with 10U of DNaseI (Roche) and the absence of chromosomal DNA contamination was confirmed by PCR using primers hrdBrt1 and hrdBrt2 (Hsiao *et al.*, 2007). To synthesize cDNA, 2 μg of RNA, 1.8 μg of random hexamers (Invitrogen) and dNTPs to a final concentration of 1 mM each were incubated at 72°C for 10 min; to this, 5x First-Strand Buffer (Invitrogen), DTT to a final concentration of 10 mM, 100U of Superscript II (Invitrogen) and RNase-free water were added to give a final volume of 30 μl and the mixture was incubated at 50°C for 100 min and 75°C for 15 min. qRT-PCR experiments were performed using the ABI Prism 7500FAST system (Applied Biosystems). Each 25 μl reaction contained 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems), 6.6 ng of cDNA, 10 pmol of each primer and 9.5 μl of water. The reaction parameters were as following: 95°C for 10 min, followed by 40 two-step amplification cycles consisting of 30 sec denaturation at 95°C and 1 min of annealing and extension at 60°C. A final dissociation step was run to generate a melting curve and consequently verify the specificity of the amplification products. Real-time PCR

was monitored and analyzed by the Sequence Detection System version 1.3 (Applied Biosystems), and relative expression levels were normalized to mRNA derived from the major vegetative sigma factor (*hrdB*). All samples were run in triplicate.

Results

Deletion of *scbR2* reveals a novel yellow diffusible secondary metabolite

A deletion mutant of *scbR2*, which encodes a protein with 32% amino acid identity to ScbR and which is located in the *cpk* gene cluster, was made to assess the role of this putative regulatory gene in secondary metabolism. The genotype of the resulting strain, LW3 (M145 *scbR2*::*aac*(3)IV), was confirmed by Southern and PCR analysis (Fig. 1, S1). LW3 was assessed initially for secondary metabolite production after growth on R2YE agar medium for 21 h, 24 h, 27.5 h, 42 h, 48 h, 3 d, 4 d and 9 days (Fig. 2). Deletion of *scbR2* resulted in a marked delay and reduction in actinorhodin production, and the appearance of a previously unseen yellow diffusible pigment. M145 and LW3 were subsequently compared on a range of agar media, including DNAgar, SMMS and R2 with essentially the same results. Production of the yellow compound was now also evident in M145, but only when grown on DNAgar, and it occurred at a much earlier time point of growth compared to the two known pigmented antibiotics and at a lower level than in LW3. Growth on MS agar, on which *S. coelicolor* sporulates well, revealed no detectable effect of the deletion of *scbR2* on morphological development or on antibiotic production (data not shown).

Since production of the yellow compound was maximal on DNAgar, where actinorhodin production was apparently abolished, this medium was used for further analysis.
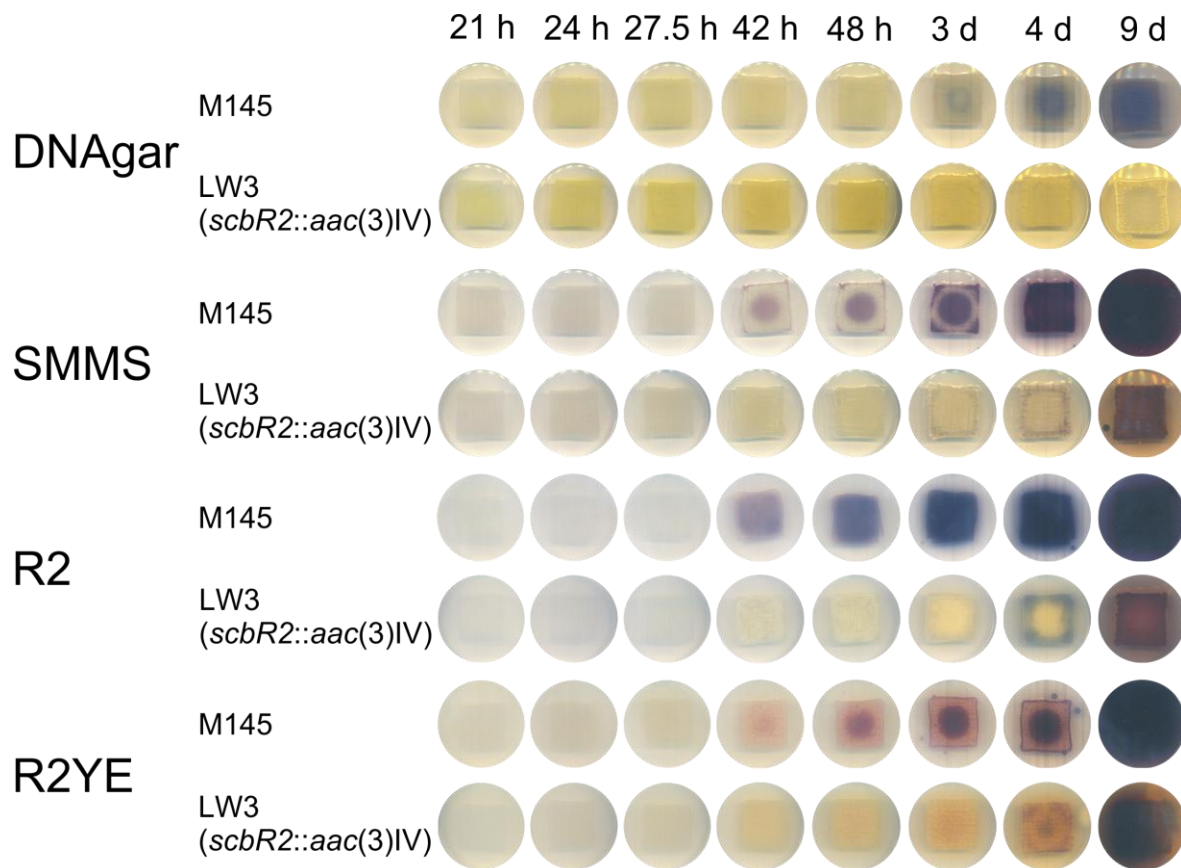
Fig. 2 Antibiotic production on different agar media

S. coelicolor strains M145 and LW3 (M145 *scbR2*::*aac*(3)IV) were patched in squares on DNAgar, SMMS, R2, R2YE medium and incubated at 30°C for 21 h, 24 h, 27.5 h, 42 h, 48 h, 3 d, 4 d and 9 days. The plates were observed from the bottom.

Secondary metabolite production in the complemented *scbR2* mutant resembles the parental strain

To confirm that production of the yellow compound and reduced actinorhodin synthesis resulted solely from deletion of *scbR2*, *scbR2* was reintroduced into LW3 using the integrative vector pIJ82 (Kieser *et al.*, 2000). The genotype of LW3 containing pIJ82 harbouring *scbR2* (pTE216) was confirmed by PCR (Fig. S1). Production of pigmented secondary metabolites by M145, LW3 (M145 *scbR2*::*aac*(3)IV), LW3/pIJ82 and LW3/pTE216 was monitored for up to six days (Fig. 3). Production of the yellow pigment was more pronounced in the *scbR2* mutants than in the *scbR2*$^+$ strains at all time points, while actinorhodin production

was restored in LW3/pTE216, although not to the level observed in M145. Production of the yellow pigment in LW3/pTE216 was restored to the same level as in M145, consistent with a role for *scbR2* in regulating its production.
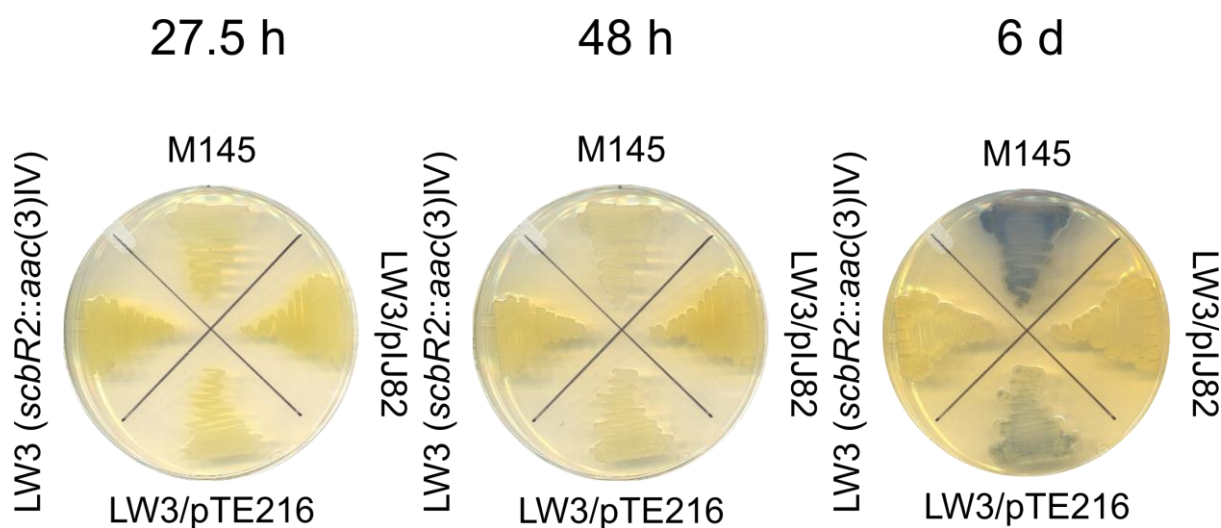
Fig. 3 Complementation of the *S. coelicolor scbR2* mutant strain LW3

S. coelicolor strains M145, LW3 (M145 *scbR2*::*aac*(3)IV) and LW3 containing the empty vector pIJ82 (LW3/pIJ82) and pIJ82 harbouring *scbR2* (LW3/pTE216) were incubated on DNAgar at 30°C. Production of pigmented secondary metabolites was monitored after 27.5 h, 48 h and 6 days of growth.

Yellow compound biosynthesis requires a functional *cpk* gene cluster

To exclude the possibility that the yellow compound was related to any of the other characterised secondary metabolites produced by *S. coelicolor*, M145 Δ*act* Δ*red* Δ*cda* (M1148) and M145Δ*act* Δ*red* Δ*cda* Δ*cpk* (M1146) were grown on DNAgar in the dark (see below) and pigment production compared with M145, LW3 (M145 *scbR2*::*aac*(3)IV), P100 (M145 *cpkC*::*aac*(3)IV) (Pawlik *et al.*, 2007), LW6 (M145 *cpkO*::*aac*(3)IV) (Takano *et al.*, 2005a), M751 (M145Δ*scbA*) and M752 (M145Δ*scbR*) (Takano *et al.*, 2001) (Fig. 4). P100 and LW6 are deletion mutants of genes encoding the biosynthetic PKS subunit CpkC and the pathway-specific activator of the *cpk* cluster, CpkO, respectively (Pawlik *et al.*, 2007, and Takano *et al.*, 2005a).

M751 and M752 are mutants in the γ-butyrolactone synthase (ScbA) and receptor (ScbR) of the *S. coelicolor* butanolide system (reviewed in Takano, 2006).

Mutants lacking a functional *cpk* cluster (i.e. M1146, P100, LW6) failed to produce the yellow pigment (henceforth called yCPK), suggesting that it is indeed the product of the orphan gene cluster. M751 and M752 also did not produce the yellow pigment, suggesting a regulatory role in yellow pigment synthesis.
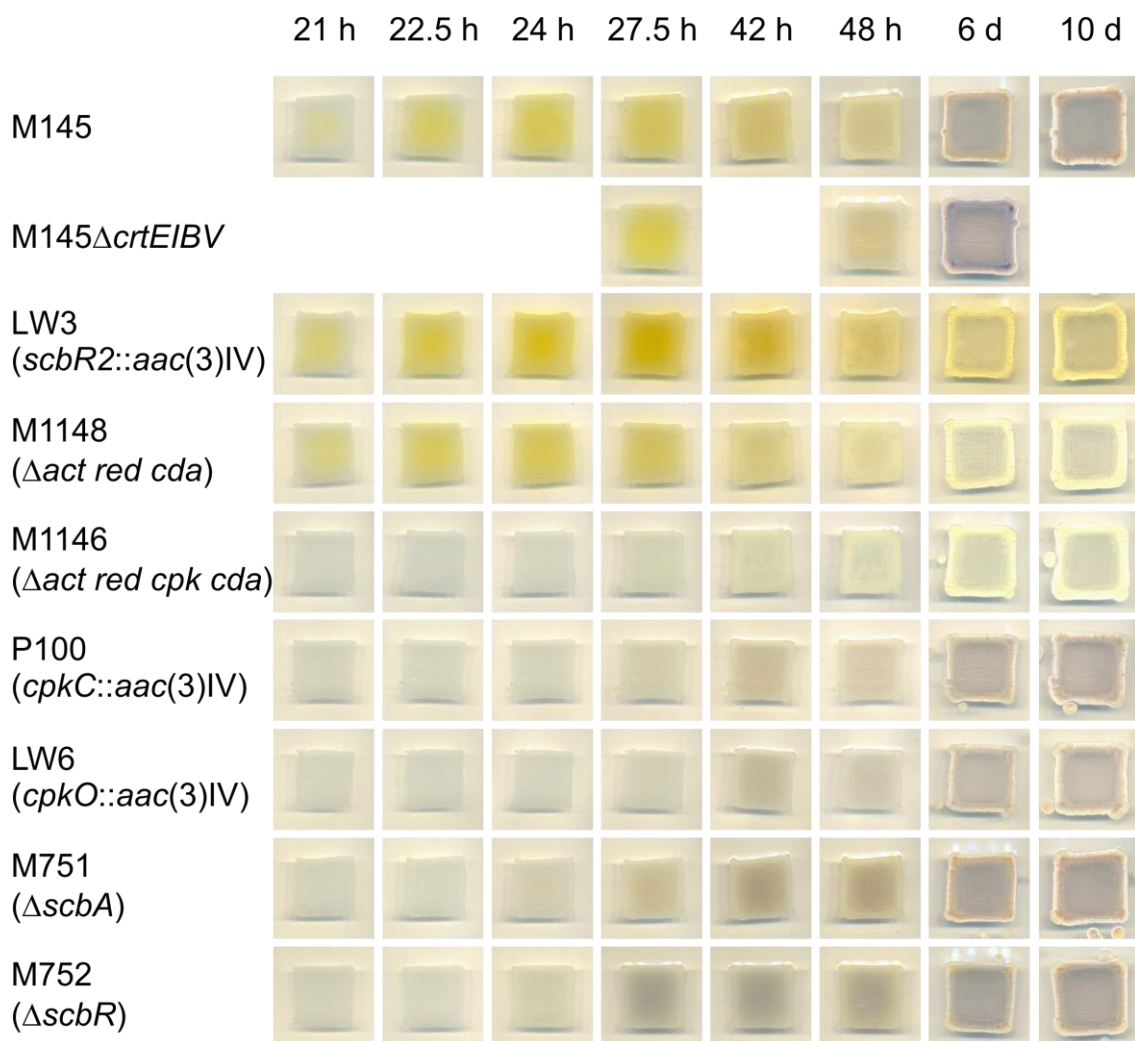
Fig. 4 Antibiotic production on DNAgar

S. coelicolor strains M145, M145Δ*crtEIVB*, LW3 (M145 *scbR2*::*aac*(3)IV), M1148 (M145Δ*act red cda cpk*), M1146 (M145Δ*act red cda*), P100 (M145 *cpkC*::*aac*(3)IV), LW6 (M145 *cpkO*::*aac(3)*IV), M751 (M145Δ*scbA*) and M752 (M145Δ*scbR*) were incubated on DNAgar at 30°C. Production of pigmented secondary metabolites was followed at 21, 22.5, 24, 27.5, 42, 48 h and 6, 10 days.

The yellow compound is produced in a carotenoid deletion mutant

S. coelicolor produces intracellular yellow-pigmented carotenoids when grown under light (Takano *et al.*, 2005b). To rule out the possibility that yCPK could reflect carotenoid production, a *S. coelicolor* mutant blocked in carotenoid synthesis (M145Δ*crtEIVB*) (Takano *et al.*, 2005b) was grown on DNAgar (Fig. 4). The mutant closely resembled M145 in yellow compound production, which was clearly visible at 27.5 h and faded afterwards (48 h). Consequently, yCPK is not related to carotenoid production. Moreover, all of the cultures shown in Fig. 4 were incubated in darkness, where carotenoid synthesis is not induced (Takano *et al.*, 2005b).

***scbR2* is temporarily expressed in M145, repressed in a *scbA* mutant and constitutively expressed in a *scbR* mutant**

In previous work the γ-butyrolactone receptor protein ScbR was shown to bind to the promoter regions of its own gene and the pathway-specific activator gene *cpkO*, repressing transcription during exponential growth. During transition to stationary phase the γ-butyrolactone SCB1 is produced, binds to ScbR and relieves repression, thus inducing *scbR* and *cpkO* expression (Takano, 2006). It was also shown that the production of SCB1 was dependent on the γ-butyrolactone synthase ScbA (Hsiao *et al.*, 2007; Takano *et al.*, 2001). To understand the relationship between ScbR2, a ScbR homologue, and ScbA and ScbR, transcription of *scbR2* was analysed in M145 and in congenic *scbA* and *scbR* mutants.

The transcriptional start site of *scbR2* was first determined by high resolution S1 nuclease protection analysis and found to lie 50 nt upstream of the ScbR translational start site (an adenine at nt 6946379 in the NCBI genome sequence NC_003888 (GenBank entry AL645882) (http://www.ncbi.nlm.nih.gov)) (Fig. 5 A and B). Potential -10 and -35 regions lie upstream of the proposed transcriptional start site. While short direct and inverted repeats were detected in the intergenic region between *scbR2* and the adjacent *scoT*, DNA sequences resembling previously reported binding sites for ScbR and its homologues ArpA, BarA and FarA (Takano *et al.*, 2005a) were not observed.

The expression of the *cpk* gene cluster was previously detected by microarray analysis using *S. coelicolor* grown in SMM (Takano *et al.*, 2005a). Therefore to determine the role of ScbR and ScbA in regulating *scbR2* expression, S1 nuclease

protection analysis was carried out on RNA isolated at different phases of growth from cultures of M145, M751 ($\triangle scbA$) and M752 ($\triangle scbR$) grown in SMM. None of the three strains produced the yellow compound (consistent with our results for M145 grown on SMMS agar medium (Fig. 2)). However, transcription of *scbR2* in M145 increased dramatically in transition phase before decreasing slightly once the culture entered stationary phase (Fig. 5 C). In the *scbA* mutant, expression of *scbR2* was abolished, while it was constitutively expressed throughout growth in the *scbR* mutant (Fig. 5 C).

Expression of the putative yCPK biosynthetic gene *cpkJ* (SCO6283) was also examined. In M145, expression was detected during transition and early stationary phase, while no or little expression was observed in M751 ($\triangle scbA$) (Fig. 5 C). In M752 ($\triangle scbR$), expression of *cpkJ* was detected at high levels during exponential growth and at a lower intensity in stationary phase.

A


```
G A T C
                C
                C
                C
                C
                A*
                C
                G
                G
```

B

```
     R   T   R   A   A   R   E   Q   K   T   M
                                                    RBS
acgcgtacgtgccgcccgctcctgcttggtcatgtcgtgctccgtggtcgcgatcgtcga
tgcgcatgcacggcgggcgaggacgaacca gta cagcacgaggcaccagcgctagcagct
              ScbR2                ←
```

```
gccccaaggcgggataagtggcacccc agtgtc acgtgccgcccccaccggg cagtaa tc
cggggttccgccctattcaccgtgggg tcacag tgcacggcggggggtggccc gtcatt ag
           ◄┄┄┄ *              -10                        -35
```

```
gcgcgaccaccacgcgctccctctggcggatctcatgc tgtcca ccagcggcagggtcca
cgcgctggtggtgcgcgagggagaccgcctagagtacg acaggt ggtcgccgtcccaggt
                                         -35
```

```
          ** ┄┄┄►
ccctggt ccgtccgctccggtcctggcccgcaccgcgtcgggttcgccgcgggcggggcc
gggacca gggcaggcgaggccaggaccgggcgtggcgcagcccaagcggcgcccgcccgg
  -10
```

```
              RBS                   ┌─────►
gcactcgtcgaacggtaagggga aaaggcg atg gggaagtgactggttcaggcggttcggt   ScoT
cgtgagcagcttgccattcccctttttccgctacccttcactgaccaagtccgccaagcca

                                M   G   S   D   W   F   R   R   F   G
```

C


```
              M145              M751            M752
                              (ΔscbA)          (ΔscbR)
        1 2 3 4 5 6 7 8  1 2 3 4 5 6 7 8 M 1 2 3 4 5 6 7 8 M
scbR2
cpkJ                                                            — 190 bp
hrdB
             E    T   S    E    T    S      E    T   S
```

Fig. 5 S1 nuclease protection analysis of *scbR2* and *cpkJ*

(A) High resolution S1 nuclease protection analysis of the transcriptional start site of *scbR2*. An asterisk indicates the probable start site; the sequence of the template strand is shown. Lanes G, A, T and C are sequence ladders derived from the same labelled primers used to generate the PCR products.

(B) The promoter regions of *scbR2* and *scoT*. Possible -10 and -35 region sequences for *scbR2* and *scoT* are indicated by solid and dashed boxes, respectively. Possible ribosomal binding sites (RBS) are shaded. Transcriptional start points are indicated by asterisks and the direction of transcription is indicated by arrows with the promoter of each gene indicated. Partial amino acid sequences of ScbR2 and ScoT are given; start codons are marked with arrowed boxes.

(C) S1 nuclease protection analysis of *scbR2, hrdB* and *cpkJ* transcripts using RNA isolated from liquid SMM cultures of *S. coelicolor* M145, M751 and M752. Numbers 1-8 denote the samples taken at different time points (see *Materials and Methods*). E, T and S indicate the exponential, transition and stationary phases of growth, respectively. M denotes a 190 bp size marker derived from pBR322 digested with *Msp*I. The *hrdB* data have been published previously (Takano *et al.*, 2005a).

scbR2, *cpkC* and *cpkE* are not expressed in a *cpkO* mutant
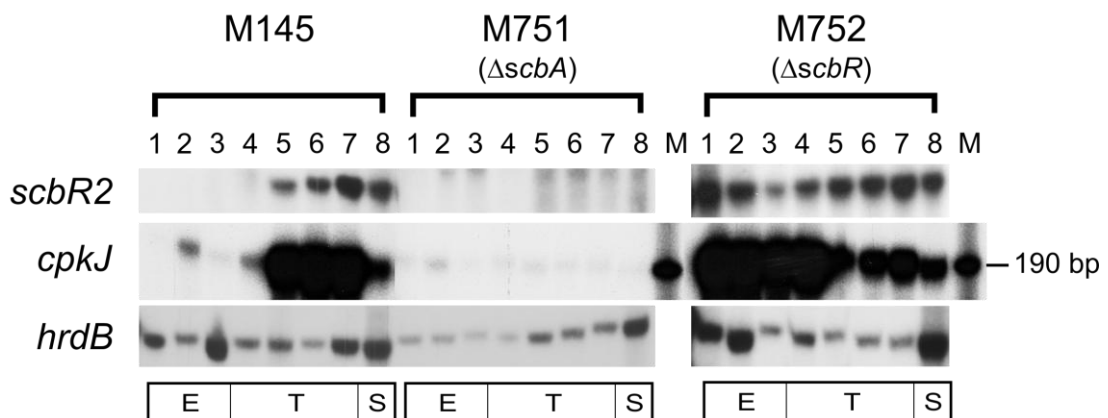
To obtain further insight into the regulation of the *cpk* gene cluster, expression of the putative regulatory genes *cpkO* (SCO6280) and *scbR2* (SCO6286), and the predicted biosynthetic genes *cpkC* (SCO6273) and *cpkE* (SCO6277), was assessed by qRT-PCR. RNA was isolated at three different time points from M145, LW6 (∆*cpkO*) and LW3 (∆*scbR2*) grown in SMM liquid media. cDNA was synthesized from 2 µg of RNA and cDNA equivalent to 6.6 ng RNA was used in each qRT-PCR with different primers (Material and Methods and Table S1). Levels of gene expression are shown in Fig. 6 for *cpkC*, *cpkE* and *cpkO* as fold-change relative to the M145 time point 1 exponential growth phase sample (Fig.6 A), and for *scbR2* relative to the M145 time point 2 sample (Fig. 6 B), since with M145 time point 1 no *scbR2* transcripts were detected.

In agreement with the S1 nuclease protection results (Fig. 5 C), qRT-PCR analysis revealed transcription of *scbR2* and all three *cpk* genes during transition phase in M145 (Fig. 6 A and B). Expression of *scbR2, cpkE* and *cpkC* was not observed in the *cpkO* mutant LW6 (Fig. 6 A and B), consistent with previous results (Takano *et al.*, 2005a) and with the role of CpkO as a transcriptional activator of the *cpk* cluster.

As expected, transcription of *cpkO* was not detected in the Δ*cpkO* deletion mutant LW6 (Fig. 6 A) since the primers used corresponded to the deleted region.

A
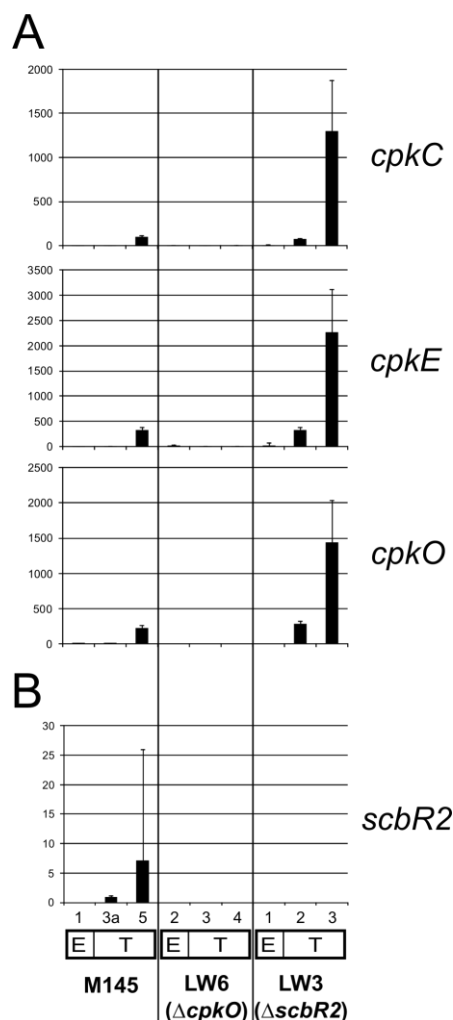
Fig. 6 Quantitative RT-PCR analysis of *cpkO*, *cpkC, cpkE, and scbR2*

qRT-PCR analysis of transcription of *cpk*O, *cpk*C, *cpk*E, and *scbR2* using cDNA synthesized from RNA isolated from liquid SMM cultures of *S. coelicolor* M145, LW6 (Δ*cpkO*) and LW3 (Δ*scbR2*). Numbers at the bottom denote samples taken at different phases of growth (E, exponential and T, transition phase). Gene expression is shown as fold-change relative to the M145 time point 1 exponential growth phase sample (A) or to the M145 time point 3a early transition phase sample (B) and is indicated on the left. Error bars indicate the standard deviation.

cpkC, *cpkE* and *cpkO* are overexpressed in a *scbR2* mutant

Expression of *cpkC*, *cpkE* and *cpkO* occurred in M145 during late transition phase (M145 time point 5 in Fig. 6 A), while in the *scbR2* mutant LW3, all three genes were transcribed earlier (LW3 time point 2 in Fig. 6 A) and eventually at much higher levels (12.9, 6.8 and 6.6 fold, respectively; LW3 time point 3 compared to M145 time point 5, Fig. 6A). This indicates a negative role for ScbR2 in regulating the expression of all three *cpk* genes.

Discussion

Discovery of a novel yellow pigmented secondary metabolite in *S. coelicolor* by deletion of a regulatory gene

Deletion of the gene encoding the γ-butyrolactone receptor homologue ScbR2 resulted in a marked change in secondary metabolite production in *S. coelicolor*. In addition to loss of actinorhodin synthesis, the mutation induced the synthesis of a previously unseen yellow metabolite, yCPK. Analysis of several different mutants strongly suggests that yCPK is the product of the orphan *cpk* gene cluster. Subsequent growth of the parental strain M145 on the agar medium optimal for yCPK production also revealed yellow pigment production, but at much lower levels.

There are several reports where manipulation of regulatory genes enhanced the production of a known antibiotic (e.g., (Bunet *et al.*, 2008; Rigali *et al.*, 2008)). More interestingly, Bok *et al.* disrupted and overexpressed the pleiotropic regulator LeaA in *Aspergillus nidulans* and identified the *tdi* gene cluster which is responsible for the production of terrequinone A, a previously undefined metabolite in the parental strain (Bok *et al.*, 2006). Also in *A. nidulans*, Bergmann and co-workers overexpressed the pathway-specific activator gene *apdR* of a cryptic PKS-NRPS gene cluster resulting in the production of a group of metabolites not detected in the parent strain (Bergmann *et al.*, 2007). The latter is the only current example of the activation of a silent gene cluster by the manipulation of a regulatory gene (Zerikly and Challis, 2009). Thus, to our knowledge, the work reported here represents the first successful application of this approach beyond *Aspergillus* and is the first example of the deletion of a regulatory gene resulting in the production of a novel metabolite that had not been previously recognised in the parental strain. The emergence of multiple antibiotic resistant bacterial pathogens has created an urgent need for new antibiotics. The strategy reported here may provide an important route to the discovery of novel antibiotics that are currently "hidden" within the genomes of actinomycetes, where as many as 25 secondary metabolite clusters have been identified by sequence analysis (Omura *et al.*, 2001).

ScbR2 is under the control of the *S. coelicolor* butanolide system and represses yellow compound production

The absence of *scbR2* expression in a *scbA* mutant and the constitutive expression of *scbR2* in a *scbR* mutant (Fig. 5 C) suggests that *scbR2* expression is controlled by the ScbA/R butanolide system. Previously, the pathway specific activator gene of the *cpk* cluster, *cpkO*, was shown to be negatively regulated by ScbR, which binds to the *cpkO* promoter region and represses transcription. The expression profile of *scbR2* resembles that of *cpkO* ((Udwary *et al.*, 2007), Fig. 5 C, and Fig. 6 A and B). However, direct regulation of *scbR2* by ScbR seems unlikely since no ScbR binding sites could be detected in the *scbR2* promoter region (Takano *et al.*, 2005a). The absence of a *scbR2* transcript in the ∆*cpk*O mutant (this study) suggests that s*cbR2* is part of the CpkO regulon.

The onset of *scbR2* transcription in M145 occurs approximately 2 hours later than that of *cpkO, cpkJ* during early transition phase ((Takano *et al.*, 2005a) and Fig. 5 C). Interestingly, the yCPK pigment decreased markedly after 27.5 hours, while in the *scbR2* deletion mutant it was sustained for a much longer period of time (Fig. 4). Consequently, it is possible that ScbR2 may serve to ultimately suppress expression of the *cpk* gene cluster. This role is also implied by the overexpression of *cpkO*, *cpkC* and *cpkE* in the *scbR2* mutant (Fig. 6 A), where *cpkC* and *cpkE* may be indirectly regulated by ScbR2 via the derepression of the positive regulator CpkO. Since CpkO activates both *scbR2* and the biosynthetic *cpk* genes, this would constitute negative feedback regulation of yCPK biosynthesis.

Actinorhodin production was highly impaired in the *scbR2* mutant on all media tested and not visible on DNAgar even after 10 days of growth (Fig. 2 and 4). Although we cannot exclude direct or indirect transcriptional regulation of the actinorhodin gene cluster by ScbR2, this may reflect competition for precursor supply as actinorhodin and yCPK are known and predicted (Pawlik *et al.*, 2007), respectively, to be derived from acetate.

There are several other *Streptomyces* species in which pairs of putative γ-butyrolactone binding proteins are involved in the regulation of secondary metabolite production (reviewed in (Takano, 2006)). For example, in *Streptomyces ambofaciens,* AlpZ and AlpW are involved in the regulation of alpomycin and orange

pigment synthesis (Bunet *et al.*, 2008)). In *Streptomyces virginiae,* BarA is the primary negative transcriptional regulator of virginiamycin biosynthesis (Kinoshita *et al.*, 1997;Nakano *et al.*, 1998;Nakano *et al.*, 2000). Binding of the virginiae butanolide autoregulator VB to BarA derepresses antibiotic production and also a corresponding resistance mechanism. However, in a strain mutated in the second γ-butyrolactone homologue BarB, virginiamycin is produced 2-3 h earlier. This suggests that BarB may also have a negative role in virginiamycin production, retarding synthesis until the cells acquired resistance against the antibiotic (Matsuno *et al.*, 2004). Interestingly, in many of these examples, only one of the pair of homologous proteins (for example, ScbR and BarA) has been shown to be a true γ-butyrolactone binding protein, and it is conceivable that the other partner has lost this ability but instead acquired an alternative function to fine tune the production of secondary metabolism.

Acknowledgements

Chapter 3

51

abCPK

Submitted for publication together with chapter 2

"Deletion of a regulatory gene within the *cpk* gene cluster reveals novel antibacterial activity in *Streptomyces coelicolor* A3(2)"

Marco Gottelt, Stefan Kol, Juan Pablo Gomez-Escribano, Mervyn Bibb, and Eriko Takano

Content of this chapter is filed as

European patent application no. 09172463.3-2406

"Antibiotic compositions, methods for providing the same, and *Streptomyces coelicolor* mutants for use in such methods"

Eriko Takano, Marco Gottelt, Stefan Kol (inventors)

Abstract

After the recent discovery of yCPK, a yellow pigment related to the previously orphan *cpk* gene cluster in *Streptomyces coelicolor* A3(2), growth conditions were systematically altered and optimized in terms of maximizing yCPK production. Feeding of the amino acid glutamate drastically increased the production of the yellow substance. Only then we could show that *S. coelicolor* strains that produce yCPK in high amounts also show antibacterial activity. However, we have evidence that this activity is not related to yCPK, but to another *cpk* metabolite, abCPK (antibiotic coelicolor polyketide). The novel antibiotic depends on a functional *cpk* gene cluster and is not related to Act, Red or CDA, three bioactive secondary metabolites from *S. coelicolor*. Active abCPK could be obtained independent from the yellow pigment. Furthermore, production of yCPK and the novel antibiotic could be separated by deletion of a putative secreted biosynthetic gene of the *cpk* cluster, *scF*. Deletion of the *scF* homologue *cpkH* resulted in the loss of yCPK and abCPK production. We therefore assume that late steps of the *cpk* biosynthetic pathway take place outside the cell where a precursor is converted into the active antibiotic and subsequently into the yellow pigment. abCPK showed bacteriostatic activity against Gram+ and Gram- bacteria. Both *cpk* cluster products are stable, polar compounds and further physiochemical properties were determined.

Introduction

Streptomycetes are mycelial soil bacteria with a complex life cycle and morphology. Streptomycetes are of major interest in the search for new drugs since the family is the richest source of antibiotics and other bioactive compounds used in medicine and agriculture (Hopwood, 2007). Estimates suggest up to several ten thousands of yet unidentified secondary metabolites of pharmaceutical interest (Berdy, 2005; Watve *et al.*, 2001).

In classical screening programs strains are isolated from their natural habitats and tested for production of antibiotic agents. Entering the post-genomic era, with currently more than a thousand complete microbial genome sequences available, has revolutionised the field of natural product research. Together with the dramatic increase in our understanding of the genetics and enzymology of microbial natural product biosynthesis, and the availability of bioinformatics tools based on this knowledge, it is now possible to identify and analyse groups of related genes located in close vicinity – "gene clusters" – likely to encode natural product biosynthetic pathways in sequenced microbial genomes ("genome mining"; reviewed in Challis, 2008).

Genome analysis of the model Streptomycete *Streptomyces coelicolor* A(3)2 revealed several secondary metabolite gene clusters (Bentley *et al.*, 2002), among them 11 well-characterised compounds including the three known *S. coelicolor* antibiotics, the blue-pigmented polyketide (gamma-)actinorhodin (Act) (Bystrykh *et al.*, 1996; Rudd and Hopwood, 1979), the red-pigmented (undecyl-)prodigiosins (Red) (Feitelson *et al.*, 1985) and the calcium dependent antibiotic (CDA) (Hopwood and Wright, 1983; Lakey *et al.*, 1983). A fourth antibiotic, methylenomycin, is not encoded chromosomally as the others listed, but on a plasmid (SCP1) lacking in the sequenced strain M145 (Bentley *et al.*, 2004). There were also 12 additional so-called "orphan" secondary metabolite clusters found to which no products could be assigned yet (Challis and Hopwood, 2003).

Recently, we described a novel *S. coelicolor* metabolite as product of one of these hitherto orphan gene clusters, a yellow-pigmented compound (yCPK) related to the *cpk* (*coelicolor polyketide*) gene cluster (formally named *kas* cluster) (Gottelt *et al.*,

submitted). The *cpk* cluster contains a Type I polyketide synthase and was described in detail in Pawlik *et al.*, 2007. Until the discovery of yCPK, the cluster remained orphan and the chemical structure of the *cpk* cluster end product has not been determined yet. yCPK was conditionally produced in the parental strain M145 and overproduced in a *scbR2* mutant, a γ-butyrolactone receptor homologue located in the *cpk* cluster. yCPK was shown to be related to the *cpk* cluster by antibiotic production analysis in a newly created deletion mutant of the entire *act*, *red* and *cda* clusters and a quadruple mutant where in addition also the *cpk* cluster was deleted (Gottelt *et al.*, submitted).

In this study, yCPK production in *S. coelicolor* was increased by the addition of glutamate to the medium. Under optimised culturing conditions for the production of the yellow pigment, an antibacterial agent (abCPK) was discovered which coincides with yCPK production. Like yCPK, also the antibiotic compound could be related to the *cpk* gene cluster by analysing mutants in the *act*, *red*, *cda* and the *cpk* gene clusters. In liquid culture yCPK was found only secreted into the medium. abCPK, however, could be isolated best from the cells which lacked yellow pigmentation. Furthermore, we prepared a knockout mutant in a *cpk* cluster gene that is unable to make yCPK, but still produces abCPK. This implies that yCPK and abCPK are two distinct metabolites of the *cpk* biosynthetic pathway. abCPK was shown to be active against Gram+ and Gram- bacteria and is stable in methanol.

Results

yCPK production in *S. coelicolor* is enhanced in glutamate supplemented medium.

As the detection of the yellow-pigmented *cpk* product (yCPK) in the sequenced *S. coelicolor* strain M145 was very difficult (Gottelt *et al.*, submitted), media conditions were optimised for maximal production of yCPK. Glutamate (325 mM (final conc.) L-glutamic acid monosodium salt (Glu)) was added to minimal SMMS medium and to DNAgar additionally or to replace the casamino acids (CA) in SMMS. On SMMS with CA (CA-SMMS) yCPK production was not observed (Fig. 1) both with the parental M145 and the overproducing *scbR2* mutant strain LW3 (M145 *scbR2*::*aac(3)*IV). This is consistent with our results presented in Gottelt *et al.*, submitted. On SMMS with glutamate in addition to the casamino acids (CA+Glu-SMMS) the yellow pigment was detected, however, it was first observed only after 40 h of growth (Fig. 1). If the casamino acids were replaced by glutamate (Glu-SMMS), and thus glutamate is the sole nitrogen source, and complex DNAgar medium supplemented with glutamate (Glu-DNAgar) gave the best production (Fig. 1 and Fig. 2, respectively). An increased amount of phosphate in (modified) SMMS showed no effect (data not shown). Both on Glu-SMMS and on Glu-DNAgar, after 24 to 26 h of incubation at 30°C the diffusible yCPK was produced and the observed amounts and the onset of production were comparable between M145 and LW3. Nonetheless, whereas in M145 production seemed to stop after a comparably short time, with LW3 the yellow pigment accumulated to persistent high amounts in the agar (Fig. 1 and Fig. 2). These results are highly consistent with the findings and conclusions presented in Gottelt *et al.*, submitted. However, production of yCPK was clearly enhanced in glutamate supplemented medium. With LW3, in contrast to DNAgar cultures (Gottelt *et al.*, submitted), the yellow pigment was still visible in high amounts even after almost 6 days of growth on Glu-DNAgar (Fig. 2). A direct comparison between DNAgar and Glu-DNAgar cultures is shown in Figure 3 where LW3 was grown without (0 mM) and with (10 to 400 mM) glutamate. Also Figure 4 shows high yCPK production with LW3 on Glu-DNAgar, which is greatly reduced on DNAgar.

Higher final production levels and later onset of yCPK production with higher glutamate concentrations.

To further characterise the effect of glutamate and to optimise yCPK production, *S. coelicolor* LW3 was grown on DNAgar supplemented with different amounts of glutamate and yCPK production was followed at 22, 24 and 26 hours of growth. Production on 10 mM Glu-DNAgar did resemble that on DNAgar without glutamate (0 mM Glu; Fig. 3). Addition of 50, 100 and 200 mM Glu-DNAgar resulted in higher yCPK production at all time points. However, with 300 and 400 mM Glu, onset of yCPK production seemed increasingly delayed with higher amounts of glutamate. No yellow pigmentation was observed after 22 h on 400 mM Glu-DNAgar (Fig. 3). As a compromise between increased and prompt production, further experiments were conducted using 325 mM (final conc.) L-glutamic acid monosodium salt in glutamate supplemented media.
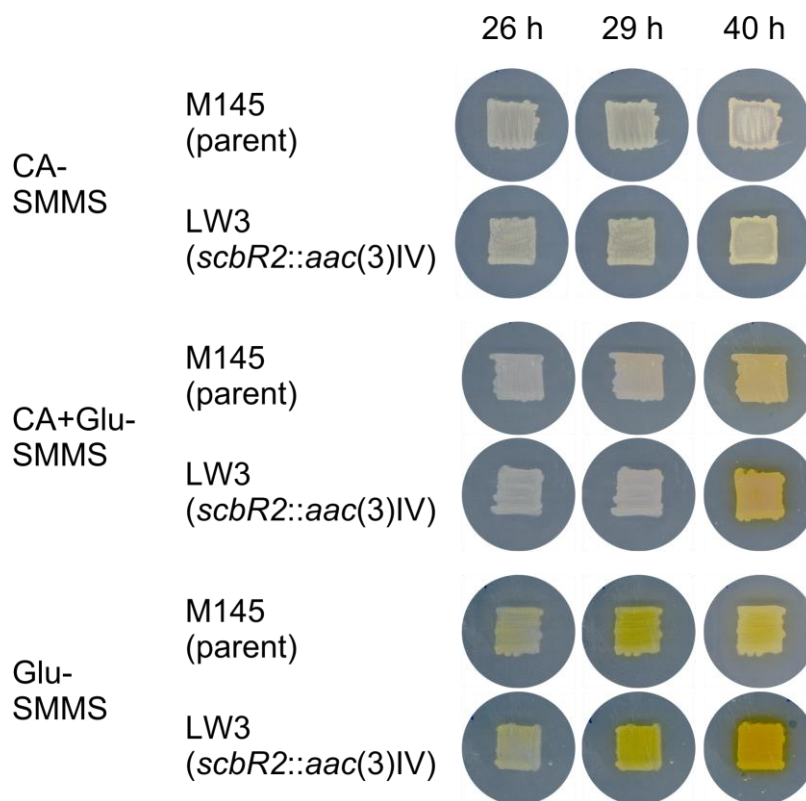
Figure 7 yCPK production on (modified) SMMS medium. *S. coelicolor* was incubated at 30°C on SMMS medium containing casamino acids (CA-SMMS) and on modified SMMS containing glutamate additionally (CA+Glu-SMMS) or replacing the casamino acids (Glu-SMMS). Pictures were taken from the bottom after 26, 29 and 40 hours of growth.
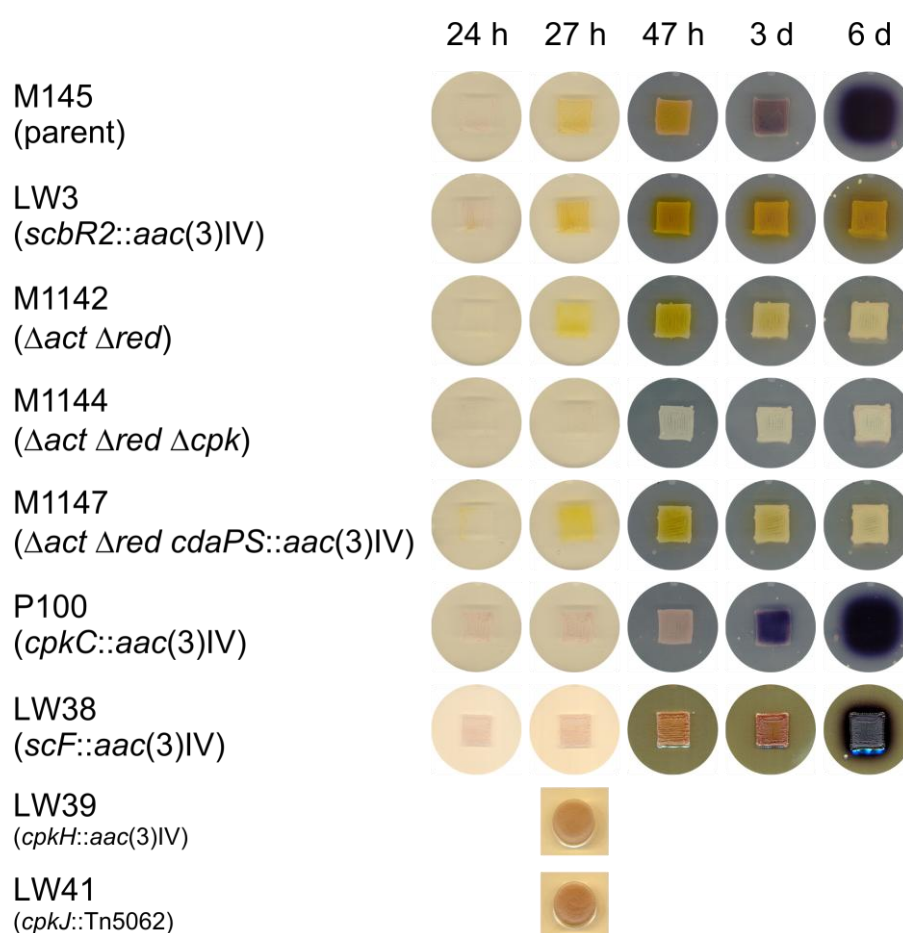
Figure 8 yCPK production on DNAgar supplemented with glutamate. *S. coelicolor* was grown on glutamate supplemented DNAgar (Glu-DNAgar) at 30°C. Pictures were taken from the bottom after 24 h, 27 h, 47 h, 3 d and 6 d of growth. Onset of yellow CPK compound (yCPK) production was observed around 24 h of growth with strains M145, LW3, M1142 and M1147.
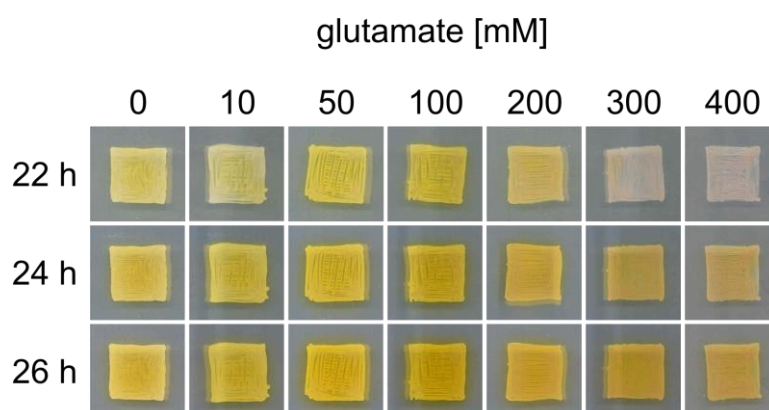
Figure 9 yCPK production on DNAgar supplemented with different amounts of glutamate. The *S. coelicolor scbR2* mutant LW3 was incubated on DNAgar supplemented with up to 400 mM (final conc.) glutamate at 30°C. Pictures were taken from the bottom after 22, 24 and 26 hours of growth.

Also under optimised production conditions yCPK production is related to the *cpk* gene cluster.

In Gottelt *et al.*, submitted, we showed in DNAgar cultures that yellow pigment production is related to the *cpk* gene cluster. To confirm these results under the optimised production conditions, the parental *S. coelicolor* strain M145, LW3, M1142 (M145 Δ*act* Δ*red*), M1144 (M145 Δ*act* Δ*red* Δ*cpk*), M1147 (M145 Δ*act* Δ*red* cdaPS::*aac(3)*IV) and P100 (M145 cpkC::*aac(3)*IV) were now grown on Glu-DNAgar plates (Fig. 2). Yellow compound production was observed only with M145, LW3, M1142 and M1147 and not with the *cpk* mutant strains. Yellow pigmentation was first seen after 24 h of incubation. In contrast to LW3, in the other production strains yellow pigmentation faded after several hours of production and could not be detected even if no Act and Red was produced (M1142 and M1147; Fig. 2). These results are consistent with our results on DNAgar presented in Gottelt *et al.*, submitted and clearly suggest that yellow compound production depends on a functional *cpk* cluster.

In addition to the deletion of the PKS subunit *cpkC* in strain P100, we recently disrupted another hypothetical biosynthesis gene of the *cpk* cluster, *cpkJ*, which shows homology to nucleoside-diphosphate-sugar epimerases (Pawlik *et al.*, 2007). The resulting in strain LW41 also did not produce yCPK after 27 hours of growth on Glu-DNAgar (Fig. 2).

yCPK production in *S. coelicolor* LW3 is slightly enhanced in N-acetylglucosamine supplemented medium.

Van Wezel and co-workers suggested a putative positive effect of N-acetylglucosamine (GlcNAc) on the expression of the *cpk* genes via the pleiotropic transcriptional regulator, DasR, and the activator of the *cpk* cluster, CpkO (Rigali *et al.*, 2008). The effect of GlcNAc on the production of yCPK was tested with *S. coelicolor* M145, LW3 and P100 grown on DNAgar supplemented with 50 mM GlcNAc and/ or 325 mM Glu. Antibiotic production after two days of growth is shown in Fig. 4. As expected from our previous results (Gottelt *et al.*, submitted), with none of the strains strong secondary metabolite production was observed on DNAgar. However, with LW3 a faint yellow pigmentation was still visible. On Glu-DNAgar, Red was produced in M145 and P100 and yCPK was observed in M145 and LW3 where LW3 clearly overproduces the yellow pigment. Compared to Glu-DNAgar, production

of yCPK in M145 and LW3 was lower on DNAgar supplemented with GlcNAc, but was still enhanced compared to DNAgar cultures. In agreement with Rigali *et al.*, 2008, GlcNAc stimulated Red production in M145 and P100, but not in LW3. Under the conditions tested, GlcNAc seems to have a stronger effect on the production of Red than on that of yCPK. Glutamate, on the other hand, rather stimulated yCPK than Red production. Pigmentation in DNAgar supplemented with both Glu and GlcNAc resembled that of the Glu-DNAgar cultures.
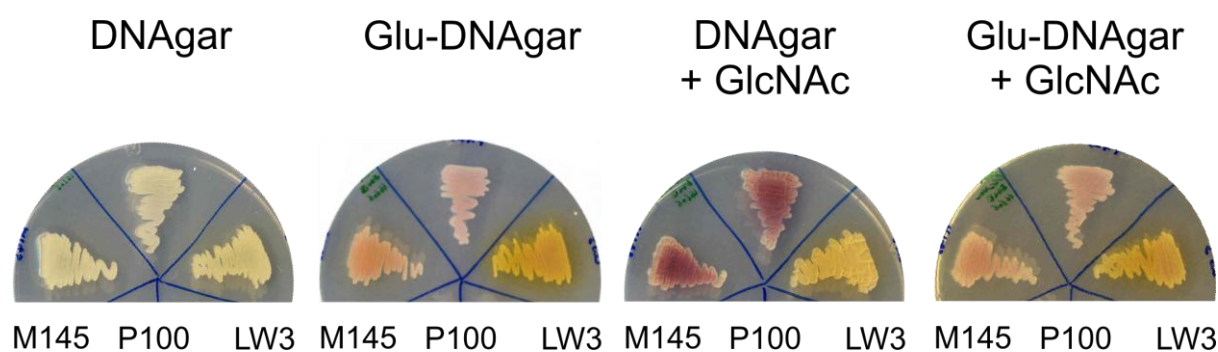
Figure 10 Secondary metabolite production on DNAgar supplemented with N-acetylglucosamine and glutamate. *S. coelicolor* strains M145 (parent), P100 (M145 *cpkC*::*aac(3)*IV) and LW3 (M145 *scbR2*::*aac(3)*IV) were incubated on DNAgar supplemented with glutamate (Glu) and/ or N-acetylglucosamine (GlcNAc) at 30°C. Pictures were taken from the bottom after 45.5 hours of growth.

yCPK is found in liquid culture supernatant and not inside the cells.

S. coelicolor strains M145, LW3, M1142, M1144, M1146 (M145 Δ*act* Δ*red* Δ*cpk* Δ*cda*) and M1147 were grown in liquid Glu-DNB medium. Growth and secondary metabolite production was followed at 20, 24 and 28 hours (M145, LW3, M1142, M1144 (Tab. 1, Fig. 5A)) and at 22 to 46 hours (M145, LW3, M1146, M1147 (Fig. 6A)). The yellow pigment was produced by *S. coelicolor* M145, LW3, M1142 and M1147 and not by strains M1144 and M1146 (Fig. 5A and 6A) which again confirms the dependency of yCPK on the *cpk* cluster. When cells were separated from the medium by centrifugation, these were found unpigmented despite the production of yCPK in strain M1142 (M145 Δ*act* Δ*red*), whereas the yellow compound was visible in the culture supernatant. In strains also producing Red (M145 and LW3), only red

and no yellow pigmentation of the cells was visible (Fig. 5A). Thus, yCPK seems to be present extracellularly. This is consistent with the yellow pigmented halos around yCPK producing strains in solid culture (Fig. 1 to 4).

Under the conditions tested, typically, yCPK became visible in the culture supernatant after 26 to 28 h (Fig. 5A and 6A). The yellow pigment could be detected by measuring OD_{450} of the cell free culture supernatant and was quantified to the cell biomass (OD_{450} of a cell suspension in yCPK-free buffer). The results for M145, LW3, M1142 and M1144 are shown in Tab. 1 and Fig. 5B, indicating that in Glu-DNB compared to the parental M145, both the *scbR2* mutant LW3 and strain M1142 overproduce yCPK, whereas the *cpk* cluster deletion mutant M1144 lacks yellow pigment production.

Table 1 Growth and yCPK production. Explanation in the text; also see Fig. 5.

	M145			LW3			M1142			M1144		
	20 h	24 h	28 h	20 h	24 h	28 h	20 h	24 h	28 h	20 h	24 h	28 h
OD450 (c)	0.62	0.92	1.48	0.57	0.92	1.26	0.26	0.92	1.4	0.71	0.86	1.35
OD450 (s/n)	0	0	0.67	0	0	0.74	0	0	0.61	0	0	0
OD450 (c) / OD450 (s/n)	0	0	0.19	0	0	0.42	0	0	0.53	0	0	0

A

B
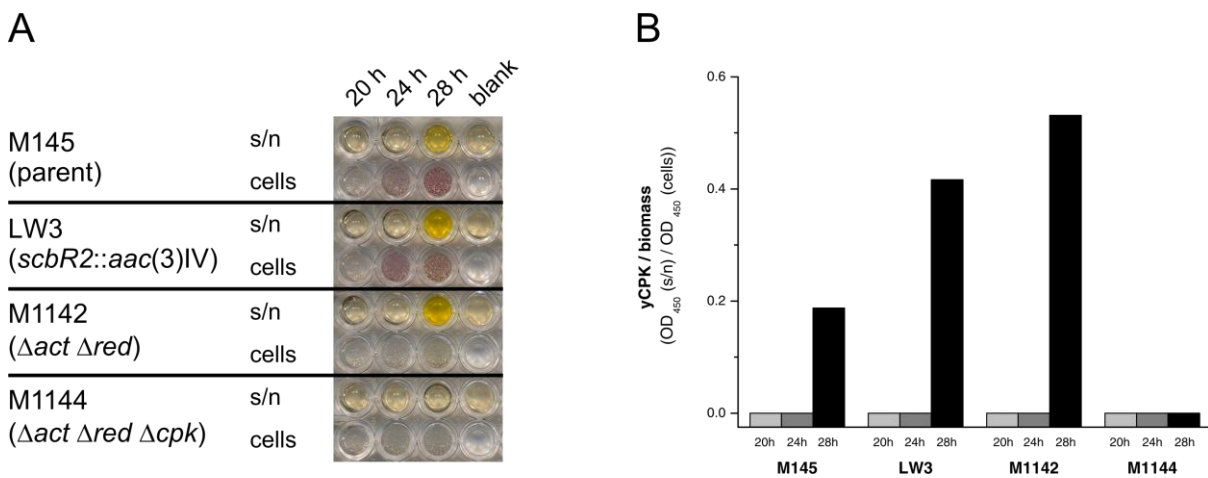
Figure 11 Production and quantification of yCPK production in liquid Glu-DNB culture. *S. coelicolor* was grown in glutamate supplemented DNB medium (Glu-DNB). Samples were taken

after 20, 24 and 28 h. Cells were separated from the culture supernatant (s/n) and resuspended in a colourless buffer. Supernatant and cells are shown in a 96 well plate, culture medium and buffer are given as blank, respectively **(A)**. To quantify the cell mass and the yellow pigment produced in the culture, OD_{450} of the cell suspension and the culture supernatant was determined, respectively. The graph shows the relative amount of yellow CPK compound (yCPK) produced (OD_{450} (s/n)) depending on the biomass (OD_{450} (cells)) at the three sampling time points **(B)**.
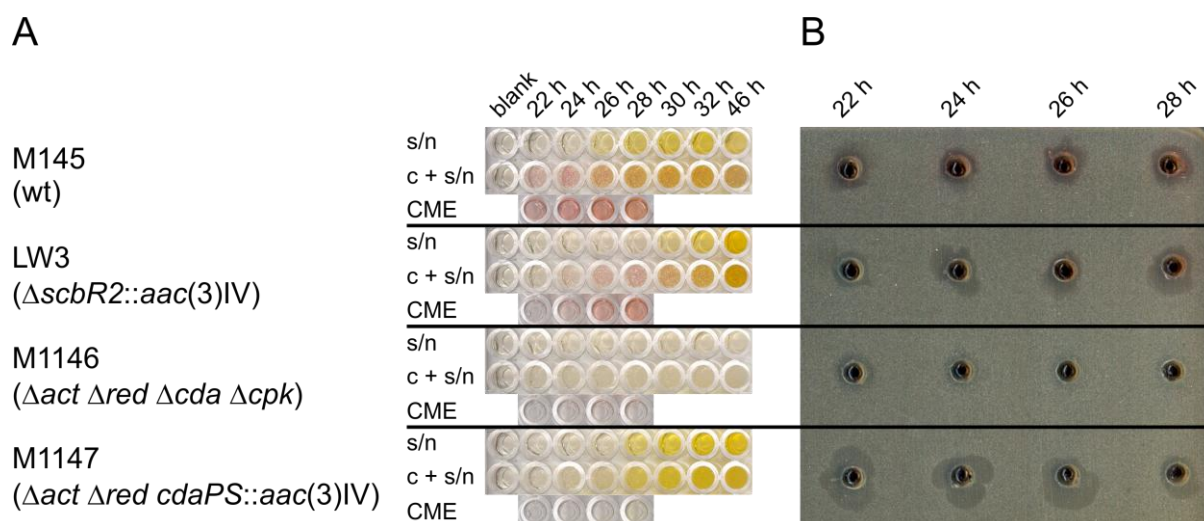
Figure 12 *cpk* gene cluster dependent production of an antibacterial compound in liquid culture. *S. coelicolor* was grown in glutamate supplemented DNB medium (Glu-DNB). Samples were taken between 22 and 46 h at the given time points and cell-free culture supernatant (s/n) and cells in culture medium (c + s/n) are shown. Culture medium is given as blank reference sample. After 22 to 28 h of growth cell methanol extracts (CME) were prepared as described in e*xperimental procedures* and are also shown **(A)**. The CME were tested for antibiotic activity. CME obtained after 22, 24, 26 and 28 hours of growth were applied to holes in a *B. subtilis* indicator plate. To determine inhibition of bacterial growth, the plate was incubated at 30°C overnight and pictures were taken from the top **(B)**.

S. coelicolor shows antibacterial activity dependent on the *cpk* gene cluster and enhanced by glutamate.

Strains M145, LW3, LW41, M1142, M1144, M1147 and P100 were grown for 27 h on Glu-DNAgar plates when yCPK was clearly visible (Fig. 7). The plates were then completely overlaid with Soft Nutrient Agar containing 0.4% of the indicator strains *Bacillus subtilis*, *Micrococcus luteus* (Gram+) and *Escherichia coli* (Gram-) and further incubated overnight. Growth of *B. subtilis* was inhibited with the yCPK producing strains (M145, LW3, M1142, and M1147), but not with the *cpk* mutant

strains LW41, M1144 and P100 (Fig. 7A). The same results were obtained with *M. luteus* and *E. coli* and only the results for LW3 are shown in Fig. 7B. Growth inhibition of the Gram- *E. coli*, however, was weaker than that of the Gram+ indicator strains (Fig. 7A, B, C). Not only yCPK production but also antibacterial activity seemed to be increased in the *scbR2* mutant strain LW3 when compared to the parental strain M145 (Fig. 7A). These results suggest that the observed antibiotic activity coincides with the production of yCPK and that it is also related to the *cpk* gene cluster. Bioactivity against *B. subtilis* and *M. luteus* could be shown in DNAgar cultures without additional glutamate. However, the inhibition zones were significantly smaller than on Glu-DNAgar plates and no activity could be shown against the Gram- *E. coli* under these conditions (data not shown). Thus, addition of glutamate to the medium seems to increase not only the production of the yellow pigment yCPK, but also antibacterial activity of *S. coelicolor*.
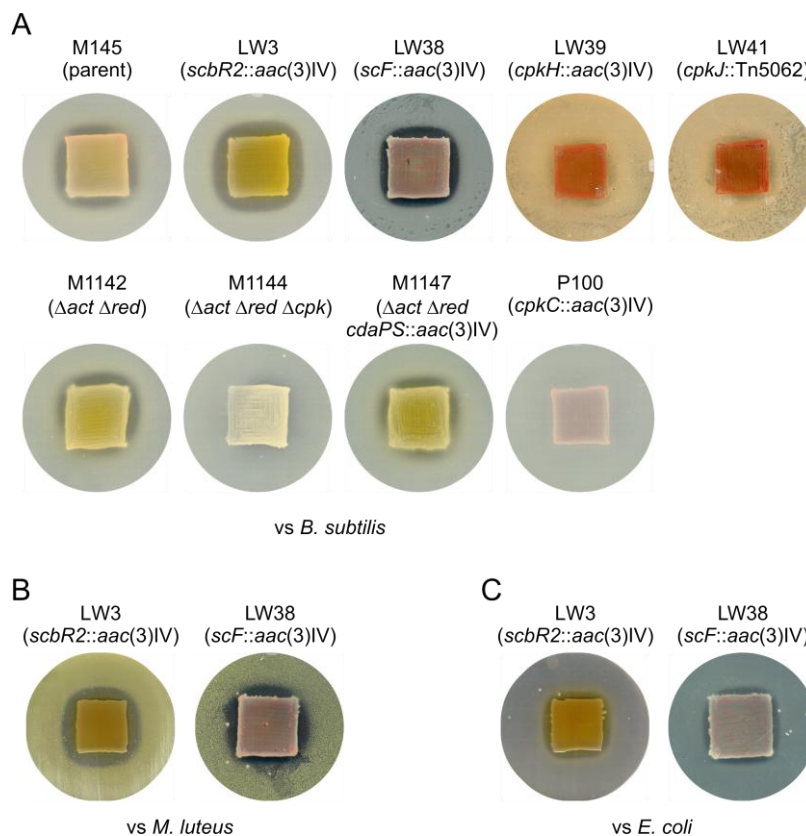
Figure 13 *cpk* cluster related bioactivity of *S. coelicolor* against *B. subtilis*, *M. luteus* and *E. coli*. *S. coelicolor* was grown on glutamate supplemented DNAgar (Glu-DNA) for 27 h and overlaid with soft nutrient agar containing *B. subtilis* **(A)**, *M. luteus* **(B)** and *E. coli* **(C)**. To determine inhibition of bacterial growth, the plates were further incubated at 30°C overnight and pictures were taken from the bottom.

Antibacterial activity does not require the yellow pigment.

However, these data do not necessarily imply that the yellow pigmented compound is the actual bioactive agent. We prepared knockout mutants of *scF* (strain LW38) and *cpkH* (strain LW39) in the *cpk* cluster. The two homologues show 53 and 49% identity to AclO, a putative oxidoreductase involved in aclacinomycin production in *Streptomyces galilaeus*. They are described as secreted FAD-binding proteins (Pawlik *et al.*, 2007) and have been shown to be substrates of the *S. coelicolor* Tat protein export system (Widdick *et al.*, 2006). Thus, ScF and CpkH might be involved in late steps of the *cpk* biosynthesis pathway outside of the cell.

As expected, no yellow compound was observed in LW38 (M145 *scF*::*aac(3)*IV), whereas Red and Act were still produced (Fig. 2). In contrast to any other mutant in the *cpk* cluster, despite the lack of yCPK production, LW38 still inhibited growth of *B. subtilis*, *M. luteus* and *E. coli* (Fig. 7A, B, C, respectively). This strongly suggests the existence of a colourless substance, different from yCPK, which is the actual antibiotic and also derives from the *cpk* biosynthetic pathway. Therefore, we named this substance abCPK (antibiotic coelicolor polyketide). Our results indicate a putative role for ScF in the conversion of the antibacterial abCPK into the yellow pigment yCPK. In agreement with this, LW38 may have a slightly higher antibiotic activity than the parental strain M145 (Fig. 7A). Also strain LW39 (M145 *cpkH*::*aac(3)*IV) did not produce the yellow pigment (Fig. 2). However, in contrast to LW38 and resembling other *cpk* mutant strains (LW41, M1144, P100), no antibiotic activity was observed with LW39 (Fig. 7A). These observations correspond with a model in which an inactive precursor is secreted or diffuses from the cells and is subsequently converted to the antibiotic abCPK and the yellow-pigmented yCPK by the extracellular enzymes CpkH and ScF.

abCPK is produced in liquid cultures and can be extracted from cells with methanol.

Production and isolation conditions for the abCPK compound were optimised. Cell and media samples from solid and liquid cultures were treated with different solvents and bioactivity tests were conducted in several ways (data not shown). Best results were obtained when cells were isolated from liquid culture and extracted with each 500 µl methanol per 5 ml culture sample. 50 µl cell methanol extract (CME) was concentrated to 5-10 µl and spotted on the surface of a LB plate containing an indicator strain. Results for *S. coelicolor* strain LW3 are shown in Fig. 8. CME was prepared from 5 ml Glu-DNB culture samples every two hours between 20 and 30 and at 44 hours (Fig. 8A) and tested for antibacterial activity against *B. subtilis* as described above (Fig. 8B). Bioactivity was observed from 22 hours onwards. Lack of activity at 20 h of growth, however, may be due to the low amount of extracted cells at this early time point and not due to missing abCPK production. Also, we cannot exclude that the increasing size of the inhibition zones at later time points is rather due to the increasing cell mass over time than to an increase in production of the antibiotic agent. Interestingly, antibiotic activity could be observed even before yCPK production became visible (after 22 h (Fig. 8B) and 28 h (Fig. 8A), respectively). Also, so far, it was not possible to isolate a bioactive agent from the culture supernatant where yCPK is found (Fig. 5A), but only from the cells. In addition to the yCPK independent production of abCPK obtained with a *scF* mutant strain described above, these data suggest that the yellow pigment and the antibiotic agent are indeed two distinct *cpk* compounds. Nonetheless, lack of bioactivity in the supernatant samples used may well be due the high dilution or the inactivation of the antibiotic agent in the course of upconcentrating the supernatant. Thus, despite the results reported here, we explicitly do not exclude the presence of abCPK in liquid culture supernatant. On the contrary, from the observation of extracellular antibacterial activity in solid cultures (Fig. 7) and the proposed production of abCPK outside the cells, we would expect the antibiotic to be found in the medium.
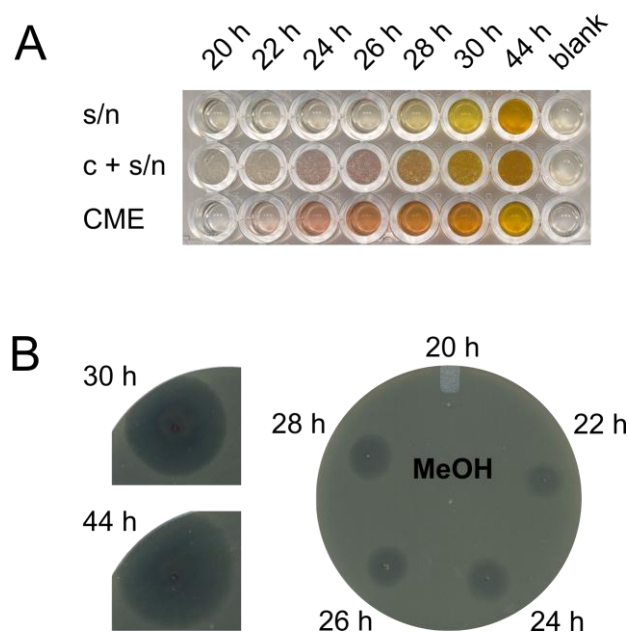
Figure 14 Optimised abCPK extraction and bioactivity test. *S. coelicolor* LW3 was grown in glutamate supplemented DNB medium (Glu-DNB). Samples were taken between 20 and 44 h at the given time points. Cell-free culture supernatant (s/n), cells in culture medium (c + s/n) and cell methanol extracts (CME) are shown in a 96 well plate. Blank culture medium and methanol are given as reference samples, respectively. The CME were prepared with 500 µl methanol from a 5 ml culture sample, independent from the cell mass **(A)**. The CME were tested for antibiotic activity. Each 50 µl CME from the indicated sampling time points were reduced to 5 to 10 µl in a SpeedVac and dropped onto a *B. subtilis* indicator plate. To determine inhibition of bacterial growth, the plate was incubated at 30°C overnight and pictures were taken from the top **(B)**.

Cell methanol extracts also contain Red (Fig. 6; also Kieser *et al.*, 2000) and maybe other *S. coelicolor* secondary metabolites that stay inside the cells. Prodigiosins (Red) were shown to possess considerable antibiotic activity towards Gram-positive organisms (Gerber, 1975). Therefore it was important to show that the antibacterial activity observed with cell methanol extracts is related to the *cpk* cluster and thus independent from the other known *S. coelicolor* antibiotics (Act, Red, CDA). *S. coelicolor* strains M145, LW3, M1146 and M1147 were grown in liquid Glu-DNB medium and CME was prepared after 22, 24, 26 and 28 hours of growth (Fig. 6A). Antibacterial activity could be observed with M145 and LW3 and the Δ*act* Δ*red* Δ*cda* mutant strain M1147 (Fig. 6B), but not for the Δ*act* Δ*red* Δ*cpk* Δ*cda* mutant strain M1146. This result is consistent with the bioactivity tests in solid cultures (Fig. 7) and

confirms that the described antibiotic activity is most likely dependent on the *cpk* cluster only.

abCPK is stable in a methanol extract.

To test the stability of the extracted abCPK, cell methanol extracts obtained from strain LW3 grown in Glu-DNB was prepared. Aliquots of the fresh CME were stored at 4°C and room temperature (RT) or were dried-up completely using a SpeedVac, stored at -80°C or RT, and eventually resuspended in methanol. Bioactivity against *B. subtilis* could be shown under all conditions tested (Fig. 9).

abCPK shows bacteriostatic activity.

To further characterize the antibiotic activity of abCPK, cells in- and outside the inhibition zone observed with abCPK against *B. subtilis* (Fig. 10A, compare to Fig. 9) were transferred to a LB plate without antibiotics (Fig. 10B). Cells from the control area ("3" in Fig. 10A), but also from a clear ("1", next to the filter disc) and turbid ("2", further away from the filter disc) area of the inhibition zone were found to grow on the fresh medium (Fig. 10B). The turbid area of the inhibition zone is most probably caused by a decreasing concentration of the diffusible abCPK dependent on the distance from the filter disc it was applied to. Re-growth of cells from a sample picked inside the clear inhibition zone may indicate a bacteriostatic rather than a bactericidal effect of the abCPK antibiotic.

The *cpk* biosynthesis pathway products are smaller than 10 kDa.

To further characterise both yCPK and abCPK, liquid culture supernatant containing yCPK and CME containing abCPK were run over 10 kDa size fractionation columns (Fig. 11). The yellow compound was detected in the culture supernatant (s/n) and the column flow-through (FT) by OD_{450} determination (Fig. 11A). Two thirds of the yellow pigment was found back in the FT fraction containing compounds smaller 10 kDa. The antibiotic activity was detected by bioassays against *B. subtilis* in the fresh cell methanol extract (CME) and the column flow-through (FT). Also the size fractionation membrane was applied to the activity test taking into consideration that abCPK may attach there. However, no antibacterial activity was observed with the membrane and a methanol control (Fig. 11B). These results suggest that both *cpk* cluster metabolites are smaller than 10 kDa and that abCPK does not bind to the

size fractionation membrane. Furthermore, yCPK was shown to sustain yellow pigmentation over a wide pH range (pH 1 to 14; data not shown).
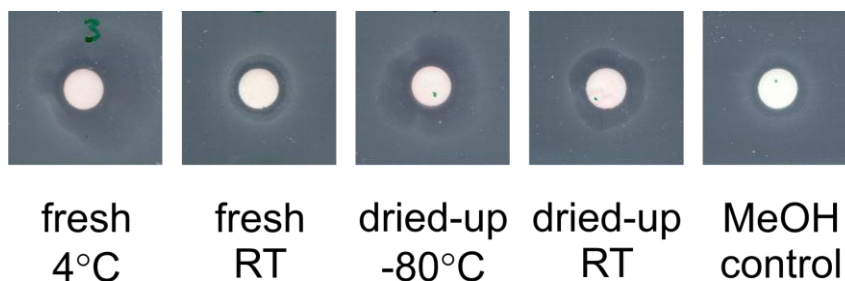
Figure 15 Stability of abCPK. Cell methanol extract (CME) from *S. coelicolor* LW3 grown in glutamate supplemented DNB medium (Glu-DNB) for 28 h was prepared and tested for bioactivity after storage at 4°C or room temperature (RT). 50 µl CME aliquots were dried-up completely in a SpeedVac and stored at -80°C and RT before being resuspended in the same amount of methanol and dropped on filter disks on a *B. subtilis* indicator plate. Also containing a methanol control, to determine inhibition of bacterial growth, the plate was incubated at 30°C overnight and pictures were taken from the bottom.
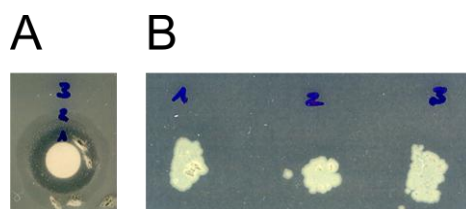
Figure 16 Bacteriostatic activity of abCPK. An abCPK antibacterial activity assay was conducted as shown in Fig. 9. Cells in- and outside the observed inhibition zone **(A)** were transferred to fresh medium and incubated overnight **(B)**.
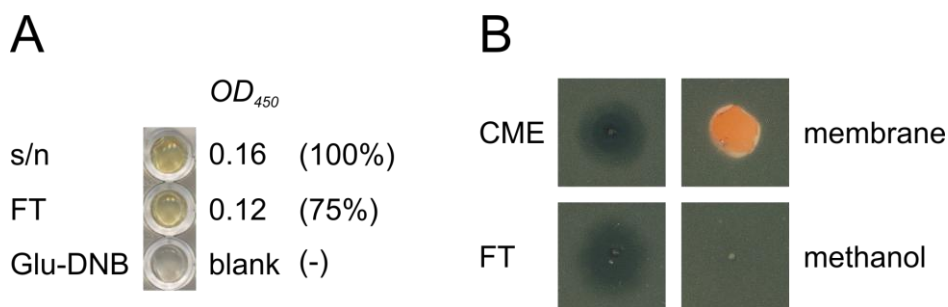
Figure 17 Molecular mass determination of *cpk* gene cluster products. Supernatant from a 28 h *S. coelicolor* M145 Glu-DNB culture **(A)** and *S. coelicolor* LW3 cell methanol extract (CME) from a 28 h Glu-DNB culture sample **(B)** were spun through a 10 kDa size fractionation column. The yellow-pigmented yCPK was detected in the culture supernatant (s/n) and the column flow-through (FT) by OD_{450} determination against a blank culture medium reference (Glu-DNB). Results are given on the right; numbers in brackets indicate relative OD_{450} values (A). The antibiotic abCPK was detected by bioactivity assays against *B. subtilis* in fresh CME and the column flow-through (FT). Also the size fractionation membrane was applied to the activity test, as well as a methanol control. To determine inhibition of bacterial growth, the indicator plate was incubated at 30°C overnight and pictures were taken from the bottom (B).

Discussion

For various reasons the number of genuinely new antibiotics discovered has been declining for decades while development of resistance among pathogens advances (Hopwood, 2007). Therefore, the search for pharmaceutically active natural products has moved back into the centre of scientific interest. The capacity of *Streptomyces* species to produce new types of secondary metabolites serving as new drug candidates seems to be almost inexhaustible (Berdy, 2005; Watve *et al.*, 2001). Hence, although already studied for a long time, the genus is still one of the most promising natural sources for novel bioactive compounds.

A novel antibiotic, abCPK, from the model actinomycete *Streptomyces coelicolor*

In this study, a novel antibiotic with activity against *B. subtilis*, *M. luteus* and *E. coli* was identified in *S. coelicolor* produced at an early stage of growth (Fig. 6, 7 and 8). Under all conditions tested, this bioactivity was never observed with four independent mutants in the *cpk* gene cluster (LW39, LW41, M1147 and P100). Using mutants in which the entire gene clusters are deleted (M1142, M1144 and

M1147) antibacterial activity was shown to be independent from the three well characterised *S. coelicolor* secondary metabolites Act, CDA and explicitly from Red (Fig. 6 and 7). Red was reported before to have slight antibacterial activity against Gram+ bacteria (Gerber, 1975; Watve *et al.*, 2001) and is extractable from cells with methanol, properties that resemble those of the newly discovered antibiotic. Our results strongly imply an antibiotic agent dependent on a functional *cpk* gene cluster (abCPK (antibiotic coelicolor polyketide) with presumably bacteriostatic activity (Fig. 10) against Gram+ and Gram- bacteria (Fig. 7).

yCPK and abCPK are distinct compounds, both related to the *cpk* gene cluster
In Gottelt *et al.*, submitted, we presented a novel yellow-pigmented secondary metabolite (yCPK) related to the *cpk* cluster. However, we have strong evidence suggesting that yCPK is not the bioactive agent (abCPK), but that they are rather two distinct metabolites, however, both deriving from the *cpk* biosynthetic pathway. We could separate yCPK and abCPK production by manipulating the ScF protein, which is part of the *cpk* cluster, and this may have lead to the accumulation of the antibiotic (Fig. 2 and 7). Furthermore, cells grown in liquid culture lack an apparent yellow pigmentation, whereas the antibiotic agent can be extracted from the cells (Fig. 5, 6 and 8). We did see bioactivity with the yellow-pigmented culture supernatant (data not shown). This, however, does not mean that abCPK can only be found intracellular, since the inhibition zones in solid culture bioactivity tests clearly suggest diffusion or secretion of the antibacterial agent into the medium. Lack of activity in liquid culture supernatant thus may rather be due to a too low concentration of abCPK or due to instability of the compound when concentrating the medium to conduct bioactivity tests.

With single mutants in the predicted *cpk* biosynthesis genes *cpkC*, *cpkJ* and *cpkH* we observed no yellow pigment and no bioactive compound production (Fig. 2 and 7). CpkH and its homologue ScF posses signal sequences for the Tat secretion system (Widdick *et al.*, 2006) and have high homology to a secreted oxydoreductase involved in antibiotic production in *Streptomyces* galilaeus (Pawlik *et al.*, 2007). We therefore hypothesize that after synthesis of the polyketide backbone by the type 1 PKS subunits *cpkABC* and modification by enzymes like CpkJ, a precursor polyketide is transported over the membrane (presumably by the CpkF protein, a

putative transmembrane efflux protein located in the *cpk* cluster (Pawlik *et al.*, 2007)). This intermediate is then further converted in a two step process performed by the secreted enzymes CpkH and ScF outside the cell: First, the precursor is transformed into the antibacterial abCPK by CpkH. Subsequently, the antibiotic is converted into the yellow pigment yCPK by ScF.

A more detailed analysis of the role of ScF and CpkH in the *cpk* biosynthesis pathway is currently under investigation in our laboratory, as is the role of the transporters and modifying enzymes. To date the proposed dependency of the two CPK compounds must be regarded speculative, but recent results confirm that yCPK and abCPK can indeed be separated from each other during isolation (data not shown). Although we suggest formation of the active antibiotic outside the cell and growth inhibition halos indicate diffusion into the medium (Fig. 7), abCPK may to a certain extend stay attached to the cells since antibiotic activity could be shown with extracts obtained from cell pellets (Fig. 6 and 8).

Despite great efforts, the chemical structure of neither yCPK nor abCPK could be elucidated yet. Partially purified samples of the yellow pigment and the antibiotic compound will be used to reveal first insights and to determine whether abCPK represents an entirely new bioactive agent or if we were able to link a known compound to the *cpk* gene cluster. However, from our results physiochemical properties of the two *cpk* products can be suggested. The aliphatic, unsaturated C_{12} polyketide backbone is predicted to be formed from six malonyl-CoA units (Pawlik *et al.*, 2007) which then is modified by tailoring enzymes of the *cpk* cluster. Both abCPK and yCPK are small molecules (<10 kDa) and diffusible in water-based medium. The hydrophilic antibacterial agent can be dissolved in methanol (this study) and other polar solvents such as DMSO and DMF (data not shown). In the experiments presented here abCPK was temperature stable (-80°C to room temperature) and showed presumably bacteriostatic activity against Gram+ and Gram- bacteria. The yellow pigmentation is pH stable and derives from an absorption maximum of yCPK between 430 and 460 nm. Furthermore, yCPK is fluorescent (data not shown).

Regulatory role of ScbR2 for CPK production

More obvious in solid than in liquid culture, disruption of the γ-butyrolactone receptor homologue in the *cpk* cluster, ScbR2, in strain LW3 lead to the overproduction of the

bioactive agent and the yellow compound and impaired the production of the other pigmented secondary metabolites, Act and Red. To date, neither a direct regulatory role of ScbR2 for the *cpk* cluster or other targets, nor its ability to bind to γ-butyrolactones is determined. However, previous results indicated negative feedback regulation of yCPK production constituted by CpkO (activator) and ScbR2 (repressor) (Gottelt *et al.*, submitted). The decreased and delayed production of Act, but also Red, in LW3 could be indirect and due to a shift of common precursors into the *cpk* biosynthetic pathway. This hypothesis is to some extend supported by finding yCPK overproduced in the Δ*act* Δ*red* mutant compared to the parental M145 and thus resembling strain LW3 (Fig. 5). Further investigation will be needed to reveal the mode of action of ScbR2 in the regulation of the *S. coelicolor* secondary metabolome.

Glutamate increases CPK production

Addition of glutamate to SMMS and DNAgar increased both the production of the yellow compound and the growth inhibitory effect of *S. coelicolor*. The *cpk* cluster contains the CpkG protein which is similar to class III aminotransferases, but does not show any homology to known antibiotic biosynthetic genes (Pawlik *et al.*, 2007). Class III aminotransferases are known to directly metabolise the amino group from amino acids (Yonaha *et al.*, 1992) which may explain the observed effect of glutamate. However, since we do not know the chemical properties of the *cpk* cluster metabolites yet and thus also not whether it contains any nitrogen, the role of CpkG is still speculative, as well as we consequently cannot explain the positive effect of glutamate on yCPK and abCPK biosynthesis. Since we used L-glutamic acid monosodium salt in comparably high amounts (typically 325 nM (final conc.)) also an effect of putative osmotic stress cannot be excluded. However, in independent experiments using even higher concentrations of NaCl in a different medium, yCPK production was not induced by osmotic stress (Takano, unpublished).

Production of the yellow CPK compound in *S. coelicolor* M145 was never seen on the standard antibiotic production test medium SMMS (Fig. 1). On DNAgar, M145 yellow compound production was visible only for a couple of hours before it faded and later the pigmented Red and Act became predominant (Gottelt *et al.*, 2009). Only the overproducing strain LW3 allowed discovery of yCPK in the first place and

thus made optimisation of the culture medium by the addition of glutamate possible. These findings were even more apparent with the antibiotic CPK compound: After the discovery of yCPK, first tests on DNAgar hardly revealed any bioactivity, only very small inhibition zones were observed (data not shown). Only under improved conditions for the production of – apparently both – *cpk* cluster products (strain LW3 on Glu-DNAgar), antibacterial activity became clearly detectable and also bioactivity test conditions could be optimised. This could explain why abCPK was not described and related to the *cpk* gene cluster earlier.

Experimental procedures

Bacterial strains and growth conditions.

Bacterial strains and plasmids used are listed in Tab. 2. *Streptomyces* was manipulated as previously described (Kieser *et al.*, 2000). *E. coli* strain JM101, strain ATCC 9341 (*Micrococcus luteus*, reclassified to *Kocuria rhizophila* (Tang and Gillevet, 2003), *Bacillus subtilis* DB104 (Kawamura and Doi, 1984) and strain ATCC 6633 (*Bacillus subtilis* subsp. *spizizenii*) were cultured at 30°C in LB medium (Sambrook and Russell, 2001). DNAgar and DNBroth (DNB) are described in Kieser *et al.*, 2000. SMMS is the agar (1.5%, w/v) version of SMM (Kieser *et al.*, 2000) as described in (Takano *et al.*, 2001). In some cases, 10 to 400 mM (final conc.) of L-glutamic acid monosodium salt (glutamate or Glu) from Sigma was added to DNB, DNAgar and SMMS additionally or to replace the casaminoacids contained in standard SMMS. Typically, 325 mM Glu was used. When N-acetylglucosamine was added to DNAgar 50 mM (final conc.) was used. MS agar (Kieser *et al.*, 2000) was used to grow cultures for preparing spore suspensions, the spore concentration was determined on LB plates. Soft nutrient agar (SNA) (Kieser *et al.*, 2000) was used for antibacterial activity tests.

Secondary metabolite production and antibacterial activity assay in solid culture.

For solid cultures, each 4 x 10^7 spores from -20°C spore stocks in 20% glycerol of *S. coelicolor* were streaked out for a 2.5 x 2.5 cm square or an irregular patch (Fig. 4). In some cases a drop of a spore solution was directly applied to the plate (Fig. 2)

prior to incubation at 30°C in darkness. For the antibacterial activity assay, plates were overlaid after 27 hours of growth with 2 ml SNA containing 0.4% of the indicator strains *E. coli*, *B. subtilis* or *M. luteus* from overnight cultures in 3 ml LB medium where the SNA completely covers the patch of *Streptomyces*. After further incubation overnight at 30°C pictures were taken from the bottom to determine zones of bacterial growth inhibition.

Table 2 Bacterial strains used in this study.

Bacterial strains	Comments	References
Bacillus subtilis		
ATCC 6633		(Nakamura *et al.*, 1999)
DB104		(Kawamura and Doi, 1984)
Escherichia coli		
JM101		(Sambrook and Russell, 2001)
Micrococcus luteus		
ATCC 9341	reclassified to *Kocuria rhizophila*	(Tang and Gillevet, 2003)
Streptomyces coelicolor **A3(2)**		
LW3	M145 *scbR2*::*aac(3)*IV	Gottelt *et al.*, submitted
LW38	M145 *scF*::aac(3)IV	This study
LW39	M145 *cpkH*::aac(3)IV	This study
LW41	M145 *cpkJ*::Tn5062	This study
M1142	M145 Δ*act* Δ*red*	Gottelt *et al.*, submitted
M1144	M145 Δ*act* Δ*red* Δ*cpk*	Gomez-Escribano (unpublished)
M1146	M145 Δ*act* Δ*red* Δ*cda* Δ*cpk*	Gottelt *et al.*, submitted
M1147	M145 Δ*act* Δ*red cdaPS*::*aac(3)*IV	Gomez-Escribano (unpublished)
M1148	M145 Δ*act* Δ*red* Δ*cda*	Gottelt *et al.*, submitted
M145	A3(2) SCP1- SCP2- derivative	(Kieser *et al.*, 2000)
P100	M145 *cpkC*::*aac(3)*IV	(Pawlik *et al.*, 2007)

Secondary metabolite production in liquid culture, preparation of cell methanol extracts and antibacterial activity assay.

For liquid cultures, each 2 x 10^8 spores from -20°C spore stocks in 20% glycerol of *S. coelicolor* were incubated in 50 or 60 ml DNB supplemented with 325 mM (final conc.) Glu and sometimes with 2 ml/L of a trace element solution (for SMM (Kieser *et al.*, 2000)) in a 250 ml Erlenmeyer flask (unbaffled, with spring) shaking at 220 rpm at 30°C overnight.

At sampling time points, cells of a 1 ml culture sample were collected by centrifugation. 200 µl of the (yellow pigmented) culture supernatant (s/n) were transferred to a 96 well plate, the rest of the liquid was discarded. The cell pellet was resuspended in 1 ml colourless Tris/EDTA buffer (Kieser *et al.*, 2000) and 200 µl were transferred to a 96 well plate. Left over 800 µl cell suspension was diluted with buffer to OD$_{450}$ below 1 to determine growth of the culture. Sometimes, the 96 well plate samples derived from a full culture sample with cells in the culture medium (c + s/n (Fig. 6 and 9)).

To prepare a cell methanol extract (CME), cells of a 5 ml culture sample were collected by centrifugation at 4°C, the liquid was removed completely and the cell pellet was resuspended in 500 µl methanol. After incubation on ice for 10 min with occasional mixing, cells were spun down and the supernatant was collected as CME and typically stored at 4°C in darkness. For documentation, 200 µl of the CME were transferred to a 96 well plate.

The 96 well plate was scanned from the bottom with a blank culture medium and a pure methanol sample. In some cases, OD$_{450}$ of the culture supernatant samples was determined using a plate reading photospectrometer.

To test the antibiotic activity of the cell methanol extract, typically, a 50 µl aliquot of fresh CME was upconcentrated to 5-10 µl in the SpeedVac in approximately 10 min and dropped directly onto the surface of a freshly prepared 20 ml LB plate containing 0.4% *B. subtilis* from a 3 ml liquid LB overnight culture. The plates were allowed to dry and incubated overnight at 30°C. Pictures were taken from the bottom to determine inhibition of bacterial growth. For Fig. 6 the CME was applied to holes in the indicator plate and the picture was taken from top. For Fig. 9 changes in CME

preparation are indicated in the text and the CME are applied on 0.5 mm filter disks on the indicator plate.

Size fractionation.

Each 2 ml of a 28 hours liquid Glu-DNB culture supernatant (s/n) and a cell methanol extract obtained from a 45 ml culture sample using 2 ml methanol (CME) as described above were run over 10 kDa size fractionation columns (Microsep 10k Omega, PALL Life Sciences; centrifugation at 4°C and 5000 g). The yellow CPK compound was detected in the s/n and the column flow-through (FT) by OD_{450} determination against a blank culture medium reference. The antibiotic CPK compound was detected by bioactivity assays against *B. subtilis* as described above with the fresh CME and with the column flow-through (FT). For the latter, the size fractionation membrane was cut from the column and applied to the activity test, as well as a methanol control.

Chapter 4

ScbR$_{M600}$

Submitted for publication

"A second form of the γ-butyrolactone receptor ScbR in *Streptomyces coelicolor* A3(2) shows impaired DNA binding ability and affects the expression of pathway-specific antibiotic regulatory genes"

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Abstract

Antibiotic production in *Streptomyces coelicolor* A3(2) is regulated by the *S. coelicolor* butanolide system. γ-Butyrolactones serve as signalling molecules and bind to the receptor protein ScbR that acts as a negative transcriptional regulator by binding to specific DNA sites upstream of its target genes. The autoregulatory ScbR affects production of the two pigmented antibiotics Act and Red, as well as transcription of the *cpk* antibiotic biosynthesis gene cluster and synthesis of the γ-butyrolactones. We identified a variant of ScbR in *S. coelicolor* (ScbR$_{M600}$) that differs from ScbR in the genome sequenced strain M145 (ScbR$_{M145}$) by a single amino acid change, R120S. ScbR$_{M600}$ is impaired in its DNA binding ability *in vitro* and *in vivo*, and alters the expression of the pathway-specific regulatory genes of the *act*, *red* and *cpk* antibiotic biosynthesis gene clusters. Also expression of the γ-butyrolactone biosynthesis gene *scbA* and production of the signalling molecules is slightly reduced.

Introduction

Streptomycetes show a complex morphological differentiation and produce a vast variety of secondary metabolites with great value in the pharmaceutical, chemical and agricultural industries (Champness, 2000; Miyadoh, 1993). The genome sequence of the model streptomycete *Streptomyces coelicolor* A3(2) strain M145 has been determined and is publicly available (Bentley *et al.*, 2002). *S. coelicolor* A3(2) strains M145 and M600 are two of many strains independently derived from *S. coelicolor* A3(2). Both are prototrophic plasmid-free derivatives, but M145 was derived using both mutagenesis and recombination while creation of M600 did not involve any mutagenesis (Weaver *et al.*, 2004). Genetically, M600 differs from M145 in that it possesses long terminal inverted repeats (TIRs) at both ends of the chromosome resulting in the duplication of 1005 genes compared to M145. This does not, however, appear to significantly affect total expression of the duplicated genes since highly similar transcript levels could be observed when comparing the two strains (Weaver *et al.*, 2004). M145 and M600 differ phenotypically, with M600 showing a delay in production of the pigmented antibiotics actinorhodin (Act) and undecylprodigiosin (Red) compared to M145 (Takano, unpublished).

In several *Streptomyces* species small autoregulatory molecules called γ-butyrolactones are involved in controlling the onset of secondary metabolite production and morphological differentiation (reviewed in Takano, 2006). There are numerous diverse and complex regulatory systems involving γ-butyrolactones of which the signalling cascade for A-factor in *S. griseus* is the best studied (Horinouchi, 2002; Horinouchi & Beppu, 1992). In *S. coelicolor*, γ-butyrolactones with Act and Red stimulatory ability have been identified (Takano *et al.*, 2000), together with the genes involved in γ-butyrolactone synthesis (*scbA*) and γ-butyrolactone binding (*scbR*). ScbR regulates transcription of both *scbA* and itself by binding to the divergent promoter region controlling both genes, and the γ-butyrolactone SCB1 inhibits this binding (Takano *et al.*, 2001). The regulatory influence of ScbR has been characterised by DNA microarray analysis, and a role in directly regulating a cryptic Type I polyketide biosynthetic gene cluster (*cpk* cluster) by binding to the promoter of its pathway-specific regulator *cpkO* was identified (Pawlik *et al.*, 2007; Takano *et al.*, 2005). We recently reported two metabolites derived from the hitherto orphan *cpk* biosynthetic pathway, the yellow pigment yCPK and an antibiotic compound, abCPK (Gottelt *et al.*, submitted). ScbR does not however bind to the promoter regions of the pathway-specific regulatory genes for Act and Red synthesis (Takano *et al.*, 2001), and it appears that SCB1 and *scbAR* do not regulate the production of these antibiotics directly. Nevertheless, a M145Δ*scbR* mutant (M752) is delayed in the production of Act and Red (Takano *et al.*, 2001), and in this sense it resembles the phenotype of strain M600 (Takano, unpublished).

ScbR is a member of the TetR protein family (Ramos *et al.*, 2005) in which the *Streptomyces* γ-butyrolactone receptors show significant similarity to each other (30-40 % amino acid sequence identity). The crystal structure of a ScbR homologue in *S. coelicolor*, CprB, has been determined and is assumed to generally represent the structure of γ-butyrolactone receptors (Natsume *et al.*, 2004). Active as homodimers, they bind to highly specific DNA binding sites in the promoter region of their target genes and typically repress their transcription. The regulatory region of the dimeric regulator contains one independent ligand binding pocket in each subunit. One γ-butyrolactone receptor thus binds two ligand molecules. Binding of

γ-butyrolactones causes conformational changes and DNA binding is relieved (Natsume *et al.*, 2004).

In this study, the *S. coelicolor* γ-butyrolactone receptor ScbR in strain M600 (ScbR$_{M600}$) was found to differ from that in the sequenced strain M145 (ScbR$_{M145}$) by a single amino acid change. The effect of the M600-type protein on the production of both pigmented antibiotics, as well as the yellow *cpk* compound, and the γ-butyrolactones was tested *in vivo*, including an analysis of the influence on the expression of genes involved in the butanolide system and secondary metabolism. The hypothesis that the amino acid substitution may affect DNA and/or γ-butyrolactone binding ability of ScbR was investigated *in vivo* and *in vitro.* Sequence analysis was used to determine the prevalence of the two forms of ScbR among strains of *S. coelicolor* and *S. lividans*.

Methods

Bacterial strains, plasmids and cosmids

Strains and vectors used in this study are listed in Table 1 and Table S1. *Streptomyces* was manipulated as described previously (Kieser *et al.*, 2000). *Escherichia coli* was grown and transformed according to (Sambrook & Russell, 2001).

Table 1 Bacterial strains used in this study

Name	Description	*scbR* type	Reference
Escherichia coli			
JM101	*supE thi* Δ(*lac-proAB*) F′ [*traD*36 *proAB$^+$ lacI^q lacZ* ΔM15]	-	(Sambrook & Russell, 2001)
ET12567	Non-methylating strain used for conjugation with *Streptomyces*	-	(MacNeil *et al.*, 1992)
Streptomyces coelicolor A3(2)			
11	*pheA1*	*scbR$_{M145}$*	(Weaver *et al.*, 2004)
13	*uraA1*	*scbR$_{M145}$*	(Weaver *et al.*, 2004)

210	*ura-3*	$scbR_{M145}$	(Weaver *et al.*, 2004)
290	*cys-4*	$scbR_{M145}$	(Weaver *et al.*, 2004)
380	*glu-3*	$scbR_{M145}$	(Weaver *et al.*, 2004)
505	*cysC3*	$scbR_{M145}$	(Weaver *et al.*, 2004)
A3(2) N1	A3(2) isolate	$scbR_{M145}$	(Weaver *et al.*, 2004)
A3(2) N2	A3(2) isolate	$scbR_{M600}$	(Weaver *et al.*, 2004)
A3(2) N3	A3(2) isolate	$scbR_{M145}$	(Weaver *et al.*, 2004)
A3(2)-Stanford	A3(2) isolate	$scbR_{M145}$	(Weaver *et al.*, 2004)
CH999	*proA1 argA1 redD60 Δact::ermE* SCP1⁻ SCP2⁻	$scbR_{M145}$	(Weaver *et al.*, 2004)
J1501	*hisA1 uraA1 strA1 pgl-1* SCP1⁻ SCP2⁻	$scbR_{M145}$	(Kieser *et al.*, 2000)
LW33	M752 + $scbR_{M600}$	$scbR_{M600}$	This study
LW34	M752 + $scbR_{M145}$	$scbR_{M145}$	This study
M132	*pheA1* SCP1⁻ SCP2⁻	$scbR_{M145}$	(Weaver *et al.*, 2004)
M145	SCP1⁻ SCP2⁻	$scbR_{M145}$	(Kieser *et al.*, 2000)
M600	SCP1⁻ SCP2⁻	$scbR_{M600}$	(Chakraburtty & Bibb, 1997)
M752	M145 Δ*scbR*	-	(Takano *et al.*, 2001)
W3443	wild type	$scbR_{M145}$	(Weaver *et al.*, 2004)
Streptomyces lividans			
1326	wild type	$scbR_{M145}$ homologue	(Kieser *et al.*, 2000)

Culture conditions

For culturing *E. coli*, liquid LB (Sambrook & Russell, 2001) or LB agar supplemented with appropriate antibiotics was used. For genomic DNA isolation *Streptomyces* was grown in liquid YEME/TSB (1:1) medium as described (Kieser *et al.*, 2000). MS medium (Kieser *et al.*, 2000) was used to harvest spores according to (Kieser *et al.*, 2000). To determine the viable spore concentration, dilution series of spore suspensions were plated on MS or DNAgar (Kieser *et al.*, 2000) and the number of colony forming units was determined. For liquid *Streptomyces* cultures, strains were cultivated using SMM as previously described (Kieser *et al.*, 2000;Takano *et al.*, 2001). Interspecific conjugation was done as described previously (Takano *et al.*, 2001).

PCR and DNA sequencing

Amplification of DNA by PCR (Mullis *et al.*, 1986) was done with Taq polymerase (Fermentas), ProofStart polymerase (Qiagen) or the Expand High Fidelity DNA System (Roche). DNA sequence analysis was carried out by Sequence Laboratories, Göttingen, Germany.

DNA manipulation, plasmid transformation and intergeneric transfer

Plasmid DNA isolation, restrictions and cloning experiments were carried out as described in (Sambrook & Russell, 2001). *Streptomyces* was manipulated as described in (Kieser *et al.*, 2000), genomic DNA was isolated according to (Leblond *et al.*, 1996).

2D gel electrophoresis and MALDI-TOF mass spectrometry

Protein extracts were prepared from typically 25 ml culture samples according to (Hesketh *et al.*, 2002) and mycelial pellets were stored at -80 °C until use. To prepare total protein extracts from spores, spores harvested from cultures grown on MS plates (Kieser *et al.*, 2000) were thawed on ice, washed once with ice cold wash buffer (Hesketh *et al.*, 2002), then transferred to a mortar submerged in liquid nitrogen together with an equal volume of washed glass beads (Sigma G-8893, 106 microns). The spores were then ground thoroughly under liquid nitrogen until an even colour and fine consistency (about 5 min), and the resulting frozen powder stored at -80 °C until use. Frozen aliquots were suspended in spore protein buffer (50 mM DTT, 4 mM Pefabloc SC protease inhibitor, 40 mM Tris pH 9·0, 1 mM EDTA, 1 mM EGTA and 2 % (w/v) SDS), sonicated briefly (Sanyo Soniprep 150; 2 x 5 second bursts at amplitude 7·5 microns), and then boiled for 10 min. After cooling, cell debris and glass beads were removed, the protein extract was cleaned up, and protein pellets were finally dissolved and stored frozen in aliquots at -80 °C until use as described in (Hesketh *et al.*, 2002). Protein extracts from mycelia and spores were subjected to 2D gel electrophoresis as detailed in (Hesketh *et al.*, 2002).

Protein spots of interest were excised from stained gels using the Investigator ProPic robot from Genomic Solutions, and identified by tryptic or chymotryptic digestion and MALDI-TOF mass spectrometry using a Micromass Q-TOF 2 mass spectrometer as previously described (Hesketh *et al.*, 2002). Identification of proteins from peptide mass fingerprint data was performed according to (Hesketh *et al.*, 2002).

Construction of the ScbR$_{M145}$-MalE-tagged expression plasmid; overexpression in *E. coli* and purification of ScbR$_{M145}$ for the generation of ScbR antibodies

The *scbR*$_{M145}$ coding sequence was amplified by PCR from the cosmid SCAH10 (Redenbach *et al.*, 1996) using primers MalE-ScbR1 and MalE-ScbR2 (Table S2). The PCR product was gel-purified and ligated to pDRIVE (Qiagen), yielding pDRIVE-ScbR. This plasmid was digested with *Bam*HI and *Hind*III and the fragment corresponding to *scbR*$_{M145}$ was ligated to the digested *Bam*HI/*Hind*III vector pTST101 (J. Altenbuchner, unpublished) to allow the translational fusion of *scbR*$_{M145}$ with *malE*, leading to pTE88. The sequence of *scbR*$_{M145}$ and the translational fusion were confirmed by DNA sequencing.

E. coli JM101 was transformed with pTE88. LB with 50 µg ampicillin ml^{-1} was inoculated to 1/100 volume with the overnight pre-culture and cells were grown at 37 °C until OD$_{600}$ reached 0·5. L-rhamnose (0·2 % (w/v) final concentration) was added for induction. After a further 2 h of incubation the induced cells were harvested and washed twice with chilled Column Buffer (CB) (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA), resuspended in chilled CB and disrupted using a French-Press. The soluble fraction containing MalE-ScbR$_{M145}$ was aliquoted and frozen at -70 °C until use. The MalE-ScbR$_{M145}$ was further purified by affinity chromatography with amylose resin (New England Biolabs) using a Bio-Logic FPLC system (Bio-Rad). After elution with CB supplemented with 10 mM maltose, positive fractions (0·5-5 mg ml^{-1}) detected by UV absorbance at 280 nm and shown to contain MalE-ScbR$_{M145}$ (66·4 kDa) by SDS-PAGE were pooled. ScbR$_{M145}$ was cleaved from MalE by the specific protease Factor Xa (10 µg ml^{-1} final concentration, New England Biolabs). Complete cleavage was determined by SDS-PAGE and cleavage was stopped by adding 2x Protease Inhibitor Cocktail (Roche). ScbR$_{M145}$ (23·8 kDa) was further purified to homogeneity by affinity chromatography using a heparin column (Amersham Biosciences) coupled to the Bio-Logic FPLC system (Bio-Rad). ScbR$_{M145}$ was eluted using a continuous salt gradient (0·2-2 M NaCl in CB). Positive fractions were checked on SDS-PAGE for presence and purity of ScbR$_{M145}$ (Fig. S2a). The purified protein (0·8 mg) was used to generate antibodies in rabbits (Eurogentec S.A., Belgium).

SDS-PAGE and Western analysis

Cell-free extracts (CE) or purified ScbR were resolved by SDS-PAGE (12 % (w/v) resolving SDS-polyacrylamide gels) according to Laemmli's procedure (Laemmli, 1970). Following electrophoresis, resolved bands were visualized by Coomassie brilliant blue staining. For Western analysis, proteins separated on SDS-PAGE gels were transferred to a nitrocellulose membrane by immersion or semi-dry blotting. Immunodetection of ScbR was carried out by using rabbit antiserum raised against ScbR$_{M145}$ (this study) and horseradish peroxydase-conjugated goat anti-rabbit IgG (Bio-Rad) as a secondary antibody with Roche's CSP-*Star* (Fig. 8b and Fig. S2b) or NBT/BCIP (Fig. 5b) as a substrate. In the former case, Super RX Medical X-ray Film (NIF100) (Fujifilm) and a Lumi-Imager F1 (Roche) (Fig. 8b) or a Konica QX-150U Medical Film Processor (Fig. S2b) were used for detection.

Construction of the ScbR$_{M600}$ expression plasmid and expression of both forms of ScbR in *E. coli*

A *scbR*$_{M600}$ expression vector, pTE58, was constructed as described in detail in Fig. S3. Plasmid pTE58 and the *scbR*$_{M145}$ expression construct pIJ6120 (Takano *et al.*, 2001) contain the two forms of *scbR* with its own promoter region cloned behind the *lacZ* promoter in a pIJ2925 background. Expression constructs pTE58 and pTE6120 were partially sequenced and differ only by the natural point mutation in *scbR*$_{M600}$ (this study). *E. coli* JM101 was transformed with pTE58 and pTE6120 for heterologous expression of ScbR$_{M600}$ and ScbR$_{M145}$ and CE was used for Western hybridisation analysis and gel retardation assays.

Cell-free extract preparation.

For *E. coli* cell-free extract (CE), 10 ml LB overnight cultures of *E. coli* JM101 harbouring pIJ2925, pIJ6120 and pTE58 were inoculated at a 1/100 concentration in 50 ml LB without glucose. Cultures were incubated at 37 °C for 2·5 h or until OD$_{600}$ 0·7-0·8 and induced with 1 mM (final concentration) IPTG. After 3 h of further incubation cells were harvested and washed twice with ice cold disruption buffer (50 mM Tris-HCl pH 7·9, 1 mM EDTA pH 8.0, 1 mM DTT, 1x (final conc.) complete EDTA-free protease inhibitor (Roche)) before being resuspended in 400 µl disruption buffer. For the *E. coli* CE used as positive control in Fig. 5 the disruption buffer contained 20% (v/v; final conc.) glycerol. Cells of 100 µl aliquots were collected by centrifugation, the supernatant was removed completely and the cell pellet was

frozen at -80 °C. To prepare CE, cells were resuspended in 150 or 200 µl disruption buffer and were disrupted by sonication. The cell lysate was clarified by centrifugation. Total protein concentration of the supernatant was determined using the BCA Protein Assay kit (Pierce) (for Fig. 7 and Fig. S2b) or a NanoDrop spectrophotometer (Thermo Fisher Scientific) (for Fig. 5 and Fig. 8). The freshly prepared CE was used immediately (Fig. 7 and Fig. S2b (100 µg total CE protein used), and Fig. 5 (50 µg)). For Fig. 8, the CE (130 µg total protein) was frozen and thawed up to three times at -20 °C (15 min) and 37 °C (5 min) before being applied to gel retardation assays and Western hybridisation analysis.

For *S. coelicolor* CE, cells from 25 ml samples of individual 60 ml SMM liquid cultures were collected at different phases of growth (GC 1 in Table S4). Fresh CE was prepared with all cells obtained as described for *E. coli* using 100, 150 or 200 µl disruption buffer with glycerol. 500 µg total CE protein was used for Western analysis and gel retardation assays shown in Fig. 5.

Gel retardation analysis

Gel retardation experiments were carried out as described previously (Takano *et al.*, 2001) using the Roche DIG Gel Shift Kit (Roche cat No. 1635352). From a genomic DNA isolate of *S. coelicolor* M145, a 177 bp PCR fragment containing the ScbR binding site in the promoter region of *scbR* (Takano *et al.*, 2001) was amplified using primers ETS6 and ETS10 and then was DIG-labelled according to the manufacturer's manual. For each sample, approximately 0·23 ng (Fig. 8a) and 1·8 ng (Fig. 7) were used. For the results shown in Fig. 5, primer ETS10 was replaced by ETS10_DIG(5') with digoxygenin linked to the 5'-end of the primer to obtain a labelled probe directly from PCR of which 5 ng was used in each sample (all primers are listed in Table S2). In some cases, 7·8 or 62·5 ng of chemically synthesised *S. coelicolor* γ-butyrolactone SCB1 was added to the mixture prior to incubation (Fig. 7). For Fig. 7, a Super RX Medical X-ray Film (NIF100) (Fujifilm) and a Konica QX-150U Medical Film Processor were used for detection. Pictures for Fig. 5(a) and Fig. 8(a) were obtained with the Lumi-Imager F1 (Roche) and the Luminescent Image analyzer LAS-4000 (Fujifilm), respectively.

Complementation of a *S. coelicolor* M145Δ*scbR* mutant (M752) with ScbR$_{M145}$ and ScbR$_{M600}$ by chromosomal replacement

scbR$_{M145}$ and *scbR*$_{M600}$ complementation vectors were constructed as described in detail in Fig. S4. Resulting plasmids pTE212 and pTE214 contain a 2·4 bp fragment of *scbR*$_{M600}$ and *scbR*$_{M145}$ and its flanking regions, respectively, in the conjugative, non-integrative vector pKC1132 (Bierman *et al.*, 1992) which is non-replicating in *S. coelicolor*. The 2·4 bp inserts were sequenced and differ only by the natural point mutation in *scbR*$_{M600}$ (this study) and an additional silent point mutation, c636t, in *scbR*$_{M145}$ of pTE214 with no effect on the amino acid sequence of ScbR$_{M145}$. pTE212 and pTE214 were transferred into *S. coelicolor* M752 by conjugation via *E. coli* ET12567/pUZ8002. Single- and double-crossover mutants were selected as described in (Takano *et al.*, 2001), but using DNA agar for non-selective growth, yielding strains LW33 and LW34 in which the truncated *scbR*$_{M145}$ region of the M145Δ*scbR*$_{M145}$ mutant M752 was replaced by *scbR*$_{M600}$ and *scbR*$_{M145}$, respectively, in a M145 chromosomal background. Presence of full-length *scbR* genes at the right chromosomal location was confirmed by PCR using primers ETseq3 and ETS7 and Southern analysis (Kieser *et al.*, 2000) using a PCR-generated 484 bp probe (primers scbArt1 and scbArt2 labelled with a DIG DNA labelling kit (Roche) binding to a 2·37 kb DNA fragment of *Nco*I digested chromosomal DNA in the presence of full-length *scbR* (Fig. S1a, b). With the in-frame deletion in *scbR*$_{M145}$ in M752, a *Nco*I restriction site disappears and the probe binds to a 3·2 kb DNA fragment. The two variants of *scbR* in LW33 and LW34 were confirmed by specific PCR using primers RCseq31 and scbR-M145_c358 or scbR-M600_c358a (Fig. S1c) and by DNA sequence analysis of the full-length *scbR* PCR product shown in Fig. S1(a) (data not shown). All primers are listed in Table S2.

Reverse transcription and quantitative RT-PCR

RNA was isolated as previously described (Takano *et al.*, 2005) from LW33 and LW34 grown in liquid SMM at different times of growth as indicated in Table 2 and S4. cDNA synthesis and quantitative RT-PCR was conducted as reported in Gottelt *et al.*, submitted.

Table 2 Growth and antibiotic production of LW34 and LW33. *S. coelicolor* LW34 and LW33 were grown *in duplo* in liquid SMM (growth curves (GC) 1 and 2). Samples were taken at different phases of growth (early, mid and late transition (eT, mT, lT), and stationary (S) phase). The sampling time is given in hours of growth in brackets underneath the determined OD_{450}. Production of actinorhodin (Act) and (undecyl-) prodiginines (Red) is given in µg /dry cell weight mg^{-1}.

		GC 1				GC 2			
time point		1	2	3	4	1	2	3	4
growth phase		eT	mT	lT	S	eT	mT	lT	S
OD_{450} (hours)	LW34	1·00 (21)	1·21 (23)	1·.26 (24·5)	1·45 (43)	1·25 (18)	1·49 (20)	1·62 (22)	1·92 (40)
	LW33	1·04 (21)	1·18 (23)	1·25 (24·5)	1·42 (43)	1·56 (18)	1·38 (20)	1·58 (21·5)	1·23 (40)
Red	LW34	0·33	0·59	0·89	3·89	0·11	0·17	0·63	0·76
	LW33	0·54	0·69	1·03	3·73	0·03	0·23	1·14	2·07
Act	LW34	0·00	0·00	0·00	0·97	0·20	0·11	0·06	0·46
	LW33	0·00	0·00	0·00	1·01	0·08	0·05	0·11	0·71

Detection of γ-butyrolactone production in liquid *S. coelicolor* cultures

S. coelicolor LW33 and LW34 were grown in liquid SMM at 30 °C. At four time points (GC 2 in Table 2), γ-butyrolactones were extracted from the culture supernatant as described in (Takano *et al.*, 2001), and detected by the kanamycin bioassay (Hsiao *et al.*, 2009). Each 10^9 spores of the indicator strain were plated out for confluent lawns on DNA agar plates containing 5 µg kanamycin ml^{-1}. Each 5 µl of the γ-butyrolactones extracts, as well as 125 ng of the γ-butyrolactone SCB1 as positive control and the solvent methanol as negative control were spotted in the middle of the plates. After incubation for 3 days at 30 °C growth of the indictor strain was determined; pictures were taken from the bottom (Fig. 4).

Determination of secondary metabolite production

Each 4×10^7 spores of *S. coelicolor* were streaked out for a 2·5 x 2·5 cm square on DNAgar and pictures were taken from the bottom at different phases of growth to determine production of pigmented secondary metabolites (Fig. 6). Antibiotic production in liquid cultures was determined as described previously (Strauch *et al.*, 1991).

Results

Two forms of the γ-butyrolactone receptor ScbR in strains of *S. coelicolor*

2D gel based proteomic analysis of transition phase cultures of liquid grown *S. coelicolor* strain M145 identified ScbR at a position consistent with its theoretical molecular weight and isoelectric point values (spot 1 in Fig. 1a). However, in an extensive analysis of strain M600 grown under the same conditions ScbR was never detected (data not shown). In an analysis of protein extracts prepared from spores of M600, ScbR was detected (spot 2 in Fig. 1b) but at coordinates corresponding to a significantly more acidic isoelectric point compared to that observed in M145. This difference was confirmed by performing a separation of an equal mixture of the M600 spore extract and the M145 transition phase mycelial extract (Fig. 1c), and indicates the occurrence of a modified form of ScbR in *S. coelicolor* strain M600.

ScbR_M600 from *S. coelicolor* M600 carries a single amino acid change, R120S, compared to ScbR_M145 from strain M145

To identify the M600 ScbR modification, the chymotryptic peptides detected for the ScbR proteins in M145 spot 1 and M600 spot 2 in Fig. 1 were compared (Fig. 1d and Table S3). Peptides corresponding to all the ScbR amino acid sequence except RRWHETLL and FHFQSKEELAL (indicated by black bars in Fig. 2), were detected in the ScbR spot from M600 (spot 2), but the RRWHETLL peptide was readily detected in the M145 ScbR sample (spot 1) at 1110·62 Da (Table S3). Peptides containing tryptophan can produce up to four peptide peaks in MALDI-TOF analysis due to two successive oxidations of the tryptophan residue (i.e. parent mass +16·0 Da and +32·0 Da), followed by spontaneous deformylation to kyneurenine (parent mass +4·0 Da) (Finley *et al.*, 1998), and peaks at 1126·62 Da and 1142·62 Da in the data for the M145 spot correspond to the oxidised forms of the parent ion detected at 1110·62 Da. Interestingly, these three peptides are absent in the peptide mass

fingerprint for the M600 ScbR spot, but three new peptide peaks apparently corresponding to a tryptophan-containing peptide can be detected at 1041·48 Da, 1045·49 Da (parent +4·0 Da), and 1073·48 Da (parent +32·0 Da; Fig. 1e, f). ScbR contains only one tryptophan residue, and the data therefore indicates that in M600 spot 2 ScbR has been modified on the RRWHETLL peptide resulting in a surprising mass loss of 69·09 Da (and an acidic shift in the isoelectric point value of the protein). This data corresponds to the replacement of an arginine residue with a serine (69·069 Da). Analysis of the parent peptide ion at 1041·48 Da using Q-TOF mass spectrometry confirmed the sequence of this peptide as RSWHETLL (data not shown). This has also subsequently been verified via sequencing of the *scbR* gene in strain M600 (see below). The M600-type protein (ScbR$_{M600}$) therefore contains a single amino acid change (R120S) compared to the M145-type protein (ScbR$_{M145}$) (Fig. 2).

(a)

M145 mycelia

(b)

M600 spores

(c)

M145 mycelia + M600 spores
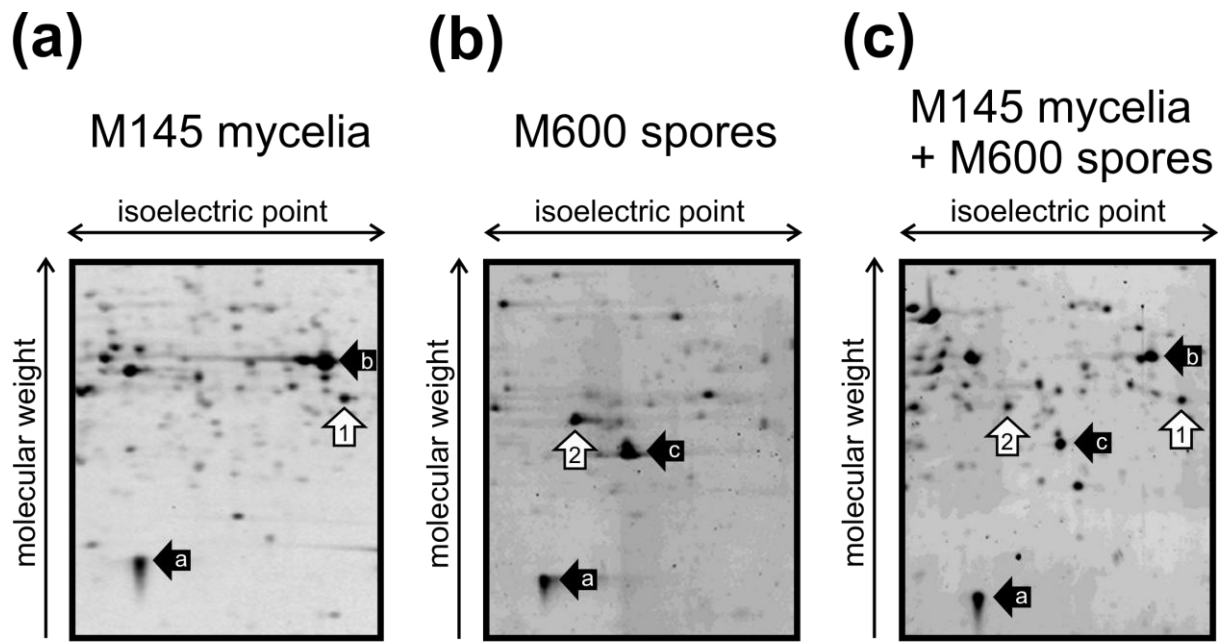
(d)

MALDI-TOF of tryptic (and chymotryptic) digest

Spot	Matched peptides	Sequence coverage	MOWSE score
1	11 (22)	52 % (87 %)	146 (100)
2	7 (21)	39 % (90 %)	88 (103)

(e)

ScbR$_{M145}$
(spot 1 in A)
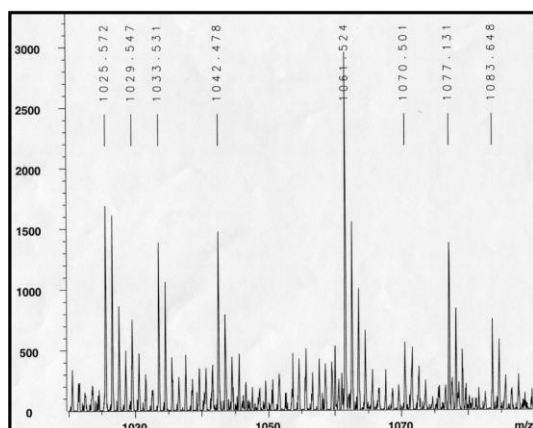
(f)

ScbR$_{M600}$
(spot 2 in B)
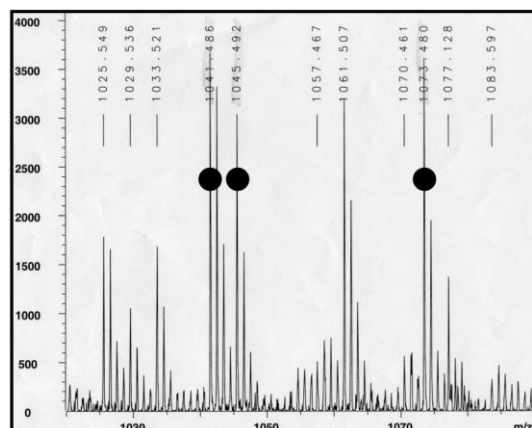
Figure 1 2D gel and MALDI-TOF peptide mass fingerprint analysis of *S. coelicolor* protein extracts showing the presence of two distinct forms of ScbR in strains M145 and M600.

(**a, b, c**) A part of the gels is shown with separation horizontally by isoelectric point and vertically by molecular weight. Numbered arrows indicate ScbR from M145 (1) and M600 (2), while black arrows highlight landmark spots (a, b, c) known to be the same in at least one of the other gels. (**d**) Identification of ScbR spots 1 and 2 by mass spectrometry after tryptic and chymotryptic digest. Data from the chymotryptic digests are given in brackets. (**e, f**) Detail of the 1020 Da to 1090 Da mass range showing three extra peptides in ScbR$_{M600}$ (spot 2 in (a)) (at 1041·486, 1045·492 and 1073·480 Da, marked with circles). The extra peak at 1041·486 Da was identified as RSWHETLL by Q-TOF mass spectrometry, and the other peaks are believed to correspond to the same peptide but with the tryptophan modified to kyneurenine (1045·492 Da) and formylkyneurenine (1073·480 Da).

Figure 2 Amino acid sequence alignment and deduction of functional domains and residues of CprB, ScbR$_{M145}$ and ScbR$_{M600}$.

The deduction of functional domains and residues is based on CprB data from (Natsume *et al.*, 2004). The amino acid sequences represent monomers of each protein. Peptides readily detected by MALDI-TOF analysis in ScbR$_{M145}$ (spot 1 in Fig.1a) but not in ScbR$_{M600}$ (spot 2 in Fig. 1b) are indicated by black bars (ScbR amino acids 48-58 and 119-126). Residues forming α-helices are boxed and labelled. Boxes are shaded for α-helices 4-8 involved in the formation of the γ-butyrolactone binding pocket. Bold boxes for α-helices 8 and 9 indicate their role in dimerisation. Dashed boxes mark the DNA binding domain (α1-3) with α2 and α3 forming a helix-turn-helix motif, and the regulatory domain (α5-10). A highly conserved tryptophan residue directly involved in ligand binding (W127 in CprB; W121 in ScbR) is underlined. The mutated amino acid residue 120 is indicated in bold for ScbR$_{M145}$ (R120) and for ScbR$_{M600}$ (S120).

ScbR$_{M145}$ is the predominant form in *S. coelicolor*

To survey the distribution of the dimorphism identified in ScbR, part of the *scbR* coding region of 16 *S. coelicolor* strains was amplified by PCR using primers ScbRrt1 and ScbRrt2 and the sequence was determined. In addition, for *S. coelicolor* M600 and *S. lividans* 1326, the entire *scbR* coding sequence and promoter region was amplified using primers ScbR2 and ETS10 (all primers used are listed in Table S2). In scbR$_{M600}$ in M600, a single base pair change, c358a was identified, confirming the amino acid mutation R120S observed in the proteomics analysis. The same mutation was also found in *S. coelicolor* strain A(3)2 N2, but was absent in the other 14 *S. coelicolor* strains tested which all possessed the M145 genotype (Table 1). No other variants of *scbR* were observed in the strains tested. The *scbR* homologue in *S. lividans* differs from *scbR*$_{M145}$ only by two silent point mutations, g402a and g582t ("gaa" and "gct", Glu and Ala triplets, respectively, instead of "gag" and "gcg" in M145). Thus, the *S. lividans* protein amino acid sequence is identical to that of ScbR$_{M145}$. The *scbR* promoter regions from 251 bp upstream of *scbR,* covering the ScbR binding sites in front of *scbA* and *scbR*, are identical in *S. coelicolor* strains M145 and M600 and also in the *scbR* homologue in *S. lividans* 1326 (data not shown). Our sequencing data were confirmed by the publically available genome sequence of *S. lividans* (www.broadinstitute.org/annotation/genome/streptomyces_ group/Regions.html).

In vivo analysis of the effect of the ScbR$_{M600}$ point mutation in a M145 background

The antibiotic production phenotypes of strains M145 and M600 differ, with M600 being notably delayed in the onset of Act and Red biosynthesis, and this is comparable to the phenotypic change observed in strain M145 following deletion of *scbR* (Takano *et al.*, 2001). To determine whether the M600 phenotype may be attributable to the point mutation observed in ScbR$_{M600}$, the M145 derivatives LW34 and LW33 were constructed for comparison, expressing the native and mutant forms of the protein respectively.

Plasmids pTE212 and pTE214 carrying *scbR*$_{M600}$ and *scbR*$_{M145}$, respectively, were used to replace the mutated *scbR*$_{M145}$ locus in the M145 Δ*scbR*$_{M145}$ in-frame deletion mutant strain M752 (Takano *et al.*, 2001) to give strains LW33 and LW34. LW33 therefore encodes ScbR$_{M600}$ in the M145 genetic background, and LW34 is the

control strain expressing ScbR$_{M145}$. Correct construction of the strains was verified using PCR and Southern analysis (Fig. S1a, b). Sequence analysis revealed a silent point mutation, c636t (leading to an "act" triplet instead of "acc", both resulting in Thr) in *scbR*$_{M145}$ of strain LW34 (pTE214, see Fig. S4e). Besides this mutation, strains LW33 and LW34 only differ by c358a in *scbR*.

Expression of *scbR*, *scbA*, *redD* and *cpkO* is altered in strain LW33 containing the mutant ScbR$_{M600}$

Strains LW33 and LW34 were grown in liquid SMM and samples for RNA and protein isolation, and γ-butyrolactone and antibiotic analysis, were collected at different phases of growth (Table 2 and S4). Quantitative real time PCR (qRT-PCR) was used to measure the expression levels of the pathway-specific regulators of the *act*, *red* and the *cpk* antibiotic biosynthesis gene clusters (*actII-4*, *redD*, *cpkO*) and of *scbA* and *scbR* of the *S. coelicolor* butanolide system in the presence of the two forms of ScbR (Fig. 3, Table S4). Compared to the control strain LW34, *redD* expression was delayed and was 2-3-fold lower in LW33 harbouring the mutant ScbR at mid transition phase (mT), but maintained this level during late transition phase where it became >2-fold higher than in LW34. The onset of *actII-4* transcription was also delayed in LW33. Expression of *cpkO* was higher in LW33 but it was only transcribed in late transition phase (lT), and thus later than in LW34. Also the onset of transcription of *scbA* was delayed in LW33 and shifted from mid to late transition phase while the expression level was similar to LW34. Also with *scbR*, maximum expression was reached only at late transition phase in LW33 compared to mid transition phase in LW34 (Fig. 3, GC 2 in Table S4).
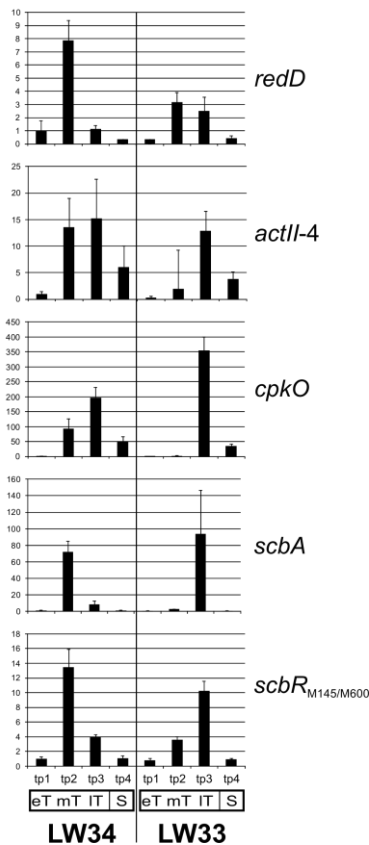
Figure 3 Transcriptional analysis of *redD, actII-4, cpkO, scbA* and *scbR*$_{M145/M600}$ using qRT-PCR.

qRT-PCR analysis of the transcription of *scbA* and *scbR*$_{M145/M600}$ using cDNA synthesized from RNA isolated from liquid SMM cultures of *S. coelicolor* LW34 and LW33. Samples were taken at four time points (tp 1-4) during different phases of growth indicated with eT, mT, lT and S (early, mid, and late transition, and stationary phase; GC 2 in Table 2 and S4). Gene expression is shown as fold-change relative to the LW34 time point 1 early transition phase sample. Error bars indicate the standard deviation.

γ-butyrolactone production is slightly delayed in LW33

γ-butyrolactone production of *S. coelicolor* strains LW34 and LW33 was determined using a kanamycin bioassay (Hsiao *et al.*, 2009) at different phases of growth corresponding to the time point samples used in the transcription studies (GC 2 in Table 2 and S4). Kanamycin resistance of a *Streptomyces* indicator strain is induced by the presence of γ-butyrolactones in extracts from the tested strains, and the extent of growth of the indicator strain on media containing kanamycin reflects the amount of γ-butyrolactones produced. Extracts from stationary phase cultures of LW33 and LW34 produced similar halos of growth, indicating the presence of similar levels of γ-butyrolactones. Slightly higher levels have been detected in both the mid and late transition phase samples in strain LW34 (Fig. 4). This indicates that ScbR$_{M600}$ in strain LW33 may lead to a minor reduction and delay in γ-butyrolactone production compared to LW34, and is consistent with the delay in *scbA* transcription observed in LW33 (Fig. 3).
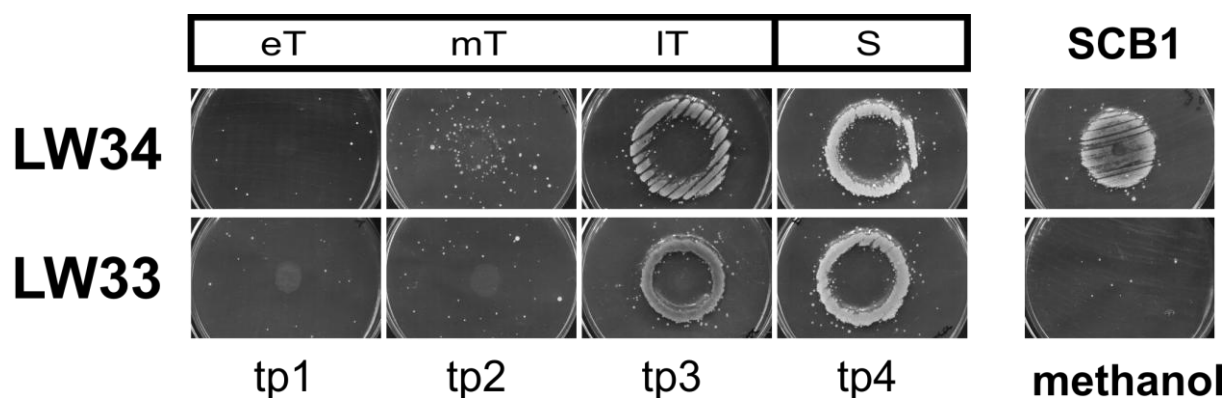
Figure 4 γ-Butyrolactone production in liquid *S. coelicolor* cultures using the kanamycin bioassay.

The kanamycin bioassay (Hsiao *et al.*, 2009) was used to detect γ-butyrolactone production in *S. coelicolor* in liquid SMM at 4 time points (tp) at different phases of growth (<u>e</u>arly, <u>m</u>id and <u>l</u>ate <u>t</u>ransition (eT, mT, lT), and <u>s</u>tationary (S) phase; GC 2 in Table 2). Growth of a bioassay indicator strain on kanamycin supplemented medium is shown. Kanamycin resistance and thus growth is induced by the presence of γ-butyrolactones. Extracts from strains LW34 and LW33 resembled each other in the induction of growth, however, with LW34 slender growth seems to be induced already at time point 2 and the area of growth is increased compared to LW33 with the late transition phase sample. Chemically synthesised *S. coelicolor* γ-butyrolactone (SCB1) was used as positive, the solvent methanol as negative control.

Protein extracts from strain LW33 show a reduced ability to bind to the *scbR* promoter sequence

CE of LW34 ($scbR_{M145}$) and LW33 ($scbR_{M600}$) were prepared from samples taken at time points corresponding to those indicated for the transcription analysis (GC 1 in Table S4). DNA binding abilities were determined in gel retardation assays using a digoxygenin-labelled *scbR* promoter fragment containing the ScbR binding site upstream of its own promoter (Takano *et al.*, 2001) and freshly prepared protein extracts (Fig. 5a). Equivalent amounts of total protein used for the gel retardation analysis were also analysed by Western hybridization using an antibody to ScbR to determine the relative abundance of ScbR in the extracts (Fig. 5b). Extracts from LW34 from late transition and stationary phase completely shifted the operator DNA in the binding assay (Fig. 5a). This is consistent with the appearance of ScbR as detected by Western blotting (Fig. 5b). In contrast, all LW33 extracts failed to

produce a full shift. Even in the presence of a higher amount of ScbR$_{M600}$ compared to ScbR$_{M145}$ (LW33 in stationary and LW34 in transition phase, respectively; Fig. 5b) the observed shift of ScbR$_{M600}$ was weaker, suggesting that the DNA binding ability of the mutant protein in LW33 is reduced.
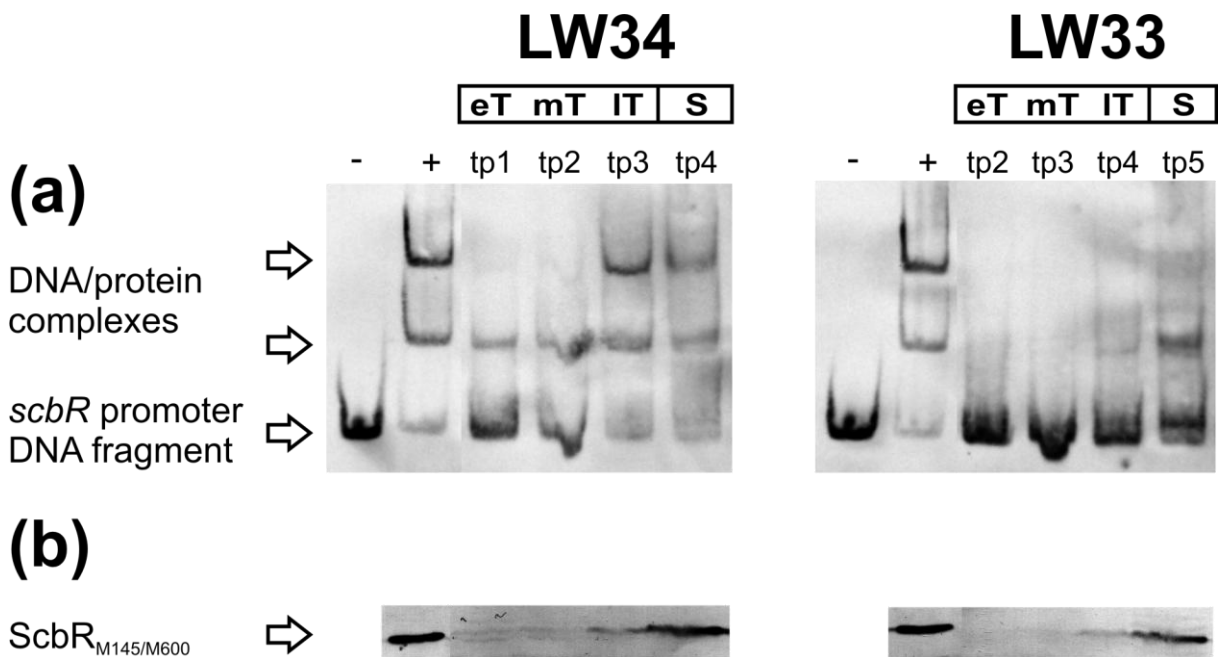
Figure 5 Gel retardation assay and Western analysis using *S. coelicolor* cell-free extracts.

Cell-free extracts (CE) of *S. coelicolor* LW34 and LW33 obtained at 4 time points (tp) at <u>e</u>arly, <u>m</u>id and <u>l</u>ate <u>t</u>ransition (eT, mT, lT) and <u>s</u>tationary (S) phase of growth (GC 1 in Table 2) were used to determine presence and DNA binding ability of ScbR$_{M145/M600}$. CE from *E. coli* JM101/pIJ6120 harbouring ScbR$_{M145}$ was used as a positive control (+). (**a**) Gel retardation assay. DNA binding abilities of ScbR$_{M145}$ and ScbR$_{M600}$ from *S. coelicolor* CE were tested using a DIG-labelled *scbR* promoter DNA fragment. The *scbR* promoter DNA fragment and DNA/protein complexes are indicated by arrows. The DNA probe alone was used as a negative control (-). (**b**) Western analysis of ScbR. ScbR$_{M145}$ (+, LW34) and ScbR$_{M600}$ (LW33) was detected in similar amounts at corresponding time points in the two *S. coelicolor* strains. ScbR signals are indicated by an arrow.

Antibiotic production in *S. coelicolor* LW34 and LW33 is comparable

Act and Red production of LW34 (*scbR*$_{M145}$) and LW33 (*scbR*$_{M600}$) was determined in liquid SMM (Table 2). No difference could be determined for Act, whereas Red

production was slightly increased in LW33. This was consistent between two independent growth experiments. However, in one of them, LW34 also produced Red in high amounts during stationary phase (Table 2). We recently identified a yellow pigment (yCPK) and an antibiotic compound (abCPK) as metabolites of the hitherto orphan *cpk* gene cluster (Gottelt *et al.*, submitted) that may be the primary target of the *S. coelicolor* butanolide system. Therefore, in addition to Act and Red, we compared production of the yellow compound in LW33 and LW34 grown on solid Difco Nutrient agar where yCPK was found to be produced by the parental strain M145 (Gottelt *et al*, submitted). All three pigmented secondary metabolites were observed in equal amounts during ten days of growth (Fig. 6). From all these data there seems to be no significant difference in antibiotic production between LW33 and LW34 under the conditions tested.
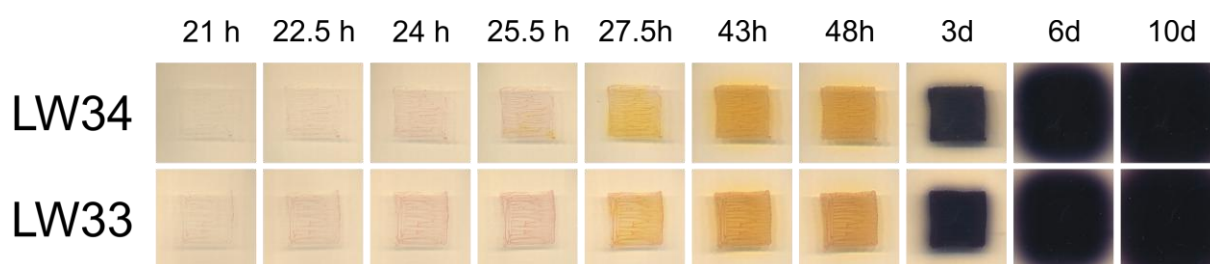
Figure 6 Secondary metabolite production on DNAgar.

S. coelicolor strains LW34 (*scbR*$_{M145}$) and LW33 (*scbR*$_{M600}$) were incubated on DNAgar at 30 °C. Production of pigmented secondary metabolites was followed at 21, 22·5, 24, 25·5, 27·5, 43, 48 h and 3, 6, 10 days.

In vitro analysis using fresh protein expressed in *E. coli* indicates that the DNA and γ-butyrolactone binding abilities of ScbR$_{M145}$ and ScbR$_{M600}$ are comparable

In vivo analysis indicated that expression of mutant ScbR$_{M600}$ in *S. coelicolor* leads to altered patterns of expression of the three pathway-specific regulatory genes for Act, Red and CPK, the *scbA* and *scbR* genes known to be controlled by ScbR in response to altered butyrolactone concentrations, and to a decreased ScbR$_{M600}$ DNA binding affinity. To investigate in more detail the effect that the point mutation may

have on the ability to bind both butyrolactones and its cognate DNA operator sequence, ScbR$_{M145}$ and ScbR$_{M600}$ were overexpressed in *E. coli*. Fresh *E. coli* CE containing equal amounts (see Methods and Figure S2b) of the two forms of ScbR showed comparable DNA binding abilities in gel retardation analysis (Fig. 7, lanes 3 and 7, and Fig. 8a, lanes 2 and 6, respectively).

Addition of low (7·8 ng; Fig. 7, lanes 5 and 9) and high (62·5 ng; Fig. 7, lanes 4 and 8) amounts of the cognate γ-butyrolactone ligand SCB1 led to a partial and a complete loss of DNA binding, respectively, with both ScbR$_{M145}$ and ScbR$_{M600}$. Therefore, under the conditions tested, the γ-butyrolactone binding affinity of the two forms of ScbR were comparable.
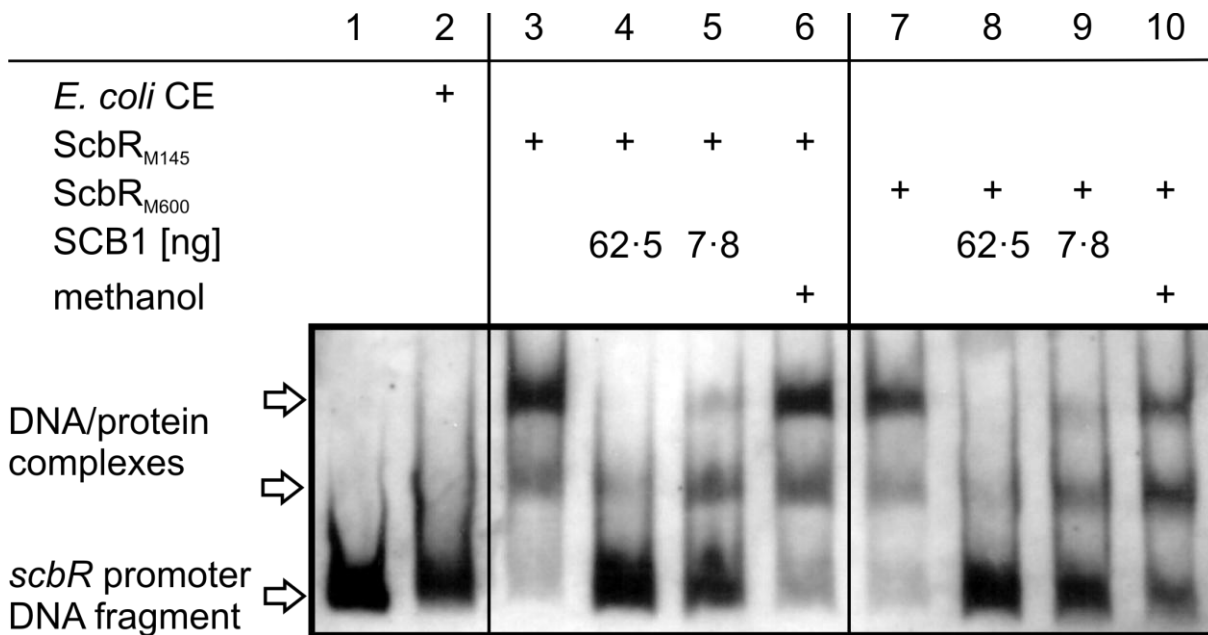
Figure 7 Gel retardation assay and Western analysis using ScbR$_{M145}$ and ScbR$_{M600}$ supplemented with SCB1.

(**a**) DNA and γ-butyrolactone binding abilities of ScbR$_{M145}$ and ScbR$_{M600}$ from *E. coli* cell-free extracts (CE) were tested using a DIG-labelled *scbR* promoter DNA fragment and the *S. coelicolor* γ-butyrolactone SCB1 in a gel retardation assay. All samples contained the labelled DNA probe. Sample two contained CE of *E. coli*/pTE2925 ("*E. coli* CE"), samples 3-6 and 7-10 of *E. coli* JM101/pTE6120 and pTE58 ("ScbR$_{M145}$" and "ScbR$_{M600}$"). To samples 4, 5, 8 and 9 SCB1 dissolved in methanol was added in high (62·5 ng) and low (7·8 ng) amounts. Samples 6 and 10 were

supplemented with same volumes of pure methanol. The *scbR* promoter DNA fragment and DNA/protein complexes formed with $ScbR_{M145/M600}$ are indicated by arrows. DNA binding abilities of the two variants of ScbR were shown to be the same in the absence and the presence of the γ-butyrolactone.

DNA binding ability of ScbR$_{M600}$ expressed in *E. coli* is unstable

In gel retardation analysis using *E. coli* CE supplemented with 4 % (v/v) glycerol, DNA binding of the mutated $ScbR_{M600}$ was reduced when stored at -20 °C for a longer time (data not shown). To further analyse this difference, fresh CE without glycerol was frozen and thawed up to three times at -20 °C and 37 °C, respectively, and the DNA binding affinity was tested by gel retardation analysis. To ensure equal amounts of protein for both $ScbR_{M145}$ and $ScbR_{M600}$, the total protein concentrations of the CE were measured, and the abundance of ScbR present was confirmed by Western analysis (Fig. 8b). Both proteins were active in fresh CE (Fig. 8a, lanes 2 and 6), but freezing and thawing of the proteins lead to a decrease (lane 7 and 8) and eventually to the loss (lane 9) of DNA binding with the mutant $ScbR_{M600}$. Native $ScbR_{M145}$ was not affected by the treatment (lanes 3 to 5). Western analysis indicated that the freezing and thawing had no effect on the amount of protein detected (Fig. 8b) indicating that the loss of DNA binding ability was not due to protein degradation. This suggests that compared to $ScbR_{M145}$, the DNA binding ability of $ScbR_{M600}$ is significantly less stable. This is also supported by the decrease in DNA binding affinity observed with the mutant protein, but not with $ScbR_{M145}$, in the presence of methanol (Fig. 7, lanes 6 and 10).

(a)
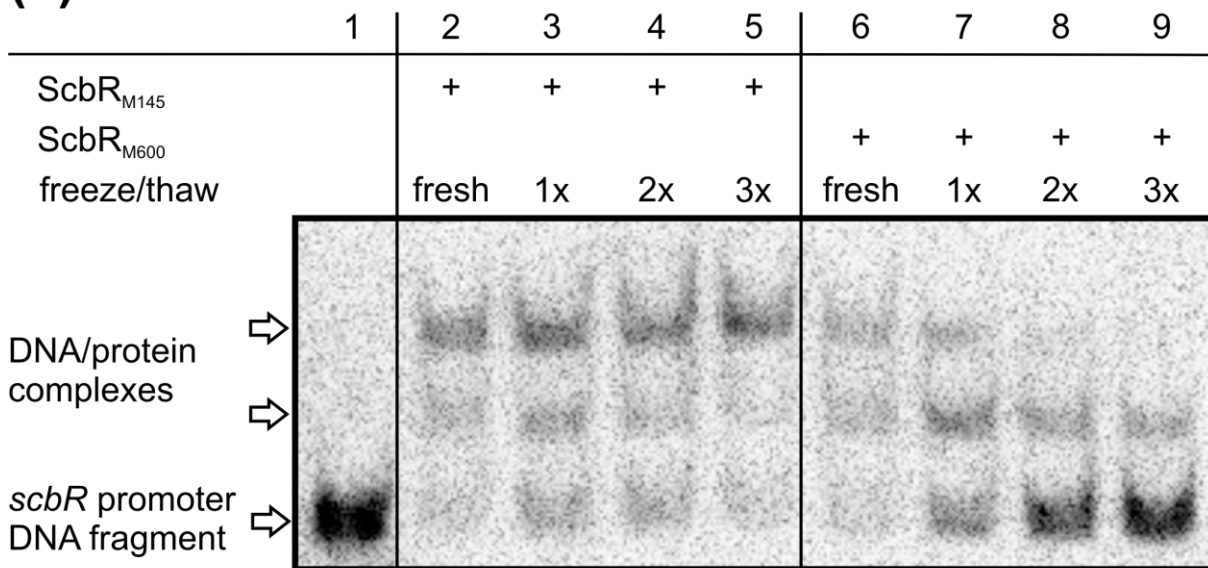
(b)
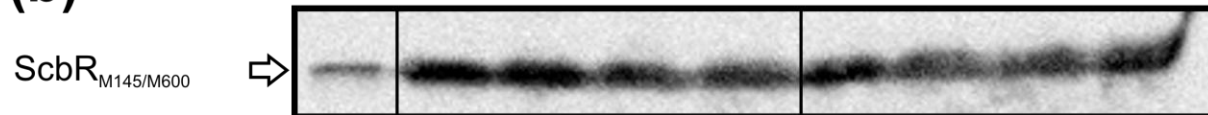
Figure 8 Gel retardation assay and Western analysis using ScbR$_{M145}$ and ScbR$_{M600}$ after freezing and thawing.

(**a**) Gel retardation assay. DNA binding abilities of ScbR$_{M145}$ and ScbR$_{M600}$ from *E. coli* cell-free extracts (CE) were tested using a DIG-labelled *scbR* promoter DNA fragment in a gel retardation assay. All samples contained the labelled DNA probe. Samples 2-5 and 6-9 contained CE of *E. coli* JM101/pTE6120 and pTE58 ("ScbR$_{M145}$" and "ScbR$_{M600}$"), respectively, frozen and thaw one to four times before added to the DNA probe. The *scbR* promoter DNA fragment and DNA/protein complexes formed with ScbR$_{M145/M600}$ are indicated by arrows. In contrast to ScbR$_{M145}$, DNA binding of ScbR$_{M600}$ was shown to be instable under the conditions tested. (**b**) Western analysis of ScbR. ScbR$_{M145}$ was detected with a positive control sample of recombinant ScbR$_{M145}$ ("rScbR$_{M145}$", lane 1) and in comparable amounts in all gel retardation assay samples harbouring ScbR$_{M145}$ or ScbR$_{M600}$ as described in (a) (lanes 2-9). ScbR bands are indicated by an arrow.

Discussion

Among at least five homologues in *S. coelicolor* (CprA, CprB, SCO6323 and ScbR2 in addition to ScbR), ScbR is the only γ-butyrolactone receptor experimentally shown to bind a cognate signalling molecule. Acting as transcriptional regulator, ScbR plays

a key role in the *S. coelicolor* butanolide system involved in the regulation of the antibiotics Act and Red, and of the *cpk* secondary metabolite gene cluster (reviewed in (Takano, 2006)). We identified a variant ScbR in *S. coelicolor* M600 that is clearly impaired in its DNA binding ability and that alters the expression of the pathway-specific regulators of all three antibiotic gene clusters.

A variant form of the γ-butyrolactone receptor ScbR in *S. coelicolor*

In addition to ScbR from the sequenced strain M145 (ScbR$_{M145}$), a second form of the protein, ScbR/R120S (ScbR$_{M600}$), was found in strain M600 (this study). The underlying point mutation, c358a, was also found in strain A3(2) N2, another *S. coelicolor* A3(2) derivative independent from both M145 and M600 (Weaver *et al.*, 2004). All 14 further strains tested showed the M145 genotype, including *S. coelicolor* W3443 the proposed progenitor of all strains described (Weaver *et al.*, 2004), and *scbR*$_{M600}$ is thus regarded as a mutant form. Relying on the lineage of *S. coelicolor* described by Kao and co-workers (Weaver *et al.*, 2004), our results indicate that the point mutation c358a occurred on two independent occasions, in strains M600 and A3(2) N2, perhaps indicating a selective pressure for this change under certain conditions.

The mutation in ScbR$_{M600}$ has no detectable effect on γ-butyrolactone binding

The crystal structure of a ScbR homologue in *S. coelicolor*, CprB, is regarded as representative for γ-butyrolactone receptors in streptomycetes. Functional domains for dimerization, DNA binding by helix-turn-helix motifs, and γ-butyrolactone binding have been proposed. DNA binding of CprB was shown experimentally (Sugiyama *et al.*, 1998), but a putative cognate γ-butyrolactone ligand has yet to be identified (Natsume *et al.*, 2004). From the CprB amino acid sequence, amino acid 120 in ScbR is proposed to form part of a central α-helix (α7) in the regulatory region (Fig. 2). The adjacent tryptophan residue W121 is predicted to be directly involved in forming the γ-butyrolactone binding pocket and is highly conserved among *Streptomyces* γ-butyrolactone receptors (Natsume *et al.*, 2004). Mutation of the corresponding W119 in the A-factor receptor protein ArpA, a ScbR homologue in *S. griseus*, abolished γ-butyrolactone binding (Sugiyama *et al.*, 1998). It therefore seems reasonable to suggest that a change in the amino acid residue 120 adjacent

to the crucial W121 in ScbR, from a large (174 Da) basic arginine to a small (105 Da) neutral serine, may affect ligand binding affinity.

However, no difference in ligand binding was observed between ScbR$_{M145}$ and ScbR$_{M600}$ (Fig. 7), and it is interesting to note that ScbR amino acid 120 is not conserved among γ-butyrolactone receptor proteins. The corresponding residues in other ScbR homologues consist of members of all classes of amino acids and of hugely differing sizes (e.g., aspartate (acidic, 133 Da) in ArpA (*S. griseus*); arginine (basic, 174 Da) in ScbR$_{M145}$; asparagine (hydrophilic, 132 Da) in FarA (*S. lavendulae*); alanine (hydrophobic, 89 Da) in SpbR (*S. pristinaespiralis*)), all adjacent to the conserved tryptophan residue (Natsume *et al.*, 2004). The high level of variability at this position suggests residue 120 does not in fact play a significant role in γ-butyrolactone binding.

The mutation in ScbR$_{M600}$ impairs DNA binding

Compared to ScbR$_{M145}$, DNA binding was reduced with ScbR$_{M600}$ (from *S. coelicolor* LW34 and LW33, respectively; Fig. 5). This difference is surprising since the functional localization of the altered amino acid 120 does not suggest an effect of the mutation on the DNA binding domain of the protein. Furthermore, Horinouchi and co-workers described the helix-turn-helix motifs of mutant ArpA/W119A as still able to bind DNA (Sugiyama *et al.*, 1998). However, in the ArpA study the intensity of the shifted band observed in the absence of A-factor was noticeably weaker for the mutated ArpA/W119A than for the wild type ArpA (Fig. 6b in (Sugiyama *et al.*, 1998)). This also implies a reduced DNA binding affinity of ArpA/W119A, and thus suggests an effect of the amino acid change W119A in ArpA similar to that observed with ScbR$_{M600}$ in this study.

Structural effect of amino acid change R120S on the DNA binding domain of ScbR$_{M600}$

DNA binding of ScbR$_{M600}$ was impaired in both the natural producer *S. coelicolor* and, after heterologous expression, in *E. coli* CE. The decreased binding ability is therefore most probably not related to specific properties of the *S. coelicolor* cellular background, e.g., a hypothetical "deactivation" mechanism of the transcriptional regulator ScbR, and it is more likely that the amino acid change is causing a structural change affecting the DNA binding domain. This is also supported by the

fact that a loss of ScbR$_{M600}$ DNA binding could be induced by freezing and thawing of the protein (Fig. 8a), and the observation that this loss was prevented by the addition of 43 % (v/v) of glycerol (data not shown). Western analysis data shown in Fig. 5b and Fig. 8b clearly exclude degradation of the protein as an explanation for these observations.

ScbR$_{M600}$ leads to altered gene expression and may affect γ-butyrolactone production

Expression of *scbA* is delayed in LW33 (Fig. 3). This could be a direct effect of the impaired DNA binding ability of ScbR$_{M600}$ since ScbR was previously shown to be necessary for *scbA* expression (Takano *et al.*, 2001). Consistently, late expression of the γ-butyrolactone synthase gene coincides with a slight delay in the production of the small signalling molecules in LW33 (Fig. 4). However, DNA binding was determined using a *scbR* promoter fragment and not the ScbR binding site in the *scbA* promoter region.

Also *scbR* expression is somewhat delayed at similar transcription levels in the presence of the M600-type protein, but not as clearly as with *scbA* (Fig. 3). This is at first glance surprising, since a reduction in DNA binding of the auto-repressor might be expected to result in a higher or early expression of *scbR*$_{M600}$. However, also in Western analysis no increase in the amount of ScbR$_{M600}$ in LW33 was observed (Fig. 5b). γ-butyrolactones abolish the DNA binding of ScbR and are active in nM concentrations (Fig. 7) (Takano *et al.*, 2001). The small reduction in γ-butyrolactone production observed in LW33 (Fig. 4) may promote binding of ScbR$_{M600}$ to its target sites *in vivo*, counteracting the impaired binding ability of the mutant protein.

In LW33, expression of the pathway-specific regulator genes for the *act*, *red* and the *cpk* antibiotic biosynthesis gene clusters was delayed compared to that in LW34 (Table 2 and Fig. 3). Despite these differences, antibiotic production was similar in the two strains. Regulation of production of these antibiotics is complex and known to be affected by many additional factors (Bibb, 2005). The *cpk* gene cluster, for example, was shown to be also regulated by the RapA1/A2 two-component system (Lu *et al.*, 2007) and the global regulator DasR (Rigali *et al.*, 2008). It appears that the changes in expression of the regulatory genes observed here are over-ridden by other control mechanisms and so do not result in any observable change in the

antibiotic phenotype. Furthermore, production of other, known and unknown, secondary metabolites in *S. coelicolor* may impair the synthesis of Act, Red and yCPK, e.g. by competition for common precursors from primary metabolism. Further experiments will be needed to reveal the detailed effects of the mutation in ScbR$_{M600}$ on the delicately balanced butanolide system in *S. coelicolor*.

Despite the somewhat impaired DNA binding affinity of the mutant ScbR$_{M600}$, our result show that strain LW33 clearly differs from a M145Δ*scbR* in-frame deletion mutant (M752). In comparison to M145, the latter strain is delayed in Red production, transcribes *scbR* early, and expresses *cpkO* early and constitutively (Takano *et al.*, 2001;Takano *et al.*, 2005). Evidently, the single R120S amino acid change has a different impact on the *S. coelicolor* butanolide system and related secondary metabolites when compared to complete deletion.

The mutation in ScbR$_{M600}$ is not responsible for the absence of the protein in vegetative mycelium of strain M600

To our surprise and in contrast to M145, ScbR$_{M600}$ was absent during vegetative growth in M600 as shown by 2D gel (Fig. 1) and Western analysis (data not shown). However, ScbR$_{M600}$ was present in vegetative mycelium of *S. coelicolor* LW33 harbouring *scbR*$_{M600}$ in a M145 genetic background. The amounts were comparable to ScbR$_{M145}$ in the control strain LW34 (Fig. 5b) and in M145 (data not shown). This suggests that it is the M600 genetic background, and not the amino acid change in ScbR$_{M600,}$ that is the reason for the absence of ScbR during vegetative growth of M600. Whether the altered properties of ScbR$_{M600}$ are an adaptation to its synthesis in the spores of *S. coelicolor* M600 and/or to a putative modified function of the regulator remains to be determined. Detailed knowledge of the butanolide regulatory system in *S. coelicolor* may allow rational construction of strains with improved timing and levels of antibiotic production.

Acknowledgements

Chapter 5

Summary and concluding remarks

Submitted for publication together with chapter 1

"γ-Butyrolactones and their role in regulation"
in "Streptomyces Molecular Biology and Biotechnology"
Marco Gottelt and Eriko Takano

The search for new drugs against pathogenic bacteria causing infectious diseases will probably never end. At best, we are always one step ahead in the race between resistant pathogens and novel antibiotics. In recent years, reports of "superbugs", deadly pathogens against which no known drug is effective any longer, alarmed both the scientific community as well as the general public. For various reasons the number of genuinely new antibiotics on the market has been declining for decades (Fig. 1 in chapter 1), while development of resistance among pathogenic bacteria increased. Therefore, the search for pharmaceutically active natural products has moved back into the centre of scientific interest (Hopwood, 2007).

More than three quarters of the clinically used antibiotics are natural products or slightly modified natural products (Newman *et al.*, 2003). These drugs originally derive mostly from fungi and a group of soil bacteria, the streptomycetes, which produce antibiotics as secondary metabolites (Fig. 1 in chapter 1) (Hopwood, 2007). However, since their biosynthesis is complex and "costly" for the producing organism, secondary metabolite production is strictly regulated in the microbial cell.

The aim of this thesis was (1) to gain further insight into the *Streptomyces coelicolor* butanolide system, a central antibiotic regulatory system in the model organism of its genus (reviewed in Takano, 2006), and (2) to trigger production of novel antibiotics by manipulation of the regulation of their biosynthetic gene clusters.

The role of ScbR in a bacterial hormone system of *Streptomyces coelicolor*

Secondary metabolite production in *Streptomyces* is controlled by a complex regulatory network. One of the regulatory systems of antibiotic production is based on "bacterial hormones", the γ-butyrolactones. The small signalling molecules are produced in and spread among the cells of a *Streptomyces* colony. This may ensure that all cells start simultaneously with the production of antibiotics and therefore increase its efficiency (Takano, 2006). Furthermore, mechanisms of self-resistance may be activated prior to the production of antibiotics (Y. Ohnishi, personal communication).

In the model streptomycete *S. coelicolor* this regulatory system is formed by three main components: first, a "sender", the ScbA protein, involved in the production of the signalling molecules. Secondly, the bacterial hormones themselves; and, thirdly, a "receiver" or receptor, the ScbR protein, the <u>S</u>treptomyces <u>c</u>oelicolor <u>b</u>utanolide <u>r</u>eceptor (Fig. 1; Fig. 4 and 5 in chapter 1) (Takano, 2006).

Fig. 1 illustrates the mode of action of ScbR: A ScbR homodimer acts as negative transcriptional regulator by binding to specific sites upstream of its target genes via a DNA binding domain (Fig. 1a). In the presence of the γ-butyrolactones, the signalling molecules bind to the ligand binding pocket of ScbR, altering the structural conformation of ScbR and in particular the DNA binding domain so that the receptor can no longer bind to DNA (Horinouchi, 2007). Consequently, the bacterial hormones have caused a de-repression of the ScbR target genes and the initially silenced genes are now expressed (Fig. 1b) (Takano *et al.*, 2001; Takano, 2006).
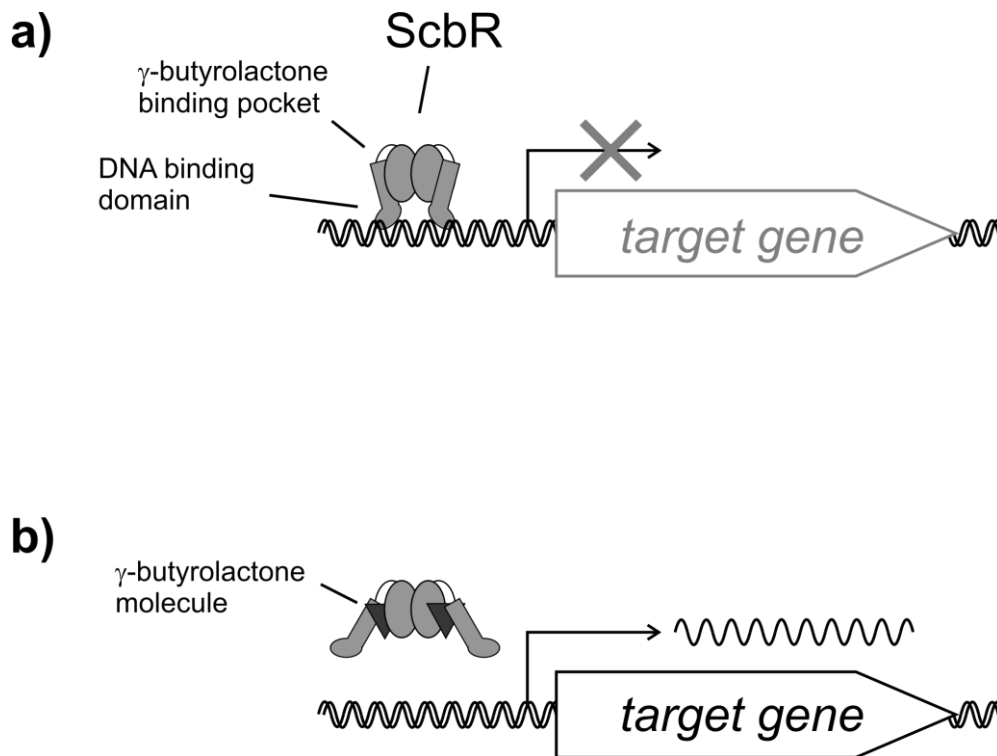
Figure 1 Mode of action of the γ-butyrolactone receptor and transcriptional regulator ScbR in _Streptomyces coelicolor_ (explanation in the text)

ScbR, _scbA_ and _cpkO_ have been experimentally identified as target genes of ScbR (Fig. 5 in chapter 1) (Takano _et al._, 2001; Takano _et al._, 2005a). ScbA most likely catalyses the condensation of dihydroxyacetone phosphate (DHAP) and a β-keto acid derivate which comprises the first step in the biosynthesis of the γ-butyrolactones (Fig. 2) (Hsiao _et al._, 2007). CpkO is the pathway specific regulator of the _cpk_ secondary metabolite gene cluster (Fig. 2 in chapter 1) (Pawlik _et al._, 2007). Thus, in addition to its own expression, ScbR also regulates the production of the signalling molecules of the _S. coelicolor_ butanolide system, as well as a putative antibiotic biosynthesis gene cluster. ScbR was also shown to affect production of two pigmented _S. coelicolor_ antibiotics, the blue-coloured actinorhodin (Act) (Bystrykh _et al._, 1996; Rudd and Hopwood, 1979), and the red-pigmented prodigiosins (Red) (Feitelson _et al._, 1985) (Fig. 4 in chapter 1).

In total four binding sites were identified in front of the three ScbR target genes: Site R in front of _scbR_, site A in front of _scbA_ and site O$_A$ and site O$_B$ in front of _cpkO_ (Takano, 2006). Site R and site O$_A$ show an identical inverted repeat sequence,

whereas site O_B and even more site A are less congruent with this ScbR binding sequence. *In silico* analysis predicted a single additional fully conserved ScbR binding site in the *S. coelicolor* chromosome in front of *orfB* (Takano *et al.*, 2005a). *OrfB* is located in close vicinity of *scbR*, separated only by two genes, *scbA* and *scbB*. Binding of ScbR in front of *orfB* was confirmed in gel retardation assays (Takano, personal communication). The function of the predicted histidine kinase OrfB, however, is not known. Recent preliminary results indicate that OrfB is involved in a ScbR deactivation mechanism by modification (possibly phosphorylation) of ScbR (Takano, personal communication). Interestingly, only for the conserved binding sites, site R and site O_A, ScbR has been confirmed as transcriptional repressor. ScbR binding at site A was suggested to possibly promote activation (Takano *et al.*, 2001), and preliminary *in vitro* data indicate a corresponding function of ScbR also at site O_B (Takano, personal communication). However, the putative dualistic function of ScbR at each two binding sites (repressor at site R and site O_A, activator at site A and site O_B) is not consistent with the position of the sites. Site A and site O_A overlap with the -10 and -35 region of the transcriptional start site of *scbA* and *cpkO*, respectively, whereas site R and site O_B are located -42 nt to -68 nt and -222 nt to -244 nt upstream of *scbR* and *cpkO*, respectively.

$ScbR_{M600}$ - Discovery and investigation of a second form of the *S. coelicolor* γ-butyrolactone receptor

After decades of *S. coelicolor* research, a whole family tree of strains of the species is available, all deriving from the original isolate (Waksman strain W3443) (Weaver *et al.*, 2004). *S. coelicolor* M145 is the typical laboratory strain that was fully sequenced (Bentley *et al.*, 2002), but also strain M600 is often used in many research groups.

When analysing the proteome of these two *S. coelicolor* strains by 2D gel electrophoresis, we identified in M600 a variant of ScbR ($ScbR_{M600}$) that is mutated in the vicinity of the γ-butyrolactone binding pocket compared to ScbR from M145 ($ScbR_{M145}$). A single amino acid change, Arg120Ser, in $ScbR_{M600}$ was revealed in MALDI-TOF analysis. A corresponding point mutation was confirmed by DNA sequence analysis in $scbR_{M600}$ of independent isolates of strain M600 provided by

several laboratories. The same mutation has also been found in the *scbR* gene of strain A3(2) N2.

Possible effects of the mutation in ScbR$_{M600}$ were examined *in vitro* and *in vivo*. For *in vitro* characterisation, we cloned and expressed both *scbR* variants in *E. coli* and used crude cell extracts to compare the properties of ScbR$_{M145}$ and ScbR$_{M600}$ in gel retardation assays. Contrary to our expectations, the mutation resulted in an instable DNA binding activity of the transcriptional regulator. This occurs despite the mutation's location near the ligand binding pocket and not in the vicinity of the DNA binding domain. Surprisingly, binding of the γ-butyrolactones, on the other hand, was not affected in ScbR$_{M600}$ (chapter 4).

From the protein structure of the ScbR homologue CprB (Horinouchi, 2007; Natsume *et al.*, 2004), amino acid 120 in ScbR (Glu126 in CprB (Fig. 6 in chapter 1)) was deduced to be part of α-helix 7 in the regulatory region of the protein (Fig. 2 in chapter 4). The residue faces the protein surface and is distant from the DNA binding and the dimerisation domain; it's only functional feature seems to be that it is the neighbouring residue to Trp121 in ScbR (Trp127 in CprB (Fig. 6 in chapter 1), a residue crucial for ligand binding (Sugiyama *et al.*, 1998) (D. Linke, personal communication, based on (Natsume *et al.*, 2004)). Therefore, the nature of the structural effect of the mutation in ScbR$_{M600}$ leading to the quick loss of DNA binding *in vitro* (chapter 4), remains unclear. Protein crystallization of the γ-butyrolactone receptor homologues is difficult and consequently the structure of CprB remains the only one available. Co-crystallization of both variants of ScbR with its cognate ligand and also with its DNA target region would lead to a better understanding of the structure-function relationship of these proteins and is therefore of great interest in future work.

For *in vivo* characterisation, and to avoid the influence of a different chromosomal background and the effects of putative further mutations in the genome of *S. coelicolor* M600, the mutated *scbR* locus in a M145Δ*scbR*$_{M145}$ in-frame deletion mutant (M752) (Takano *et al.*, 2001) was replaced by *scbR*$_{M600}$ and *scbR*$_{M145}$ yielding strains LW33 and LW34, respectively. LW33 therefore encodes ScbR$_{M600}$ in the M145 genetic background; LW34 is the control strain expressing the native ScbR$_{M145}$. *In vivo*, in agreement with our *in vitro* results, DNA binding activity of

ScbR$_{M600}$ was impaired. Quantitative RT-PCR analysis showed delayed transcription of the pathway-specific regulatory genes of the *act*, *red* and the *cpk* biosynthesis gene clusters in strain LW33. However, production levels of the related secondary metabolites did not differ significantly between strains LW33 and LW34. Also, expression of *scbA* involved in the synthesis of the γ-butyrolactones was delayed in LW33, and consistently a γ-butyrolactone bioassay indicated a minor decrease in the production of the signalling molecules (chapter 4).

Regulation of the *act*, *red* and *cpk* gene clusters by the butanolide system is intertwined with the complex regulatory network of secondary metabolism in *S. coelicolor*. The *cpk* gene cluster, for example, was shown to be also regulated by the RapA1/A2 two-component system (Lu *et al.*, 2007) and the global regulator DasR (Rigali *et al.*, 2008). PhoP, the response regulator of the PhoR/P two-component system related to phosphate metabolism, binds to an intragenic DNA region of a *cpk* biosynthesis gene and is required for the transcription of the *cpk* genes (Colin Smith, personal communication). Furthermore, production of other, known and unknown, secondary metabolites in *S. coelicolor* may impair the synthesis of Act, Red and the *cpk* end product, e.g. by competition for common precursors from primary metabolism. Precursor competition would affect production of Act, Red and the *cpk* product even when the related biosynthesis genes are highly expressed, thereby overriding the transcriptional regulation of the *act*, *red* and *cpk* gene clusters. All this may explain why the comparably small changes in gene expression of their pathway-specific regulatory genes caused by the mutation in ScbR$_{M600}$ did not result in an apparent change in the antibiotic phenotype (chapter 4).

ScbR$_{M600}$ was initially identified in 2D gel analysis in the proteome of spores of *S. coelicolor* M600. Surprisingly, ScbR$_{M600}$ was never detected in mycelial samples of M600, whereas in M145 ScbR$_{M145}$ is present in the mycelium (chapter 4), but was never found in spores (A. Hesketh, personal communication). This striking difference is most probably not caused by the mutation in ScbR$_{M600}$, but is due to the M600 genetic background, since ScbR$_{M600}$ was present in the mycelium when expressed in a M145 genetic background in LW33 (chapter 4). Intracellular conditions in the dormant spores may differ from those in growing mycelial cells. Although studied in

vegetative mycelium, the ScbR$_{M600}$ specific protein characteristics may therefore represent an adaptation of the protein to the spores where expression of ScbR$_{M600}$ was detected and thus to its natural cellular environment. *In vivo* activity of ScbR$_{M600}$ in spores has not been shown yet, and one could further speculate that the role of ScbR$_{M600}$ in the spores, where no antibiotics are produced, differs from that described for ScbR$_{M145}$ in the mycelium. A M600Δ*scbR*$_{M600}$ mutant, as well as the expression of *scbR*$_{M145}$ in a M600Δ*scbR*$_{M600}$ genetic background would be useful to address these hypotheses in future work. Putative effects on development, especially on sporulation and germination, but also changed properties of the spores (e.g. heat resistance) would be of special interest.

In the lineage of the *S. coelicolor* laboratory strains, two major changes in the genome are most apparent: the loss of the two naturally occurring *S. coelicolor* plasmids, the linear SCP1 and the circular SCP2, and the duplication of a 1.06 Mb region from the left chromosome end. The duplication contains 1005 genes, among them four gene clusters known or predicted to be related to secondary metabolites (eicosapentaenoic acid, isorenieratene, deoxysugar synthases/glycosyl transferases, and coelichelin) (Bentley *et al.*, 2002). Transcript levels of the duplicated genes, however, are similar in strains with or without the duplication, and few obvious phenotypic differences exist (Weaver *et al.*, 2004). On the *S. coelicolor* plasmids, we find secondary metabolite genes, i.e. the methylenomycin (Mm) biosynthesis gene cluster on SCP1 (Bentley *et al.*, 2004). Mm production is controlled by a recently discovered regulatory system encoded in the Mm gene cluster that is similar to the *S. coelicolor* butanolide system. This system is based on a novel class of antibiotic biosynthesis inducers, the 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids (AHFCAs), collectively called Mm furans (MMFs), with analogous functions to the γ-butyrolactone regulatory molecules. Also ScbR-like receptor and transcriptional regulator proteins (MmyR and MmfR) were identified (Corre *et al.*, 2008). Given the encoded functions, the chromosomal duplication and the presence or absence of SCP1 plasmid may influence antibiotic production and regulation. Therefore one could speculate that the mutation in ScbR$_{M600}$ is related to these genomic alterations. *S. coelicolor* M145 and M600 lost the plasmids; strain A3(2) N2 possesses SCP1 and SCP2. Interestingly, strains M600 and A3(2) N2 (*scbR*$_{M600}$) possess the duplication, whereas M145 (*scbR*$_{M145}$) is the only one without the prolonged

chromosome. A putative correlation between the duplication and the mutant *scbR*$_{M600}$, however, could not be confirmed, since in 7 additional *S. coelicolor* strains with the duplication (Weaver *et al.*, 2004), *scbR*$_{M145}$ was identified (chapter 4).

Although unknown, a natural relevance for the amino acid change Arg120Ser in ScbR of strain M600 and A3(2) N2 seems likely, since this was the only variant form of the protein found and based on the lineage of *S. coelicolor* (Weaver *et al.*, 2004) the mutation occurred independently in the two strains. Also, strains M600 and A3(2) did not undergo any mutagenic treatment (Weaver *et al.*, 2004).

The *cpk* gene cluster as the actual target of the *Streptomyces coelicolor* butanolide system

Usually, production of the two coloured antibiotics Act and Red is investigated in *S. coelicolor*. There is only one additional antibiotic known in *S. coelicolor* M145, the colourless c̲alcium-d̲ependent a̲ntibiotic, CDA (Hopwood and Wright, 1983; Lakey *et al.*, 1983). Although Act and Red production is influenced by the *S. coelicolor* butanolide system, it becomes more and more evident that none of these antibiotics is the actual target of the regulatory system (Takano, 2006).

Genome analysis of *S. coelicolor* M145 has identified many other secondary metabolite gene clusters in addition to those required for the production of Act, Red and CDA (Bentley *et al.*, 2002). Most of them, however, are still "orphan" (Gross, 2007) and the metabolites produced by the encoded biosynthetic pathways are unknown. Most likely, these gene clusters are not expressed under typical growth conditions. Alternatively, the biosynthesis genes may well be expressed, but the related compound has not been identified. One of these orphan gene clusters is the *cpk* gene cluster. CPK denotes for c̲oelicolor p̲oly̲ketide. Polyketides compose a group of chemical compounds to which numerous antibiotics are assigned and it can also be assumed that the *cpk* gene cluster encodes for a polyketide. However, no details about the chemical structure of the substance can be predicted, nor was it known when this secondary metabolite is produced by the bacterium and whether the natural compound shows antibiotic activity (Pawlik *et al.*, 2007).

There is evidence that the butanolide system in *S. coelicolor* primarily provides regulation for the *cpk* cluster. The previously observed effects on the production of Act and Red seem to be downstream effects only, e.g. by competition for common precursors (chapters 2 and 3, and (Takano, 2006). The γ-butyrolactone receptor ScbR binds upstream of *cpkO*, the pathway-specific activator of the *cpk* cluster (Takano *et al.*, 2005a) and represses its expression. For Act and Red, on the other hand, such direct regulation could not be shown (Takano *et al.*, 2001). Interestingly, *scbR2* was identified in the *cpk* gene cluster (Fig. 2 in chapter 1). ScbR2 shows 32% amino acid identity with ScbR$_{M145}$. Based on amino acid sequence similarity, ScbR2, together with e.g. TylQ from *S. fradiae* and BarB from *S. virginiae*, appear to belong to a subfamily of γ-butyrolactone receptor homologues that act as transcriptional regulators in the absence of ligand binding (Takano *et al.*, 2001).

The presence of several γ-butyrolactone receptor homologues in a *Streptomyces* strain or even in a single gene cluster is not unusual. The tylosin biosynthetic gene cluster in *S. fradiae* contains two, TylP and TylQ (Bignell *et al.*, 2007), of which TylQ is proposed to be a pseudo-receptor without ligand binding ability. AlpW and the pseudo-receptor AlpZ in the alpomycin gene cluster in *S. ambofaciens* (Bunet *et al.*, 2008) are another example. In addition to ScbR and ScbR2, which are both involved in the regulation of the *cpk* gene cluster (Takano *et al.*, 2005a; chapter 2), *S. coelicolor* possesses at least 3 other chromosomally encoded ScbR homologues (CprA, CprB, SCO6323). CprA and CprB have been reported to affect Act and Red production and sporulation in *S. coelicolor* (Onaka *et al.*, 1998) and based on their typical alkaline pI values (pI 9.53 and 9.77, respectively) are supposed to be pseudo-receptors (Kitani *et al.*, 2008). CprA and CprB have been shown *in vitro* to bind to site O$_A$ in front of *cpkO* in *S. coelicolor* and thus share at least one target site with ScbR (Takano, personal communication). Furthermore, CprA and CprB bind the DNA target of ArpA from *S. griseus* (Sugiyama *et al.*, 1998). Horinouchi and co-workers even suggested that most of the receptor homologues recognize and bind the same target DNAs because of the great similarity of the amino acid sequence consisting of the helix–turn–helix DNA recognition domain (Nishida *et al.*, 2007). Assuming corresponding functions for CprA, CprB and putatively SCO6323 also *in vivo* would add a whole new level of complexity to the regulatory processes taking place at the ScbR target sites in *S. coelicolor*.

yCPK - Triggering the production of a novel natural product in *Streptomyces coelicolor*

Given the important role of ScbR for antibiotic production in *S. coelicolor* we investigated the function of ScbR2. A *scbR2* deletion mutant was created and conditionally produced a yellow pigment which was not observed previously, replacing the red (Red) and blue (Act) antibiotics. To confirm that production of the yellow compound resulted solely from the deletion of *scbR2*, *scbR2* was reintroduced into M145Δ*scbR*. Act, Red and yellow compound production was restored to the levels observed in the parental strain M145. Under specific growth conditions, the yellow pigment was also produced in M145, however, in much lower levels. Using a newly created *act*, *red*, *cda* triple antibiotic biosynthesis gene cluster deletion mutant, the yellow substance was shown not to be related to the three characterized *S. coelicolor* secondary metabolites. Additional deletion of single *cpk* genes or of the entire *cpk* cluster, however, resulted in disappearance of the yellow colour (Fig. 4 in chapter 3). Therefore, we concluded that production of the yellow pigment (yCPK (y̲ellow c̲oelicolor p̲oly̲k̲etide)) requires a functional *cpk* gene cluster and thus assigned yCPK to the hitherto orphan gene cluster.

yCPK production in the parental M145 was observed for only a few hours during an early stage of growth, whereas in the *scbR2* mutant production was clearly prolonged (Fig. 4 in chapter 3). This may be the reason why, despite comprehensive metabolite analysis of *S. coelicolor*, the yellow pigment has not been described earlier and the *cpk* gene cluster remained orphan. In agreement with the observed yCPK production, transcription studies using quantitative RT-PCR analysis indicated that ScbR2 represses expression of *cpkO* and consequently also of biosynthetic genes in the *cpk* cluster (Fig. 6 in chapter 3). Together with the activator CpkO, ScbR2 constitutes "negative feedback regulation" of yCPK production: CpkO activates the *cpk* genes that will provide for the production of the yellow pigment. Slightly delayed, CpkO also activates *scbR2*. ScbR2 eventually represses *cpkO* and thus the production of yCPK and its own expression. And since *cpkO* is regulated by ScbR this control cycle is directly interlinked with the *S. coelicolor* butanolide system (Fig. 2; chapter 3).

abCPK - A new antibiotic from *Streptomyces coelicolor*

After the discovery of the novel yellow pigment, growth conditions were optimized for the production of yCPK. In particular, supplementing the culture media with glutamate increased yCPK production significantly (Fig. 2 in chapter 3). On the contrary, in medium with casamino acids yCPK production was suppressed (Fig.1 in chapter 3). This clearly suggests an effect of the nitrogen source on the *cpk* biosynthetic pathway (chapter 3). A possible explanation for the stimulating effect of glutamate is discussed later in this chapter. Different nitrogen sources including other amino acids should be used to characterise this dependency and possibly further increase the production of the *cpk* metabolite. Feeding of radio-labelled glutamate might serve to demonstrate incorporation of the provided nitrogen in yCPK.

Coinciding with yCPK production under the optimised conditions we also observed a previously undetected antibacterial activity against Gram+ and Gram- bacteria in *S. coelicolor*. Bioactivity tests using, for example, the Δ*act red* (*cpk*) *cda* mutants revealed that also the antibiotic activity is related to the *cpk* gene cluster (Fig. 6 in chapter 3). Furthermore, increased antibiotic production was observed with the yCPK overproducing *scbR2* mutant (Fig. 7 in chapter 3). Thus, at first, it seemed that yCPK is not only a novel pigment, but actually a yet undiscovered antibiotic in *S. coelicolor*.

The yellow substance was partially purified to further investigate its properties. Surprisingly, the yellow pigment yCPK, as well as an antibacterial substance (abCPK (antibiotic coelicolor polyketide)) were isolated separately, and thus these are not identical metabolites. In HPLC analysis the yellow and the antibacterial *cpk* compounds were detected in different fractions, and yCPK could never be shown to have antibiotic activity (data not shown). Furthermore, yCPK is only found secreted into the medium in *S. coelicolor* liquid cultures (Fig. 5 in chapter 3), whereas active abCPK can be isolated from cells lacking the yellow pigmentation with polar solvents such as methanol (Fig. 8 in chapter 3). However, we explicitly do not exclude the possibility that abCPK is present outside, or attached to, the cell. Even more importantly, yCPK and abCPK production was uncoupled by deletion of the *cpk* biosynthesis gene *scF*. In the *scF* mutant, yCPK production is abolished whereas

abCPK activity was still observed. In contrast, a mutant in the *scF* homologue *cpkH* produced neither the yellow pigment nor the antibiotic (Fig. 7 in chapter 3). ScF and CpkH are predicted secreted oxidoreductases (Pawlik *et al.*, 2007) and are substrates of the *S. coelicolor* Tat protein secretion system (Widdick *et al.*, 2006). We therefore propose that the last steps of the biosynthesis of the *cpk* products take place outside of the cell. There, CpkH catalyses the formation of abCPK that is subsequently converted to yCPK by ScF. We have stopped yCPK synthesis and simultaneously increased the production of abCPK (chapter 3) which may facilitate high-yield extraction, purification and structure elucidation of the antibacterial *cpk* metabolite in future work. Subsequently, the potential of the novel antibiotic agent as a pharmaceutical product will be further characterised.

Our results suggest that the antibiotic compound (abCPK) is an intermediate and not the end product of the *cpk* biosynthetic pathway (Fig. 2; chapter 3). This raises questions about the natural function of abCPK. Due to the immediate conversion of the antibiotic into a yellow pigment, for which no antibacterial activity could be shown, a specific role as antibacterial agent secreted by *S. coelicolor* seems unlikely. It may well be that the intermediate abCPK is nothing more than a "shunt product" of the *cpk* biosynthetic pathway. Therefore, the unknown role of the putative *cpk* end product yCPK is of high interest and remains to be further investigated. As mentioned above, results presented in chapters 2 and 3 suggest that yCPK does not possess antibacterial activity. It is produced by M145 grown in darkness and only during a short period of growth. Therefore also a role in the protection of cells from photodamage, similar to the function of yellow carotenoids in *S. coelicolor* (Takano *et al.*, 2005b), appears unlikely. It has been suggested that the majority of low-molecular-weight organic compounds made and secreted by microbes, and even many of those that show antibiotic activity, play roles as cell-signalling molecules in the environment and regulate gene expression in microbial populations, and possibly the interactions of these populations with the surrounding organisms (Yim *et al.*, 2007). This idea is particularly fascinating when conferred on the *cpk* products which themselves are controlled by the γ-butyrolactone signalling molecules. However, no experimental data are available and thus one can only speculate about the function of yCPK as well as about a putative additional or alternative role for the antibacterial abCPK.

A hypothetical model of the *cpk* biosynthesis pathway and its regulation

Figure 2 shows a comprehensive hypothetical model of the *cpk* biosynthetic pathway and its regulation. The model summarizes the results presented in this thesis (chapters 2 and 3) and in relevant publications, amino acid sequence (homology) analysis of the *cpk* gene products (using blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi (Ye *et al.*, 2006), http://www.expasy.org/tools/blast/ (Gasteiger *et al.*, 2003)), Tatfind (http://signalfind.org/tatfind.html (Rose *et al.*, 2002)), SignalP (http://www.cbs.dtu.dk/services/SignalP/ (Bendtsen *et al.*, 2004)) (data not shown) and (Pawlik *et al.*, 2007)) and computational prediction of the functions of Cpk proteins in cellular processes of *S. coelicolor* (using KEGG (Kanehisa *et al.*, 2009) and BRENDA (Chang *et al.*, 2009)).

CpkPα and CpkPβ are predicted to form together the branched-chain α-keto acid dehydrogenase (BCDH) complex. The CpkP$\alpha\beta$ holoenzyme is mapped to catalyse the conversion of 2-oxoglutarate into succinyl-CoA in the TCA cycle of *S. coelicolor* (KEGG). Interestingly, homologues of the putative class-III aminotransferase CpkG were shown to form 2-oxoglutarate from glutamate (BLAST, ExPASy, BRENDA). Thus, CpkG may catalyse a direct anaplerotic reaction of the TCA cycle. However, both reactions are reversible and thus, CpkP$\alpha\beta$ and CpkG could also provide glutamate from TCA cycle intermediates. Glutamate and other amino acids could also be used by CpkG as donor substrate in a direct transamination (Pawlik *et al.*, 2007;Yonaha *et al.*, 1992) of a metabolite of the *cpk* biosynthesis pathway. A corresponding hypothetical role for CpkG is discussed in detail later and is denoted in Fig. 2. Any of these proposed enzymatic functions may be involved in the stimulating effect of glutamate on the production of the *cpk* products reported in chapters 2 and 3. Clearly, more work is needed to elucidate the dependency of yCPK and abCPK production on primary metabolism and on specific nitrogen sources in particular.
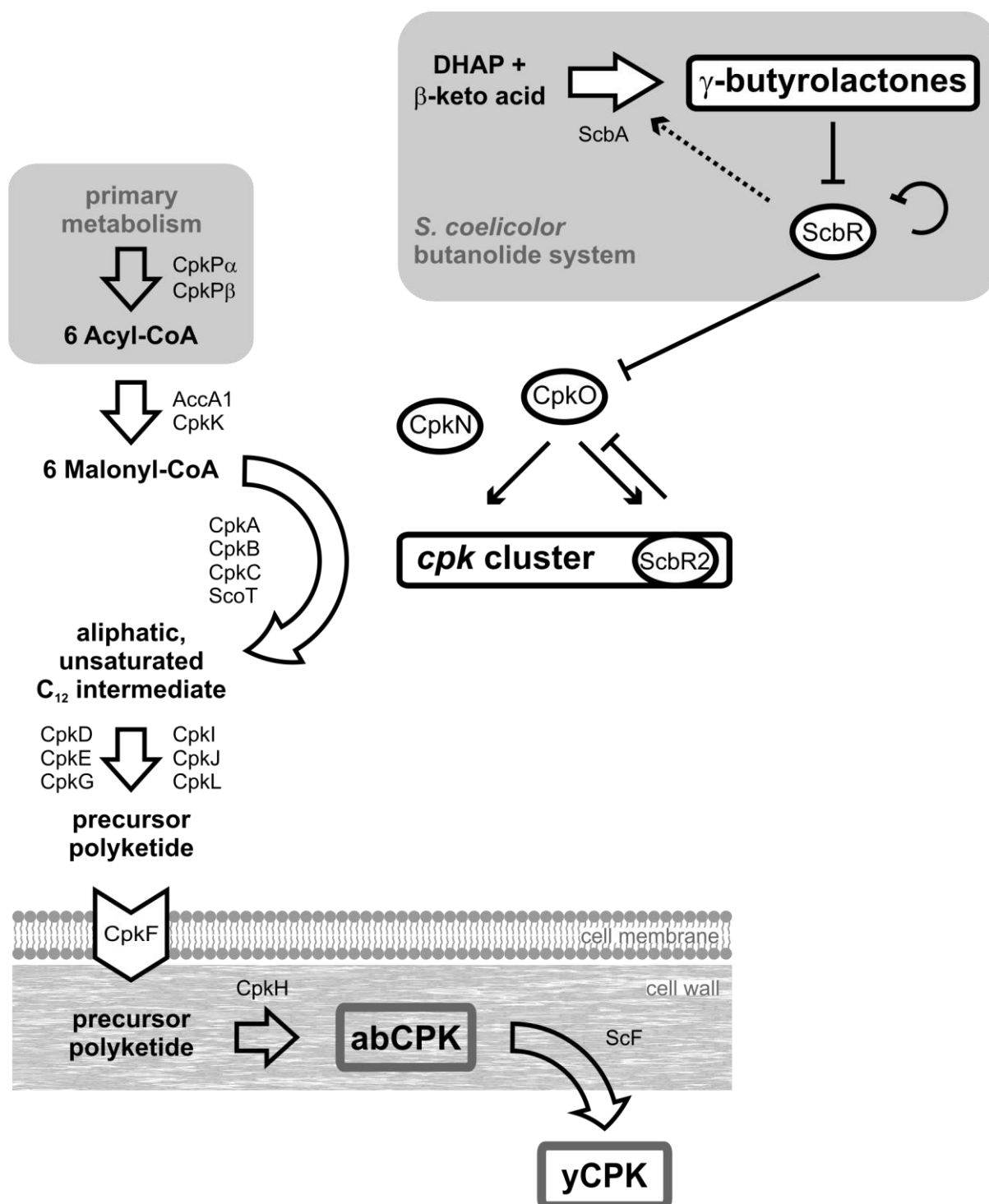
Figure 2 Hypothetical model of the cpk biosynthetic pathway and its regulation (explanation in the text)

During antibiotic production the acetyl-CoA flux may be redirected from entering the TCA cycle into the biosynthesis of the polyketide precursor malonyl-CoA (de Carvalho Lima Lobato *et al.*, 2007). The putative AccA1/CpkK Acyl-CoA carboxylase complex (Pawlik *et al.*, 2007) is predicted to form malonyl-CoA from acetyl-CoA and

other acyl-CoA species (Rodriguez and Gramajo, 1999). In this case, homeostasis of the TCA cycle may be impaired and its metabolic balance then depends on 3-phosphoglycerate and anaplerotic reactions (de Carvalho Lima Lobato *et al.*, 2007), such as the one suggested for CpkG.

In total six malonyl-CoA units are used as starter and extender units in the formation of a first aliphatic, unsaturated C_{12} CPK polyketide intermediate by the Type I polyketide synthase (PKS) subunits CpkA, CpkB and CpkC (Pawlik *et al.*, 2007). The thioesterase ScoT most probably removes acyl residues different from malonyl-CoA that block the PKS extension modules in CpkABC (Kotowska *et al.*, 2002; Kotowska *et al.*, 2009). The primary polyketide may subsequently be further modified by CpkE, a predicted epoxide hydrolase, CpkI, a putative 3-oxoacyl-ACP reductase, CpkJ, a nucleoside-diphosphate-sugar epimerase homologue, the hypothetical protein CpkL, and by CpkD (Pawlik *et al.*, 2007). Although CpkD is predicted to be a secreted FAD-binding protein in view of a Twin-Arginine motif in its putative signal peptide (Signal P and (Pawlik *et al.*, 2007)), experimentally, it could not be verified as substrate for the Tat secretion system (Widdick *et al.*, 2006).

The tailored *cpk* precursor polyketide could then be transported out of the cell via the putative transmembrane efflux protein CpkF (Pawlik *et al.*, 2007). CpkH and ScF are Tat-dependent, cell wall-associated (Widdick *et al.*, 2006) FAD-binding oxidoreductase homologues (Pawlik *et al.*, 2007). Based on the phenotype of a *scF* mutant that produces abCPK, but not yCPK (chapter 3), we propose that the precursor is first converted into the antibiotic abCPK by CpkH, and then subsequently modified to the yellow pigment yCPK by ScF. In *S. coelicolor* liquid cultures, abCPK could be extracted best from the cells, in contrast to the secreted yCPK (chapters 2 and 3). This is in agreement with the localisation of CpkH and ScF in the cell wall: although also found diffusing into solid media (chapter 3), the intermediate abCPK may mainly stay attached to the cell before being further converted to yCPK.

ScF and CpkH show high sequence homology (blastp) and protein structure similarity (M. W. Fraaije, personal communication) to AknOx, a secreted oxidoreductase in *S. galilaeus* involved in aclacinomycin biosynthesis. AknOx catalyses the modification of the sugar residue of this antibiotic (Alexeev *et al.*,

2007). Also, pyridoxal phosphate (PLP) dependent transaminases, such as CpkG, are involved in the synthesis of aminosugars that subsequently serve as glycone moiety in *Streptomyces* secondary metabolites (Liu and Thorson, 1994) (e.g. DnmJ in daunosamine biosynthesis in the doxorubicin producer *S. peuceticus* (Hutchinson and Colombo, 1999)). Interestingly, with FdtB from *Aneurinibacillus thermoaerophilus*, among several amino group donors tested, including glutamine, alanine, and aspartate, only glutamate resulted in a turnover (Pfoestl *et al.*, 2003). This may suggest a putative role of glutamate and CpkG in aminosugar synthesis in the *cpk* biosynthetic pathway. This would be consistent with a function of CpkG in the direct transamination of a *cpk*-related metabolite proposed above. Furthermore, also the epimerase CpkJ is predicted to act on sugars (Pawlik *et al.*, 2007). A likely function of CpkJ is the conversion of a C-1 activated nucleoside diphospho-sugar, typically the TDP-D-glucose derivative TDP-4-keto-6-deoxy-D-glucose. Usually, the resulting activated deoxy-L-hexose isomer is then subsequently incorporated in glycosylated antibiotics (Walsh *et al.*, 2003). All these findings suggest the glycosylation of an intermediate of the *cpk* biosynthetic pathway and the presence of a sugar moiety attached to the polyketide aglycon of yCPK and abCPK.

On the other hand, the *cpk* gene cluster does not provide various additional gene functions needed for, e.g., glycon synthesis and also no glycosyltransferase is predicted in the *cpk* gene cluster (Pawlik *et al.*, 2007). Generally, the genes encoding the dedicated glycosyltransferases are found in the same gene cluster that contains the genes to make the particular activated deoxysugars and the aglycon (Walsh, 2002).

However, we cannot exclude that the missing enzymatic functions for the putative glycosylation of the *cpk* product are encoded elsewhere on the *S. coelicolor* chromosome, outside of the *cpk* cluster. In the elloramycin producer *Streptomyces olivaceus* it has been demonstrated that a small glycon biosynthesis gene cluster and the polyketide aglycon biosynthesis gene cluster are present on separated chromosomal loci (Ramos *et al.*, 2008). In *Saccharopolyspora spinosa*, only one set of biosynthetic genes is present providing rhamnose for both primary structural components (cell walls) and a secondary metabolite (spinosyns). Also in *S. spinosa* the sugar biosynthesis genes are found far away from the spinosyn aglycon gene

cluster (Madduri *et al.*, 2001). Biosynthesis of the heterocyst envelope polysaccharide (HEP) in the filamentous cyanobacterium *Anabaena sp.* is mainly encoded in the HEP genetic island. Glycosyltransferases essential for HEP production, however, are located outside the HEP island (Wang *et al.*, 2007). The given examples suggest that also in yCPK and abCPK production yet unidentified gene functions may be encoded outside of the *cpk* gene cluster, involved in synthesis and attachment of a putative glycon moiety. CpkG, CpkJ, and possibly also glutamate, may be involved in deoxy-sugar biosynthesis; CpkH and ScF may successively modify the glycon resulting in abCPK and yCPK, respectively. Structure elucidation of the *cpk* metabolites may eventually reveal whether they have one or more sugars attached, or whether a sugar-like structure is present in an unglycosylated *cpk* polyketide.

CpkN is a predicted *Streptomyces* antibiotic regulatory protein (SARP) and overexpression of CpkN leads to enhanced production of yCPK (Pawlik *et al.*, poster 47, Biology of *Streptomyces*, Münster, Germany, 2009). Its function may therefore resemble that of CpkO, the other SARP regulator and pathway-specific activator of the gene cluster. A third, presumably negative, regulatory gene, ScbR2, together with CpkO may constitute negative feedback regulation of the *cpk* genes (chapter 2, Fig. 2). As yet, for none of these transcriptional regulators DNA binding sites and direct target genes have been characterised.

Via CpkO that is under the direct control of ScbR (Takano *et al.*, 2005a), regulation of the *cpk* gene cluster is linked to the *S. coelicolor* butanolide system (Fig. 2). The results presented in this thesis thus provide for the first time a continuous regulatory cascade from the bacterial hormones (the γ-butyrolactones) to secondary metabolites (abCPK and yCPK) in *S. coelicolor* (chapters 2 and 3). Furthermore, the identification of abCPK is the first successful example of the induced production of a hitherto undescribed antibiotic in a streptomycete by the manipulation of a regulatory gene (*scbR2*) (chapter 3). This concept could be applied not only to the *cpk* gene cluster, but to virtually all orphan gene clusters; not only in *S. coelicolor* but essentially in all streptomycetes and even beyond the genus. Hence, awakening these sleeping genes affords a promising chance for the discovery of countless novel secondary metabolites and therewith potentially new antibiotics.

Chapter 6.1

Nederlandse samenvatting

Voorwoord

Dit hoofdstuk bevat een vereenvoudigde samenvatting van dit proefschrift. Het is bedoeld voor leken die geïnteresseerd zijn en die zich altijd al hebben afgevraagd wat wij doen en waarom. Om deze reden zal deze samenvatting geen aanspraak maken op volledigheid noch zullen wetenschappelijke details besproken worden. Er zal een inzicht gegeven worden in wetenschappelijk denken en werken, de resultaten die bevonden zijn in dit proefschrift en de waarde van deze resultaten voor de wereld buiten het laboratorium.

Nieuwe antibiotica in de strijd tegen de toenemende aantallen resistente ziekteverwekkers

De zoektocht van de mens naar nieuwe geneesmiddelen in het gevecht tegen infectieziekten veroorzakende bacteriën zal hoogstwaarschijnlijk nooit eindigen. In het beste geval zullen we in deze strijd altijd een stapje voor liggen op resistente bacteriën. Ondanks alle alternatieve (geneeskundige) benaderingen en passende kritiek op het gebruik en misbruik van antibiotica kunnen we nauwelijks ontsnappen aan deze wapenwedloop. In de afgelopen jaren is onze voorsprong beangstigend geslonken en nieuws over 'superbugs', dodelijke ziekteverwekkers waar tegen (vrijwel) geen geneesmiddel voorhanden is, vindt zijn weg, óók in de massamedia. Het aantal werkelijk nieuwe antibiotica dat beschikbaar komt is om verschillende redenen al tientallen jaren aan het verminderen (Fig. 1 in hoofdstuk 1), terwijl de ontwikkeling van resistentie in de ziekteverwekkers doorgaat. Alleen al om deze redenen is de zoektocht naar medisch actieve natuurstoffen weer in het middelpunt van de wetenschappelijke interesse komen te staan.

Meer dan driekwart van de klinisch gebruikte antibiotica zijn natuurstoffen of licht gemodificeerde natuurstoffen. Deze worden tegenwoordig echter normaliter niet meer uit hun natuurlijke producenten verkregen, maar vooral door een biotechnologisch of puur chemisch synthese proces. Niettemin zijn de grondstoffen afkomstig uit schimmels en bepaalde bodembacteriën, voornamelijk uit de Streptomyceten. Deze produceren ze als zogenaamde secundaire metabolieten. In tegenstelling tot de primaire metabolieten zijn stoffen die niet noodzakelijk zijn voor

de groei van Streptomyceten, maar die onder bepaalde omstandigheden wel voordelen met zich meebrengen. In het geval van antibiotica kan men zich gemakkelijk voorstellen dat ze dienen als "chemische wapens" tegen concurrerende micro-organismen in de strijd om voedingsstoffen. Echter, de productie van secundaire metabolieten is ingewikkeld en "duur" voor de natuurlijke producenten (de *Streptomyces* bacteriën) en daarom onderhevig aan een strenge regulering in de levende cel. Voor biotechnologische productie van antibiotica is detailkennis nodig van deze regulatie processen, idealiter gevolgd door hun uitschakeling resulterend in sterk verhoogde productie.

Een bacterieel hormoon regelt de productie van antibiotica in bodembacteriën

Een van de regulatie systemen voor antibioticaproductie in *Streptomyces* is gebaseerd op "bacteriële hormonen". Deze signaalmoleculen worden geproduceerd door de bacterie zelf en verspreiden zich over de miljarden cellen van een *Streptomyces* kolonie die groeit op een vaste voedingsbodem (Fig. 2). Deze signaalmoleculen reguleren dat alle cellen tegelijkertijd beginnen met de productie van antibiotica waardoor de doeltreffendheid ervan zal toenemen. Bovendien worden er in alle cellen van de kolonie voorafgaand aan de productie mechanismen geactiveerd om de producerende cellen te beschermen tegen hun eigen antibiotica; met andere woorden: om "zelfmoord" te voorkomen.

In het modelorganisme *Streptomyces coelicolor* bestaat dit regulerende hormoonsysteem in principe uit drie belangrijke componenten: ten eerste een "zender", het ScbA eiwit, dat de signaalmoleculen produceert; ten tweede de hormonen zelf (chemisch gezien zijn dit γ-butyrolactonen of butanoliden), en ten derde een "ontvanger" of receptor eiwit, genaamd ScbR, een afkorting van *Streptomyces coelicolor* butanolide receptor. Normaliter voorkomt de hormoonreceptor ScbR de productie van antibiotica in de bacterie. Echter, in de aanwezigheid van de γ-butyrolactonen herkent en bindt ScbR deze hormonen. Hierdoor verandert de vorm van het ScbR eiwit zodat deze receptor de productie van antibiotica niet langer kan onderdrukken; de bacteriële hormonen hebben de

antibioticaproductie aldus geïnduceerd. Daarnaast reguleert ScbR ook de productie van de hormonen zelf.
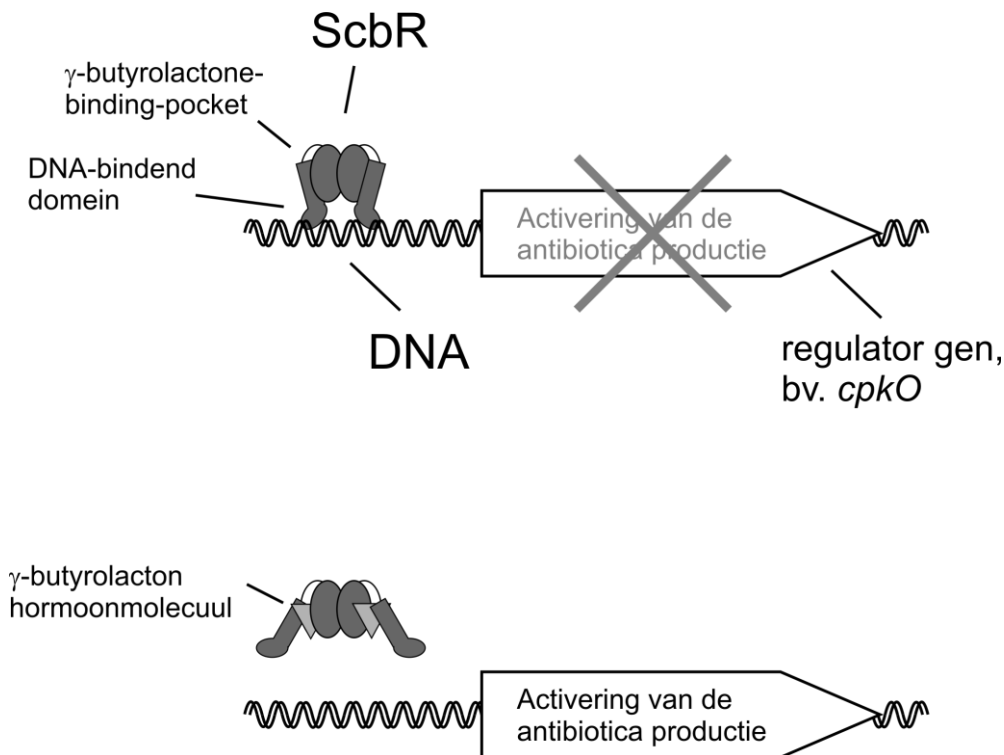
Figuur 1 **Werkingswijze van de hormoon receptor ScbR in *Streptomyces coelicolor*** (Omschrijving in de tekst)

Figuur 1 laat de werkingswijze van ScbR zien: in het begin van de groei wordt productie van antibiotica geblokkeerd door binding van ScbR op specifieke locaties aan het bacteriële DNA. Hier onderdrukt ScbR de aflezing van genen, kleine stukjes van het erfelijk materiaal DNA, die verantwoordelijk zijn voor bepaalde functies in de cel. In *S. coelicolor* activeren deze genen de antibioticaproductie, maar niet als ze worden geblokkeerd door de binding van ScbR. ScbR heeft een specifieke rumtelijke structuur om te kunnen binden aan het DNA, het zogenaamde DNA-bindende domein. Daarnaast zit in het receptoreiwit een speciale holte (Eng. pocket) waarin de γ-butyrolactonen passen (Fig. 6 in hoofdstuk 1). Na een periode van actieve groei worden deze hormoonmoleculen geproduceerd en binden ze in deze holte in ScbR waardoor de ruimtelijke structuur van dit receptoreiwit veranderd, met name in de organisatie van het DNA-bindende domein. Het domein is daardoor niet meer

functioneel met als gevolg dat ScbR niet langer kan binden aan het DNA. Op deze manier worden de eerder onderdrukte genen nu geactiveerd en komt de antibioticaproductie op gang.

ScbR$_{M600}$ - Ontdekking van en onderzoek naar een variant van de bacteriële hormoon receptor ScbR

Al decennia lang is *S. coelicolor* het onderwerp van studie in laboratoria over de hele wereld. Ondertussen kan een hele stamboom van natuurlijke en gekweekte stammen van deze bacteriesoort getekend worden. De typische laboratorium stam is *S. coelicolor* M145, maar vaak wordt ook *S. coelicolor* M600 gebruikt.

We hebben in M600, en alleen in M600, een variant van de hormoonreceptor ScbR (ScbR$_{M600}$) ontdekt. Vergeleken met de originele ScbR (ScbR$_{M145}$) laat ScbR$_{M600}$ een kleine verandering (mutatie) zien in de nabijheid van de γ-butyrolactone-bindende holte. Aangezien de antibioticaproductie in M600 sterk verschilt van die in M145 en, zoals hierboven beschreven, ScbR een centrale rol speelt in de regulering hiervan, hebben we de effecten van de mutatie in ScbR$_{M600}$ onderzocht.

Daartoe hebben we de genen die coderen voor ScbR eiwitten overgebracht van deze *S. coelicolor* stammen in een andere bacterie, *Escherichia coli*. *E. coli* is het werkpaard in de microbiële genetica en in ons geval heeft het geholpen bij de productie van relatief grote hoeveelheden van beide ScbR eiwitvarianten. Op deze manier konden hun eigenschappen worden bepaald "in de reageerbuis" (*in vitro*). Tot onze verrassing bleek de mutatie in ScbR$_{M600}$ invloed te hebben op de stabiliteit en de DNA-bindende eigenschappen van de receptor, ondanks zijn ligging dichtbij de hormoon-bindende holte en niet in de nabijheid van het DNA-bindende domein. De binding van de γ-butyrolactonen aan ScbR, echter, is verassenderwijze niet beïnvloed (hoofdstuk 4).

Idealiter wilden we de effecten van de waargenomen verschillen ook in het levende organisme (*in vivo*) ophelderen. Hiertoe hebben we een *S. coelicolor* stam gecreëerd die identiek is aan stam M145, maar die wel de verandering in ScbR draagt die we gevonden hebben in ScbR$_{M600}$. Ieder waar te nemen verschil tussen de uitgangsstam M145 en de nieuwe stam kan om deze reden toegeschreven worden

aan de verandering in het receptoreiwit. Zoals hierboven beschreven, deactiveert ScbR bepaalde genen door binding aan het DNA en het is in feite deze capaciteit tot binding die verschilt in de ScbR$_{M600}$.variant. Daarom hebben we in de nieuwe stam de activiteit van enkele genen onderzocht die door ScbR gecontroleerd worden. Hierbij hebben we daadwerkelijk een verschil vastgesteld: Het gen voor de "zender" ScbA (verantwoordelijk voor de productie van de bacteriële hormonen) was minder of later actief. Dit resulteerde ook in een afname of vertraging in de productie van de γ-butyrolactonen. De mutatie in ScbR$_{M600}$ had echter geen groot effect op de productie van antibiotica in *S. coelicolor* (hoofdstuk 4).

De *cpk* genen als het eigenlijke doel van het bacteriële hormoonsysteem

In *S. coelicolor* is veel onderzoek gedaan naar de productie van twee gekleurde antibiotica: het rood (Eng. red) gepigmenteerde prodigiosin, afgekort als "Red", en het blauw gekleurde actinorhodine, afgekort als "Act" (Fig. 2). Natuurlijk vergemakkelijkt de kleur van deze stoffen hun opsporing omdat er geen complexe instrumenten nodig zijn. Verder is er slechts nog één ander antibioticum bekend in *S. coelicolor* M145, het kleurloze calcium-afhankelijke antibioticum CDA (Eng. calcium dependent antibiotic). Hoewel de productie van Act en Red beïnvloed wordt door het bacteriële hormoonsysteem, wordt het meer en meer duidelijk dat geen van deze antibiotica het eigenlijke doel van het regulatiesysteem is.

Figuur 2 **Gepigmenteerde antibiotica in *Streptomyces coelicolor***

Te zien zijn drie koloniën van de bacterie. De lichte kleuring van de linker kolonie wordt veroorzaakt door het rood gekleurde antibioticum Red dat in de cellen blijft. De middelste kolonie scheidt het blauw gekleurde antibioticum Act uit in kleine druppels.

De gehele genetische informatie (het genoom) van *S. coelicolor* is ontcijferd. Naast de groepen van genen, of "clusters", die nodig zijn voor de productie van Act, Red en CDA konden er nog diverse andere genclusters voor secundaire metabolieten geïdentificeerd worden. De meeste van deze zijn echter nog steeds "wezen": alleen

het bestaan van de genclusters is bekend, maar niet welke stoffen deze produceren. Een van deze verweesde genclusters is het *cpk* gen cluster. CPK staat voor <u>c</u>oelicolor <u>polyk</u>etide. Polyketides zijn een groep chemische verbindingen waaronder verscheidene met antibiotica activiteit zijn. Aangenomen wordt dat ook het *cpk* gencluster codeert voor een polyketide. Er kunnen echter geen verdere details betreffende de chemische structuur van de stof worden voorspeld. Bij de aanvang van ons onderzoek was het onbekend of en wanneer dit secundaire metaboliet door de bacterie geproduceerd wordt, en ook of deze natuurstof eventueel antibiotische activiteit had.

Er zijn aanwijzingen dat het hormoonsysteem in *S. coelicolor* voornamelijk de regulering van het *cpk* cluster dient. De eerder waargenomen effecten op de productie van Act en Red lijken slechts ondergeschikte effecten te zijn. Dat kan, naast aanvullende bewijzen, geconcludeerd worden doordat de γ-butyrolactone receptor ScbR direct aan het *cpkO* gen bindt (Fig. 1). Het CpkO eiwit is de activator van het gehele *cpk* cluster. Voor Act en Red kon zo'n rechtstreeks effect nooit worden aangetoond. Het *cpk* cluster wordt nog interessanter door de identificatie van *scbR2* als deel van deze groep van genen (Fig. 2 in hoofdstuk 1). Zoals al aangegeven met zijn naam, ScbR2 is zeer vergelijkbaar met de hormoonreceptor ScbR, zodat we er van uitgaan dat ook het ScbR2 eiwit een regulerende rol heeft.

yCPK – het voorgenomen teweegbrengen van de productie van een nieuwe natuurstof

Wetend dat ScbR een prominente rol heeft in antibioticaproductie in *S. coelicolor* hebben we ook de exacte functie van ScbR2 nader onderzocht. Daartoe hebben we met genetische technieken het *scbR2* gen verwijderd uit *S. coelicolor*. Deze *scbR2* mutant vertoont een opmerkelijke verandering: onder bepaalde voorwaarden produceert deze in plaats van de rode (Red) en blauwe (Act) antibiotica een gele kleurstof die nog nooit eerder is waargenomen in *S. coelicolor* (Fig. 4 in hoofdstuk 2). Is dit pigment een onbekend (eind)product van het *cpk* cluster? En daarmee ook het eigenlijke doel van het gehele bacteriële hormoonsysteem? In een andere mutant werden de genclusters van alle eerder bekende *S. coelicolor* antibiotica (Act, Red en CDA) verwijderd; de gele stof werd echter nog steeds geproduceerd. Indien er

echter ook genen van het *cpk* cluster worden verwijderd, verdwijnt de gele kleur. De productie van de gele (Eng. yellow) stof, nu genaamd yCPK (yellow *coelicolor* polyketide) hangt dus inderdaad uitsluitend af van het *cpk* cluster. Hiermee is de rol van ScbR2 voor de productie van yCPK echter nog niet opgehelderd. Uit het tijdsverloop van de productie en een analyse van de genactiviteit blijkt dat ScbR2 normaliter de productie van yCPK blokkeert. De activator CpkO vormt samen met ScbR2 vermoedelijk een bepaalde "negatieve terugkoppeling": CpkO alleen activeert echter de *cpk* genen die vervolgens zorgen voor de productie van de kleurstof. Enigszins vertraagd activeert CpkO ook ScbR2. ScbR2 van zijn kant deactiveert CpkO en daarmee de productie van yCPK en – na het gedane werk – ook productie van zichzelf. En aangezien *cpkO* ook door de γ-butyrolactone receptor ScbR gereguleerd wordt is deze controle cyclus direct verbonden met het bacteriële hormoon systeem (Fig. 2 in hoofdstuk 5).

Deze resultaten zijn met name ook belangrijk omdat hier voor het eerst in *S. coelicolor* alle schakels van de keten tussen de bacteriële hormonen en de productie van een natuurstof (yCPK) worden beschreven. Bovendien is deze studie het eerste succesvolle voorbeeld voor het teweegbrengen van de productie van een tot nu toe onbekende natuurstof in een streptomyceet door de relatief simpele verandering van een regulator (ScbR2) eiwit. Dit concept zou niet alleen kunnen worden toegepast op het *cpk* gencluster, maar op vrijwel alle verweesde genclusters, en dit niet alleen in *S. coelicolor*, maar in principe in alle streptomyceten. Het wekken van deze slapende genen biedt dus veelbelovende kansen voor de ontdekking van honderden nieuwe secundaire metabolieten, waaronder potentiële nieuwe antibiotica (hoofdstuk 2).

abCPK – Een nieuw antibioticum van *Streptomyces coelicolor*

Na de ontdekking van de voorheen onbekende gele stof (yCPK) werden de groeiomstandigheden voor de producerende bacteriële stam systematisch veranderd en geoptimaliseerd voor een gemaximaliseerde yCPK productie. Met name het toevoegen van het aminozuur glutamaat verhoogde de productie van yCPK aanzienlijk. Alleen dan zagen we dat *S. coelicolor* stammen, welke de gele kleurstof in grote hoeveelheden produceren, ook een antibiotische activiteit laten zien. Aan de andere kant zijn mutanten die geen yCPK meer produceren ook niet meer in staat andere bacteriën te doden. En net als de productie van yCPK is ook de antibiotische

activiteit afhankelijk van een functioneel *cpk* gencluster en wordt deze verhoogd door het toevoegen van glutamaat (Fig. 7 in hoofdstuk 3).

Deze waarnemingen geven dus aan dat we met het yCPK onderzoek niet alleen de productie van een nieuwe gele kleurstof teweeg hadden gebracht (hetgeen zoals hierboven beschreven van groot fundamenteel wetenschappelijk belang is), maar dat we bovenal een tot dusver onbekend antibioticum gevonden hadden (hetgeen tot eventuele commerciële toepassingen zou kunnen leiden). De gele kleurstof werd geïsoleerd van de vele andere stoffen die geproduceerd worden in een bacteriële cel om yCPK in de meest pure vorm te verkrijgen zodat zijn specifieke eigenschappen onderzocht konden worden. Hierbij bleek dat zowel de gele stof yCPK als een antibacteriële stof geïsoleerd konden worden, maar dat deze niet identiek aan elkaar waren. Het gele yCPK vertoonde echter geen antibiotische activiteit. Daarom moet er een andere - kleurloze - stof aanwezig zijn die net als yCPK afhangt van het *cpk* gen cluster en die het werkelijke antibioticum is. Om deze reden noemen we deze stof abCPK (<u>a</u>nti<u>b</u>iotisch <u>c</u>oelicolor <u>p</u>oly<u>k</u>etide). De aanmaak van de *cpk* cluster producten is een stapsgewijs proces. Ten minste twee van deze stappen vinden waarschijnlijk plaats buiten de bacteriële cel. Als we de CPK productie in één van deze twee stappen onderbreken verdwijnen zowel de gele kleurstof (yCPK) als ook het antibioticum (abCPK). Als we de andere stap onderbreken wordt er geen yCPK meer gevormd, maar de antibacteriële activiteit kan nog wel worden bepaald. Deze antibacteriële activiteit wordt hierdoor zelfs nog iets hoger wat op een verhoogde aanmaak van abCPK wijst.

Al onze waarnemingen kunnen worden verklaard met de volgende hypothese: een voorloper van het antibioticum wordt gevormd binnen de cellen en naar buiten getransporteerd. Daar wordt de voorloper omgezet in het actieve antibioticum, abCPK, wat effectief is tegen concurrerende bacteriën. Het abCPK kan echter ook verder omgezet worden in de gele kleurstof yCPK (Fig. 2 in hoofdstuk 5).

Op basis van dit model proberen we nu de productie van de gele kleurstof te voorkomen en tegelijkertijd de productie van het antibioticum verder te verhogen zodat de winning, zuivering en nadere karakterisering van abCPK beter mogelijk zal worden. Vervolgens kan de relevantie en het potentieel van het nieuwe antibioticum als een medicijn bepaald worden (hoofdstuk 3).

Chapter 6.2

Deutsche Zusammenfassung

Vorwort

Dieses Kapitel enthält eine vereinfachte Zusammenfassung dieser Doktorarbeit. Es richtet sich an interessierte Laien, die sich schon immer gefragt haben, was wir da eigentlich machen und warum. Diese Zusammenfassung erhebt daher weder den Anspruch auf Vollständigkeit, noch sollen wissenschaftliche Details besprochen werden. Vielmehr soll ein Einblick in naturwissenschaftliches Denken und Arbeiten, in die während dieser Doktorarbeit erzielten Ergebnisse und deren Bedeutung für die Welt außerhalb des Labors gegeben werden.

Neue Antibiotika für den Kampf gegen zunehmend resistente Krankheitserreger

Die Suche nach neuen Medikamenten im Kampf gegen Infektionskrankheiten wird wohl niemals enden. Bestenfalls sind wir im ewigen Wettlauf zwischen resistenten Krankheitserregern und neuen Wirkstoffen immer einen kleinen Schritt voraus. Und trotz aller alternativ(medizinisch)er Ansätze und angebrachter Kritik am Ge- und Missbrauch von Antibiotika können wir uns diesem Hochrüsten nur schwerlich entziehen. In den letzten Jahren ist unser Vorsprung erschreckend geschmolzen und Berichte über "*superbugs*", tödliche Keime gegen die kein bekanntes Arzneimittel mehr wirkt, finden Ihren Weg auch in die Massenmedien. Die Zahl wirklich neuer Antibiotika auf dem Mark sinkt aus den verschiedensten Gründen seit Jahrzehnten (Abb. 1 in Kapitel 1), während die Resistenzentwicklung der Erreger fortschreitet. Nicht zuletzt aus diesen Gründen ist die Suche nach medizinisch wirksamen Naturstoffen wieder ins Zentrum des wissenschaftlichen Interesses gerückt.

Mehr als drei Viertel der medizinisch eingesetzten Antibiotika sind Naturstoffe oder leicht veränderte Naturstoffe. Diese werden heute jedoch meist nicht mehr direkt aus ihren natürlichen Produzenten gewonnen, sondern biotechnologisch oder, seltener, in rein chemischen Verfahren produziert. Nichtsdestotrotz stammen diese Wirkstoffe eigentlich aus Pilzen oder bestimmten Bodenbakterien, vor allem aus den Streptomyceten. Von diesen werden sie als so genannte Sekundärmetabolite produziert. Metabolite sind Substanzen, die an den biochemischen Reaktionen beteiligt sind, die in allen Lebewesen ablaufen. Im Gegensatz zu Primärmetaboliten

sind Sekundärmetabolite zwar nicht Teil lebenswichtiger Prozessen in den Bakterienzellen, unter bestimmten Umständen können sie aber von Vorteil sein. Im Falle der Antibiotika, zum Beispiel, kann man sich diese leicht als „chemische Waffen" gegen konkurrierende Mikroorganismen im Kampf um Nährstoffe vorstellen. Allerdings ist die Herstellung dieser Sekundärmetabolite aufwendig und „teuer" für die natürlichen Produzenten (die Bodenbakterien) und unterliegt daher in der lebenden Zelle einer strengen Kontrolle. Für die biotechnologische Herstellung von Antibiotika ist Detailwissen über diese Regulationsprozesse notwendig. Bestenfalls kann dann diese Regulation „ausgeschalten" werden, resultierend in einer stark erhöhten Antibiotikaproduktion.

Ein bakterielles Hormonsystem reguliert die Antibiotikaproduktion in Bodenbakterien

Ein Kontrollsystem der Antibiotikaproduktion in Streptomyceten basiert auf „bakteriellen Hormonen". Diese Signalstoffe werden von den Bakterien selbst produziert und verbreiten sich zwischen den vielen Milliarden Zellen einer Streptomyceten-Kolonie. Dadurch wird sichergestellt, dass alle Zellen gleichzeitig mit der Antibiotikaproduktion beginnen, wodurch deren Effizienz erhöht wird. Außerdem werden dadurch in allen Zellen der Kolonie vor der Antibiotikaproduktion Mechanismen zur Eigenresistenz aktiviert, sozusagen um einen „Selbstmord" zu verhindern.

Im Modell-Streptomyceten *Streptomyces coelicolor* besteht dieses regulatorische Hormonsystem im Wesentlichen aus drei Komponenten: Erstens, einem „Sender", ScbA, der die Signalstoffe herstellt; zweitens, den Hormonen selbst (chemisch gesehen sind diese so genannte γ-*Butyrolaktone* oder *Butanolide*); und drittens, einem „Empfänger" oder *Rezeptor*, genannt ScbR, eine Abkürzung für <u>S</u>*treptomyces coelicolor* <u>B</u>utanolid-<u>R</u>ezeptor. Dieser Hormon-Rezeptor ScbR verhindert normalerweise die Produktion der Antibiotika in den Bakterien. Werden nun allerdings die γ-Butyrolaktone synthetisiert, erkennt und bindet ScbR an die Hormonmoleküle. Dadurch verändert sich die Form des Rezeptors und ScbR kann die Antibiotikaproduktion nicht mehr unterdrücken; die bakteriellen Hormone haben

die Antibiotikaproduktion eingeleitet. Darüber hinaus beeinflusst ScbR auch die Produktion der Hormone selbst.
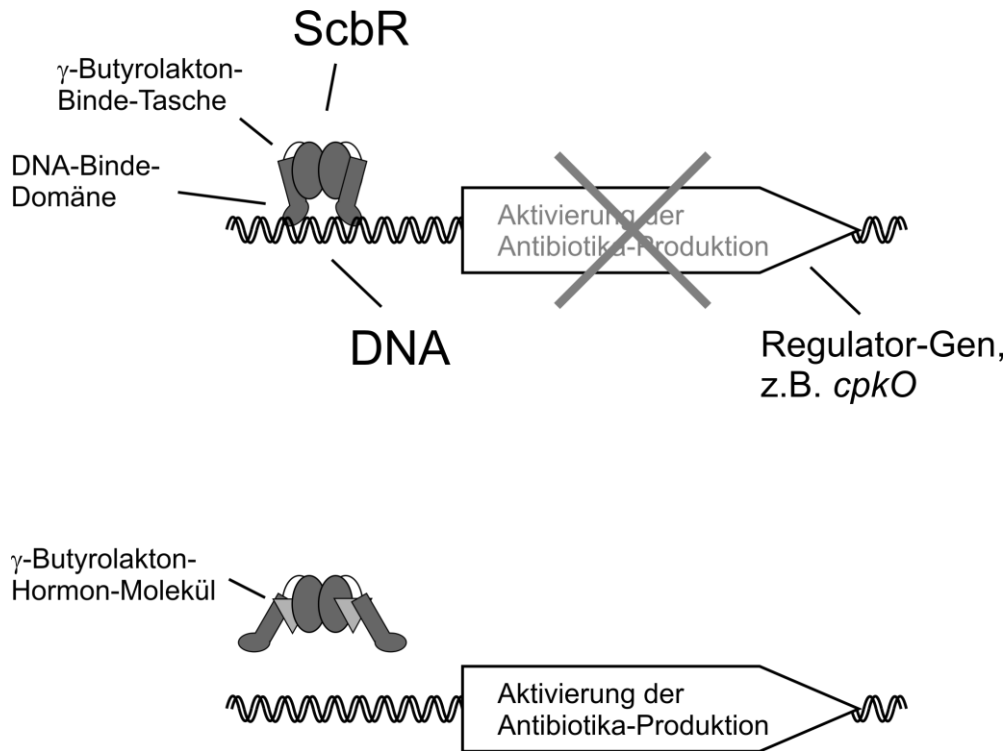
Abbildung 2 **Wirkungsweise des Hormonrezeptors ScbR in** *Streptomyces coelicolor*

(Erklärung im Text)

In Abbildung 1 ist die Wirkungsweise von ScbR veranschaulicht: Um die Antibiotikaproduktion anfangs zu verhindern, bindet ScbR an bestimmten Stellen in die Erbsubstanz der Zelle, die *DNA*. Dort blockiert ScbR bestimmte *Gene,* kleine Abschnitte der DNA die für bestimmte Funktionen der Zelle verantwortlich sind. In unserem Fall sorgen diese Gene für den Beginn der Antibiotikaproduktion; nicht jedoch, wenn sie durch ScbR deaktiviert werden. Um an die DNA binden zu können, bildet ScbR eine bestimmte Struktur aus, die so genannte DNA Binde-Domäne. Außerdem besitzt der Rezeptor eine speziell geformte „Tasche" in die die γ-Butyrolakton-Hormone passen (Abb. 6 in Kapitel 1). Befindet sich ein Hormonmolekül in dieser Tasche verändert dies die Form des Rezeptors,

insbesondere die Anordnung der DNA Binde-Domäne. Diese ist so nicht mehr funktionsfähig, ScbR kann nicht mehr an die DNA binden, die bisher blockierten Gene werden aktiv und die Antibiotikaproduktion beginnt.

ScbR$_{M600}$ - Die Entdeckung und Untersuchung einer Variante des bakteriellen Hormonrezeptors ScbR

Streptomyces coelicolor ist seit Jahrzehnten Forschungsobjekt in Laboren in der ganzen Welt. Mittlerweile kann ein ganzer Stammbaum von natürlichen oder im Labor gezüchteten Stämmen dieser Art aufgestellt werden. Der typische Labor-Stamm ist *S. coelicolor* M145, oft wird aber auch *S. coelicolor* M600 verwendet.

Wir haben in M600, und nur in M600, eine Variante des Hormonrezeptors ScbR entdeckt (ScbR-M600), die im Vergleich zu ScbR vom Stamm M145 (ScbR$_{M145}$) eine einzige kleine Veränderung in der Nähe der γ-Butyrolakton-Binde-Tasche aufweist. Da die Antibiotikaproduktion in M600 stark von der in M145 abweicht und da ScbR die beschriebene zentrale Rolle in deren Regulation spielt, haben wir die Auswirkungen dieser Mutation untersucht. Dafür haben wir das Gen, das für ScbR codiert, aus *S. coelicolor* in ein anderes Bakterium, *Escherichia coli*, übertragen. *E. coli* ist das „Arbeitspferd" in der mikrobiellen Genetik und in unserem Fall hilft es bei der Herstellung von vergleichsweise großen Mengen beider ScbR-Varianten. So konnten deren Eigenschaften in Reagenzglasversuchen (*in vitro*) untersucht werden. Entgegen unserer Erwartung hat die Mutation Auswirkungen auf die Stabilität und die DNA-Bindung des Rezeptors. Und das, obwohl sie nicht im Bereich der DNA-Binde-Domäne, sondern in der Nähe der Hormon-Binde-Tasche zu finden ist. Die γ-Butyrolakton-Bindung dagegen ist überraschenderweise nicht beeinflusst (Kapitel 4).

Nun wollten wir die Auswirkungen der beobachteten Unterschiede auch im lebenden Organismus (*in vivo*) untersuchen. Dazu haben wir einen *S. coelicolor* Stamm geschaffen, der identisch ist mit Stamm M145, jedoch die Mutation in ScbR trägt, die wir in M600 gefunden haben. Jeder Unterschied zwischen dem „elterlichen" Stamm M145 und dem neuen Stamm kann also auf die Veränderung in dem Rezeptor zurückgeführt werden. Wie gesagt deaktiviert ScbR bestimmte Gene durch Bindung an die DNA (Abb. 1), und eben diese Bindeaktivität weicht in ScbR$_{M600}$ ab. Deshalb haben wir die Aktivität einiger durch ScbR kontrollierter Gene untersucht und

tatsächlich eine Veränderung festgestellt. Zum Beispiel war das Gen für den „Sender" ScbA (verantwortlich für die Produktion der bakteriellen Hormone) schwächer oder später aktiv. Folgerichtig konnten wir auch eine Abnahme oder Verzögerung der γ-Butyrolakton-Produktion messen. Ein signifikanter Einfluss der Mutation in $ScbR_{M600}$ auf die Antibiotikaproduktion in *S. coelicolor* war allerdings nicht festzustellen (Kapitel 4).

Die *cpk* Gene als eigentliches Ziel des bakteriellen Hormonsystems

In *S. coelicolor* wird üblicherweise die Produktion zweier farbiger Antibiotika untersucht, den rot (engl. „red") pigmentierten Prodigiosinen, abgekürzt mit „Red", und dem blau gefärbten Actinorhodin, kurz „Act". Abb. 2 in Kapitel 6.1 zeigt drei Kolonien des Bakteriums. Die leichte Pigmentierung der linken Kolonie stammt von dem rot gefärbten Antibiotikum Red, das in den Zellen verbleibt. Die mittlere Kolonie scheidet das blau gefärbte Antibiotikum Act als kleine Tröpfen aus. Die Farbigkeit dieser Substanzen erleichtert deren Nachweis natürlich ungemein, da keine aufwendigen Messinstrumente benötigt werden. Zusätzlich ist in *S. coelicolor* M145 nur noch ein weiteres Antibiotikum bekannt, das farblose kalziumabhängige Antibiotikum (<u>c</u>alcium <u>d</u>ependent <u>a</u>ntibiotic), CDA. Obwohl die Act- und Red-Produktion durch das bakterielle Hormonsystem beeinflusst wird, kristallisiert sich immer mehr heraus, dass keines dieser Antibiotika das eigentliche Ziel des Regulationssystems ist.

Seit die gesamte Erbinformation, das *Genom*, von *S. coelicolor* entschlüsselt ist, konnten neben den Gen-Gruppen (Cluster), die für Act, Red und CDA benötigt werden, noch weitere Sekundärmetabolit-Gen-Cluster identifiziert werden. Die meisten davon sind jedoch immer noch „Waisen": zwar ist die Existenz der Gen-Cluster bekannt ist, jedoch nicht welche Substanzen sie produzieren. Eines dieser verwaisten Gen-Cluster ist das *cpk* Gen-Cluster. CPK steht für <u>c</u>oelicolor <u>P</u>oly<u>k</u>etid. Polyketide sind eine Gruppe chemischer Verbindungen, der etliche heute eingesetzter Antibiotika zuzuordnen sind. Es kann davon ausgegangen werden, dass auch das *cpk* Gen-Cluster für ein Polyketid codiert. Jedoch können weder Details der chemischen Struktur der Substanz vorhergesagt werden, noch war

bekannt wann dieser Sekundärmetabolit von den Bakterien gebildet wird und ob der Naturstoff eventuell eine antibiotische Wirkung hat.

Nun gibt es hinweise, dass das Hormonsystem in *S. coelicolor* in erster Linie der Regulation des *cpk* Clusters dient. Die bisher beobachteten Auswirkungen auf die Produktion der Antibiotika Act und Red scheinen nur nachgelagerte Effekte zu sein. Das kann unter Anderem daraus geschlossen werden, dass der γ-Butyrolakton-Rezeptor ScbR wie in Abb. 1 gezeigt direkt an das *cpkO* Gen bindet, den Aktivator des gesamten *cpk* Clusters. Für Act und Red dagegen konnte dieser unmittelbare Einfluss nie gezeigt werden. Noch interessanter macht das *cpk* Cluster, dass *scbR2* als Teil dieser Gen-Gruppe identifiziert wurde (Abb. 2 in Kapitel 1). Wie schon der Name andeutet, ist ScbR2 dem Hormonrezeptor ScbR sehr ähnlich, und man kann von einer einer regulatorischen Funktion auch für ScbR2 ausgehen.

yCPK - Das gezielte Auslösen der Produktion eines neuen Naturstoffs

Mit dem Wissen um die herausragende Rolle von ScbR für die Antibiotikaproduktion in *S. coelicolor*, haben wir die genaue Funktion von ScbR2 untersucht. Dazu haben wir das *scbR2* Gen aus *S. coelicolor* entfernt (deletiert). Diese *scbR2* Deletionsmutante zeigt eine erstaunliche Veränderung: Unter bestimmten Bedingungen produziert sie anstelle der roten (Red) und blauen (Act) Antibiotika einen gelben Farbstoff, der niemals zuvor in *S. coelicolor* gefunden wurde (Abb. 4 in Kapitel 2). Ist dieses Pigment das unbekannte Endprodukt des *cpk* Clusters? Und damit auch das Ziel des gesamten bakteriellen Hormonsystems? In einer weiteren Mutante wurden die Gen-Cluster aller bisher bekannter *S. coelicolor* Antibiotika (Act, Red und CDA) entfernt; die gelbe Substanz wird immer noch gebildet. Werden allerdings zusätzlich Gene des *cpk* Clusters entfernt, verschwindet die gelbe Färbung. Die Produktion der gelben Substanz, jetzt yCPK (<u>y</u>ellow (dt. „gelbes") *coelicolor* <u>P</u>ol<u>y</u>ketid) genannt, hängt also tatsächlich nur vom *cpk* Cluster ab. Die Rolle von ScbR2 bei die Herstellung von yCPK war damit allerdings noch nicht geklärt. Der zeitliche Verlauf der Produktion und eine Genaktivitätsanalyse weißt darauf hin, dass ScbR2 normalerweise die Herstellung von yCPK abschaltet. Zusammen mit dem Aktivator CpkO bildet ScbR2 vermutlich eine Art „negative

Rückkopplung": CpkO schaltet die *cpk* Gene ein, die dann für Produktion des Farbstoffs sorgen. Ein wenig verzögert aktiviert CpkO auch *scbR2*. ScbR2 seinerseits deaktiviert *cpkO* und damit die Produktion von yCPK, und – nach getaner Arbeit – auch von sich selbst. Und da *cpkO* durch ScbR kontrolliert wird, steht dieser Regelkreis in direkter Verbindung mit dem bakterielle Hormonsystem (Abb. 2 in Kapitel 5).

Diese Ergebnisse sind deshalb so wichtig für zukünftige Projekte, weil damit erstmals in *S. coelicolor* lückenlos alle Schritte des Zusammenhangs zwischen den bakteriellen Hormonen bis hin zur Produktion eines Naturstoffes (yCPK) beschrieben werden. Diese Arbeit ist aber auch das erste erfolgreiche Beispiel dafür, dass in einem Streptomyceten durch die Manipulation eines Regulators (ScbR2) die Produktion einer bisher unbekannten Substanz ausgelöst wurde. Dieses Konzept könnte nicht nur auf das *cpk* Gen-Cluster sonder auf praktisch alle verwaisten Gen-Cluster angewendet werden. Und dies nicht nur in *S. coelicolor* sondern in praktisch allen Streptomyceten. So bietet sich durch das Aufwecken dieser „schalfenden Gene" die Chance auf die Entdeckung hunderter neuer Sekundärmetabolite und damit potentieller neuer Antibiotika (Kapitel 2).

abCPK - Eine neues Antibiotikum aus *Streptomyces coelicolor*

Nach der Entdeckung der bisher unbekannten gelben Substanz (yCPK) wurden die Wachstumsbedingungen für den produzierenden Bakterien-Stamm systematisch verändert und im Hinblick auf eine maximierte yCPK-Produktion optimiert. Insbesonders die Zufütterung der Aminosäure Glutamat erhöht die yCPK-Produktion merklich. Danach konnten wir zeigen, dass bei *S. coelicolor* Stämmen, die das gelbe Pigment in großer Menge bilden, auch eine antibiotische Aktivität zu beobachten ist. Mutanten dagegen, die kein yCPK mehr produzieren, können auch keine anderen Bakterien mehr abtöten. Und wie die Produktion von yCPK, hängt auch die antibiotische Aktivität auf einem funktionsfähigen *cpk* Gen-Cluster ab und ist durch die Zugabe von Glutamat erhöht (Abb. 7 in Kapitel 3). Zunächst sah also Alles danach aus, dass wir mit yCPK nicht nur einen neuen gelben Farbstoff zur Produktion gebracht hatten (was zwar wie oben beschrieben durchaus schon an sich von Bedeutung für die Grundlagenforschung ist), sondern tatsächlich ein bisher unbekanntes Antibiotikum entdeckt hatten (das möglicherweise von medizinischem

und kommerziellem Interesse sein könnte). Das gelbe Pigment wurde von den vielen anderen Substanzen die eine Bakterien-Zelle produziert isoliert, um so yCPK in möglichst reiner Form zu erhalten und dessen Eigenschaften näher zu untersuchen. Dabei stellte sich heraus, dass wir sowohl den gelben Farbstoff yCPK, als auch eine antibakterielle Substanz isolieren konnten, diese jedoch nicht identisch sind. Das gelbe yCPK zeigt keine antibiotische Wirkung. Stattdessen muss es also eine weitere – farblose - Substanz geben die wie yCPK vom *cpk* Gen-Cluster abhängt und die das eigentliche Antibiotikum darstellt. Diese Substanz nennen wir deshalb abCPK (antibiotisches *coelicolor* Polyketid). Die Herstellung der *cpk* Produkte verläuft schrittweise. Mindestens zwei dieser Schritte finden höchstwahrscheinlich außerhalb der Zelle statt. Unterbrechen wir die CPK-Produktion an einem dieser Schritte, verschwindet sowohl die gelbe Färbung (yCPK), als auch das Antibiotikum (abCPK). Wird der andere Produktionsschritt blockiert, wird zwar kein yCPK mehr synthetisiert, die antibakterielle Aktivität lässt sich jedoch noch immer nachweisen. Diese ist sogar stärker, was auf eine erhöhte Menge an abCPK hindeutet.

Alle unsere Beobachtungen sind mit der folgenden Hypothese zu erklären: Ein Vorläufer des Antibiotikums wird innerhalb der Zelle gebildet und nach Aussen transportiert. Dort wird der Vorläufer zum aktiven Antibiotikum, abCPK, umgewandelt und wirkt gegen konkurrierende Bakterien. Anschliessend wird abCPK in den gelben Farbstoff yCPK umgesetzt (Abb. 2 in Kapitel 5).

Basierend auf diesem Model versuchen wir nun die Produktion des Farbstoffes zu verindern und gleichzeitig die des *cpk* Antibiotikums weiter zu steigern, um so die Gewinnung, Aufreiningung und Charakterisierung von abCPK zu ermöglichen. Danach kann dann die Relevanz und das Potential des neuen Antibiotikums als Arzneimittel ermittelt werden (Kapitel 3).

Chapter 6.3

English summary

Preface

This chapter contains a simplified summary of this thesis. It is geared towards interested non-scientists who have always wondered what we are actually doing here, and why. Therefore, this summary neither claims to be exhaustive nor discusses scientific details in depth. Rather, this summary presents an insight into scientific thinking and working, the results obtained during this thesis and their significance for the world outside the laboratory.

New antibiotics to combat increasingly resistant pathogenic bacteria

The search for new drugs against infectious diseases will probably never end. At best, we are always one step ahead in the race between resistant pathogens and novel antibiotics. And despite all alternative (medicinal) approaches and appropriate criticism about the (mis)use of antibiotics we can hardly escape this arms race. In recent years, reports of *"superbugs"*, deadly germs against which no known drug is effective any longer, found their way into the mass media. For various reasons the number of genuinely new antibiotics on the market has been declining for decades (Fig. 1 in chapter 1) while development of resistance among the pathogens advances. Therefore, the search for pharmaceutically active natural products has moved back into the centre of scientific interest.

More than three quarters of the clinically used antibiotics are natural products or slightly modified natural products. Usually, however, these are no longer obtained directly from their natural producers, but are produced biotechnologically or, less commonly, in purely chemical processes. Nonetheless, these drugs originally derive mostly from fungi and certain soil bacteria, the streptomycetes. From these, antibiotics are produced as so-called secondary metabolites. Metabolites are substances that are part of the biochemical processes taking place in any living organism. In contrast to primary metabolites, secondary metabolites are not part of vital processes in the bacterial cells, but, nevertheless, can be beneficial under certain circumstances. In the case of antibiotics, for example, one can easily imagine these as "chemical weapons" against competing microorganisms in the struggle for

nutrients. However, the production of secondary metabolites is complex and "costly" for the natural producers and therefore is strictly regulated. Detailed knowledge of these regulatory processes may allow construction of strains that have lost this strict control of antibiotic synthesis and thus produce increased amounts, possibly resulting in a biotechnological production process.

A bacterial hormone system regulates antibiotic production in soil bacteria

One of the regulatory systems of antibiotic production in *Streptomyces* is based on "bacterial hormones". These signalling molecules are produced by the bacterium itself and spread among the billions of cells of a *Streptomyces* colony (Fig. 2). This ensures that all cells start to produce antibiotics simultaneously, thus increasing its efficiency. Furthermore, mechanisms of self-resistance are activated in all cells of the colony prior to the production of antibiotics, in order to prevent "suicide", so to speak.

In the model organism *Streptomyces coelicolor* this regulatory hormone system comprises three key players: first, a "sender", the ScbA protein, that is involved in the production of the signalling molecules; secondly, the hormones themselves (chemically these are γ-butyrolactones or butanolides); and, thirdly, a "receiver" or receptor protein, named ScbR, an abbreviation of <u>S</u>treptomyces <u>c</u>oelicolor <u>b</u>utanolide <u>r</u>eceptor. The hormone receptor ScbR normally prevents production of antibiotics in *S. coelicolor*. In the presence of the γ-butyrolactones, however, ScbR recognizes and binds the hormones. Thereby the shape of the ScbR protein changes and the receptor can no longer suppress the production of antibiotics: the bacterial hormones thus have induced antibiotic production. In addition, ScbR also regulates the production of the hormones themselves.
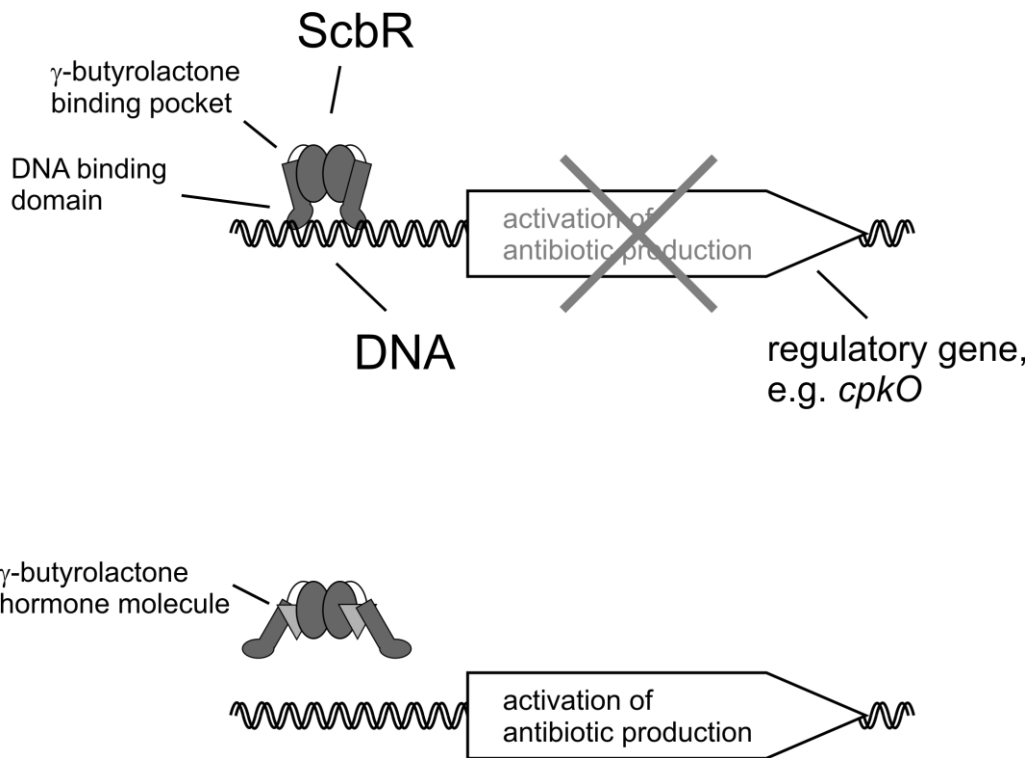
Figure 3 **Mode of action of the hormone receptor ScbR in** *Streptomyces coelicolor*

(Explanation in the text)

Figure 1 illustrates the mode of action of ScbR: To initially prevent the production of antibiotics, ScbR binds at specific sites to the bacterial DNA. When ScbR is bound, it represses genes, small portions of the DNA responsible for distinct functions of the cell. In our case, these genes cause the activation of antibiotic production, but not if they are blocked by the binding of ScbR. To be able to bind to the DNA, ScbR forms a particular structure, the so-called DNA binding domain. In addition, the receptor protein has a specially shaped "pocket" in which the γ-butyrolactone hormones fit (Fig. 6 in chapter 1). If a hormone molecule gets into the pocket, this changes the shape of the receptor, in particular the arrangement of the DNA binding domain. The domain is not functional anymore and ScbR can no longer bind to the DNA. Thus, the previously silenced genes are now active and the production of antibiotics begins.

ScbR$_{M600}$ - Discovery and investigation of a variant of the bacterial hormone receptor ScbR

For decades, the soil bacterium *Streptomyces coelicolor* has been studied in laboratories throughout the world. Therefore, a whole family tree of natural and genetically engineered strains of this species is available for research. The typical laboratory strain is *S. coelicolor* M145, but often *S. coelicolor* M600 is also used.

We have discovered in M600, and only in M600, a variant of the hormone receptor ScbR (ScbR$_{M600}$) that shows a small change (a mutation) in the vicinity of the γ-butyrolactone binding pocket compared to ScbR from strain M145 (ScbR$_{M145}$). Since antibiotic production in strain M600 differs from that in M145 and, as described above, ScbR plays a central role in its regulation, we examined the effects of the mutation in ScbR$_{M600}$. To do so, we transferred the gene that codes for ScbR into another bacterium, *Escherichia coli*. *E. coli* is the "workhorse" in microbial genetics and in our case, it served as host for the production of comparably large amounts of both ScbR variants. Thus, the properties of both ScbR variants could be determined "in the test tube" (*in vitro*). Contrary to our expectations, the mutation reduced the stability and the DNA binding ability of ScbR$_{M600}$. This is surprising since the mutation's location is near the hormone binding pocket, not in the vicinity of the DNA binding domain. Surprisingly, binding of the γ-butyrolactones was not affected in the ScbR$_{M600}$ mutant protein (chapter 4).

In the next step we wanted to investigate the effects of the mutation in ScbR$_{M600}$ also in the living organism (*in vivo*). For this purpose, we constructed a *S. coelicolor* strain which is identical to strain M145, but carries the mutation in ScbR that we detected in ScbR$_{M600}$. Any difference between the "parental" strain M145 and the new strain can therefore be attributed to the variant receptor protein. As described above, ScbR disables certain genes by binding to the DNA (Fig. 1), and it is this binding activity that differs in ScbR$_{M600}$. Therefore, we studied the activity of some genes controlled by ScbR and, indeed, we determined a change in the gene activity. For example, the gene coding for the "sender" ScbA (involved in production of the bacterial hormones) was activated later or at a lower level. Consequently, we could also see a delay or decrease in γ-butyrolactone production. A significant influence of the mutation in

ScbR$_{M600}$ on antibiotic production in *S. coelicolor* M145, however, was not evident (chapter 4).

The *cpk* genes as the actual target of the bacterial hormone system

Usually, the production of two coloured antibiotics, the red-pigmented prodigiosins, abbreviated as "Red", and the blue-coloured actinorhodin, "Act", is investigated in *S. coelicolor*. Fig. 2 in chapter 6.1 shows three colonies of the bacterium. The slight pigmentation of the left colony derives from the red-pigmented antibiotic Red that remains in the cells. The middle colony secretes the blue-coloured antibiotic Act as small droplets. Of course, the pigmentation of these substances makes their detection much easier, since no complex instruments are needed. There is only one additional antibiotic known in *S. coelicolor* M145, the colourless calcium-dependent antibiotic, CDA. Although Act and Red production is influenced by the bacterial hormone system, it is becoming clearer and clearer that none of these antibiotics is the actual target of the regulatory system.

In recent years the entire genetic information stored in the genome of *S. coelicolor* has been decoded. In addition to the groups of genes (gene clusters) required for the production of Act, Red and CDA, further secondary metabolite gene clusters were identified. Most of them, however, are still "orphan": only the existence of the gene cluster is known, but not what substances they produce *in vivo*. One of these orphan gene clusters is the *cpk* gene cluster. CPK denotes for coelicolor polyketide. Polyketides compose a group of chemical compounds to which numerous antibiotics are assigned. It appeared likely that also the *cpk* gene cluster encodes for a polyketide. However, no details about the chemical structure of the substance could be predicted, nor was it known when this secondary metabolite is produced by the bacterium and whether the natural compound shows antibiotic activity.

There is evidence that the hormone system in *S. coelicolor* primarily provides regulation for the *cpk* cluster. The previously observed effects on the production of the antibiotics Act and Red seem to be downstream effects only. In addition to other evidence, this can be concluded from the finding that the γ-butyrolactone receptor ScbR binds in front of the *cpkO* gene (Fig. 1). CpkO is the activator of the entire *cpk* cluster. For Act and Red, on the other hand, such a direct effect could never be

shown. The *cpk* cluster became even more interesting when *scbR2* was identified as part of this group of genes (Fig. 2 in chapter 1). As already indicated by its name, ScbR2 is very similar to the hormone receptor ScbR and one can also expect a regulatory role for ScbR2.

yCPK - Triggering the production of a novel natural product

Knowing of the importance of ScbR for antibiotic production in *S. coelicolor* we decided to investigate the role of ScbR2 in detail. Therefore, we removed (deleted) the *scbR2* gene from *S. coelicolor*. This *scbR2* deletion mutant showed a remarkable change: under certain conditions replacing the red (Red) and blue (Act) antibiotics it produces a yellow pigment that has never been observed in *S. coelicolor* before (Fig. 4 in chapter 2). Is this pigment the unknown end product of the *cpk* cluster? And hence the actual target of the entire bacterial hormone system? In another mutant strain the gene clusters of all previously known *S. coelicolor* antibiotics (Act, Red and CDA) were removed; the yellow compound was still produced. If, however, genes of the *cpk* gene cluster were also deleted, the yellow colour disappeared as well. Production of the yellow substance, now called yCPK (yellow coelicolor polyketide), thus indeed depended solely on the *cpk* gene cluster. Nevertheless, the role of ScbR2 in yCPK production remained to be clarified. The time course of production and analysis of the activities of several genes related to the *cpk* gene cluster indicated that ScbR2 normally shuts down the production of yCPK. Together with the activator CpkO, ScbR2 constitutes "negative feedback regulation": CpkO activates the *cpk* genes that will provide for the production of the yellow pigment. CpkO also activates *scbR2*, however, this happens slightly delayed. ScbR2 deactivates *cpkO*, and thus terminates the production of yCPK and - after the work has been done - also of itself. Since *cpkO* is regulated by the γ-butyrolactone receptor ScbR this control cycle is directly interlinked with the bacterial hormone system (Fig. 2 in chapter 5).

These results are of particular importance because here for the first time in *S. coelicolor* all links of the chain between the bacterial hormones and the production of a natural compound (yCPK) are described. Furthermore, this study is the first successful example of the induced production of a hitherto unknown natural compound in a streptomycete by the manipulation of a regulator (ScbR2). This

concept could be applied not only to the *cpk* gene cluster, but to virtually all orphan gene clusters; and this not only in *S. coelicolor* but essentially in all streptomycetes. Hence, awakening these sleeping genes affords a promising chance for the discovery of hundreds of novel secondary metabolites and therewith potential new antibiotics (chapter 2).

abCPK - A new antibiotic from *Streptomyces coelicolor*

After the discovery of the previously unknown yellow substance (yCPK), growth conditions for the producing bacterial strain were systematically altered and optimized in terms of maximizing yCPK production. In particular, feeding of the amino acid glutamate increased yCPK production significantly. Only then we could show that *S. coelicolor* strains that produce the yellow pigment in high amounts also show antibacterial activity. On the other hand, mutants that do not produce yCPK any more are also no longer able to kill other bacteria. And just as the production of yCPK, the antibiotic activity requires a functional *cpk* gene cluster and is increased by the addition of glutamate (Fig. 7 in chapter 3). Thus, at first, it seemed that yCPK is not only a novel pigment (which is, as described above, already of importance for basic research), but actually a yet undiscovered antibiotic (potentially of pharmaceutical and commercial interest) in *S. coelicolor*. To further investigate its properties, the yellow substance was separated from the many other compounds produced by the bacterial cells in order to obtain yCPK in the purest possible form. It turned out that both the yellow pigment yCPK, as well as an antibacterial substance could be isolated, but these were not identical. The yellow yCPK showed no antibiotic activity. Thus, there must be another - colourless - substance instead that just as yCPK requires the *cpk* gene cluster for synthesis, and which is the actual antibiotic. Therefore, we call this substance abCPK (antibiotic coelicolor polyketide). Synthesis of the *cpk* cluster products is a stepwise process. At least two of the intermediate steps most probably take place outside the bacterial cells. When interrupting CPK production in one of these steps, both the yellow pigmentation (yCPK) and the antibiotic (abCPK) disappeared. When blocking the other step, no yCPK was formed any longer, whereas antibacterial activity could still be observed.

All our observations could be explained by the following hypothesis: A precursor of the antibiotic is formed inside and transported out of the cell. There, the precursor is

converted into the active antibiotic, abCPK, and is effective against competing bacteria. The abCPK molecule is then further transformed into the yellow pigment yCPK (Fig. 2 in chapter 5).

Based on this model, we are now trying to prevent production of the pigment aiming to simultaneously increase the production of the *cpk* antibiotic. Following extraction and purification of abCPK, the compound will be subjected to structural characterization. Of strong interest, also, the relevance and the potential of the novel antibiotic agent as a pharmaceutical product will be determined (chapter 3).

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N.H. Hsiao, M. Gottelt, and E. Takano. Regulation of antibiotic production by bacterial hormones. *Methods Enzymol*. 2009;458:143-57.

E. Takano, M. Gottelt, S. Kol (Inventors). Antibiotic compositions, methods for providing the same and *Streptomyces coelicolor* mutants for use in such methods. *Patent No. 09172463.3-2406* filed on 07.10.2009 by Rijksuniversiteit Groningen.

M. Gottelt, S. Kol, J.P. Gomez-Escribano, M. Bibb, and E. Takano. Deletion of a regulatory gene within the *cpk* gene cluster reveals novel antibacterial activity in *Streptomyces coelicolor* A3(2). Submitted.

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Appendix 1.1

Supplementary data for chapter 2

Supplementary tables for chapter 2

Table S1 Bacterial strains, plasmids and cosmids used in this study

Primers	Primer sequence (5'-3')	Amplified gene*
cpkCrtfw	cgtaccttccacctcttcaac	*cpkC* (SCO6280)
cpkCrtrv	acctcgtccagctcgtatc	
cpkErtfw	gaggtcctgtccgtcatcga	*cpkE* (SCO6277)
cpkErtrv	ccctgggcaccgagttc	
cpkOrtfw	acgatgtggccggaactc	*cpkO* (SCO6280)
cpkOrtrv	gccgcaccgcagctt	
hrdBrtoutfw	catgcgcttcggactca	*hrdB* (SCO5820)
hrdBrtoutrv	actcgatctggcggatg	
ksEF	ctcgatcgagtggaccgtctc	*cpkIJ* (SCO6283)
ksER	ggatcgccgtcgttctggacg	
mscbR2F	ccttggggctcgacgatcgcgaccacggagcacgacatgattccggggatccgtcgacc	*scbR2* (SCO6286) and pIJ773
mscbR2R	gacgcggggtccgggtcagtgcggcgcgtcctgccgctctgtaggctggagctgcttc	
scbR2A_*Xba*I	cattctagagcgtagcaacgtgcggtggcc	*scbR2* (SCO6286)
scbR2FW3_*Xba*I	ctgtctagaccttaccgttcgacgagtgcg	
scbR2-REV	tggcggaacgcggcgtagc	
scbR2rtfw	cggtgctccggcagatc	
scbR2rtrv	ccgcagcacgacatcgt	
scbRA2	gcgtagcaacgtgcggtggcc	
SCBR-FW	cgtccaccagcaggccgatgt	
scbRFW2	gtcgaactcgggcgtcagctc	
scbRFW3	ccttaccgttcgacgagtgcg	

* Description of the amplified gene. The SCO numbers given in brackets are taken from EMBL Accession No. AL645882.

Supplementary figures for chapter 2
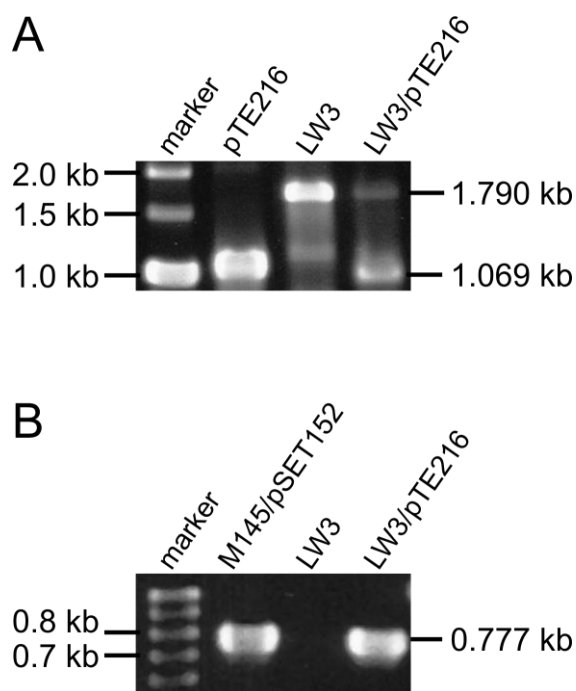
Figure S1 Genotype analysis of *S. coelicolor* strains LW3 (M145 *scbR2*::*aac*(3)IV) and LW3/pTE216 (pIJ82 harbouring *scbR2*) using PCR. (A) PCR was carried out using primers scbR2A and scbR2FW3 to amplify a 1069 bp *scbR2* and a 1790 bp *scbR2*::*aac*(3)IV fragment. The *scbR2* fragment was amplified from pTE216 (pIJ82 harbouring *scbR2*), from genomic DNA of LW3 and LW3/pTE216 (*scbR2* complemented LW3). Relevant marker bands are given in kb on the left. **(B)** PCR was carried out using primers JGattB-fwd and JGattPint-rev to give a 777 bp product when pSET152 or its derivatives integrated into the chromosome. PCR was conducted using genomic DNA of a M145/pSET152, LW3 and LW3/pTE216 (*scbR2* complemented LW3). Relevant marker bands are given in kb on the left.

Appendix 1.2

Supplementary data for chapter 4

Supplementary tables for chapter 4

Table S1 Plasmids and cosmids used in this study

Name	Description	Reference
pDrive	PCR product cloning vector	Qiagen
pDrive-ScbR	pDrive harbouring $scbR_{M145}$	This study
pGEM-T Easy	PCR product cloning vector	Promega
pIJ2925	pUC18 derivative with a multi-cloning site flanked by *Bgl*II	(Janssen & Bibb, 1993)
pIJ6120	pIJ2925 derivative containing $scbR_{M145}$	(Takano *et al.*, 2001)
pKC1132	conjugative plasmid bearing Apr^r *rep*^{pUC}	(Kieser *et al.*, 2000)
pTE51	pDrive containing $scbR_{M600}$ with additional mutations in $scbR_{M600}$ (t587a) and the $scbR_{M600}$ promoter region (a30g in *scbA*)	This study
pTE53	pIJ2925 containing $scbR_{M600}$ with additional mutations in $scbR_{M600}$ (t587a) and the $scbR_{M600}$ promoter region (a30g in *scbA*)	This study
pTE56	pIJ2925 containing $scbR_{M600}$ with an additional mutation in the $scbR_{M600}$ promoter region (a30g in *scbA*)	This study
pTE58	pIJ2925 containing $scbR_{M600}$	This study
pTE63	pGEM-T Easy containing the $scbR_{M145}$ region with additional mutations in $scbR_{M145}$ (c636t) and *scbA* (c308t)	This study
pTE64	pGEM-T Easy containing the $scbR_{M600}$ region with an additional mutation in *scbA* (t77c)	This study
pTE88	pST101 with *gfp* replaced by *Bam*HI/*Hind*III $scbR_{M145}$ fragment from pDrive-ScbR	This study
pTE203	pGEM-T Easy containing the $scbR_{M600}$ region with an additional mutation in *scbB* (c644t)	This study
pTE211	pGEM-T EASY containing the $scbR_{M600}$ region and an additional 1·4 kb fragment containing truncated *scbA* and $scbR_{M600}$ genes	This study
pTE212	pKC1132 containing the $scbR_{M600}$ region	This study
pTE213	pGEM-T EASY containing the $scbR_{M145}$ region	This study
pTE214	pKC1132 containing the $scbR_{M145}$ region	This study
pTST101	expression plasmid containing *malE-gfp* fusion under T7and *rhaBAD* control	J. Altenbuchner, unpublished
pUZ8002	RK2 derivative with a defective *oriT* (*aph*); not self-transmissible mobilization vector for *oriT*-containing plasmids	(Flett *et al.*, 1997)

Table S2 Primers used for (qRT-)PCR experiments

Primers	Primer sequence (5'-3')
actII-4rtfw	gacgcgggactggatctct
actII-4rtrv	tgcgcgatattgctttcg
BamETseq1	catggatcctcgagcagcagcatgcccggta
BamRCseq31enh	catggatccatgcgtgcacatgggacgagg
ETS10	cttcggtatccagctgaccggga
ETS10_DIG(5')	DIG-cttcggtatccagctgaccggga
ETS3	tatccagctgaccgggaacgcgtc
ETS6	atacagaacagctcggcatcac
ETS7	tgatgccgagctgttctgtatg
ETseq3	ttcggcggtcagtccttcccggtc
hrdBrtoutfw	catgcgcttcggactca
hrdBrtoutrv	actcgatctggcggatg
MalE-ScbR1	caaggacggatccatggccaagc
MalE-ScbR2	ggtgcggaagcttcggcggtcag
RCseq31	ggagcaggccggtacgtcctc
redDrtfw	tcatgggagtgcggagaac
redDrtrv	catcccccgaagttgtacag
scbArt1	tctgcgtccgatgccaactcg
scbArt2	ggtagacttgaggactggtga
scbArtfw	cgtacagggacaggtggactg
scbArtrv	cggtagacttgaggactggtgaa
scbR2	cacggcgggtcggtatccggt
scbR-M145_c358	cgaggacccttccgtc
scbR-M600_c358a	cgaggacccttccgta
ScbRrt1	caggatgtgcttctgcagcag
ScbRrt2	gcaggtcttcgagaagcaggg
scbRrtfw	tgaaccaggccaaggagaa
scbRrtrv	tctgggacacgacctgtatcc

Table S3 Additional MALDI-TOF data

Unmodified M145 ScbR (chymotrypsin digest):

75 ppm mass accuracy; 22 matched peptides; 87% coverage; MOWSE score = 100

```
  1 MAKQDRAIRT RQTILDAAAQ VFEKQGYQAA TITEILKVAG VTKGALYFHF

 51 QSKEELALGV FDAQEPPQAV PEQPLRLQEL IDMGMLFCHR LRTNVVARAG

101 VRLSMDQQAH GLDRRGPFRR WHETLLKLLN QAKENGELLP HVVTTDSADL

151 YVGTFAGIQV VSQTVSDYQD LEHRYALLQK HILPAIAVPS VLAALDLSEE

201 RGARLAAELA PTGKD
```

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
23 - 47	2652.5436	2651.5363	2651.4533	0.0830	3	F.EKQGYQAATITEILKVAGVTKGALY.F
28 - 47	2047.2069	2046.1997	2046.1724	0.0273	2	Y.QAATITEILKVAGVTKGALY.F
48 - 61	1651.9032	1650.8959	1650.8405	0.0554	4	Y.FHFQSKEELALGVF.D
48 - 75	3151.6526	3150.6453	3150.5661	0.0792	5	Y.FHFQSKEELALGVFDAQEPPQAVPEQPL.R
51 - 58	917.4995	916.4922	916.4865	0.0057	1	F.QSKEELAL.G
76 - 87	1497.7870	1496.7798	1496.7367	0.0431	3	L.RLQELIDMGMLF.C 2 Oxidation (M)
92 - 103	1311.8345	1310.8272	1310.7894	0.0378	0	L.RTNVVARAGVRL.S
104 - 118	1730.8395	1729.8322	1729.7954	0.0368	1	L.SMDQQAHGLDRRGPF.R Oxidation (HW)
113 - 118	747.4277	746.4204	746.3823	0.0381	0	L.DRRGPF.R
119 - 126	1110.6295	1109.6223	1109.6094	0.0129	2	F.RRWHETLL.K
119 - 126	1126.6293	1125.6220	1125.6043	0.0177	2	F.RRWHETLL.K Oxidation (HW)
119 - 126	1142.6222	1141.6149	1141.5992	0.0157	2	F.RRWHETLL.K 2 Oxidation (HW)
127 - 150	2605.4042	2604.3969	2604.3758	0.0211	3	L.KLLNQAKENGELLPHVVTTDSADL.Y
127 - 151	2768.5430	2767.5357	2767.4391	0.0965	4	L.KLLNQAKENGELLPHVVTTDSADLY.V
130 - 151	2414.2440	2413.2367	2413.1761	0.0607	2	L.NQAKENGELLPHVVTTDSADLY.V
152 - 175	2712.3910	2711.3837	2711.3190	0.0647	3	Y.VGTFAGIQVVSQTVSDYQDLEHRY.A
156 - 175	2308.1621	2307.1548	2307.1131	0.0417	2	F.AGIQVVSQTVSDYQDLEHRY.A
176 - 192	1783.1573	1782.1500	1782.1131	0.0369	2	Y.ALLQKHILPAIAVPSVL.A
179 - 192	1485.9502	1484.9429	1484.9078	0.0351	0	L.QKHILPAIAVPSVL.A
193 - 205	1400.7960	1399.7887	1399.7419	0.0468	2	L.AALDLSEERGARL.A
193 - 215	2354.3136	2353.3063	2353.2237	0.0826	4	L.AALDLSEERGARLAAELAPTGKD.-
196 - 215	2099.1165	2098.1093	2098.0654	0.0439	3	L.DLSEERGARLAAELAPTGKD.-

M600 ScbR (chymotrypsin digest):

75 ppm mass accuracy; 21 matched peptides; 90% coverage; MOWSE score = 103

```
  1 MAKQDRAIRT RQTILDAAAQ VFEKQGYQAA TITEILKVAG VTKGALYFHF

 51 QSKEELALGV FDAQEPPQAV PEQPLRLQEL IDMGMLFCHR LRTNVVARAG

101 VRLSMDQQAH GLDRRGPFRR WHETLLKLLN QAKENGELLP HVVTTDSADL

151 YVGTFAGIQV VSQTVSDYQD LEHRYALLQK HILPAIAVPS VLAALDLSEE

201 RGARLAAELA PTGKD
```

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
2 - 22	2372.1788	2371.1715	2371.3084	-0.1368	1	M.AKQDRAIRTRQTILDAAAQVF.E
23 - 47	2652.3385	2651.3312	2651.4533	-0.1221	3	F.EKQGYQAATITEILKVAGVTKGALY.F
28 - 47	2047.0710	2046.0638	2046.1724	-0.1086	2	Y.QAATITEILKVAGVTKGALY.F
59 - 75	1822.0098	1821.0025	1820.8944	0.1081	1	L.GVFDAQEPPQAVPEQPL.R
76 - 87	1497.6905	1496.6833	1496.7367	-0.0534	3	L.RLQELIDMGMLF.C 2 Oxidation (M)
88 - 103	1877.9897	1876.9825	1877.0642	-0.0817	1	F.CHRLRTNVVARAGVRL.S
92 - 103	1311.7528	1310.7455	1310.7894	-0.0439	0	L.RTNVVARAGVRL.S
104 - 118	1714.7468	1713.7395	1713.8005	-0.0610	1	L.SMDQQAHGLDRRGPF.R
104 - 118	1730.7224	1729.7151	1729.7954	-0.0803	1	L.SMDQQAHGLDRRGPF.R Oxidation (M)
113 - 118	747.3719	746.3646	746.3823	-0.0177	0	L.DRRGPF.R
127 - 151	2768.3271	2767.3198	2767.4391	-0.1193	4	L.KLLNQAKENGELLPHVVTTDSADLY.V
130 - 151	2414.0592	2413.0520	2413.1761	-0.1241	2	L.NQAKENGELLPHVVTTDSADLY.V
152 - 175	2712.1937	2711.1865	2711.3190	-0.1326	3	Y.VGTFAGIQVVSQTVSDYQDLEHRY.A
156 - 175	2307.9984	2306.9912	2307.1131	-0.1219	2	F.AGIQVVSQTVSDYQDLEHRY.A
156 - 178	2605.2350	2604.2277	2604.3183	-0.0906	4	F.AGIQVVSQTVSDYQDLEHRYALL.Q
176 - 192	1783.0211	1782.0139	1782.1131	-0.0992	2	Y.ALLQKHILPAIAVPSVL.A
179 - 192	1485.8562	1484.8489	1484.9078	-0.0589	0	L.QKHILPAIAVPSVL.A
193 - 205	1400.7096	1399.7023	1399.7419	-0.0396	2	L.AALDLSEERGARL.A
193 - 215	2354.1193	2353.1120	2353.2237	-0.1117	4	L.AALDLSEERGARLAAELAPTGKD.-
196 - 215	2098.9645	2097.9573	2098.0654	-0.1081	3	L.DLSEERGARLAAELAPTGKD.-
198 - 205	917.4419	916.4346	916.4726	-0.0380	0	L.SEERGARL.A

Table S4 Transcriptional analysis of LW34 and LW33.

S. coelicolor LW34 and LW33 was grown *in duplo* in liquid SMM (growth curves (GC) 1 and 2). Samples were taken at different phases of growth (<u>e</u>arly, <u>m</u>id and <u>l</u>ate <u>t</u>ransition (eT, mT, lT), and stationary (S) phase; also see Table 2). Gene expression is shown for *redD*, *actII-4*, *cpkO*, *scbA*, *scbR*$_{M145}$ (LW34) and *scbR*$_{M600}$ (LW33) as fold-change relative to the LW34 time point 1 sample of each GC; the standard deviation is given in brackets.

|		GC 1				GC 2			
time point		1	2	3	4	1	2	3	4
growth phase		eT	mT	lT	S	eT	mT	lT	S
redD	LW34	1·00 (0·62)	5·11 (2·94)	0·34 (-0·34)	0·00 (0·00)	1·00 (0·75)	7·86 (1·53)	1·12 (0·30)	0·37 (-0·37)
	LW33	0.93 (0·63)	2.17 (0·79)	1.71 (0·41)	0.00 (0·00)	0.37 (-0·37)	3.16 (0·74)	2.51 (1·06)	0.44 (0·21)
actII-4	LW34	1·00 (0·74)	18·74 (5·79)	11·06 (2·70)	5·56 (3·16)	1·00 (0·43)	13·57 (5·46)	15·20 (7·48)	6·10 (3·95)
	LW33	0·43 (0·29)	26·21 (24·59)	18·41 (4·67)	1·03 (0·08)	0·34 (0·30)	2·00 (7·24)	12·85 (3·75)	3·83 (1·31)
cpkO	LW34	1·00 (-1·00)	15·16 (4·39)	136·68 (42·56)	18·95 (8·53)	1·00 (0·75)	7·86 (1·53)	1·12 (0·30)	0·37 (-0·37)
	LW33	0·00 (0·00)	0·00 (0·00)	67·29 (17·88)	7·81 (0·77)	0·23 (0·20)	2·01 (1·58)	354·15 (46·80)	36·00 (6·69)
scbA	LW34	1·00 (0·57)	7·86 (2·00)	7·24 (4·25)	0·23 (0·10)	1·00 (0·77)	72·05 (12·91)	8·80 (3·90)	1·04 (0·75)
	LW33	0·55 (0·54)	2·68 (2·09)	16·73 (3·81)	1·42 (0·60)	0·41 (0·37)	2·83 (0·34)	93·81 (53·02)	0·54 (0·44)
scbR (M145/M600)	LW34	1·00 (0·46)	2·97 (0·75)	2·29 (0·30)	0·39 (0·14)	1·00 (0·30)	13·47 (2·49)	3·93 (0·37)	1·08 (0·34)
	LW33	0·93 (0·38)	2·96 (1·16)	5·22 (1·11)	0·49 (0·04)	0·81 (0·26)	3·62 (0·37)	10·26 (1·32)	0·90 (0·15)

Supplementary figures for chapter 4
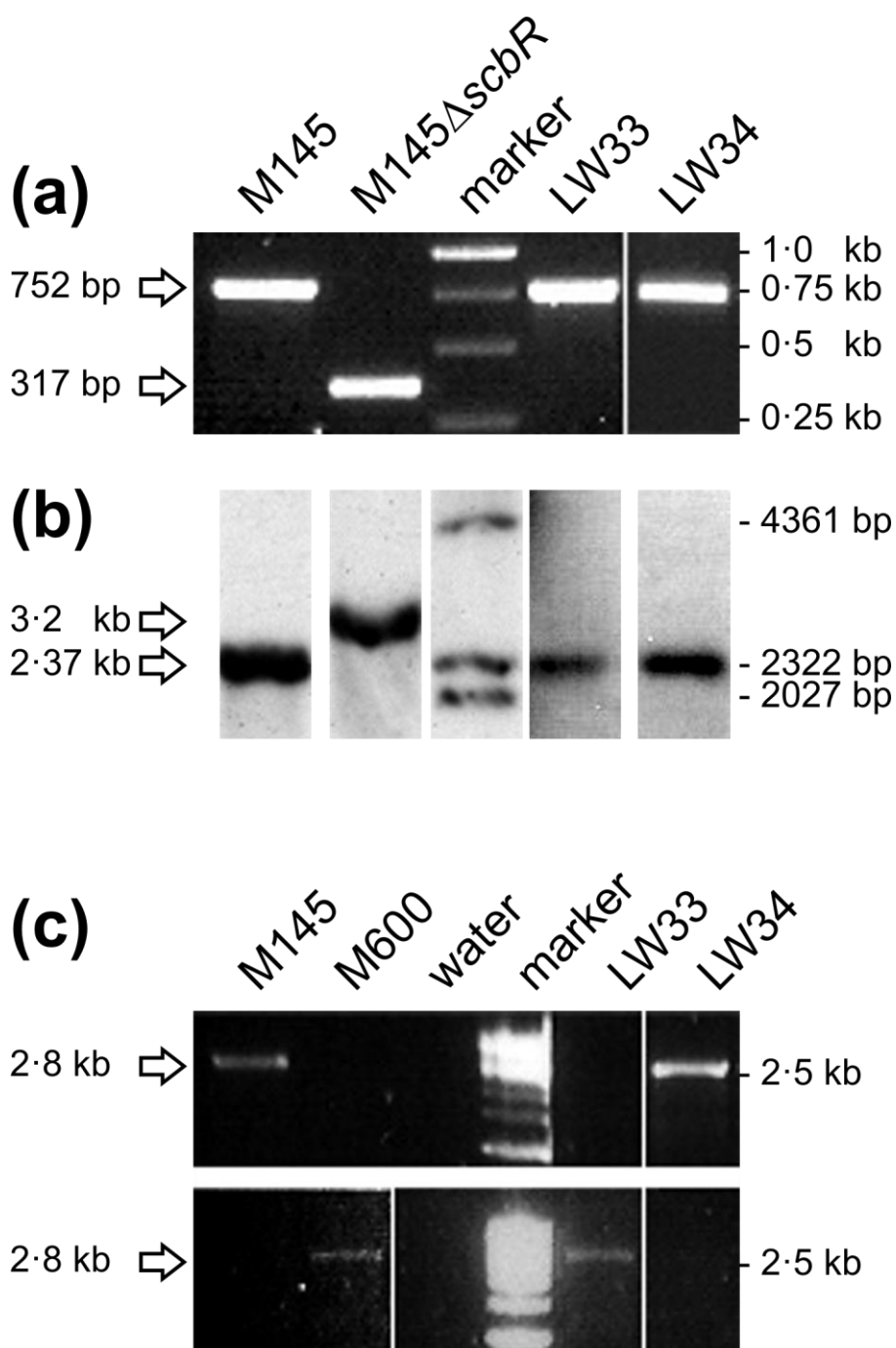
Figure S1 Identification of full-length $scbR_{M145/M600}$ in *S. coelicolor* strains LW34 and LW33.

(**a**) PCR was carried out using primers ETseq3 and ETS7 to amplify a 752 bp full-length $scbR_{M145/M600}$ fragment and a 317 bp fragment from the $\Delta scbR$ region. The 317 bp PCR product was only found with

the LW33/LW34 parental M145ΔscbR mutant. A full-length scbR$_{M145/M600}$ fragment was amplified from a wt control (M145) and from LW33 and LW34. Obtained PCR products are indicated with arrows on the left; sizes of the marker bands are given on the right. The M145-/M600-type of the gene was confirmed by DNA sequence analysis (data not shown). (**b**) Southern analysis was carried out using *Nco*I digested genomic DNA of the four strains. A DIG-labelled DNA probe was used to detect the expected 3·2 kb fragment with M145ΔscbR and 2·37 kb fragments with the wt control (M145) and LW33 and LW34. Detected DNA fragments are indicated with arrows on the left; sizes of the marker bands are given on the right. (**c**) PCR was carried out using primers RCseq31 and scbR-M145_c358 (scbR-M600_c358a) to amplify a 2·8 kb scbR$_{M145}$ (scbR$_{M600}$) fragment. A scbR$_{M145}$ fragment was only obtained with control strain M145 and with LW34, whereas control strain M600 and LW33 showed a scbR$_{M600}$ specific PCR product. Using water as template did not give any product. Obtained PCR products are labelled with arrows on the left; a 2·5 kb marker band is indicated on the right.
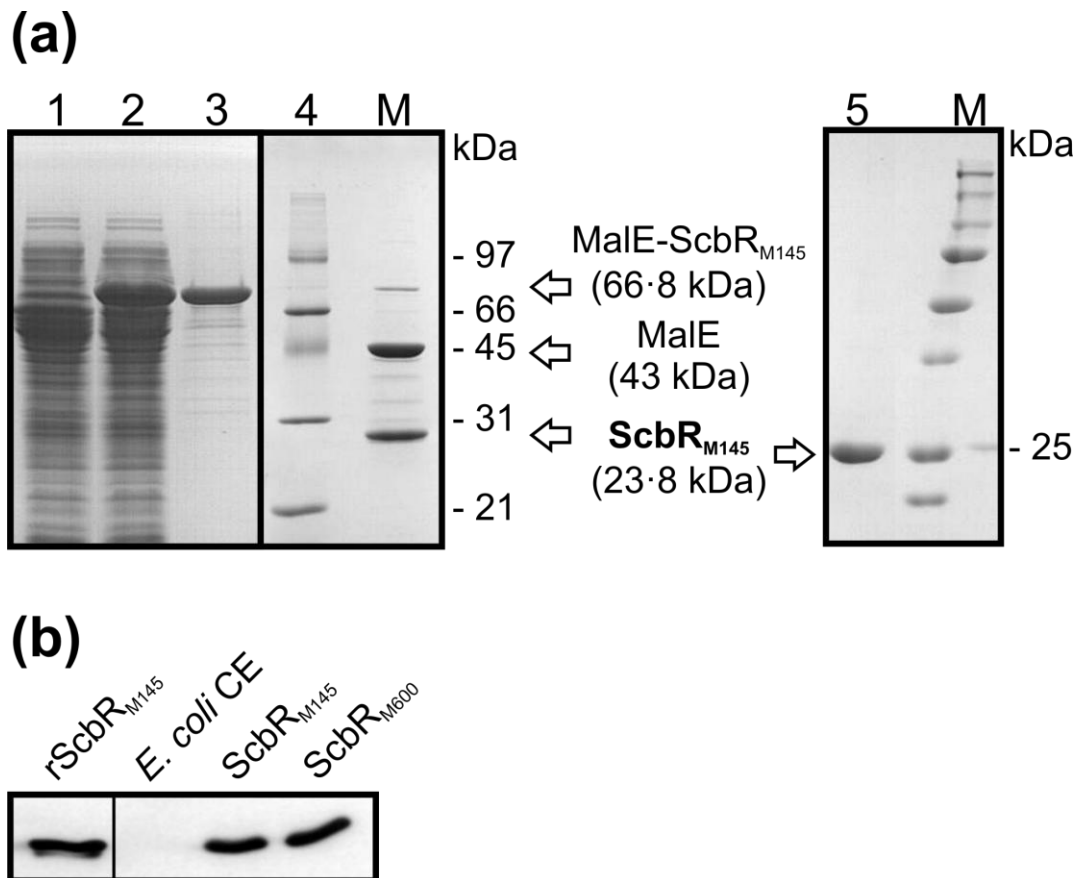
Figure S2 Heterologous expression and Western analysis of ScbR

(**a**) Heterologous expression and purification of ScbR$_{M145}$ for the creation of ScbR antibodies. Crude cell extracts from *E. coli* JM101 harbouring pTE88 before (lane 1) and after (lane 2) induction with 0·2 % (w/v) of L-rhamnose. The MalE-ScbR$_{M145}$ fusion protein present in the induced fraction was then
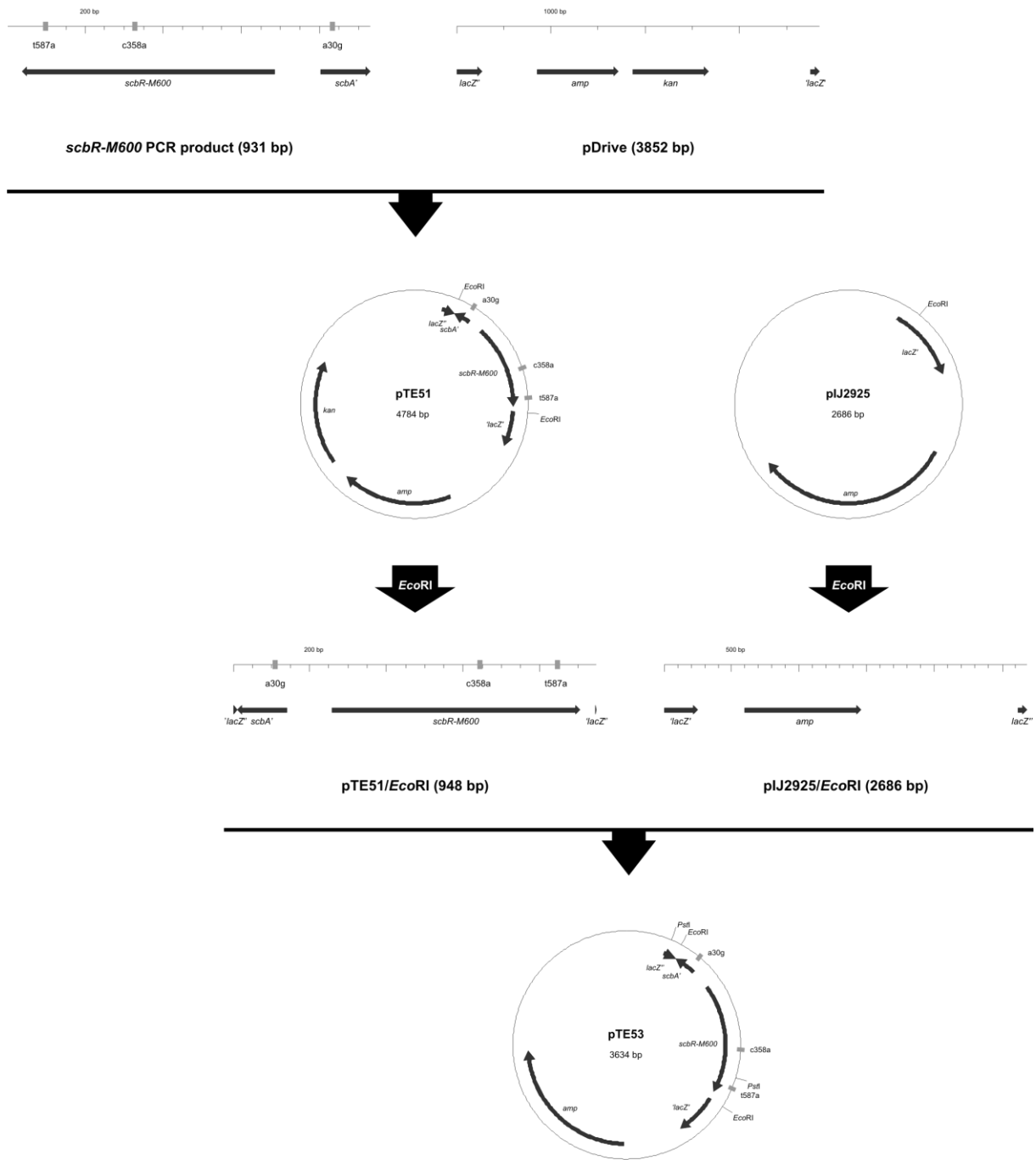
purified with an amylose resin (lane 3). MalE-ScbR$_{M145}$ was cleaved with Factor Xa to separate MalE from ScbR$_{M145}$ (lane 4). ScbR$_{M145}$ was further purified with a heparin column (lane 5). Arrows show the protein bands representing each protein. Theoretical molecular weight of MalE-ScbR$_{M145}$, MalE and ScbR$_{M145}$ are noted in brackets. All protein fractions were analysed on 12 % (w/v) SDS-PAGE followed by staining with Coomassie blue. M denotes for prestained protein molecular weight ladders. (**b**) Heterologous expression of ScbR$_{M145}$ and ScbR$_{M600}$ for gel retardation assays and Western analysis of both forms of ScbR. ScbR-antibodies were generated using recombinant ScbR$_{M145}$ shown in (a). ScbR$_{M145}$ and ScbR$_{M600}$ were expressed in *E. coli* JM101/pTE6120 and pTE58 harbouring *scbR*$_{M145}$ and *scbR*$_{M600}$, respectively. In Western analysis, ScbR was detected with a sample of the recombinant ScbR$_{M145}$ ("rScbR$_{M145}$") and in cell-free extracts (CE) of *E. coli* JM101/pTE6120 and pTE58 ("ScbR$_{M145}$" and "ScbR$_{M600}$"). In CE of *E. coli* JM101 harbouring the empty expression vector pIJ2925 ("*E. coli* CE") no ScbR was found. Comparable amounts of ScbR were detected with same amounts of total CE proteins.
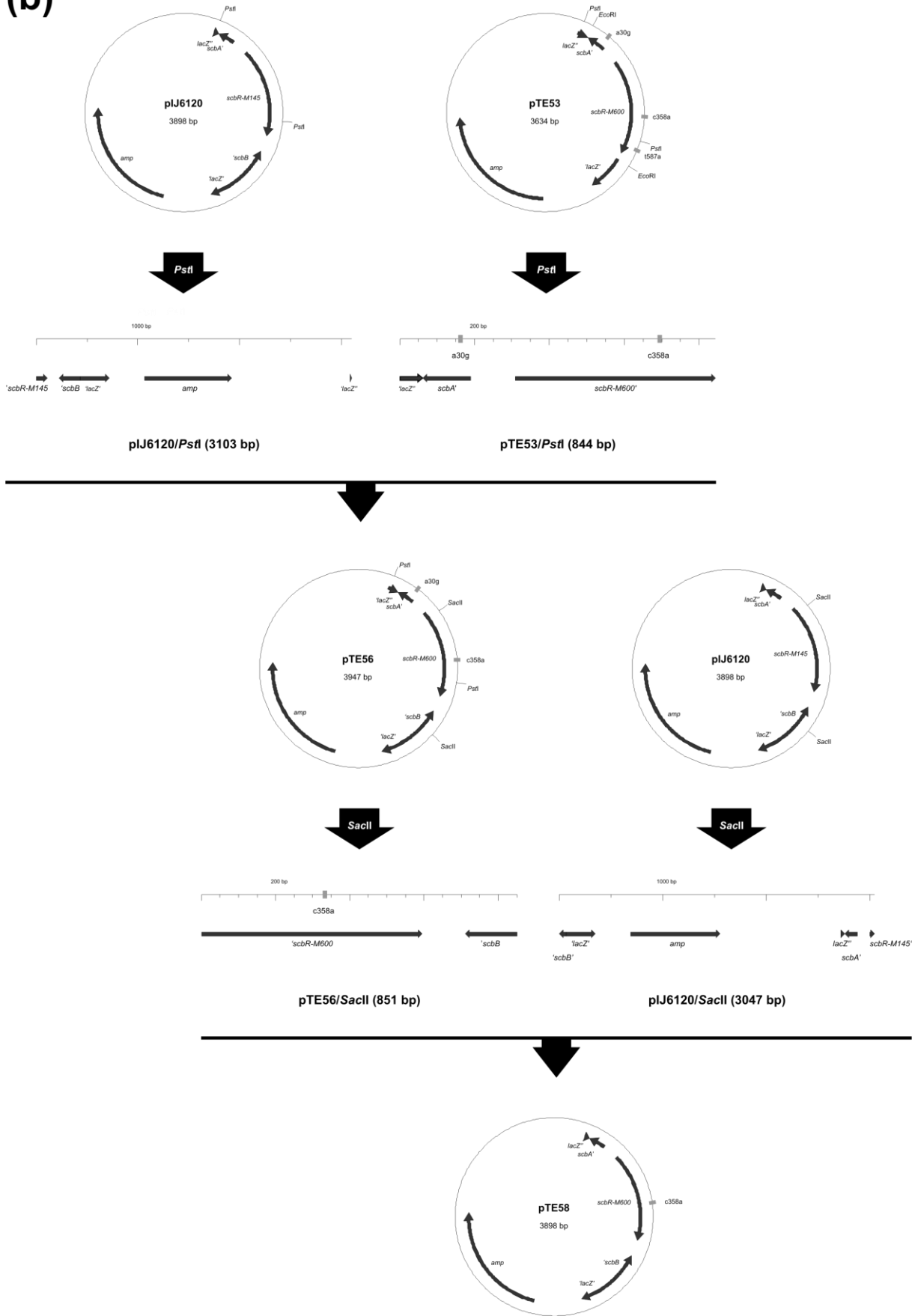
Plasmid construction

Linear DNA fragments are named and a scale is given with indicated base pair (bp) units. Plasmids are named and represented by black circles. Genes are indicated by dark arrows and labelled with gene names. For incomplete genes the missing part is indicated by an apostrophe at the beginning or the end of the gene name. Antibiotic resistance genes are abbreviated with "*amp*" for ampicillin, "*apra*" for apramycin and "*kan*" for kanamycin resistance. "*lacZ'*" denotes for the LacZ α-peptide coding sequence. In pSET152, "*oriT*" indicates the origin of transfer, "*attP*" the phage ΦC31 derived attachment site and "*int*" the integrase gene. The position of the described mutations is indicated by labelled grey boxes on the scales and in the plasmid maps; relevant restriction sites are shown with enzyme names. Thick black lines with arrows indicate ligation events; big black arrows with enzyme names indicate corresponding restriction steps.

(a)

scbR-M600 PCR product (931 bp) pDrive (3852 bp)

pTE51 4784 bp pIJ2925 2686 bp

pTE51/*Eco*RI (948 bp) pIJ2925/*Eco*RI (2686 bp)

pTE53 3634 bp

(b)

pIJ6120
3898 bp

pTE53
3634 bp

*Pst*I

*Pst*I

pIJ6120/*Pst*I (3103 bp)

pTE53/*Pst*I (844 bp)

pTE56
3947 bp

pIJ6120
3898 bp

*Sac*II

*Sac*II

pTE56/*Sac*II (851 bp)

pIJ6120/*Sac*II (3047 bp)

pTE58
3898 bp

Figure S3 Construction of the ScbR$_{M600}$ expression vector pTE58.

From a genomic DNA isolate of *S. coelicolor* strain M600, a 931 bp PCR fragment containing the *scbR$_{M600}$* coding sequence and the *scbR* promoter region was amplified using primers ETS3 and ScbR2 (Table S2). The PCR product was gel-purified and ligated to pDRIVE (Qiagen), yielding pTE51 of which the insert contains not only the natural mutation c358a in *scbR$_{M600}$*, but also additional mutations: a30g in *scbA* is located in the promoter region of *scbR*, and nucleotide change t587a in the coding sequence of *scbR$_{M600}$*, respectively. The point mutation t587a leads to an amino acid change, D196V, in ScbR$_{M600}$ (data not shown). A 948 bp pTE51/*Eco*RI *scbR$_{M600}$* fragment was cloned into the pUC18 derivate pIJ2925 (Janssen & Bibb, 1993) to gain pTE53 containing all three described mutations (**a**). A 844 bp pTE53/*Pst*I fragment containing the *scbR* promoter region and nucleotides 1-535 of the *scbR$_{M600}$* coding region was ligated into a 3103 bp pIJ6120/*Pst*I vector fragment (Takano *et al.*, 2001) containing nucleotides 536-648 of the *scbR$_{M145}$* coding region, yielding pTE56. Partial sequence analysis of pTE56 revealed only the *scbR* promoter mutation a30g in *scbA* and the expected coding sequence mutation c358a of *scbR$_{M600}$*. A 851 bp pTE56/*Sac*II fragment containing nucleotides 54-304 of *scbR$_{M600}$* was cloned into a 3047 bp pIJ6120/*Sac*II vector fragment containing the *scbR* promoter region and nucleotides 1-53 and 305-648 of the *scbR$_{M145}$* coding sequence, yielding pTE58 with *scbR$_{M600}$* and the *scbR* promoter region without additional undesired mutations in the same orientation as the IPTG-inducible *E. coli lacZ* promoter (**b**). The *scbR$_{M145}$* expression construct pIJ6120 (Takano *et al.*, 2001) and pTE58 differ only by the natural mutation, c358a, in *scbR$_{M600}$* and were used for the heterologous expression of ScbR$_{M145}$ and ScbR$_{M600}$ in *E. coli*.
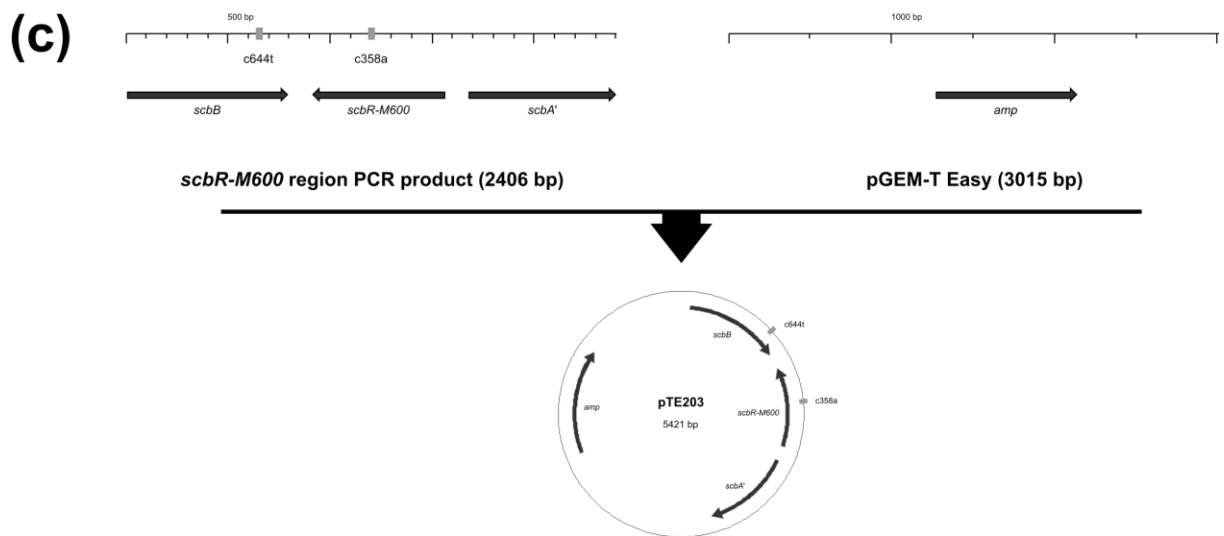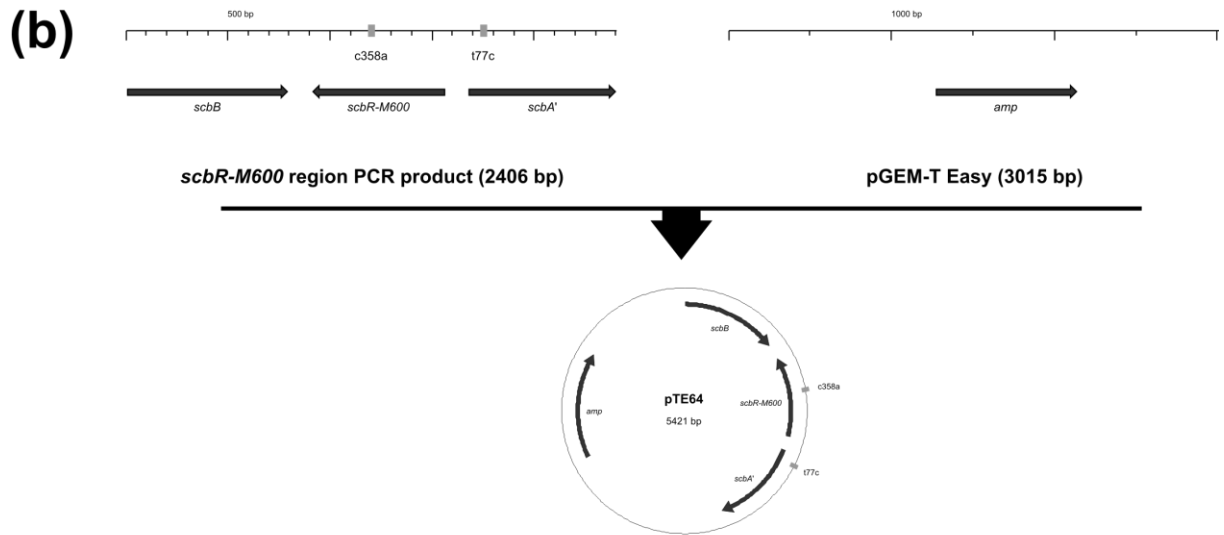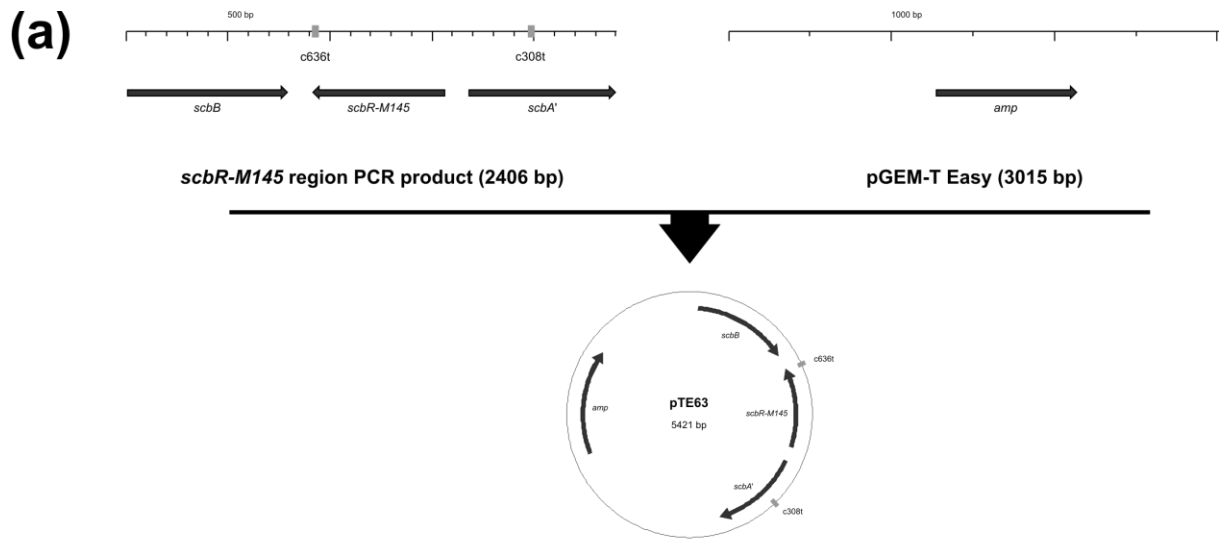
(d)
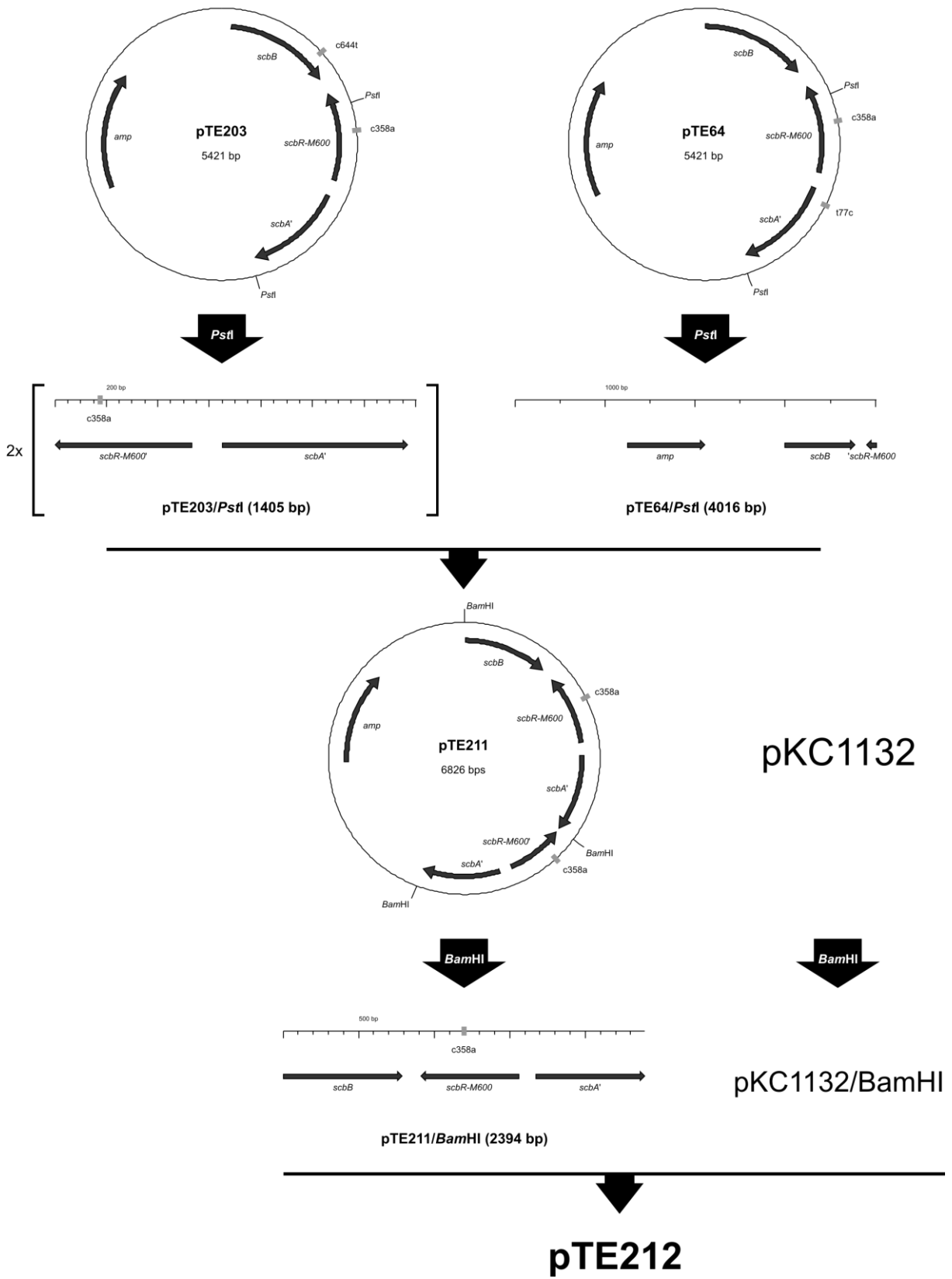
(e)
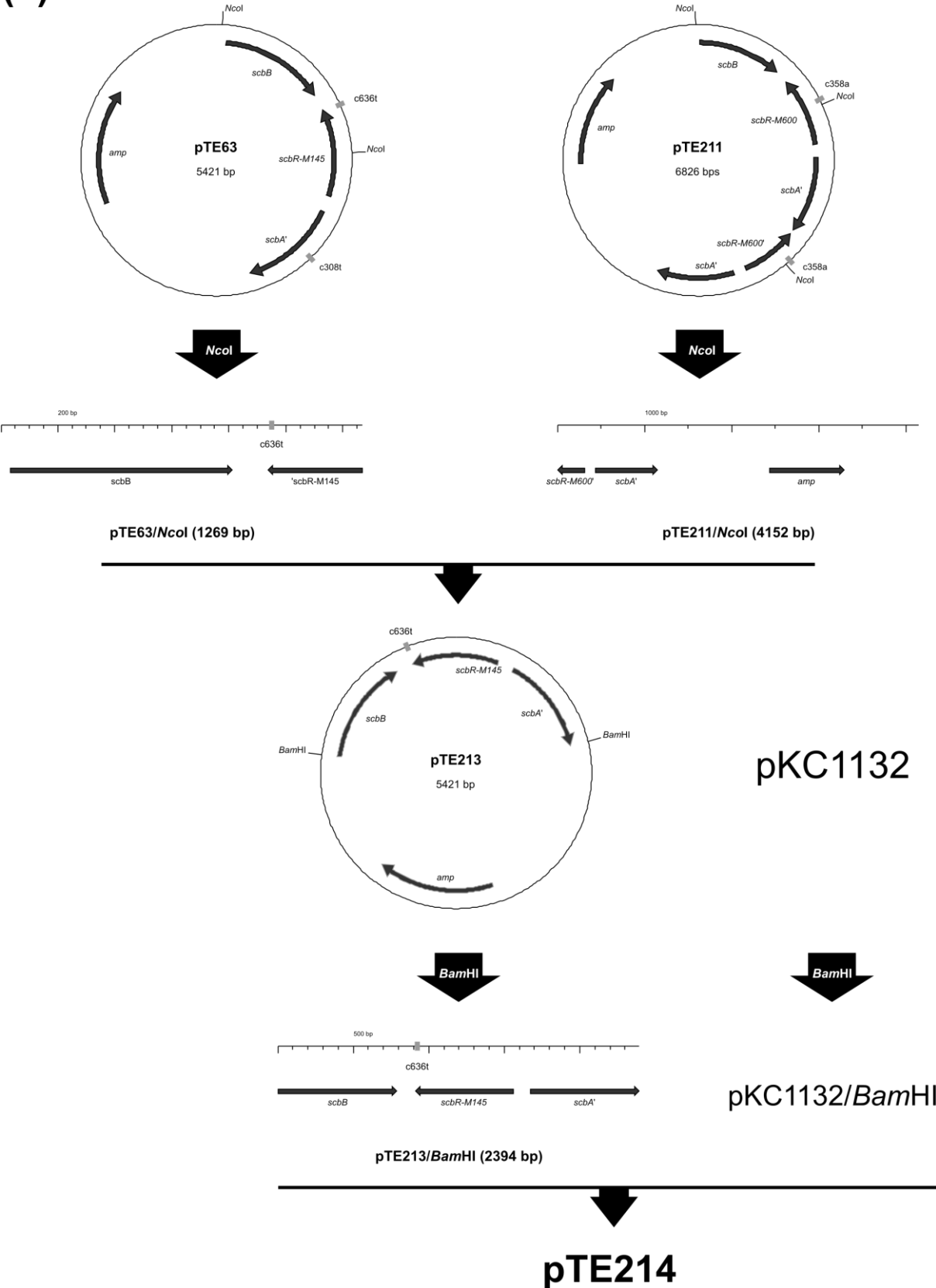
Figure S4 Construction of the complementation vectors pTE212 and pTE214.

From genomic DNA isolates of *S. coelicolor* strains M145 and M600, a 2406 bp PCR fragment containing the *scbR*$_{M145/M600}$ coding sequence and flanking regions was amplified using primers BamRCseq31enh and BamETseq1 (Table S2). PCR products were gel-purified and ligated to pGEM-T EASY (Promega), yielding pTE63 harbouring *scbR*$_{M145}$ (**a**), and pTE64 (**b**) and pTE203 (**c**) harbouring *scbR*$_{M600}$. The inserts of the plasmids were sequenced and pTE63 was found to contain mutations in *scbR*$_{M145}$ (silent mutation c636t) and *scbA* (c308t leading to A103V). pTE64 contains a mutation in *scbA* (t77c leading to M26T), and pTE203 in *scbB* (c644t leading to A215V). Two 1405 bp pTE203/*Pst*I fragments were introduced by tandem integration into a 4016 bp pTE64/*Pst*I vector fragment to give pTE211, which is pGEM-T EASY with a 2394 bp *Bam*HI *scbR*$_{M600}$ fragment without any additional mutations and an additional 1·4 kb *Bam*HI fragment. The 2394 bp pTE211/*Bam*HI fragment was cloned into pKC1132 to give pTE212 (**d**). A 1269 bp pTE63/*Nco*I fragment containing the silent mutation c636t in *scbR*$_{M145}$ was cloned into a 4152 bp pTE211/*Nco*I vector fragment to give pTE213, which is pGEM-T EASY with a 2394 bp *Bam*HI *scbR*$_{M145}$ fragment with only the silent mutation. The 2394 bp pTE213/*Bam*HI fragment was cloned into pKC1132 to give pTE214 (**e**).

Appendix 2

"Regulation of antibiotic production by bacterial hormones."

N.H. Hsiao, M. Gottelt, and E. Takano.

Methods Enzymol. 2009;458:143-57.

REGULATION OF ANTIBIOTIC PRODUCTION BY BACTERIAL HORMONES

Nai-Hua Hsiao, Marco Gottelt, *and* Eriko Takano

Contents

Abstract

Antibiotic production is regulated by numerous signals, including the so-called bacterial hormones found in antibiotic producing organisms such as *Streptomyces*. These signals, the γ-butyrolactones, are produced in very small quantities, which has hindered their structural elucidation and made it difficult to assess whether they are being produced. In this chapter, we describe a rapid small-scale extraction method from either solid or liquid cultures in scales of one plate or 50 ml of medium. Also described is a bioassay to detect the γ-butyrolactones by determining either the production of pigmented antibiotic of *Streptomyces coelicolor* or kanamycin resistant growth on addition of the γ-butyrolactones. We also describe some insights into the identification of the γ-butyrolactone receptor and its targets and also the gel retardation conditions with three differently labeled probes.

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1. INTRODUCTION

Most secondary metabolites (including antibiotics) are the products of complex biosynthetic pathways activated in the stationary phase or during slow growth, which triggers the transition from primary to secondary metabolism (Bibb, 2005). This switch is complex and poorly understood, and involves many signals, including those by small signaling molecules called γ-butyrolactones. These signaling molecules, found mainly in *Streptomyces* species, are considered to be "bacterial hormones" because they are important in the regulation of antibiotic production and in some cases morphological differentiation (Takano, 2006). The first γ-butyrolactone, A-factor, was identified from *Streptomyces griseus* in 1967 and was isolated as a compound that could stimulate the production of streptomycin and sporulation in a mutant defective in these characteristics (Khokhlov *et al.*, 1967) (Fig. 6.1). Subsequently, the group of Sueharu Horinouchi identified the A–factor receptor (Onaka *et al.*, 1995) and the gene involved in the
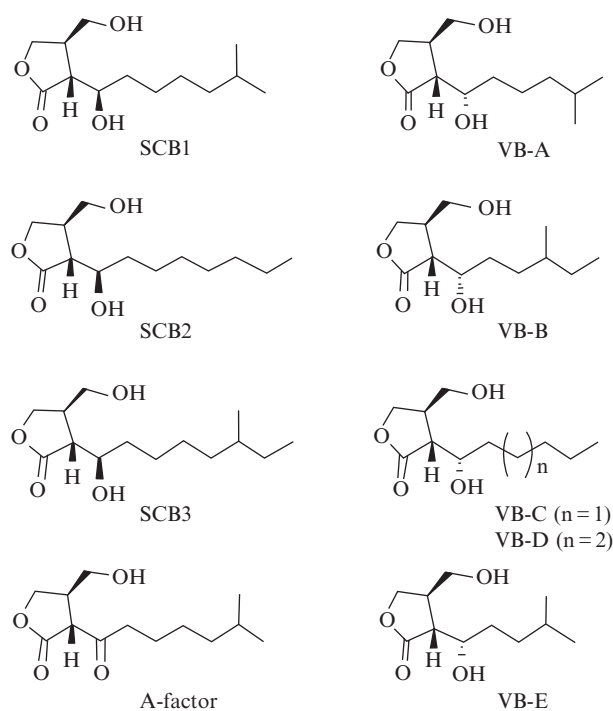
Figure 6.1 Chemical structures of the γ–butyrolactones. SCB1, 2, and 3 are produced by *S. coelicolor*; A-factor is produced by *S. griseus*; VB–A, B, C. D, and E are produced by *S. virginiae*. Figure contributed by Christian Hertweck with permission.

synthesis of A-factor (Horinouchi *et al.*, 1985) and also the pathway by which A-factor regulates streptomycin production (Ohnishi *et al.*, 1999). Other γ-butyrolactones and its receptor systems were identified in *Streptomyces virginae* (Yamada, 1999 review), *Streptomyces lavendulae* FRI-5 (Kitani *et al.*, 2008), and *Streptomyces coelicolor* (Takano *et al.*, 2005), the model organism for *Streptomyces* genetics.

The number of identified receptor homologues has more than tripled in recent years with the analysis of antibiotic biosynthetic gene cluster sequences, suggesting that the γ-butyrolactone system may be a widespread regulatory component in controlling antibiotic production in the genus *Streptomyces* and in other actinomycetes (Takano, 2006).

These signaling molecules are produced only in minute concentrations, making them challenging to purify. However, their activity in nanomolar concentration is helpful in bioactivity assays, which can be performed with small quantities of material. In all the systems that have been reported, the γ-butyrolactones have been detected by such bioassays. For example: A-factor in *S. griseus* is extracted with chloroform and detected by measuring the induction of streptomycin production on a solid culture of a non-A-factor producing mutant, *S. griseus* HHI (Hara and Beppu, 1982); virginiae butanolides (VBs) in *Streptomyces virginiae* are extracted with ethyl acetate from acidified cell-free supernatants of cultures and detected by measuring the production of virginiamycin in a liquid culture of an *S. virginiae* mutant (Nihira *et al.*, 1988); IM-2 in *S. lavendulae* FRI-5 is extracted in the same way as VBs and detected by the induction of a blue pigment (Sato *et al.*, 1989).

To quickly assess whether γ-butyrolactones are a regulatory component in the antibiotics of choice, a large-scale purification or structural elucidation is not the first preference. This chapter describes the rapid small-scale extraction and detection of γ-butyrolactones in *S. coelicolor* which will aid in solving this problem. We also aim to aid identification of γ-butyrolactone receptors and their targets.

2. Rapid Small-Scale γ-Butyrolactone Purification

Intracellularly produced γ-butyrolactones are believed to diffuse passively out of the mycelium. In most protocols, liquid culture supernatants are used for production. However, the amounts of γ-butyrolactones produced in liquid cultures vary with the growth of the mycelium and extraction can be tedious. Therefore, we have developed a method using plates of solid agar medium. Here, we describe extraction protocols for both liquid and solid cultures.

1. Grow the strain either on plates up to aerial mycelium formation or early spore formation, or in liquid medium up to early stationary phase.

   Note: Both minimal and rich media can be used in both cases; however, the stability of the γ-butyrolactones in rich media is much less than in a defined medium and they must be extracted at an earlier time.

2. For plates, cut the agar into large pieces and place in a beaker. Add 30–40 ml ethyl acetate to cover the agar pieces, and shake gently. Transfer the ethyl acetate into a rotary flask.

   Note: Avoid breaking the agar into small pieces as this makes it difficult to separate the agar from the solvent. Also, it is important to test the ethyl acetate used for extraction beforehand as some ethyl acetate may already have bioactivity.

3. For liquid cultures, add a 2–3-fold volume of ethyl acetate in a separation flask. Shake 5–10 times, then separate the ethyl acetate phase from the water phase and place the ethyl acetate phase in a rotary flask.

   Note: If a small amount of second phase separates from the ethyl acetate sample, remove it before evaporation. Normally, any second phase consists of fatty acids which cannot be evaporated will affect the diffusion ability of samples in the bioassay.

4. Evaporate all the ethyl acetate in a rotary evaporator and resuspend in 100% HPLC-grade methanol.

   Note: From 3 plates or from 50 ml cultures, resuspend sample in $\leq 50\ \mu$l of methanol.

3. Antibiotic Bioassay

The classical approach for the bioassay of γ-butyrolactones is the observation or measurement of induced antibiotic production from an indicator strain in response to the compound of interest. Based on this concept, the antibiotic bioassay in *S. coelicolor* detects early production of the pigmented antibiotics, blue actinorhodin, and red prodiginines (Takano *et al.*, 2000).

1. Prepare spore stocks of the antibiotic bioassay indicator strain *S. coelicolor* M145 (Kieser *et al.*, 2000). Determine viable counts and store spores at $-20\ °$C in 20% glycerol.
2. Prepare supplemented minimal media solid (SMMS) plates (20 ml agar/ plate; Takano *et al.*, 2001).

   Note: The source of the agar strongly affects the timing and production of the pigmented antibiotics. Try to use only agar from the same company and the same batch.

3. For each plate, add 1×10^7–10^8 M145 spores (so the plate is just about confluent) in 60–100 μl of water. Using wet sterile cotton buds, spread confluently onto the plates and dry in the laminar flow cabinet.

4. Spot a sample (<2 μl) in the middle of the plate and dry. Incubate plates at 30 °C, check them every 8–12 h and record the antibiotic production by scanning the plates on a scanner (or take a photograph) (Fig. 6.2).

Note:

- Avoid dropping a sample before a plate is completely dry or dropping more than 2 μl of the sample. If the sample volume is more than 2 μl, spot in 2 μl aliquots several times after drying each time or concentrate the sample before spotting.

- The extracted samples should be spotted onto the indicator plate within 8 h of spreading, the sooner the better. No induction of the pigmented antibiotics will be observed if the indicator is grown for more than 8 h.

- The optimal amount of the positive control is 0.25 μg for the γ-butyrolactone SCB1. When using extracts made from *S. coelicolor* M145, 20–30 μl out of the 50 μl of sample either from 50 ml liquid cultures or from three plates should give bioactivity.

- To measure the approximate concentration of γ-butyrolactones isolated: for plates start the inoculum with the exact same number of viable spore counts for each sample; for liquid cultures measure the growth phase (by OD450 nm) convert into milligrams per dry weight and also measure the volume of the supernatant; this amount then can be used for comparison between samples.

- As seen in Fig. 6.2, the color may change depending on the amount of indicator spores used and also the length of time incubated.

Extracts from
S. coelicolor J1501

Extracts from
S. lividans
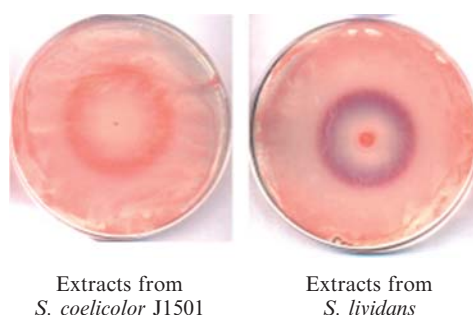
Figure 6.2   An example of a typical bioassay. Extracts from solid plates of *S. coelicolor* J1501 and *S. lividans* were spotted onto the indicator strain M145, then incubated for 36 h at 30 °C. The pigmented halos are the result of the butyrolactones stimulating pigmented antibiotic production.

4. Kanamycin Bioassay

The kanamycin bioassay was developed as an easy and direct method to detect γ-butyrolactone. It is based on the γ-butyrolactone receptor, ScbR, and its binding to the target DNA and the γ-butyrolactones. ScbR represses transcription of its own gene and that of *cpkO*, a pathway-specific regulatory gene for the Cpk cluster, by binding to the promoter regions (Pawlik *et al.*, 2007; Takano *et al.*, 2005). This repression is abolished by SCB1, resulting in transcription of the target genes. Using these principles, a γ-butyrolactone detection plasmid, pTE134, was constructed which harbors *scbR* and its own promoter region with the ScbR binding sites, together with the *cpkO* promoter with the ScbR binding sites, fused to a promoterless-kanamycin resistance gene (Fig. 6.3). pTE134, was transferred into *S. coelicolor* LW16 (*scbA* and *scbR* double deletion mutant) to obtain the kanamycin indicator strain LW16/pTE134. LW16 was chosen as a host strain for the kanamycin bioassay because it does not produce γ-butyrolactones and it lacks *scbR*, thus avoiding competition from endogenous ScbR. The kanamycin bioassay indicator strain LW16/pTE134 is sensitive to kanamycin but when γ-butyrolactones are added the repression caused by ScbR is abolished
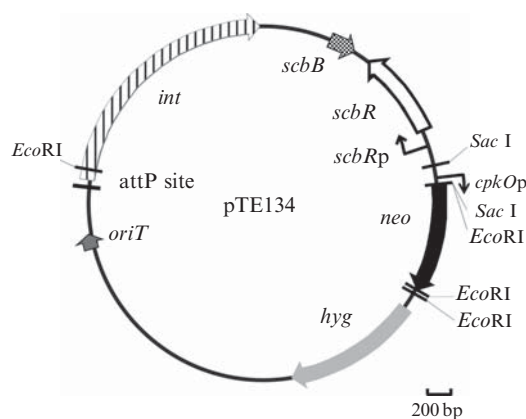
Figure 6.3 Schematic map of pTE134 used for the kanamycin bioassay. pTE134 has *scbR* (open arrow) with its own promoter region, *scbR*p (black arrow) and a *cpkO* promoter, *cpkO*p (black arrow) coupled with a promoterless kanamycin resistance gene (*neo*, solid arrow). The light gray arrow indicates *hyg* (omega hygromycin resistance gene), the deep gray arrow represents *oriT* (*RP4* origin of single-stranded DNA transfer), the hatched arrow represents *int* (phiC31 integrase), the shaded arrow indicates a partial coding region (204 bp) of *scbB* and the attP site (phage phiC31 attP site) is indicated by a vertical black line. Restriction sites used for cloning are indicated by black lines (not all *Sac*I and *Eco*RI sites are shown on the map).

and the kanamycin resistance gene is transcribed, rendering the indicator strain kanamycin resistant (Hsiao *et al.*, submitted for publication).

This assay was also tested for its ability to detect other butyrolactones than the ones produced by *S. coelicolor*. Though the sensitivity is weaker than for the native γ-butyrolactones, with enough material spotted to the plates, kanamycin resistance can be observed. Furthermore, γ-butyrolactones have been identified from those antibiotic producing *Streptomyces* strains which were not known to produce these molecules using this assay (Hsiao *et al.*, submitted for publication).

1. Prepare spore stocks of the kanamycin bioassay indicator strain LW16/pTE134 and stock spores in 50–100 μl aliquots at –20 °C in 20% glycerol.

   Note: Because this indicator strain is unstable, spores need to be collected from MS plates (Kieser *et al.*, 2000) containing 50 μg/ml hygromycin, then aliquoted. Avoid thawing and refreezing the spore stocks.

2. Prepare fresh DNAgar (Difco Nutrient Agar) plates containing kanamycin at a final concentration 3–5 μg/ml (20 ml per plate).

   Note: Because the *cpkO* promoter has very low activity (conferring resistance to <10 μg/ml kanamycin), the concentration of kanamycin should not be higher than 5 μg/ml (Fig. 6.4).

3. To each plate, add 2.6×10^6 spores of the indicator strain in about 60–100 μl of water. Using wet sterile cotton buds, spread confluently onto the plates and dry.

4. Spot the extract or sample (<2 μl) in the middle of the plate and dry. Incubate plates at 30 °C, check them after 2–3 days and record the growth by scanning the plates on a scanner. The kanamycin resistant colonies will grow around the spot where the γ-butyrolactones have diffused.
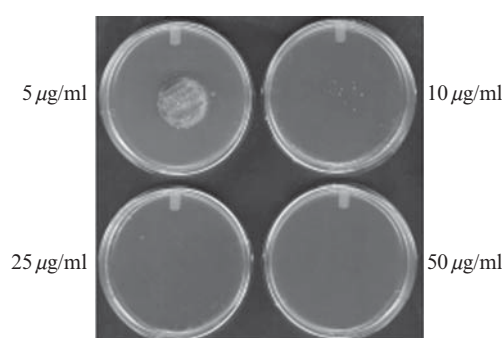
Figure 6.4   The kanamycin bioassay using different kanamycin concentrations in the indicator media. 0.1 μg of chemically synthesized SCB1 was spotted onto lawns of LW16/pTE134 (2.6×10^6 spores) on DNAgar plates containing 5, 10, 25, or 50 μg/ml kanamycin, respectively. The plates were incubated at 30 °C for 3 days.
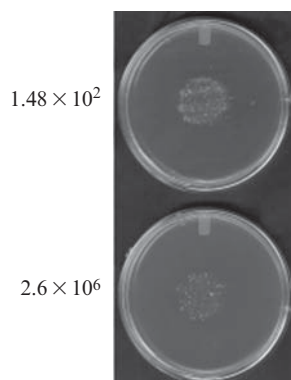
Figure 6.5 The kanamycin bioassay using different amount of indicator strains. 0.1 μg of chemically synthesized SCB1 was spotted onto lawns of LW16/pTE134 (2.6×10^6 or 1.48×10^7 spores) on DNAgar plates containing 5 μg/ml kanamycin, respectively. The plates were incubated at 30 $^\circ$C for 3 days.

Note:
- Again avoid spotting a sample before a plate is completely dry or spotting more than 2 μl of the sample. If the sample volume is more than 2 μl, spot in 2 μl aliquots several times after drying each time or concentrate the sample before spotting.
- The optimal amount of the positive control is 0.1 μg for SCB1 and 0.25 μg for A-factor. To enhance the density of the growth halo, a larger amount of indicator spores and a smaller amount of kanamycin in the indicator plate can be used (Figs. 6.4 and 6.5).

5. IDENTIFICATION OF γ-BUTYROLACTONE RECEPTORS

Several groups have used different methods to identify the γ-butyrolactone receptors. The first receptor to be identified was by Horinouchi and co-workers using an A-factor affinity column and recovering the receptor from the crude extract of *S. griseus* (Onaka *et al.*, 1995). The same technique was used to identify the γ-butyrolactone receptors from *S. virginiae* (Okamoto *et al.*, 1995) and *S. lavendulae* FRI-5 (Ruengjitchatchawalya *et al.*, 1995). Another approach was via PCR using degenerate oligonucleotides to isolate the two receptor homologues in *S. coelicolor* (Onaka *et al.*, 1998). Interestingly, Onaka and co-workers failed to identify ScbR with this approach. This may be due to the limited homologous sequence information at the time.

We have identified ScbR only by cloning the γ-butyrolactone synthase gene, ScbA, which lies divergent from the ScbR gene, using PCR (Takano *et al.*, 2001). In most of the γ-butyrolactone systems identified (apart from that in *S. griseus*) synthase and receptor genes are next to or very close to each other. It is best to use this property and to identify the γ-butyrolactone synthase gene first using degenerate oligonucleotides or by an *in silico* approach. The degenerate oligonucleotides should be designed near the active sites we have identified by mutagenesis analysis, which also correspond to the AfsA repeats (Hsiao *et al.*, 2007).

Another approach is to use any sequence information available. These γ-butyrolactone receptors will resemble TetR repressors and may be found especially concentrated close to antibiotic biosynthetic gene clusters. There are usually several γ-butyrolactone receptor homologues in one strain. To determine the "real" receptor, the amino acid identity should be more than 40% compared to the functionally proven γ-butyrolactone receptors, that is, ArpA, BarA, FarA, and ScbR. These proteins also will have a pI of around 5–6 compared to the other ScbR homologues whose pIs are much higher at around 9–11.

6. Identification of the γ-Butyrolactone Receptor Targets

The first target to be identified was from the A-factor system by use of the genomic SELEX (systematic evolution of ligands by exponential enrichment) system (Ohnishi *et al.*, 1999). This method uses purified γ-butyrolactone receptor in gel retardation assays on partially digested chromosomal DNA to find the binding sites. Several groups have now proposed the target sequences for the γ-butyrolactone receptors (Folcher *et al.*, 2001; Kinoshita *et al.*, 1999; Onaka and Horinouchi, 1997) (Fig. 6.6). However, these sequences are not sufficiently conserved compared with the identified targets for ScbR (Fig. 6.6).

We have used the properties of the TetR receptor family, which the γ-butyrolactone receptors resemble. TetR proteins regulate their own genes and/or the adjacent gene. By using this property, gel retardation was performed to identify one of the ScbR targets. To identify another target of ScbR, microarray analysis was performed, which did not result in the identification of a direct target but rather the downstream genes which represent the Cpk antibiotic biosynthetic gene cluster. But doing so, the direct target, a pathway-specific activator, for the Cpk cluster was identified. Surprisingly, of the four targets of ScbR that we have identified, two, site R and site OA, have 100% consensus sequences at 6 bp at each ends. While the

| SiteA (*scbA/R* promoter region) | GAAAAA*AA**AAC**C**G*CTC TAG TCTG*T*A TC*T*TAA |
| SiteOB (*kasO* promoter region) | CA*A***AAC**A*G*ACT TGT TAG**C***T*G TT*T* |

SiteR (*scbA/R* promoter region)	GG**AACCGG**CAATGC**GGTTTGT**TCGATC
SiteOA (*kasO* promoter region)	ACA**AACCGG**TGTGCT**GGTTTGT**AAAGTCGTGG
ScbR binding consensus	***AACCGG**NNNNNN**GGTTTGT**

ArpA binding consensus	^A_CC$^A_G$$^A_T$**A****ACCG**$^A_G$CCGG$^T_C$**CGGT**$^A_T$$^T_C$**G**$^T_G$
BarA, FarA binding consensus	aCa$^{AA}_{TG}$c$^{GAAC}_{CGGA}$CGgtc**gg****TTTG**
SpbR binding consensus	TNANA^A_T**C**NNAC^C_TNNN**GGTTT**^G_T**T**TT

Figure 6.6 Consensus sequences of different γ-butyrolactone receptors. The target sequences found so far for ScbR and its consensus sequences in *S. coelicolor* are presented on the top five lanes. The bottom three lanes are consensus sequences reported for the different receptor proteins. ArpA: Onaka and Horinouchi (1997); BarA, FarA: Kinoshita *et al.* (1999); SpbR: Folcher *et al.* (2001). Bold letters represent the conserved base pairs in ScbR binding sites. The base pairs in italic in siteA and siteOB represent the conserved base pairs between these two sites.

others, site A and site OB, only have 4 bp that are conserved with site R and site OA. In contrast, within the pair, 11 bp are conserved out of 23 bp (Fig. 6.6) (Takano *et al.*, 2005).

To identify the γ-butyrolactone receptor targets, first use the promoter region itself, and also include the promoter region of the gene adjacent to it, to perform a gel retardation assay with either a purified protein or a crude extract. The activity of the protein is stabilized when the protein is still in the crude extract and at a low concentration. As soon as the protein is purified and concentrated, it tends to aggregate and will no longer be suitable for any further experiments to analyze activity. Use the identified target sequence to identify further targets *in silico*.

7. GEL RETARDATION ASSAY TO DETECT TARGET SEQUENCES OF THE γ-BUTYROLACTONE RECEPTORS

Gel retardation is not a new technique and can be found described in many publications (e.g., Folcher *et al.*, 2001; Kinoshita *et al.*, 1999; Onaka and Horinouchi, 1997). However, we have experience in using three differently labeled γ-butyrolactone receptor target DNA fragments, and have optimized the technique for each probe using pure protein and crude extracts from either *Escherichia coli* or *Streptomyces*.

7.1. Labeling of DNA fragments
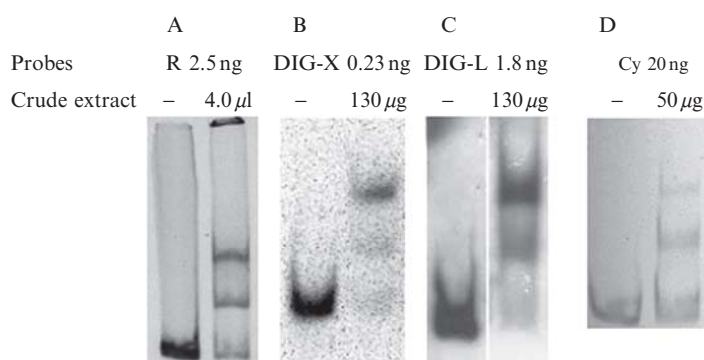
7.1.1. Radio-labeled probes

1. The oligo was first labeled at the $5'$ ends by incubation of 50 pmol of oligo, 5 μl of [γ-^{32}P]ATP (10 mCi/ml), 1 μl of $\times$10 kinase buffer, and 1 μl T4 nuclease kinase in a total volume of 50 μl incubated at 37 °C for 30 min^{-1} h. The reaction was stopped and cleaned by addition of $\times$1 volume of phenol/chloroform with vortexing, then the phenol was extracted by addition of either $\times$1 vol of chloroform or ether, twice. The oligo were precipitated using 100% EtOH with 3 M NaoAc and 1 μg of glycogen at -20 °C preferably O/N. The mixture was spun at 4 °C for 30 min, then the supernatant discarded, and the pellet air dried.

2. PCR was conducted using labeled- and unlabeled-oligos. 60 ng of template, 50 pmol of both unlabeled- and labeled-oligos, 200 μM dNTP, 5% DMSO, and PCR buffer (commercial) together with Taq polymerase were incubated to amplify in a total volume of 100 μl. The PCR conditions are: 95 °C for 5 min, then 30 cycles of 95 °C for 50 s, 55 °C for 40 s, 72 °C for 40 s, then extend at 72 °C for 5 min. Five microliters of the reaction was loaded on a gel to assure amplification. If extra bands are amplified, the fragment of interest can be purified using a commercial gel-extraction kit. The final volume should be eluted in 50 μl.

3. Gel retardation was conducted with 2.5 ng of labeled DNA (about 10 cpm), pure protein or 0–15 μl crude extract from *E. coli* or *Streptomyces*, 125 mM HEPES pH 7.5, 20 mM DTT, 20% glycerol, 200 mM KCl, 0.16 μg/μl calf thymus DNA in a final volume of 12.5–25 μl. The mixture was incubated at room temperature for 10 min, then 2–4 μl of loading dye (50%(v/v) glycerol, 0.25% (w/v) bromophenol blue, 10 mM Tris–HCl, pH 8, 1 mM EDTA) was added. To detect the binding of the receptor to the butyrolactones, the butyrolactones can be added either before or 10 min after the incubation. In the case of SCB1, 1 μg was added to see a release of the full shift.

   Note:
   - For the *E. coli* crude extract, a 10 ml LB overnight preculture of *E. coli* harboring a plasmid with the γ-butyrolactone receptor gene under the control of the *lacZ* promoter was inoculated at a 1:100 dilution in 50 ml LB and grown at 37 °C until OD$_{600}$ 0.7–0.8 when induced with 1 mM (final conc.) IPTG (Isopropyl β-D-1-thiogalactopyranoside). After another 3 h of incubation, cells were harvested by centrifugation and the cell pellet was washed twice with buffer (50 mM of Tris pH 7.0, 1 mM of EDTA, 1 mM of DTT, 100 mM of phenylmethylsulfonylfluoride (PMSF)) and resuspended in 500 μl of the same buffer. 100 μl aliquots were stored at -80 °C for later use. For crude extract

preparation, the cells were immediately disrupted by sonication. The cell lysate was clarified by centrifugation and the supernatant used as a crude extract after determination of the total protein content.

- For the *S. coelicolor* crude extract, *S. coelicolor* was grown in 50 ml of SMM then the same procedure as above was followed except the cells were resuspended in 100 μl prior to sonication and 10 μl was used for the gel retardation analysis. Due to the growth phase-dependent expression of ScbR, the time point of growth when the cells were harvested is crucial to detect any protein binding to the DNA.
- KCl was the best salt for this assay and MgCl was not suitable.

4. For detection of 100–300 bp DNA fragments, a 5% acrylamide gel (5% acrylamide: bisacrylamide = 37.5:1, 1.25 ml of ×10 TBE, 15 μl of TEMED, 87.5 μl of 10% AMPS in a total of 12.5 ml) was used in ×1 TBE (90 mM Tris–HCl, 90 mM boric acid, 2 mM EDTA, pH 8.0) running buffer. It is also possible to use a 3.5% acrylamide gel to detect a longer DNA fragment. A Bio-Rad Mini Protean kit was used for running the gel at 100 V constant for 1 h till the BPB just ran off the gel.

5. The gel was then taken off the glass and wrapped in cling film (Saran wrap) and directly placed onto an X-ray film (Super RX (Fujifilm)), and exposed for 30 min at room temperature in a cassette. The film was developed using an automated film developer (Fig. 6.7A).

	A	B	C	D
Probes	R 2.5 ng	DIG-X 0.23 ng	DIG-L 1.8 ng	Cy 20 ng
Crude extract	– 4.0 μl	– 130 μg	– 130 μg	– 50 μg

Figure 6.7 Gel retardation assay using differently labeled probes. Gel retardation using: (A) Radio-labeled probes (R); (B) DIG-labeled probes with detection using X-ray film (DIG-X); (C) DIG-labeled probes with detection using Lumi-imager (DIG-L); (D) Cy3-labeled probes (Cy). All probes were made using a 146 bp DNA fragment which includes the ScbR binding site R which is situated at −68 to −42 nt from the transcriptional start site (Takano *et al.*, 2001). The amount of each probe used is indicated next to each probe name. Crude extract (given in total protein as microl, microg) from *E. coli* harboring pIJ6120 was used and the amount is indicated on the top of the lanes.

7.1.2. DIG-labeled probes

1. DNA fragments were first amplified by PCR using both unlabeled-oligos using the PCR conditions mentioned in Point 2 of Section 7.1.1. Purified PCR product (either by a cleanup kit or by extracting from a gel), 100 ng, was used in the DIG gel shift kit 2nd generation (Roche) labeling procedure, steps 5–7 (p11 of Roche protocol). (Note: this kit labels $3'$ ends.)

2. After labeling, the probes were incubated with the protein for gel retardation assays as in Point 3 of Section 7.1.1.

   Note:
   - The labeled probes can be stored at −20 °C for a very long time.
   - Depending on the sensitivity of the detection method used (see below), the amount of DIG-labeled probe may have to be varied. In our case, eightfold less probe was used for detection with X-ray films (we used 1.8 ng of labeled probe) compared to when a Lumi-imager F1 (we used 0.23 ng labeled probe) was used for detection (Fig. 6.7B and C).
   - After optimization of the amount of probe used, the protein concentration in the assay will have to be adjusted to keep the protein/DNA ratio constant.

3. After running, the gel was taken off the glass plates for direct contact-blotting (Roche protocol 3.7.2), further crosslinking (3.7.3), and chemi-luminescent detection (3.8). The membrane was detected using an X-ray film exposed for 20 min, then developed in an automated X-ray film developer (Fig. 6.7B) or in a Lumi-imager F1 (Roche) for 40 min at room temperature (Fig. 6.7C).

7.1.3. Cy3-labeled probes

The DIG-labeling protocol will take up to 2 days. To improve the efficiency in time, Cy3-labeled oligos are now our preferred choice. A preliminary protocol is described below.

1. $5'$–Cy3-labeled oligo was purchased from Sigma–Aldrich and used for PCR along with the unlabeled-oligo as in Point 3 of Section 7.1.1. The amplified product was purified and the concentration measured by Nanodrop (Thermo scientific).

2. The gel retardation was conducted with 20 ng of Cy3-labeled DNA fragment with 50–300 μg of crude extracts from *Streptomyces* as in Points 3 and 4 of Section 7.1.1. After running, the gel with the glass plate is exposed for 30 s and detected in a LAS 4000 (Fujifilm) (Fig. 6.7D).

   Note: A flourescence compound was detected in the *Streptomyces* crude extract which may interfere with detection.

8. Conclusions

The rapid small-scale purification of the γ-butyrolactones and the kanamycin assay have simplified the detection of the γ-butyrolactones, which are found to be one of the important factors in the regulation of antibiotic production. From genome sequences and also from the sequence of many antibiotic biosynthetic gene clusters many homologues to the γ-butyrolactone synthases and receptors have been identified, but the number of γ-butyrolactones elucidated has not increased since year 2000. This method may provide an easy solution to detect the γ-butyrolactones from those strains which have the synthase and receptor homologues and also to identify new organisms that may produce γ-butyrolactones. The binding sensitivity of the different γ-butyrolactones to ScbR has been tested and found to be lower; however, with enough material kanamycin resistance was observed (Hsiao *et al.*, submitted). Identification of the receptor target is a major job. However, with the several hints obtained from previous work and also from our experience with the gel retardation assays, it may be possible to quicken the procedure.

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Stellingen

behorende bij het proefschrift

Regulation of antibiotic production in *Streptomyces coelicolor*

**A bacterial hormone receptor variant and
the awakening of a cryptic antibiotic biosynthesis gene cluster**

van Marco Gottelt

I.   In the course of a PhD project, too little time, interest, and appreciation are devoted to open-minded hypothesizing and unrestricted scientific discussions. Any effort to improve PhD education should take the personal, project-related, and academic value of these discussions into account.

II. Despite the results presented in this thesis, yellow is not my favourite colour.

III. Antibiotic substances that are used as antibacterial therapeutic drugs may in many cases serve a different purpose in the natural producer (*inspired by the work of Julian Davies and this thesis*).

IV. "What gets us into trouble is not what we don't know; it's what we know for sure that just ain't so." (*Mark Twain*).

V.   From a linguistic point of view it may be surprising that antibiotics save lives.

VI. The ideal of freedom of science is challenged by the idea that science should exist primarily to serve the needs of society. However, neither has society proven the farsightedness to define its true needs, nor can scientists predict (the usefulness of) entirely new discoveries.

VII. In most cases, the term "cryptic gene cluster" is misleading and should be replaced by "orphan gene cluster" (*inspired by Gross, Appl Microbiol Biotechnol, 2007 and this thesis*).

VIII. In future research projects, abCPK and yCPK are more eligible as indicators for the role of the *Streptomyces coelicolor* butanolide system in secondary metabolite production than the hitherto used Act and Red (*this thesis*).

IX. "Given that we can live only a small part of what there is in us – what happens with the rest?" (*Pascal Mercier*). The factual globalization of professional and private lives creates a fascinating, but also challenging variety of opportunities.