

# Two transcription factors, CabA and CabR, are independently involved in multilevel regulation of the biosynthetic gene cluster encoding the novel aminocoumarin, cacibiocin

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**Abstract** Aminocoumarins are potent antibiotics belonging to a relatively small group of secondary metabolites produced by actinomycetes. Genome mining of *Catenulispora acidiphila* has recently led to the discovery of a gene cluster responsible for biosynthesis of novel aminocoumarins, cacibiocins. However, regulation of the expression of this novel gene cluster has not yet been analyzed. In this study, we identify transcriptional regulators of the cacibiocin gene cluster. Using a heterologous expression system, we show that the CabA and CabR proteins encoded by *cabA* and *cabR* genes in the cacibiocin gene cluster control the expression of genes involved in the biosynthesis, modification, regulation, and potentially, efflux/resistance of cacibiocins. CabA positively regulates the expression of *cabH* (the first gene in the *cabHIYJKL* operon) and *cabhal* genes encoding key enzymes responsible for the biosynthesis and halogenation of the aminocoumarin moiety, respectively. We provide evidence that CabA is a direct inducer of cacibiocin production,

whereas the second transcriptional factor, CabR, is involved in the negative regulation of its own gene and *cabT*—the latter of which encodes a putative cacibiocin transporter. We also demonstrate that CabR activity is negatively regulated in vitro by aminocoumarin compounds, suggesting the existence of analogous regulation in vivo. Finally, we propose a model of multilevel regulation of gene transcription in the cacibiocin gene cluster by CabA and CabR.

**Keywords** Cacibiocin · Antibiotics · *Catenulispora acidiphila* · *Streptomyces coelicolor* · Transcription regulation · Genome mining · Gene cluster

## Introduction

Aminocoumarins are inhibitors of bacterial type II topoisomerases, such as DNA gyrase and DNA topoisomerase IV (Alt et al. 2011; Hardy and Cozzarelli 2003; Maxwell 1999; Opegard et al. 2009). DNA gyrase introduces negative supercoils, whereas topoisomerase IV is involved in chromosome decatenation. Both enzymes require ATP hydrolysis for their activities (Bates and Maxwell 2007; Champoux 2001; Schoeffler and Berger 2005). Most aminocoumarins, such as the first-discovered novobiocin, interact with the ATP binding pocket within GyrB and ParE subunits of gyrase (GyrA<sub>2</sub>GyrB<sub>2</sub>) and topoisomerase IV (ParC<sub>2</sub>ParE<sub>2</sub>) heterotetramers, respectively, to competitively block the binding of ATP to the enzymes (Gormley et al. 1996; Maxwell 1997; Mizuuchi et al. 1978; Sugino et al. 1978). Simocyclinones exhibit a different mode of action, acting by binding to the GyrA subunit similar to quinolones, which are a different class of gyrase inhibitors that, unlike quinolones, prevent DNA binding instead of blocking the resealing activity of this enzyme (Anderson et al. 1998; Flatman et al. 2005; Redgrave et al. 2014).

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Despite the efficacy of aminocoumarins in gyrase inhibition, none of these agents are currently approved for therapy in humans although they continue to be used in veterinary medicine. Novobiocin, used in the past for *Staphylococcus aureus* infections, was withdrawn by the Food and Drug Administration in 2011, owing to its side effects and limited effectiveness. Recent studies, however, suggest novel therapeutic targets for aminocoumarins, highlighting the antiproliferative activity of these compounds against cancer cells in tumor development (Audisio et al. 2014; Luo et al. 2010; Marcu et al. 2000; Wu et al. 2013). These new potential applications, as well as the demand for new antibiotics arising from the increasing number of antibiotic-resistant nosocomial strains, underscore the need for novel aminocoumarin compounds to overcome drawbacks such as high toxicity and low solubility of currently available compounds (Maxwell and Lawson 2003).

Aminocoumarins are secondary metabolites produced mostly by actinomycetes of the genus *Streptomyces*. However, the aminocoumarin moiety (3-amino-4,7-dihydroxycoumarin) is rare in nature and only few aminocoumarins have been discovered to date (Heide 2009b, 2014). Genes responsible for the synthesis of secondary metabolites, including aminocoumarins, are usually organized into clusters. The majority of these gene clusters is silent, and their products remain undetectable under laboratory conditions (Bentley et al. 2002; Ohnishi et al. 2008; Riesenfeld et al. 2004; Seyedsayamdost 2014). Genome mining is a relatively new, powerful approach that seeks to discover novel secondary metabolites by combining global genome sequence analysis and directed activation of biosynthetic gene clusters (Baltz 2008; Challis 2008; Rutledge and Challis 2015; Walsh and Fischbach 2010). This strategy has led to the identification of a large number of novel biosynthetic gene clusters (e.g., for polyketides and nonribosomal peptide synthetase products), including a gene cluster encoding novel aminocoumarins (cacibiocins A and B) in the actinomycete *Catenulispora acidiphila* (Gross 2007; Gross et al. 2007; Kersten et al. 2011; Lautru et al. 2005; Zettler et al. 2014). Both molecules are unusual in that they include unique dichlorination (cacibiocin B) and pyrrole (cacibiocins A and B) substitutions not found in other aminocoumarins.

Similar to other secondary metabolite synthesis pathways, the aminocoumarin gene clusters contain not only genes required for the biosynthesis of these compounds but also those involved in drug resistance and regulation of gene expression. Orthologs of *novHIJK* genes involved in the biosynthesis of the principal aminocoumarin skeleton of novobiocin are found in all described aminocoumarin gene clusters. The roles of these gene products in aminocoumarin biosynthesis have been extensively studied (Chen and Walsh 2001; Galm et al. 2002; Pacholec et al. 2005; Pojer et al. 2003; Wolpert et al. 2008; for review, see also Heide 2009a). Aminocoumarin

producers defend themselves against antibiotics by producing specific gyrase and/or topoisomerase IV subunits that are resistant to aminocoumarin compounds. In both cases, genes conferring resistance to antibiotics (*gyrB<sup>R</sup>* and *parY<sup>R</sup>*, respectively) are part of the corresponding biosynthetic gene clusters. Other cluster genes encoding transporter proteins responsible for aminocoumarin efflux may provide a second mechanism of resistance against these antibiotics (for review, see Heide 2009b). Genes encoding regulatory proteins, also referred to as cluster-situated regulators (CSRs), are often pathway-specific regulators responsible for activating or repressing the transcription of particular biosynthetic gene operons or resistance genes and regulating the onset of aminocoumarin production (Dangel et al. 2008, 2009; Eustaquio et al. 2005; Horbal et al. 2012; Le et al. 2009). The activity of some CSRs may also be modulated by the product of the antibiotic biosynthetic pathway, generating additional feedback regulation of gene expression (Ahn et al. 2007; Le et al. 2009; Liu et al. 2013; Tahlan et al. 2008). A better understanding of the molecular regulation of antibiotic biosynthesis may be crucial for the activation of many silent secondary metabolite gene clusters. However, despite extensive studies on aminocoumarin biosynthesis, there are only a few examples of experimentally verified regulatory circuits.

The newly identified aminocoumarin gene cluster of *C. acidiphila* DSM44928 encodes the biosynthetic proteins for the production of cacibiocins A and B and two genes, *cabR8* and *cabR7* (hereafter *cabA* and *cabR*), encoding putative cluster regulators CabA and CabR (Zettler et al. 2014). The CabA and CabR proteins belong to the LuxR and TetR families of transcriptional regulators, respectively, involved in a number of cellular processes, such as carbon metabolism, stress responses, and quorum sensing (for review, see Ramos et al. 2005). The main aim of this study was to investigate the roles of the two regulatory proteins, CabA and CabR, in regulating the expression of genes in the cacibiocin gene cluster (Zettler et al. 2014). Here, we demonstrate the multilevel regulation of transcription of the cacibiocin gene cluster by CabA and CabR. Our results also suggest the existence of a mechanism that couples the biosynthesis of cacibiocins to their export.

## Materials and methods

### Bacterial strains, plasmids, and culture conditions

The *Streptomyces coelicolor* and *Escherichia coli* strains used in this study are listed in Table S1. Culture conditions, media, antibiotic concentrations, and transformation and conjugation methods followed general procedures for *E. coli* (Sambrook et al. 1989) and *Streptomyces* (Kieser et al. 2000). Briefly, for tests of cacibiocin production, precultures of *S. coelicolor*

M1152 strains were grown for 2 days in 50 ml YMG (yeast extract 4 g/l, malt extract 10 g/l, glucose 4 g/l) in 300-ml baffled Erlenmeyer flasks containing a stainless steel spring at 30 °C. Production medium SM12 (soy flour 10 g/l, glucose 50 g/l, peptone g/l, meat extract g/l, yeast extract g/l, NaCl 2.5 g/l, CaCO<sub>3</sub> 5 g/l) was inoculated with 5 % preculture and cultivated for 11 days. Screening was performed in 24-deepwell plates (Siebenberg et al. 2010) using the same conditions as for cultivation in flasks but shaking at 300 rpm. Each well was cultivated with 3 ml medium and supplemented with 0.6 % siloxylated ethylene oxide/propylene oxide copolymer (Siebenberg et al. 2010). Triplicate measurements were performed for each medium and strain. For *C. acidiphila* cultures, YMG medium (preculture) and chemically defined medium (CDM) (main culture) were used (Kieser et al. 2000; Kominek 1972; Sambrook et al. 1989) as described previously (Zettler et al. 2014). For DNA isolation, *S. coelicolor* was cultivated in 79 liquid medium (10 g peptone, 2 g casein hydrolysate, 2 g yeast extract, 6 g NaCl, H<sub>2</sub>O to 1 l, pH 7.2–7.4).

### DNA manipulations and plasmid construction

Standard cloning procedures based on published protocols (Kieser et al. 2000; Sambrook et al. 1989) were applied for plasmid and strain construction (*E. coli* and *Streptomyces*; for details, see the Supplemental Information (SI)). All PCR-derived clones were analyzed by DNA sequencing to check their fidelity. Enzymes were supplied by Life Technologies, Promega or Roche, and oligonucleotides (Table S2) were from Sigma-Aldrich.

### Extraction, analysis, and purification of cacibiocin A

Extraction of culture was performed as described for clorobiocin (Pojer et al. 2002). The cultures were acidified to pH 4 with HCl and extracted twice with equal volumes of ethyl acetate. Afterwards, the solvent was evaporated and the dried extract was resuspended in methanol for further analysis (Pojer et al. 2002). Metabolites were analyzed by HPLC using a ReproSil-Pur C18-AQ column (5 µm, 250×4.6 mm) and a linear gradient from 30 to 100 % methanol in 0.1 % aqueous formic acid over 31 min; the flow rate was 1 ml/min, and detection was carried out at 320 nm.

### Polyacrylamide gel electrophoresis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % polyacrylamide gels as described previously (Laemmli 1970).

### CabRHis<sub>6</sub> purification protocol

Detailed cloning, expression, and purification procedures are described in the SI. Briefly, the PCR-amplified *cabR* gene was cloned into the pET-21a(+) expression vector (Merck), yielding pET-21a(+)*cabR*, and *E. coli* BL21(DE3) (Merck) was used as the host strain for overexpression of His-tagged CabR protein (CabRHis<sub>6</sub>). The recombinant protein was expressed and purified by nickel affinity chromatography (HIS-Select Nickel Affinity Gel; Sigma-Aldrich) as described previously (Wolanski et al. 2011). SDS-PAGE analysis of protein fractions showed that the fusion protein was ~95 % pure.

### MBPCabA purification protocol

The *cabA* gene was cloned into the pMAL-p2x expression vector (New England Biolabs), yielding pMAL-p2xcabA, and *E. coli* Arctic Express RIL strain (Agilent Technologies) was used to overexpress MBPCabA fusion protein (CabA fused to maltose binding protein (MBP)). The recombinant protein was purified using a two-step procedure using a HiTrap Heparin HP column and dextrin Sepharose resin. For details on cloning, expression, and purification, see the SI.

### Affinity chromatography assay

The procedure used was similar to that described previously (Jakimowicz et al. 1998). Briefly, the GSTCabR protein was immobilized on glutathione Sepharose 4B (GE Healthcare) and incubated with enzymatically digested cosmid 1E1. The DNA fragments bound by GSTCabR were eluted using buffer containing increasing salt concentration and were subsequently analyzed on agarose gels stained with ethidium bromide. For more details on the assay, see the SI.

### Electrophoretic mobility shift assay

#### For DNA-CabRHis<sub>6</sub> interactions

The electrophoretic mobility shift assay (EMSA) was carried out as described previously, with minor changes (Wolanski et al. 2011). Briefly, purified CabRHis<sub>6</sub> was incubated with 50–100 ng of nonradioactive DNA fragments for 30 min at room temperature (~25 °C) in 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) supplemented with 5 % glycerol and 5 µg/µl bovine serum albumin (BSA). Samples were subsequently loaded onto 4–5 % polyacrylamide gels in 0.25× Tris-borate-EDTA (TBE) buffer and resolved at 5–10 V/cm for 3–4 h at 4–6 °C. The gels were then stained with ethidium bromide or SYBR Green and analyzed using a G:BOX system (Syngene). Before use, the CabRHis<sub>6</sub> protein was exchanged

into 1× PBS (without glycerol and BSA) using Zeba Spin Desalting Columns (7K MWCO; Pierce).

#### For DNA-MBPCabA interactions

Purified recombinant MBPCabA protein was thawed on ice before use and subsequently incubated with either digested 1E1 cosmid DNA (750 ng) or nonradioactive PCR-amplified DNA fragments (50–150 ng). The binding reactions were conducted in buffer S (20 mM Tris-HCl, pH 8.5; 200 mM NaCl, 5 µg/µl BSA, 5 mM ATP, 5 mM MgCl<sub>2</sub>). After a 45-min incubation at room temperature (~25 °C), the samples were applied to agarose gels (1 % or 2 %) in 1× TBE buffer and resolved at 20 V at room temperature overnight. The DNA complexes were stained with SYBR Green or ethidium bromide and visualized with UV light.

#### DNase I footprinting

Footprinting experiments were performed similar to a previously described method (Wolanski et al. 2011). Briefly, ~10 fmol of DNA fragments prepared by PCR amplification using 5'-end radiolabeled oligonucleotides was incubated with different amounts of recombinant protein (CabRHis<sub>6</sub>, MBPCabA) in 1× reaction buffer (the same as that used for EMSAs) at 25 °C for 30 min. After DNase I digestion, cleavage products were separated on an 8 % polyacrylamide-urea sequencing gel. The gel was analyzed using the Typhoon 8600 Variable Mode Imager and Image Quant software.

#### Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed similar to a previously described method (Majka et al. 1999; Wolanski et al. 2011). Briefly, DNA fragments were labeled with biotin on the 5'-end using PCR and approximately 100 response units (RU) of DNA was immobilized on the streptavidin-coated sensor chip (Series S Sensor Chip SA; GE Healthcare) of the Biacore T200 apparatus according to the manufacturer's protocol. A DNA fragment lacking a CabR binding site was used as a negative control. The DNA fragments used in these studies were generated using the primer pairs Caci\_SPR\_1For-biot and Caci\_SPR\_1Rev for the *cabR-cabT* region and the primers b-ScoriC and Br3-oriC for the negative control (Table S2).

Effects of mass transport on the kinetics of protein-DNA interactions were excluded by obtaining sensograms at various CabRHis<sub>6</sub> concentrations (10 to 100 nM) at a continuous flow rate (30 µl/min) for 120 s. The dissociation time was set at 600 s. Measurements were performed in PBS buffer containing 0.05 % Tween 20 (final concentration). At the end of each cycle, the residual protein was removed from the chip with a 20-µl pulse of 0.05 % SDS (15 µl/min). In experiments

that included aminocoumarins, the respective compounds were dissolved in 100 % DMSO and added at 1/20th of the final sample volume. DMSO was added to the control samples to a final concentration of 5 % to control for the effects of organic solvent addition.

The final sensograms were obtained after automatic subtraction of the background response signal obtained in a chip channel containing control DNA. The data were evaluated using the Biacore T200 Evaluation software version 1.0.

For calculation of protein-DNA stoichiometry, we used the equation,  $n = R_{\max} \times MW_{\text{DNA}} / (R_{\text{DNA}} \times MW_{\text{protein}})$  (Majka and Speck 2007), where  $n$  is the number of protein molecules bound to DNA,  $R_{\max}$  is the response at a saturating concentration of protein,  $R_{\text{DNA}}$  is the amount of immobilized DNA (RU), and  $MW_{\text{DNA}}$  and  $MW_{\text{protein}}$  are the molecular weights of DNA and protein, respectively.

#### RNA extraction, reverse transcription, and quantitative polymerase chain reaction

For total RNA preparation, *C. acidiphila* cultures were grown in YMG medium and CDM as described previously (Zettler et al. 2014). After sample collection by vacuum filtration, the cells were immediately frozen in liquid nitrogen and stored at -80 °C. Before use, the cells were thawed on ice and ground in liquid nitrogen using a mortar and pestle. RNA was isolated by resuspending the cells in TRI Reagent (Sigma) containing 0.5 ml of 0.1-mm zirconia/silica beads and vortexing three times (3-min vortex with 1-min pause on ice between cycles). The extracts were left at room temperature for 20 min to allow phase separation. Subsequent steps were performed according to the manufacturer's protocol. RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer, and the quality of the RNA was analyzed on an agarose gel. Complementary DNA (cDNA) was obtained by reverse transcription of 1–4 µg of DNase I-treated total RNA using MultiScribe murine leukemia virus (MuLV) reverse transcriptase and random hexamer primers (High Capacity RNA-to-cDNA Master Mix; Life Technologies), following the procedures recommended by the manufacturer. Expression of genes was analyzed by quantitative polymerase chain reaction (qPCR) using an ABI system (StepOnePlus) and an SYBR Real-time 2× PCR Master Mix kit (A&A Biotechnology). qPCR was performed using 50–300 ng cDNA, and the thermocycling conditions are the following: 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The primers used for qPCR, designed using Primer Express v. 3.0 or Primer 3 web tool (<http://simgene.com/Primer3>), are listed in Table S2 (see the SI). The specificity of each primer pair was initially verified using the Primer-Blast tool (NCBI) and subsequently confirmed by qPCR reactions. At the end of each PCR, the products were verified by performing a melting curve analysis and running

the DNA fragment on a gel. Melting curves were obtained using the default manufacturer's protocol in a StepOnePlus system for SYBR Green detection-based chemistries, and the performance of each primer pair was analyzed using the StepOne software v 2.0 (Applied Biosystems). Target gene transcript levels were normalized internally to the level of the RNA polymerase sigma factor *caci\_1761* (*Streptomyces hrdB* principal sigma factor homolog) gene. Each experiment was performed at least in duplicate, and each transcript level was measured in triplicate. With each set of primers, negative-control experiments, performed in the absence of reverse transcriptase, confirmed that PCR products were amplified from a cDNA template (i.e., there was no significant contamination with chromosomal DNA).

### Luciferase assay

Promoter activity was determined using a luciferase activity assay following procedures similar to those described previously (Le et al. 2009). Construction of plasmids and strains used for luciferase assays is described in detail in the SI. Briefly, gene promoter regions (500 bp) were cloned into the conjugative vector pIJ5972 containing the luciferase reporter gene and the resulting plasmids were transferred by conjugation into *S. coelicolor* M1152 (Kieser et al. 2000). In the second conjugation step, plasmids carrying regulatory genes (*cabA* and *cabR*) under the control of the constitutive *ermE\** promoter (or an empty control plasmid) were delivered into the *Streptomyces* strains obtained in the previous step. Introduction of the respective genes and promoter regions in all constructed strains was confirmed by PCR.

Promoter activity assays were performed by first inoculating the liquid CDM with spores of the respective *Streptomyces* strains and growing them in glass tubes (5 ml medium per 25-ml glass tube) for 48 h at 30 °C with shaking (180–200 rpm). Each tube was inoculated with approximately the same amount of spores (final spore  $OD_{600}=0.04$ ). After 48 h, the cells were harvested by centrifugation and washed with PBS. The pellets were stored at –20 °C. Before measurements, mycelia were thawed on ice, resuspended in 100  $\mu$ l of PBS, and transferred to a 96-well OptiPlate (PerkinElmer). Luciferase activity was measured by adding 2  $\mu$ l of *n*-decanal to each sample and recording luminescence at 1-min intervals for 5 min using an EnVision Multilabel Reader (PerkinElmer) at default settings. Luciferase activity was expressed as luminescence (cps) per gram (wet weight) of mycelium. Values (Fig. 4a, b) correspond to the means  $\pm$  standard errors (calculated using the Gnumeric software) of three biological replicates. Charts were composed using the Excel software.

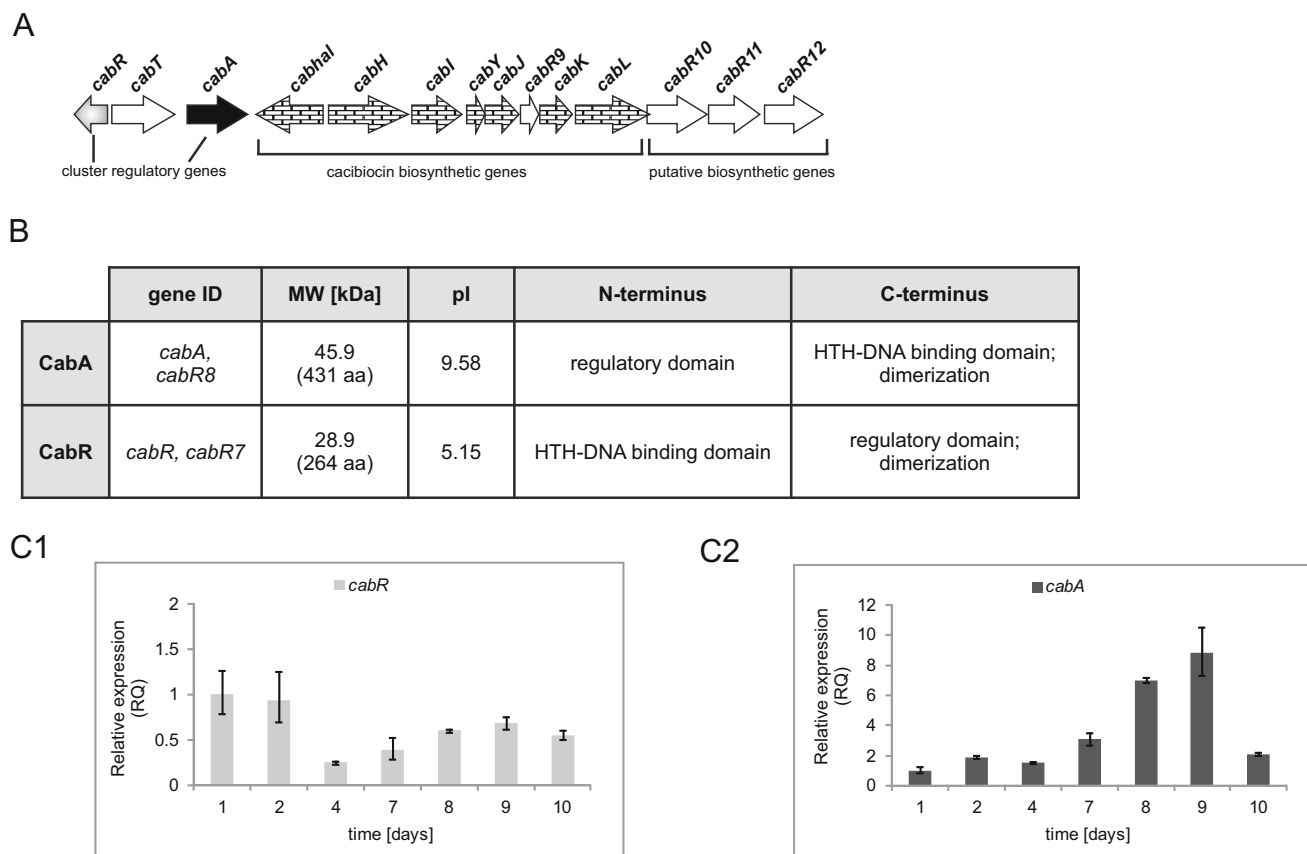
## Results

### Expression of one of the two putative gene cluster regulators positively correlates with cacibiocin production

Both *cabA* and *cabR* genes encoding putative gene cluster regulatory proteins, CabA and CabR, respectively, are located upstream of the cacibiocin biosynthetic genes (*cabhal* and *cabHIJKL*) and are separated by a single gene, *cabR5*, encoding a putative transporter protein (here termed CabT) (Fig. 1a). A sequence alignment analysis using the NCBI BLASTP algorithm and CDD search (Conserved Domain Search) revealed that CabA and CabR proteins are classified as members of the HTH-XRE superfamily of transcriptional regulators, which encompasses proteins containing a DNA-binding helix-turn-helix (HTH) motif and a xenobiotic response element (XRE).

The CabA belongs to the LuxR family of transcriptional factors that usually act as positive regulators. Therefore, the CabA protein is predicted to be an activator of the cacibiocin gene cluster. LuxR-like proteins exhibit a two-domain organization, where the N-terminal region contains a regulatory domain and the C-terminal domain is responsible for DNA binding and dimerization (Fig. 1b). The activity of these proteins is often modulated by binding of signal molecules to the N-terminal domain or by posttranslational modifications of this domain (for a review of the regulation of LuxR regulators, see Chen and Xie 2011). However, the CDD search showed no resemblance of the N-terminal region of the CabA protein to any conserved regulatory domain. An amino acid sequence BLASTP search revealed that CabA is similar to RubRg2 (43 % identity for 75 % sequence coverage, *E* value  $9e-74$ ), a putative regulator present in the aminocoumarin gene cluster for rubradirin biosynthesis. However, the role of the *rubRg2* gene product has not yet been investigated (Sohng et al. 1997). CabA does not possess sequence homologs encoded in other aminocoumarin gene clusters.

The CabR protein consists of the N-terminal DNA binding domain and a C-terminal regulatory domain (Fig. 1b). Since CabR belongs to the TetR family of regulators, the *cabR* gene was predicted to encode a negative transcriptional regulator of the cacibiocin gene cluster (Ramos et al. 2005). The common feature of these regulators is that their binding is modulated by interactions with a ligand, leading to derepression of the regulated gene(s). Although CabR orthologs are widely distributed among actinomycetes, mainly in the genus *Streptomyces*, among the aminocoumarins, only the cacibiocin and simocyclinone gene clusters encode such regulators. CabR exhibits 31 % similarity to its counterpart SimR (for 90 % coverage, *E* value  $2e-27$ ), encoded by the *simR* gene (referred formerly as *simReg2*) in the simocyclinone biosynthetic gene cluster (Le et al. 2009; Trefzer et al. 2002).



**Fig. 1** CabA and CabR—putative regulators of the cacibiocin gene cluster. **a** Schematic organization of the cacibiocin gene cluster (modified from Zettler et al. 2014). **b** Basic characteristics of CabA and CabR proteins. **c** Time course of qPCR for *cabR* (1) and *cabA* (2) transcription. The relative expression levels of the corresponding genes

were measured at different time points during *C. acidiphila* growth as described in the [Materials and methods](#) section. Means were calculated from triplicate samples of two independent experiments. *Error bars* indicate standard deviations calculated with StepOne software using default settings

Recently, Apel and coworkers showed that the synthesis of cacibiocins A and B by their natural producer, *C. acidiphila*, remained at low levels over the entire period of cultivation in most of tested media and the highest amounts of cacibiocins in culture extracts were reached after 10 days of cultivation (Zettler et al. 2014). These authors also demonstrated that deletion of a fragment of the cacibiocin gene cluster upstream of the putative operon *cabHIYJKL* that included *cabA* and *cabhal* genes abolished cacibiocin production, indirectly suggesting a possible role of CabA in the activation of cacibiocin biosynthesis. In order to relate *cabA* and *cabR* gene expression profiles to cacibiocin production in *C. acidiphila* (in a CDM production medium), we investigated transcriptional profiles of these genes using real-time quantitative polymerase chain reaction (qPCR). These experiments showed that transcript levels of both genes were low during the exponential growth phase. The cycle threshold ( $C_t$ ) values obtained for *cabA* and *cabR* gene transcripts were high ( $C_t \approx 32$ – $36$ ) compared with those for the RNA polymerase principal sigma factor (*caci\_1761*) transcript ( $C_t \approx 27$ – $31$ ), used as an endogenous reference gene (data not shown). To calculate the relative

quantity (RQ) of transcripts expressed, we internally normalized the cDNA levels for *cabA* and *cabR* genes to those of *caci\_1761* cDNA. The resulting analysis indicated that the *cabR* gene was expressed most efficiently in very early cultures, reaching RQ values of  $\sim 1$  at days 1 and 2 and then dropping to  $\sim 0.25$  at day 4, before rising slightly again at days 7–10 (RQ  $\approx 0.4$ – $0.7$ ; Fig. 1c(1)). In contrast, the *cabA* gene transcript remained at relatively low levels (RQ  $\approx 1$ – $2$ ) between days 1 and 4 of culture, significantly increased on days 7–9 (RQ  $\approx 3$ – $9$ ), and then decreased again at day 10 (RQ  $\approx 2$ ) (Fig. 1c(2)). The increase in *cabA* and *cabR* transcript levels observed here preceded the onset of cacibiocin production reported previously under the same culture conditions (Zettler et al. 2014). These results suggest that, as predicted, the CabA protein positively influences the expression of the biosynthetic genes (*cabHIYJKL* and *cabhal*) involved in aminocoumarin production. Interestingly, both transcription of the *cabR* gene, encoding the putative negative regulator, and cacibiocin production increased, suggesting that cacibiocins are involved in regulating *cabR* expression, rather than the converse. On the basis of previous studies (Zettler

et al. 2014) and to confirm a direct positive influence of CabA on cacibiocin production, we measured cacibiocin levels in a *Streptomyces* host harboring a 10E2int cosmid encompassing the cacibiocin gene cluster, lacking the region upstream of the *cabHIYJKL* genes, and a pXL11 plasmid containing the *cabA* gene under the control of a constitutive *ermE\** promoter. These experiments revealed that the production of cacibiocin A occurred only in the presence of pXL11 and not with empty control plasmid pUWL201 (Fig. 4d), showing that *cabA* is essential for cacibiocin biosynthesis.

Collectively, these findings indicate that the recently discovered cacibiocin gene cluster encodes two putative transcriptional regulators, CabA (activator) and CabR (repressor), belonging respectively to the extensively characterized LuxR and TetR families of transcriptional factors. In *C. acidiphila*, transcription of both *cabA* and *cabR* genes positively correlated with the biosynthesis of cacibiocins. Moreover, *cabA* encodes a protein responsible for the activation of cacibiocin biosynthesis.

### CabA specifically binds to the bidirectional promoter controlling the genes for cacibiocin biosynthesis

To elucidate the regulation of gene expression in the cacibiocin gene cluster in greater detail, we sought to identify CabA and CabR binding sites within the cluster. For this purpose, we first cloned and expressed the *cabA* gene, purified recombinant protein, and subsequently applied electrophoretic mobility shift assays (EMSAs) to identify the promoter regions bound by CabA.

In these interaction studies, we used the CabA protein fused with maltose binding protein (MBP) at the N-terminus (MBPCabA; 92.2 kDa). Although other systems such as pGEX-6P-2 and pET21a(+) have been tested, we were only able to obtain soluble fractions of the protein using the pMAL expression system (Fig. S1A). To identify the binding sites, we incubated the MBPCabA protein with different sets of restriction-digested 1E1 cosmids harboring the entire cacibiocin gene cluster and used agarose gel electrophoresis to identify selectively bound fragments (Fig. S2A). A thorough inspection of MBPCabA-bound DNA fragments led to the identification of a 1494-bp DNA fragment (Fig. S2B) encompassing inter-alia the entire *cabhal* and *cabH* intergenic region (165 bp) (Fig. 2a). To determine more precisely the CabA binding region, we subdivided the 1494-bp DNA stretch into shorter fragments for use in additional EMSA analyses. These experiments allowed us to substantially narrow the initial 1494-bp region to 815- and 463-bp fragments (Fig. 2b(1)) and, finally, to a 182-bp fragment that still contained the entire *cabhal-cabH* (*cabhal-H*) intergenic region (Fig. 2b(2)). EMSA results indicated that MBPCabA specifically interacted with the 182-bp DNA fragment but did not bind a negative control (Fig. 2b(2)). Interestingly,

typical mobility shifts were not observed in any EMSA and MBPCabA-DNA complexes remained in the wells. This may reflect the formation of high molecular weight nucleoprotein complexes, possibly resulting from protein oligomerization and/or interaction of the large CabA protein (92.2 kDa) with more than one binding site. To identify the MBPCabA binding sites, we applied DNase I footprinting assays using a PCR-amplified DNA fragment (249 bp) encompassing the 182-bp region identified by EMSA (Fig. 2a). Footprinting analyses revealed that five stretches of DNA were protected from DNase I digestion in the presence of recombinant CabA protein (see dotted lines in Fig. 2c). Overall, the protected DNA region covered ~100 bp on the upper strand (protected sites *Ps1*, *Ps2*, and *Ps4*) and ~60 bp on the bottom strand (*Ps3* and *Ps5*) (Fig. 2d) located ~70–170 bp upstream from the start codon of the *cabH* gene. The extensive protected DNA region suggests the presence of multiple MBPCabA binding sites within the *cabhal-H* intergenic region, corroborating EMSA results. In addition to DNA protection, we also observed that binding of MBPCabA promoted enhanced DNase I cleavage at sites flanking or between protected regions, indicating possible DNA bending at those sites (see hypersensitive sites marked by asterisks, Fig. 2c, d).

To define a putative CabA binding sequence within the *cabhal-H* region, we used the MEME searching tool (Bailey et al. 2009). For this purpose, we performed the sequence alignment of the five protected sites, *Ps1* to *Ps5*, obtained from DNase I footprinting studies (Fig. 2e), displaying the output as a sequence logo. The resulting logo revealed a 10-nucleotide sequence, 5'-C(T/C)(A/T)T(T/C)(C/T)C(C/G)(G/C)(A/G)-3', representing a putative CabA protein recognition motif (Fig. 2f). Using the established logo sequence, we annotated the positions of four CabA binding sites, A1 to A4, within the *cabhal-H* intergenic region (boxes in Fig. 2c, d). The nucleotide motif appears to be significantly relaxed since only three of 10 nucleotides, at positions 1, 4, and 7, are conserved in all four boxes (Fig. 2e).

To compare the CabA interaction with A1 and A3 boxes, which differ from each other by only two nucleotides, we performed additional EMSA experiments. These experiments indicated that the DNA fragment encompassing two boxes, A2 and A3, was bound more strongly by the MBPCabA protein than that containing the A1 and A2 pair of boxes (fragment 2 vs. 1, Fig. S5A and B), suggesting that CabA interacts with the A3 box with higher affinity than with the A1 box. The observed differences in the interaction of CabA with fragments 1 and 2 could also reflect the relative organization of CabA boxes within their corresponding DNA fragments and subsequent interaction between DNA-bound CabA molecules. Specifically, boxes A1 and A2 (fragment 1) are located on opposite DNA strand interfaces (17-bp gap) whereas boxes A2 and A3 (fragment 2) are on the same interface (11-bp gap), corresponding to approximately 1.6 and 1 DNA helix turn,

respectively. We also found that MBPCabA was capable of binding a single A4 box, albeit much more weakly than fragments containing either A2 and A3 or A1 and A2 box pairs (Fig. S5A and B).

Collectively, these analyses show that CabA binds to four sites (A1–A4) within the *cabhal-H* intergenic region of the cacibiocin gene cluster. This suggests that CabA may directly control the expression of all genes involved in cacibiocin biosynthesis (*cabhal* and *cabHIYJKL*) via this bidirectional promoter.

### CabR interacts with the *cabR-cabT* intergenic region and its DNA binding activity is modulated by aminocoumarins

To define possible CabR binding sites, we used CabR C-terminally fused with glutathione *S*-transferase (GST) (GSTCabR, 56 kDa) (Fig. S1B) and affinity chromatography, an approach similar to that described previously (Jakimowicz et al. 1998). GSTCabR protein immobilized on resin containing glutathione was incubated with three sets of restriction fragments of cosmid 1E1. An analysis of DNA fragments bound by GSTCabR allowed us to identify a CabR target region corresponding to an approximately 500-bp DNA fragment encompassing the whole intergenic region (56 bp) of the divergently transcribed *cabR* and *cabT* genes (Figs. S3A and B and 3a). This suggested that CabR may regulate promoter(s) of the corresponding genes. To confirm the identified protein-DNA interaction and gain additional insight into the binding characteristics, we applied EMSA, DNase I footprinting, and SPR techniques. For these experiments, we used C-terminally His-tagged CabR protein (CabRHis<sub>6</sub>; 31.5 kDa) (Fig. S1C). First, to narrow the CabR binding region, we PCR amplified a shorter DNA fragment (101 bp) (Fig. 3a) encompassing the *cabR-cabT* (*cabR-T*) intergenic region and used it as an EMSA probe. This analysis showed that CabRHis<sub>6</sub> specifically interacted with the 101-bp *cabR-T* region, leading to the formation of a single nucleoprotein complex; the negative control was not bound (Fig. 3b). Since only one nucleoprotein complex was always observed in EMSA experiments, we assumed that CabR binds to its binding site in the *cabR-T* intergenic region with high affinity, forming a stable complex. SPR, used to determine the kinetic parameters of the interaction of CabRHis<sub>6</sub> with the *cabR-T* region, revealed a fast association of the protein with the target DNA and a relatively fast dissociation from the DNA within the range of studied CabRHis<sub>6</sub> concentrations used (40–160 nM) (Fig. 3d). Using the Biacore build-in software 1:1 kinetics, we determined the dissociation constant ( $K_D$ ) values, which were in the range of 0.49 to 4.7 nM, depending on the experiment (Fig. S8). SPR values were in good agreement with the preliminary results obtained using MicroScale Thermophoresis ( $K_D \approx 10$  nM; data not shown). Taken together, both sets of

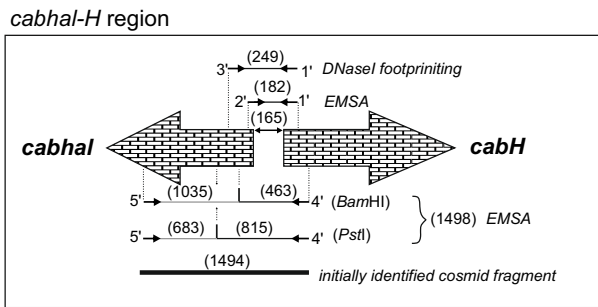
**Fig. 2** Identification of CabA binding sites within the gene cluster. **a** Schematic representation of the *cabhal-H* region. Primers used in these studies are designated by *numbers* (Table S2), and the lengths of the corresponding DNA fragments are given in *parentheses*. The 1494-bp fragment indicates the initially identified cosmid region bound by MBPCabA (Fig. S2). **b** EMSA. Interactions between MBPCabA and *cabhal-H* region were determined at protein concentrations of 25, 50, 75, and 100 nM (**(1)**) or 25, 50, 100, 120, 140, 160, 180, and 200 nM (**(2)**) using different DNA fragments encompassing the *cabhal-H* region. A 340-bp fragment, amplified using primers 2 and 7 (Fig. 3a), served as a negative control (NC). The ratio np/nD corresponds to the molar protein-to-total DNA ratio. *Short bars* along the gel indicate freely migrating DNA; *bars with asterisks* indicate DNA-protein complexes. Freely migrating fragments that were specifically bound by MBPCabA are indicated by *black bars*, whereas not bound by *light grey bars*. **c** DNase I footprinting. <sup>32</sup>P-labeled DNA fragments were incubated with increasing concentrations of MBPCabA (50, 150, 300, and 480 nM) and then subjected to DNase I digestion. *Dotted lines* correspond to regions protected from DNase I digestion. *Asterisks* indicate DNase I-hypersensitive sites. *Gray rectangles (A1 to A4)* indicate CabA binding sequences. *Lanes T, G, C, and A* refer to sequencing reactions. <sup>32</sup>P-labeled primers (1' or 3') are indicated by *small black arrows*. **d** DNase I footprinting results plotted on the sequence of the *cabhal-H* region. *Ps1–Ps5* refer to sites protected from DNase I digestion. **e** Alignment of *Ps* sequences using the MEME tool. Nucleotides for the MBPCabA binding site are shown in colors corresponding to panel f. Sequences are shown in the 5'–3' direction. **f** Sequence motif recognized by CabA

data indicate that CabR exhibits high binding affinity toward the *cabR-T* intergenic region.

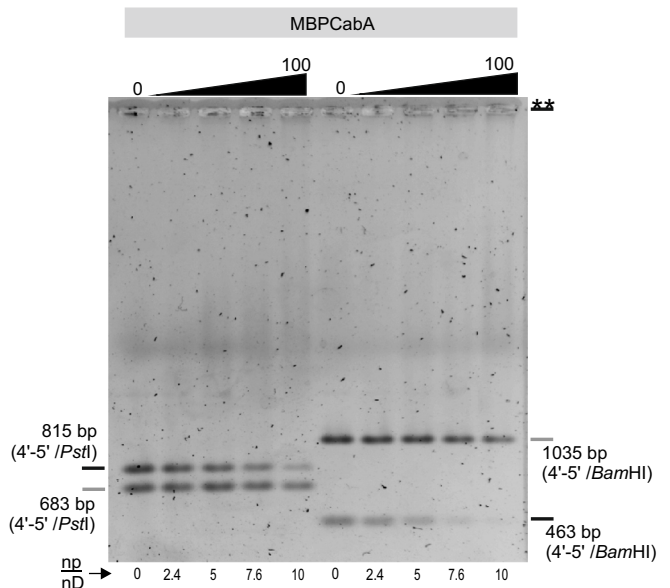
DNase I footprinting assays were subsequently used to determine CabR binding site(s). For this purpose, a 161-bp DNA fragment encompassing the *cabR-T* intergenic region was amplified (Fig. 3a). Footprinting experiments revealed that four stretches of DNA were protected from DNase I digestion in the presence of recombinant CabR protein (see dotted lines in Fig. 3c, e). These stretches were located symmetrically on complementary strands strictly within the *cabR-T* intergenic region (protection sites *Ps1*, *Ps2*, *Ps3*, and *Ps4*) (Fig. 3e). The symmetrical orientation of protected DNA regions suggested that CabR may recognize two binding sites within the studied DNA fragment. Notably, binding of CabRHis<sub>6</sub> also led to preferential digestion of DNA at sites that were exposed to DNase I, probably as a result of DNA bending introduced by assembly of CabR molecules at multiple binding sites (Fig. 3c, e). To determine the CabR binding site sequence(s) within the *cabR-T* region, we aligned the protection sites (*Ps*) identified by DNase I footprinting. Since *Ps1* and *Ps2* cover *Ps3* and *Ps4* sites, respectively, we used the MEME tool for motif repeat searches and *Ps1* and *Ps2* site sequences as input (Fig. 3f). These analyses revealed a common 17-bp nucleotide sequence within both protected sites represented by the sequence logo 5'-CG(T/C)TTAC(G/A)(T/G)TGTAATC-3' (Fig. 3g). The putative CabR binding consensus sequence allowed us to define two CabR boxes, hereafter referred to as R1 and R2, within the *cabR-T* intergenic region (Fig. 3c, e).



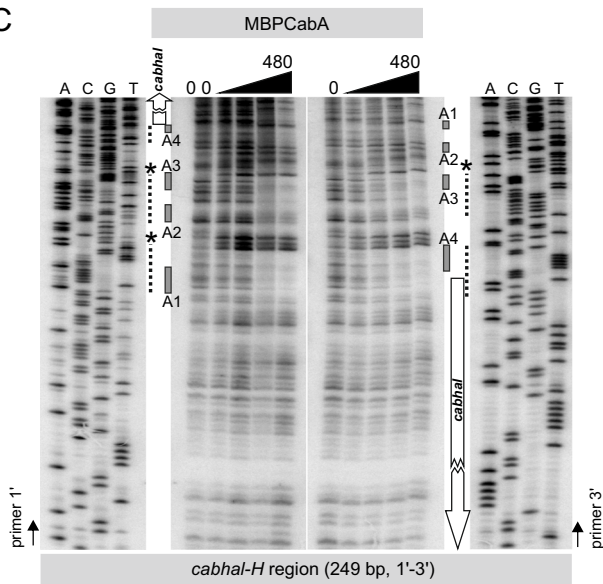
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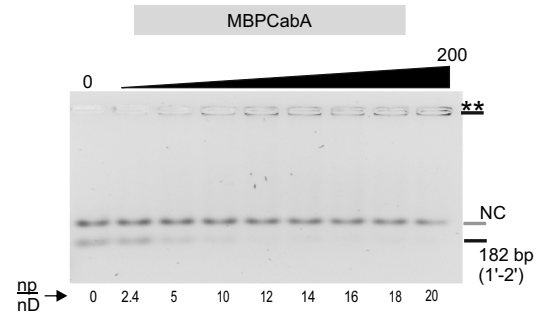
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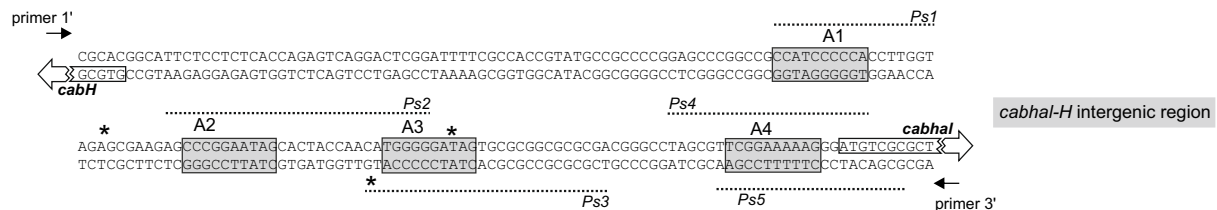
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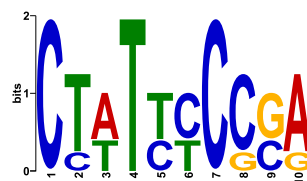
D



E

Sequence	Box
<i>Ps1</i> GTTGGTAGTG <b>CCATCCCCCA</b> CCTTGGTAA	A1
<i>Ps2</i> GTTGGTAGTG <b>CTATTCCGGG</b> CT	A2
<i>Ps3</i> GCGCCGCGCA <b>CTATCCCCCA</b> TGT	A3
<i>Ps4</i> ACATCC <b>CTTTTCCGA</b> ACGCTAGG	A4
<i>Ps5</i> CGGACATCC <b>CTTTTCCGA</b> A	

F



Interestingly, the identified binding consensus motif encompasses an inverted repeat with the sequence 5'-TTAC-3' (Fig. 3g), which may suggest that each 17-bp CabR box is

bound by a CabR dimer. Consistent with this possibility, we have shown that CabR effectively dimerizes in solution (Fig. S1D). However, CabR was not able to bind control

DNA fragments containing only the TTAC sequence, which is one half of the palindrome forming a CabR box (data not shown). Additional EMSA analyses indicated that recombinant CabR protein was able to interact and form a stable nucleoprotein complex with a DNA fragment encompassing the single R1 box (fragment 6\_cabR-T, Fig. S6A). Notably, an attempt to analyze the interaction of CabR with the single R2 box (fragment 4\_cabR-T, Fig. S6A) revealed that the relative migration distances for nucleoprotein complexes formed by CabRHis<sub>6</sub> and the DNA fragment putatively encompassing only one CabR box (R2 box on 4\_cabR-T) was similar to the fragment encompassing two boxes (R1 and R2 boxes on 7\_cabR-T). Additionally, in the presence of CabRHis<sub>6</sub>, the relative migration distances of these two DNA fragments were less than those of the fragment encompassing the single CabR box R1 on fragment 6\_cabR-T (Fig. S6A). These observations suggest that, in addition to R2, another as yet undefined CabR binding site may be present in the intergenic region. Further experiments showed that CabRHis<sub>6</sub> was able to bind, though weakly, to the DNA fragment encompassing a partial sequence of box R2 and its 3' flanking region (fragment 5\_cabR-T), supporting the possibility that an additional CabR binding site is present in that region (Fig. S6A). Interestingly, an *in silico* search using the CabR logo motif revealed the presence of a relaxed CabR box sequence, hereafter called R3 box, located in the proximity of the *cabR* gene start (Fig. S6A and B). The R3 box sequence differs from the determined 17-bp consensus by the presence of a single nucleotide mismatch in each TTAC repeat (Fig. S6B). However, we could not detect this R3 box using either DNase I footprinting (Fig. 3c) or shift assays in which a DNA fragment encompassing the R3 box sequence alone was applied (data not shown). SPR-based calculations revealed that the protein-to-DNA stoichiometry was approximately 7.6 (Fig. S7A and B), suggesting that at least three CabRHis<sub>6</sub> dimers are able to bind the *cabR-T* intergenic region and that each of the three CabR boxes is occupied by a protein dimer. Additionally, this indicates that binding of the R3 box is probably supported by cooperative interactions of the CabR dimers; the fact that the binding stoichiometry was >6 further suggests the availability of extra CabR molecules that could also be involved in the formation of the protein-DNA complex.

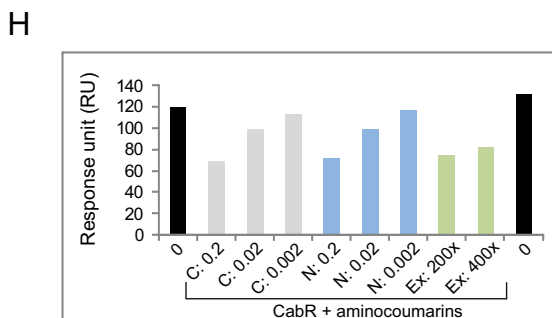
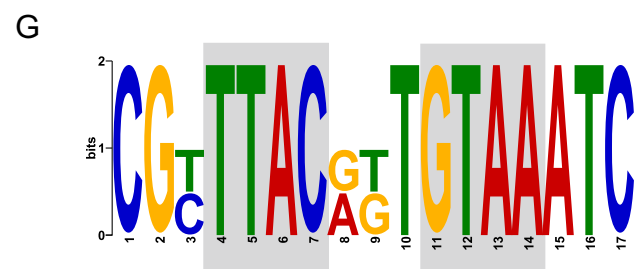
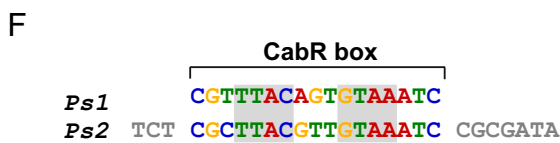
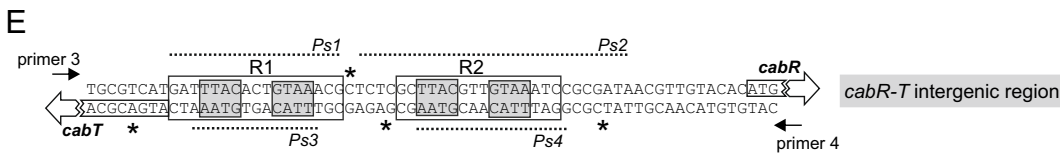
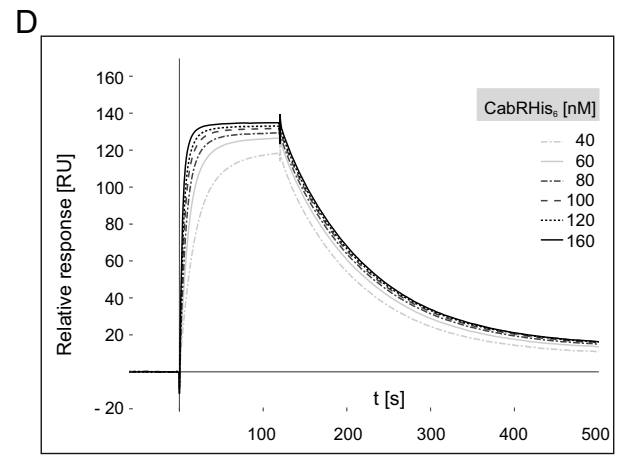
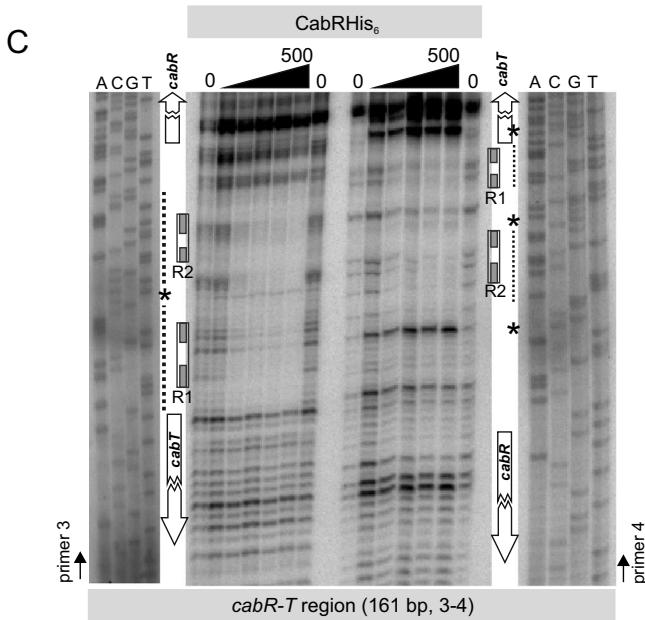
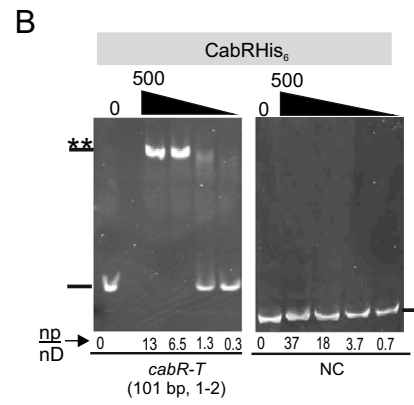
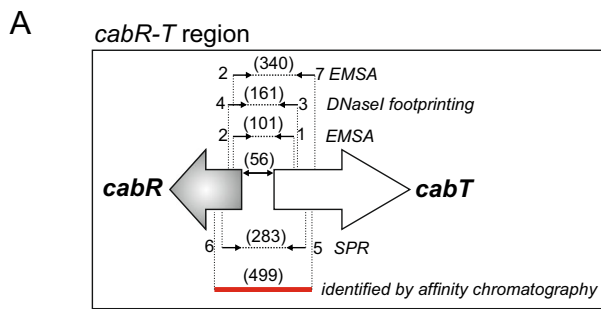
As the end products of biosynthetic pathways often regulate the activity of corresponding transcriptional negative regulators, we decided to determine whether the end product of cacibiocin gene cluster and other aminocoumarins modulate the interaction of CabR with target DNA. For this purpose, we studied the influence of clorobiocin, novobiocin, and cacibiocins (the latter as an 11-day culture extract) on CabR DNA binding activity using EMSA and SPR techniques.

In EMSAs, the CabRHis<sub>6</sub> concentration was held constant and increasing amounts of novobiocin, clorobiocin, or

**Fig. 3** Identification of CabR binding sites within the gene cluster. **a** Schematic representation of the *cabR-cabT* intergenic region. Primers used in these studies are designated by *numbers* (Table S2), and the lengths of the corresponding DNA fragments are given in *parentheses*. The 499-bp fragment (*red line*) indicates the cosmid DNA identified using affinity chromatography (Fig. S3). **b** EMSA. Different concentrations of CabRHis<sub>6</sub> protein (10, 50, 250, and 500 nM) were incubated with a constant amount (50 ng) of the corresponding DNA fragments. A 283-bp fragment amplified using oriC-Bf1 and oriC-Br3 primers served as a negative control (NC). The ratio np/nD corresponds to the molar protein-to-DNA ratio. *Short bars* along the gel indicate freely migrating DNA; *bars with asterisks* indicate DNA-protein complexes. **c** DNase I footprinting. <sup>32</sup>P-labeled DNA fragments were incubated with increasing concentrations of CabRHis<sub>6</sub> (5, 25, 50, 100, 250, and 500 nM) and then subjected to DNase I digestion. *Dotted lines* correspond to regions protected from DNase I digestion. *Asterisks* indicate DNase I-hypersensitive sites. *Empty and gray rectangles* indicate CabR binding sequences and a 4-bp TTAC repeat, respectively. *Lanes T, G, C, and A* refer to sequencing reactions. <sup>32</sup>P-labeled primers (3 and 4) are indicated by *small black arrows*. **d** SPR. Biotin-labeled, 283-bp DNA fragments, amplified using primer pairs 5/6 and b-ScoriC/Br3-oriC for the *cabR-T* region and NC, respectively, were immobilized on streptavidin-coated sensor chip of the Biacore apparatus. Sensograms were obtained at different concentrations of CabRHis<sub>6</sub> (10, 20, 40, 60, 80, and 100 nM). **e** DNase I footprinting results plotted on the sequence of the *cabR-T* region. *Ps1–Ps4* refer to sites protected from DNase I digestion. *R1* and *R2* refer to possible CabR binding boxes. **f** Alignment of *Ps* sequences using the MEME tool. Nucleotides forming a CabR binding sequence are shown in colors corresponding to panel **f**. TTAC repeats are highlighted by *gray rectangles in the background*. Sequences are shown in the 5'–3' direction. **g** Sequence motif recognized by CabR protein. **h** Interactions of CabRHis<sub>6</sub> (40 nM) with different aminocoumarins (*C* clorobiocin, *N* novobiocin, *Ex* cacibiocin extract), dissolved in 5 % DMSO, were investigated by SPR. For *C* and *N*, the *numbers* indicate final concentrations of the compound (mM); for *Ex*, the *numbers* correspond to fold dilution of the initial solution in the reaction mixture

cacibiocin extract were added to the reactions. In all cases, CabR binding to DNA ceased only at the highest concentrations of added aminocoumarins (2 mM for clorobiocin and novobiocin and 20-fold-diluted cacibiocin extract) (Fig. S8). SPR analyses showed a progressive decrease in relative response unit (RU) values as the concentration of aminocoumarins increased in the reaction mixture over a 100-fold range (0.002 to 0.2 mM for clorobiocin and novobiocin) compared with control samples containing only CabR and the aminocoumarin solvent (DMSO). Similarly, we observed a substantial decrease in RU values after the addition of cacibiocin extract (Fig. 3h).

Taking together, our studies reveal that CabR specifically interacts with two or three binding sites within the intergenic region of *cabT* and *cabR* genes, suggesting a possible regulatory role of CabR in controlling the expression of these genes. Additionally, *in vitro* studies revealed that aminocoumarins (i.e., clorobiocin, novobiocin, and cacibiocins) are capable of preventing CabR binding to DNA, indicating the existence of a regulatory circuit that controls the activity of this putative negative regulator of the cacibiocin gene cluster.



### CabA and CabR act as transcriptional activator and repressor, respectively, of the cacibiocin gene cluster

Our in vitro experiments led us to conclude that the cacibiocin gene cluster is controlled by the transcriptional regulators CabA and CabR. The genes proposed to be regulated by the CabA activator encode proteins involved in the key steps of cacibiocin biosynthesis, including the formation of the aminocoumarin moiety (*cabHIYJK*) and aminocoumarin dichlorination in cacibiocin B (*cabhal*). The putative negative regulator CabR is presumed to control its own expression and the expression of *cabT*, a gene encoding a putative cacibiocin exporter (CabT). To investigate the true influence of these regulatory proteins on the activity of the corresponding promoters, we used a previously described luciferase-based reporter assay (Le et al. 2009). Since there are no tools available for genetic manipulations in *C. acidiphila*, we performed these experiments in *S. coelicolor* M1152, a strain recently used as a heterologous host for cacibiocin production (Zettler et al. 2014). To this end, we cloned the putative promoter regions for four genes of interest (*cabT*, *cabR*, *cabH*, and *cabhal*) upstream of the luciferase genes *luxAB* in the pIJ5972 reporter plasmid. Since we did not experimentally identify the transcriptional start points, we cloned 500-bp-long DNA fragments upstream of each gene of interest to ensure that the corresponding promoters were entirely contained in the resulting reporter plasmids.

To examine the activity of *cabH* and *cabhal* promoters (*pcabH* and *pcabhal*, respectively) in the presence and absence of CabA regulator, we introduced either a plasmid carrying *cabA* under the control of the constitutive *ermE\** promoter (pXL11-Hyg) or the empty control vector (pUWL201-Hyg) into the *S. coelicolor* M1152 strains already harboring the corresponding reporter plasmids (pIJ5972*pcabH* and pIJ5972*pcabhal*) (Fig. 4a). In the case of the CabR regulator, the plasmid carrying the *cabR* gene under the control of the *ermE\** promoter (pIJ10257*cabR*) or the empty control vector (pIJ10257) was similarly introduced into *S. coelicolor* M1152 that already contained reporter plasmids bearing the *cabR* or *cabT* promoter (*pcabR* and *pcabT*, respectively) fused with the luciferase gene (pIJ5972*pcabR* and pIJ5972*pcabT*, respectively) (Fig. 4b). The respective strains were subsequently grown in liquid CDM production medium, and luciferase activities were measured as described in the Supplemental Information (SI). These experiments showed that, in the presence of CabA, both *cabH* and *cabhal* promoters were activated relative to control strains lacking the *cabA* gene. Interestingly, these two promoter regions were activated to a different extent, with *cabH* and *cabhal* exhibiting 10- and 2-fold increases, respectively (Fig. 4a, c). These results are consistent with the previously predicted role of CabA and indicate that this protein acts as an activator. In the case of CabR studies, luciferase reporter assays demonstrated that *pcabR* and *pcabT* promoter activity

**Fig. 4** Regulation of promoter activities by CabA and CabR. Promoter activities of *pcabH*, *pcabhal*, *pcabT*, and *pcabR* were measured in the presence and absence of CabA (a) or CabR (b). The corresponding gene promoter regions were cloned upstream of *luxAB* genes in an integrative luciferase promoter-probe reporter plasmid (pIJ5972) and transferred into the *S. coelicolor* M1152 strains expressing either the *cabA* or *cabR* gene under the control of the *ermE\** promoter (for details, see the SI). Luciferase activity, expressed as luminescence (cps) per wet weight of mycelium (g), was calculated as the mean±standard error of three biological replicates. c Relative promoter activity. The chart presents fold-change in activation or repression of the corresponding promoter in the presence and absence of the regulatory gene. Values given were calculated as the ratio of luciferase activity in the presence of regulator to that in the absence of regulator. The green arrows and red bar-headed lines schematically depict the upregulation and downregulation, respectively, of particular genes. d CabA-dependent cacibiocin A production in *Streptomyces* host. Aminocoumarin production was measured for *S. coelicolor* M1152 strains harboring a 10E2int cosmid, *S. coelicolor* M1152 (10E2int), and either the pXL11 plasmid carrying the *cabA* gene (*cabA* pXL11) or the pUWL201 empty vector ( $\Delta$ *cabA* pUWL201) or without plasmid vector ( $\Delta$ *cabA* no plasmid). Production was measured after 11 days of cultivation. Data are mean values of triplicate measurements. ND not detected

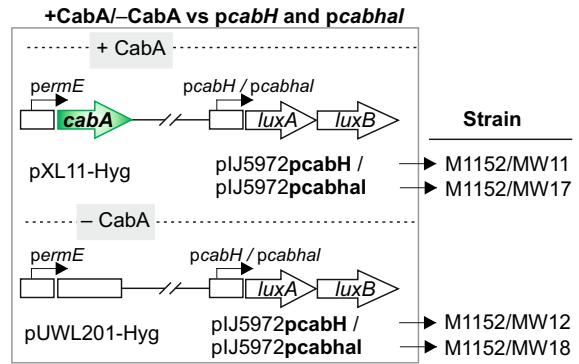
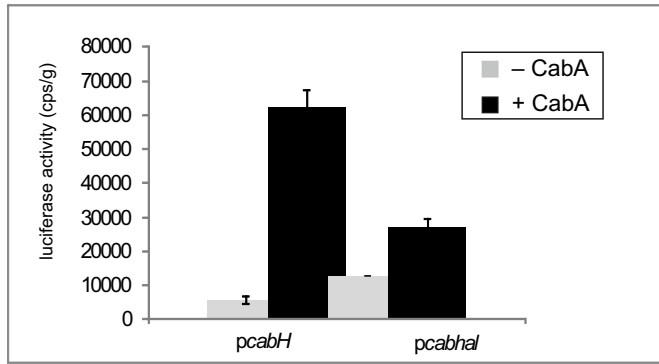
in the presence of CabR was only 0.31- and 0.37-fold that measured in the corresponding control strains without *cabR* (Fig. 4b, c). These data confirm the bioinformatics prediction for the transcriptional regulator CabR and demonstrate that it similarly downregulates its own expression and that of the divergently transcribed *cabT* gene.

Taken together, the luciferase reporter assay results clearly demonstrate that *cabA* and *cabR* genes, respectively, encode positive and negative regulators of gene expression in the cacibiocin gene cluster. The CabA protein directly activates the promoters of *cabhal* and *cabH*, whereas the CabR repressor inhibits the expression of *cabT* and autoregulates the expression of its own gene. These results indicate that CabA is important for the regulation of cacibiocin biosynthesis by activating a possible *cabHIYJKL* operon, whereas CabR probably regulates the export of these compounds.

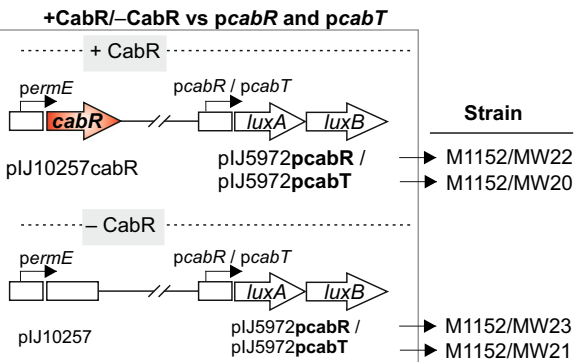
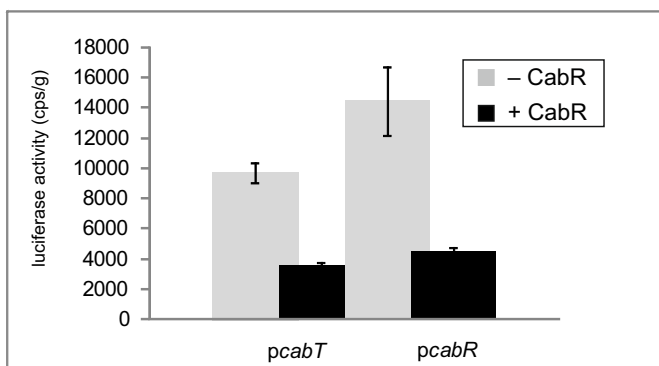
### Discussion

Compared with other secondary metabolite gene clusters, relatively little is known about the regulation of gene expression in aminocoumarin gene clusters. In addition to cacibiocin, five aminocoumarin gene clusters and products of their biosynthesis have been described to date. Few described cluster-situated regulators (CSRs) of aminocoumarin gene clusters include transcription factors that activate the expression of genes involved in aminocoumarin biosynthesis in the novobiocin gene cluster (NovE and NovG proteins) (Dangel et al. 2008, 2009; Eustaquio et al. 2005) or orthologs of these proteins identified in the clorobiocin (CloE and CloG) and coumerycin (CouE and CouG) gene clusters. However, the regulatory roles of these latter proteins have not been confirmed experimentally

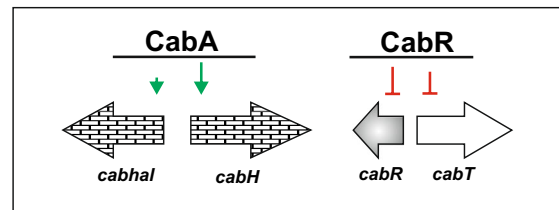
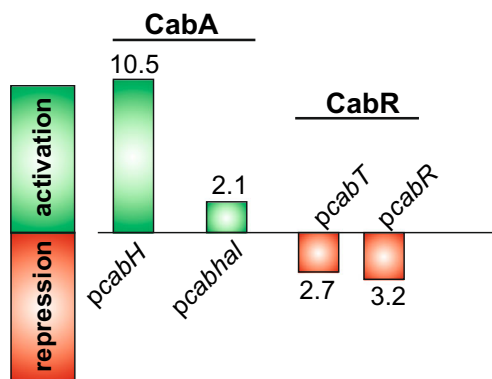
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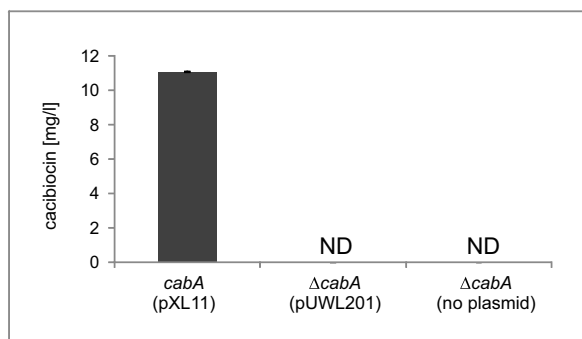
**B**



**C**



**D**



(Heide 2009a). The most-recently described and best-studied example is that of the repressor protein SimR of the simocyclinone gene cluster. SimR negatively regulates the expression of the simocyclinone transporter and that of its own gene; however, it does not appear to regulate the expression of biosynthetic genes (Le et al. 2009). A second putative CSR regulator (SimReg1) from the simocyclinone gene cluster has also been identified, although its regulatory role remains unclear (Horbal et al. 2012; Trefzer et al. 2002). Interestingly, to date, genes encoding negative regulators have been identified only in the simocyclinone and coumermycin gene clusters (*simR* and *couR6*, respectively), although the role of the latter regulator has not yet been studied (Heide 2009b). It should be noted that these positive and negative regulators are not conserved in all aminocoumarin gene clusters; thus, there is no common gene regulation pattern. In this context, studying the regulatory circuit within the newly identified cacibiocin gene cluster would be an important next step. Prior to this study, the role of both positive and negative regulators of an aminocoumarin gene cluster had not been presented in a single report. Here, we describe for the first time a rational mechanism for regulation of gene expression in the novel cacibiocin gene cluster by both negative and positive CSRs.

A genome mining strategy recently led to the identification of a new aminocoumarin gene cluster in *C. acidiphila* and to the discovery of two novel end products of its biosynthesis, cacibiocins A and B (Zettler et al. 2014). The cacibiocin gene cluster contains genes involved in the biosynthesis of the aminocoumarin moiety (*cabHIYJK*), a novel pyrrole moiety (putatively *cabR10* and *R11*), an amide synthetase (*cabL*) for chlorination of the end product (*cabhal*), a gene encoding a putative cacibiocin exporter (*cabT*), and putative gene cluster regulators (*cabA* and *cabR*) (Fig. 1a). The main aim of the current study was to identify the roles of the proteins encoded by *cabA* and *cabR* genes in regulating the expression of the cacibiocin gene cluster.

It was recently shown that both cacibiocins A and B are present in low amounts in the original producer *C. acidiphila* (Zettler et al. 2014). Our qPCR and luciferase-based transcriptional studies indicate that this low-level expression is probably attributable to late and low expression of CabA-regulated biosynthetic genes (*cabhal*, *cabJ*, *cabR11*) (Fig. S4). We showed that late expression of the *cabA* gene (Fig. 1c(1)) was positively correlated with the expression of the biosynthetic genes, *cabhal*, *cabJ*, and *cabR11*, and corresponds to the onset of cacibiocin production in *C. acidiphila* reported previously (Zettler et al. 2014). The *cabA* transcript level increased during days 7–9, consistent with reports of maximum amounts of cacibiocins in 10-day and older cultures, suggesting that CabA is responsible for induction of the expression of cacibiocin biosynthetic genes. Indeed, a direct-activating influence of CabA on cacibiocin biosynthesis was confirmed

using a heterologous *Streptomyces* host system (Fig. 4d). Using an EMSA approach, we identified the *cabhal-H* intergenic region as the target for recombinant MBPCabA and experiments based on luciferase reporter assays further showed that transcriptional activities of the corresponding *cabH* and *cabhal* promoters were increased in the presence of exogenously expressed *cabA* (under the control of the constitutive promoter *ermE\**). However, *cabH* was activated to a much greater extent (~10-fold) compared with the *cabhal* gene (~2-fold) (Fig. 4a, c). Additionally, transcriptional analyses showed that *cabJ*, presumably expressed from the same promoter as *cabH* (Fig. 1b), and *cabhal* were also induced to different degrees in the presence of CabA (~6–7-fold and ~4-fold for *cabJ* and *cabhal*, respectively) (Fig. S4). Interestingly, in *C. acidiphila* cultures, *cabhal* expression seemed to be slightly delayed compared with the expression of *cabJ*, reaching its highest levels at culture day 9, whereas *cabJ* reached its maximum at days 7 and 9. This result may explain why the nonhalogenated cacibiocin A dominated over dichlorinated cacibiocin B in cultures carried out in a CDM production medium, whereas the opposite was reported in other media (Zettler et al. 2014). Delayed expression of the *cabhal* gene led to a delay in the synthesis of the cacibiocin halogenase Cabhal, thus allowing the accumulation of nonhalogenated cacibiocin A. The transcription of *cabJ* and *cabR11* was similarly correlated with *cabA* expression, since both biosynthetic genes started exhibiting higher transcript levels at culture day 7 (Fig. S4). This result indicates that the *cabH-cabR12* genes presumably form an operon and, together with *cabhal*, are positively regulated by CabA. The *cabR10* and *cabR11* genes were previously suggested to play a role in pyrrole moiety biosynthesis, whereas *cabR12* may be involved in cacibiocin export (Zettler et al. 2014). Apart from CabA, the only other experimentally verified examples of positive regulators of the aminocoumarin gene clusters are NovE and NovG, which control the transcription in the novobiocin biosynthetic gene cluster. NovE and NovG are responsible for indirect and direct activation of gene expression in the corresponding gene cluster (Dangel et al. 2008; Eustaquio et al. 2005). Collectively, our results indicate that CabA acts as a positive regulator of the cacibiocin gene cluster by directly activating the expression of genes responsible for biosynthesis (*cabHIJKLR10R11R12* operon) and modification (*cabhal*) of the corresponding aminocoumarin compounds. In our studies, we established a consensus 10-mer CabA binding site that differs in terms of sequence similarity and length from consensus sequences of other LuxR proteins like NarL, GerE, and LuxR (Crater and Moran 2001; Ducros et al. 2001; Maris et al. 2002; Pompeani et al. 2008). Additionally, despite the resemblance of the CabA C-terminal domain sequence to that of other LuxR proteins, the orientation of the identified CabA binding sites within the *cabhal-H* intergenic region does not exhibit the inverted-repeat orientation (Fig. 2d, e) often

encountered in LuxR proteins (Ramos et al. 2005). Moreover, bioinformatics studies indicated that the N-terminal regulatory domain of CabA does not exhibit sequence similarity to any other described protein. Therefore, it is difficult to predict what type of signal this regulator may sense. Taken together, these observations indicate that CabA exhibits a mode of DNA binding and a transcription activation mechanism different from that of other LuxR regulators.

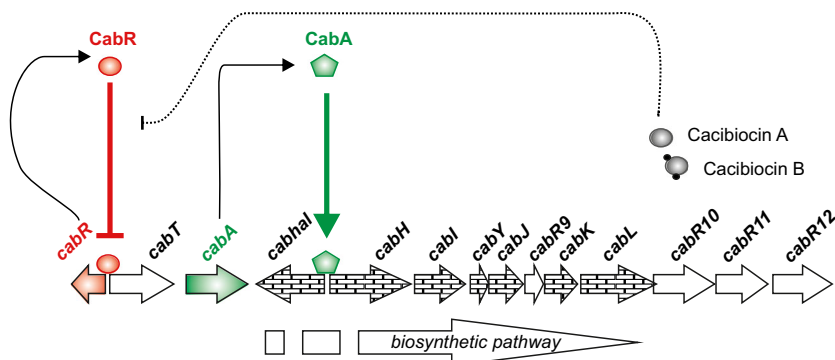
Similar to *cabA*, *cabR* transcription in *C. acidiphila* increased at days 7 to 10 but, in contrast to *cabA*, its transcription reached the highest levels in early cultures (days 1 and 2), after which it decreased (day 4) before increasing again at the onset of cacibiocin production (days 7–10) (Fig. 1c(1)). CabR does not regulate the transcription of the biosynthetic genes; however, it does downregulate the expression of its own gene and that of the *cabT* gene encoding a putative exporter for cacibiocins. Using an affinity chromatography approach, we identified the *cabR-T* intergenic region as the only binding target within the gene cluster (Fig. 3). Interestingly, the gene context and organization within the *cabR-T* region is similar to that found in simocyclinone and coumermycin gene clusters. The counterpart genes, *simR* and *simX*, were confirmed to function as repressor and transporter proteins, respectively, in the simocyclinone gene cluster (Horbal et al. 2012; Le et al. 2009; Trefzer et al. 2002). The functions of CouR6 and CouR5 in the coumermycin gene cluster have not yet been studied (Galm et al. 2002; Heide 2009b; Schmutz et al. 2003). CabR belongs to the TetR family of proteins and exhibits substantial similarity (31 % identity and 43 % positive amino acid sequence alignment) to SimR from the simocyclinone gene cluster. SimR negatively regulates the expression of both *simR* and *simX* by binding inverted-repeat sequences within their promoter regions (Le et al. 2009). Our experiments showed that CabR plays a similar role in the regulation of *cabR* and *cabT* transcription, acting as a negative regulator of both genes (Fig. 4). This is similar to other members of the TetR protein family, which often function as repressors of genes encoding drug-efflux pumps (Ramos et al. 2005). CabR appeared to repress the activities of *cabR* and *cabT* promoters to a similar extent (3.2- and 2.7-fold, respectively;

this study), whereas in the case of SimR, the corresponding counterpart promoters of the *simR* and *simX* genes were reported to be repressed to a much different extent—12- and 100-fold, respectively (Le et al. 2009). Moreover, the inhibition of *simX* expression (100-fold) was much more pronounced than that in the case of the *cabT* gene (3.2-fold). Although these comparisons reflect different experimental conditions (liquid medium vs. solid culture) and host organism (*S. coelicolor* vs. *Streptomyces lividans*), they may indicate different molecular mechanisms of actions of both proteins as a result of different arrangements of repressor binding sites with respect to promoter regions. Indeed, the *cabR-T* intergenic region (56 nucleotides) is barely half as long as the *simR-X* region (96 nucleotides), and the *cabR-T* region contains three CabR binding sites, whereas the *simR-simX* intergenic region contains only two SimR binding sites. These results indicate that both repressor proteins may exhibit differences in the mode by which they interact with their target DNA regions.

Several reports have shown that end products and/or intermediates of secondary metabolite biosynthetic pathways often interact with negative regulators of the corresponding gene clusters to release repression and allow gene expression (Ahn et al. 2007; Le et al. 2009; Novakova et al. 2010; Ostash et al. 2008; Tahlan et al. 2007, 2008). The SimR DNA binding activity is also modulated by an intermediate and by end products of simocyclinone biosynthesis (Le et al. 2009, 2011). We have shown that, similar to SimR, CabR DNA binding activity is also negatively modulated by aminocoumarin compounds, including cacibiocins A and B (Figs. 3h and S8). Thus, like simocyclinone, the end product of the cacibiocin biosynthetic gene cluster accumulates in cells, leading to CabR release from the *cabR* and *cabT* promoters and derepression of the expression of both genes.

In novobiocin and coumermycin producers, the resistance against these antibiotics is primarily conferred by an additional copy of mutated *gyrB<sup>R</sup>* or *parY<sup>R</sup>* (or both) genes encoding gyrase and topoisomerase IV subunits, respectively. The GyrB<sup>R</sup> and ParY<sup>R</sup> subunits replace the sensitive GyrB<sup>S</sup> and ParY<sup>S</sup> subunits, respectively, in the corresponding protein

**Fig. 5** Regulation of cacibiocin biosynthesis. Schematic depiction of multilevel regulation of gene expression by CabA and CabR proteins. The green arrows and red bar-headed lines reflect the activation and repression exerted by CabA and CabR, respectively



heterotetramer, providing host resistance. These additional genes are located within or in the close proximity to the biosynthetic gene cluster (Heide 2009b, 2014). However, the aminocoumarin gene cluster in *C. acidiphila* encodes neither *parY* nor *gyrB* resistance genes, suggesting that cacibiocins may not target gyrase or topoisomerase activity. Thus, if these compounds are toxic at all to a natural producer, the host must possess a different mechanism of protection against them.

An auxiliary mechanism of resistance in novobiocin and coumermycin producer strains is based on the export of these compounds by corresponding transporter proteins (NovA and CouR5, respectively) (Eustáquio et al. 2003; Schmutz et al. 2003). In the simocyclinone producer, which does not contain additional copies of *gyrB<sup>R</sup>* and *parY<sup>R</sup>* genes within the gene cluster, export by the SimX transporter serves as the only resistance mechanism (Le et al. 2009). The function of the putative cacibiocin exporter encoded by the *cabT* gene remains unclear, although it was suggested to play some role in the export of aminocoumarin compounds owing to its high similarity (50 % identity) to the putative CouR5 transporter from the coumermycin gene cluster (Schmutz et al. 2003; Zettler et al. 2014). Since neither cacibiocin A nor cacibiocin B exhibits an antibacterial activity towards *Streptomyces* strains in solid cultures (data not shown), we were unable to verify whether the *cabT* gene product confers resistance to these compounds in the heterologous host. Additionally, no genetic tools have yet been developed to manipulate *C. acidiphila*. It should be noted that cacibiocins are found in culture supernatants of both *C. acidiphila* and *S. coelicolor* producers, indicating the existence of an aminocoumarin export mechanism. However, the role of CabT in this process has not yet been studied.

In this study, we propose a rational mechanism for regulation of gene expression in the recently identified cacibiocin biosynthetic gene cluster by two regulators: the CabA activator and CabR repressor (Fig. 5). CabA-dependent production is activated late during the course of growth and continues until the intracellular concentration of cacibiocins reaches a certain level; CabR represses the expression of its own gene to some extent as well as that of putative cacibiocin exporter-encoding genes. The accumulation of cacibiocins releases CabR repression and allows the expression of the transporter protein. This mechanism may prevent the accumulation of toxic by-products or end products of cacibiocin biosynthesis.

In summary, in this report, we describe novel CSRs for aminocoumarin biosynthesis. The current study shows that the recently identified cacibiocin gene cluster involved in the synthesis of cacibiocins encodes regulatory activator and repressor proteins responsible for controlling gene expression within the cluster. The CabA protein binds to the common intergenic region of *cabhal* and *cabH* genes and regulates the expression of proteins involved in cacibiocin biosynthesis and modification. The CabR regulator negatively controls the

transcriptional activity of its own gene and that of the *cabT* gene, thought to encode a protein involved in cacibiocin export. Moreover, CabR regulator activity seems to be negatively controlled by end products of the cacibiocin biosynthetic pathway.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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