The role of the TetR-family transcriptional regulator Aur1R in negative regulation of the auricin gene cluster in *Streptomyces aureofaciens* CCM 3239

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Two regulatory genes, *aur1P* and *aur1R*, have been previously identified upstream of the *aur1* polyketide gene cluster involved in biosynthesis of the angucycline-like antibiotic auricin in *Streptomyces aureofaciens* CCM 3239. The *aur1P* gene encodes a protein similar to the response regulators of bacterial two-component signal transduction systems and has been shown to specifically activate expression of the auricin biosynthetic genes. The *aur1R* gene encodes a protein homologous to transcriptional repressors of the TetR family. Here we describe the characterization of the *aur1R* gene. Expression of the gene is directed by a single promoter, *aur1Rp*, which is induced just before stationary phase. Disruption of *aur1R* in *S. aureofaciens* CCM 3239 had no effect on growth and differentiation. However, the disrupted strain produced more auricin than its parental wild-type *S. aureofaciens* CCM 3239 strain. Transcription from the *aur1Ap* and *aur1Pp* promoters, directing expression of the first biosynthetic gene in the auricin gene cluster and the pathway-specific transcriptional activator, respectively, was increased in the *S. aureofaciens* CCM 3239 *aur1R* mutant strain. However, Aur1R was shown to bind specifically only to the *aur1Pp* promoter *in vitro*. This binding was abolished by the addition of auricin and/or its intermediates. The results indicate that the Aur1R regulator specifically represses expression of the *aur1P* gene, which encodes a pathway-specific activator of the auricin biosynthetic gene cluster in *S. aureofaciens* CCM 3239, and that this repression is relieved by auricin or its intermediates.

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INTRODUCTION

Gram-positive, filamentous, soil-dwelling bacteria of the genus *Streptomyces* are characterized by a complex morphological differentiation and by the ability to produce an extraordinary diversity of biologically active secondary metabolites, including the majority of known antibiotics. Antibiotic production is co-ordinated with morphological differentiation and often occurs in a growth phase-dependent manner, usually at the beginning of the stationary growth phase, and is also influenced by a variety of physiological and environmental factors. Most antibiotics are produced by complex biosynthetic pathways encoded by physically clustered genes. The gene clusters are usually regulated by pathway-specific transcriptional activators of different families that are located in these clusters and act as a master switch for biosynthesis of the respective secondary metabolite. In addition to the transcriptional activators, expression of the antibiotic biosynthetic genes is often negatively regulated by transcriptional regulators that belong mainly to the TetR family. Moreover, various global regulatory genes affect antibiotic production indirectly and

have pleiotropic roles in stress response and morphological differentiation. Many of these pleiotropic regulatory genes influence the activity of the pathway-specific regulatory genes (Bibb, 2005).

We have previously identified a type II polyketide synthase (PKS) gene cluster, *aur1*, which is responsible for production of the angucycline-like polyketide antibiotic auricin in *Streptomyces aureofaciens* CCM 3239 (Novakova *et al.*, 2002). Even though auricin has been clearly detected in *S. aureofaciens* CCM 3239 grown in Bennet medium (Novakova *et al.*, 2002), its purification and complete structural elucidation, which are currently under investigation, are hampered by its extremely low productivity (J. Kormanec, unpublished results). Similar problems have been recently described for several other homologous angucycline clusters (Lombo *et al.*, 2004; Metsä-Ketela *et al.*, 2004; Pang *et al.*, 2004). This indicates that these types of PKS clusters are very tightly regulated. Knowledge of the mechanisms of their regulation at the molecular level is therefore of great interest, and would potentially allow an increase in the yields of auricin in the producer strain.

Two putative regulatory genes, *aur1P* and *aur1R*, whose deduced products share significant similarity with two

Abbreviations: EMSA, electrophoretic mobility-shift assay; PKS, polyketide synthase; TSP, transcription start point.

different types of bacterial regulatory proteins, have been previously identified upstream of the *aur1* polyketide gene cluster in *S. aureofaciens* CCM 3239. The *aur1P* gene, encoding a protein similar to response regulators of bacterial two-component signal transduction systems, has been characterized in more detail. It has been shown to specifically activate expression of the auricin biosynthetic genes (Novakova *et al.*, 2005). The divergent *aur1R* gene has been shown to encode a protein homologous to transcriptional repressors of the TetR family. Members of this family function mainly as repressors and regulate genes encoding biosynthetic enzymes for antibiotics, drug-efflux pumps and other proteins. The structure of the TetR-family proteins consists of two domains: a DNA-binding domain which contains a helix–turn–helix motif and a regulatory domain that recognizes the signals via ligand binding. TetR-family proteins show high sequence similarity in their DNA-binding domains and almost no significant similarity in the regulatory domains (Ramos *et al.*, 2005).

The aim of the present study was to characterize the *aur1R* gene in *S. aureofaciens* CCM 3239. For this purpose the gene was inactivated by gene replacement and auricin production was investigated in the mutant. Gene expression analysis, involving S1 nuclease mapping, was applied to the known *aur1* promoters in the *aur1R* mutant as well as in the wild-type strain. We further showed that Aur1R directly binds one of the promoters, *aur1Pp*, and that the binding is affected by auricin or its intermediates. The results presented here show that Aur1R is a transcriptional repressor of the *aur1P* gene, which encodes a pathway-specific activator of the auricin biosynthetic gene cluster in *S. aureofaciens* CCM 3239.

METHODS

Bacterial strains, plasmids and culture conditions. *S. aureofaciens* CCM 3239 wild-type was from the Czechoslovak Collection of Micro-organisms (Brno, Czech Republic). *Escherichia coli* cosmid SuperCos-1 (Evans *et al.*, 1989) was from Stratagene. *E. coli* BW25113/pIJ790 was used as a host for PCR-targeted gene disruption using the apramycin-resistance plasmid pIJ773, and *E. coli* ET12567/pUZ8002 was used as a non-methylating host (Gust *et al.*, 2003). The *Streptomyces* integrative plasmid pPM927 (Smokvina *et al.*, 1990) was provided by Mark J. Buttner (John Innes Institute, Norwich, UK). The plasmid pJUP3, encompassing the *aur1R* and *aur1P* region, has been described previously (Novakova *et al.*, 2005). *E. coli* BL21(DE3) pLysS and the plasmid pET28a, used for *aur1R* overexpression, were obtained from Novagen. Growth and transformation of *S. aureofaciens* CCM 3239 strains were carried out as described in Kormanec *et al.* (1993). The phenotype of the *S. aureofaciens* Δ *aur1R* mutant was analysed after growth on solid minimal MM medium (Kieser *et al.*, 2000) and rich Bennet medium (Horinouchi *et al.*, 1983), and after growth in liquid minimal NMP medium (Kieser *et al.*, 2000) and rich Bennet medium. For RNA isolation, 5×10^8 c.f.u. of the particular *S. aureofaciens* CCM 3239 strain were inoculated in 50 ml liquid Bennet medium and grown at 28 °C to different growth phases. Conditions for *E. coli* growth and transformation have been described by Ausubel *et al.* (1995).

DNA manipulations. The standard DNA manipulations in *E. coli* were done as described in Ausubel *et al.* (1995), and those in *Streptomyces* according to Kieser *et al.* (2000). Chromosomal DNA from *S. aureofaciens* CCM 3239 strains was isolated according to Kieser *et al.* (2000). A cosmid library of partially *Sau*3AI-digested *S. aureofaciens* CCM 3239 chromosomal DNA fragments cloned in the *Bam*HI site of SuperCos-1 was constructed essentially as described in Redenbach *et al.* (1996). The cosmid library (5000 clones) was screened with a 670 bp *Sac*I DNA fragment comprising the *aur1M*–*aur1N* genes from the end of the previously cloned 15.1 kb region of the *aur1* cluster (Novakova *et al.*, 2002). A total of 28 positive clones were identified, and the corresponding recombinant cosmids pCosSA1–28 isolated and analysed by restriction mapping and hybridization. One cosmid, pCosSA25, containing the largest region upstream of the *aur1R* gene in the *aur1* cluster, has been used in further studies for *aur1R* disruption.

Disruption of the *S. aureofaciens* CCM 3239 *aur1R* gene. The PCR-targeted REDIRECT procedure (Gust *et al.*, 2003) was used to delete the *aur1R* gene in *S. aureofaciens* CCM 3239. In brief, the apramycin-resistance cassette was PCR-amplified using primers Aur1Rdir (5'-TTTAATAATAGAAGGAACTGATACAGGAGCTA-TGCGTGATTCCGGGGATCCGTCGACC-3') and Aur1Rrev (5'-GG-CTGCCAGACAGGGCATGACCACCGCCAGAACCCCGCTGTAG-GCTGGAGCTGCTTC-3') and the template plasmid pIJ773. The resulting PCR product was introduced by electroporation into *E. coli* BW25113/pIJ790 containing cosmid pCosSA25. The correct replacement of the *aur1R*-coding region in the resulting SuperCos1-derived cosmid pCosSA25::AmR was verified by restriction mapping. The cosmid pCosSA25::AmR was transformed into the non-methylating *E. coli* ET12567/pUZ8002 strain and introduced into *S. aureofaciens* CCM 3239 by conjugation, and mutants resistant to apramycin and sensitive to kanamycin were selected. Two apramycin-resistant and kanamycin-sensitive colonies were identified, and for each the correct double-crossover event was proved by Southern blot hybridization (data not shown). Both clones had a similar phenotype, and the first strain, *S. aureofaciens* Δ *aur1R*1, was chosen for further study. The plasmid pPM-aur1R, used for complementation of the *aur1R* mutation, was prepared by inserting a 1.25 kb *Bam*HI–*Nco*I (blunt-ended) fragment of plasmid pJUP3 (containing the *aur1R* gene with its promoter) in pPM927 cut with *Bam*HI and *Kpn*I (blunt-ended).

RNA isolation and S1 nuclease mapping. Isolation of total RNA from *S. aureofaciens* CCM 3239 and high-resolution S1 nuclease mapping were performed according to Kormanec (2001). Samples (40 µg) of RNA were hybridized to approximately 0.015 pmol of suitable DNA probe labelled at one 5' end with [γ -³²P]ATP [$\sim 10^6$ d.p.m. pmol⁻¹ (60 MBq pmol⁻¹) of probe]. The S1 probes were prepared as follows. Probe 1 (for the *aur1Rp* promoter) was prepared by PCR amplification from plasmid pJUP3 using the 5' end-labelled primer Aur1RS1 (5'-GCTCAGCGTCGCCTCTGCGTACC-3') from the *aur1R*-coding region and the unlabelled primer Aur1PS1 (5'-CCTTCGGCCGGTGAGAAACC-3') from the *aur1P*-coding region. Probe 2 (for the *aur1Pp* promoter) was prepared similarly by PCR amplification using 5' end-labelled primer Aur1PS1 and unlabelled primer Aur1RS1. Probe 3 (for the *aur1Ap* promoter) has been described previously (Novakova *et al.*, 2005), and the control *hrdBp2* promoter probe has been described in Kormanec & Farkasovsky (1993). The primers were labelled at the 5' end with [γ -³²P]ATP [ICN, 4500 Ci mmol⁻¹ (166.5 TBq mmol⁻¹)] and T4 polynucleotide kinase, as described by Ausubel *et al.* (1995). The protected DNA fragments were analysed on DNA sequencing gels together with G+A and T+C sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980).

Overexpression of *aur1R* in *E. coli* and protein purification. The *aur1R* gene was amplified by PCR using pJUP3 as a template and the

primers aur1RNde (5'-GATACAGGAGCTACATATGATCAAACA-AG-3') and aur1RHind (5'-GGGGAAGCTTCAGGAGCCGCGGC-CCGAGCGC-3'), which introduced an *Nde*I site (in bold type) overlapping the translation initiation codon and a *Hind*III site (in bold type) downstream of the stop codon, respectively. To ensure higher fidelity of DNA synthesis during PCR, *Pfu* DNA polymerase (Stratagene) was used. A 620 bp *aur1R*-containing DNA fragment was cut with *Nde*I and *Hind*III and cloned in pET28a cut with the same enzymes, resulting in pET-aur1R, which was verified by nucleotide sequencing. The host strain for pET-series expression plasmids, *E. coli* BL21 (DE3) pLysS, transformed with the plasmid pET-aur1R, was grown in LB medium containing 30 µg chloramphenicol ml⁻¹ and 40 µg kanamycin ml⁻¹ at 30 °C to OD₆₀₀ 0.5. Expression was induced with 1 mM IPTG. After 3 h, the cells were harvested by centrifugation at 12 000 g for 10 min and washed with ice-cold 0.9 % (w/v) NaCl. The lysis of cells and native purification of His-tagged Aur1R protein on His-Tag Bind resin (Novagen) were carried out as directed by the manufacturer. The eluted proteins were dialysed overnight at 4 °C against the storage buffer [12.5 mM Tris/HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 50 % (v/v) glycerol] and cleared by centrifugation at 30 000 g for 10 min, and the supernatant was stored in aliquots at -20 °C. The overproduction and purity of the eluted protein were confirmed by SDS-PAGE (Laemmli, 1970). A prominent band (*M*_r 24 500) was identified after IPTG induction and isolation by His-Tag Bind resin (data not shown). This value corresponded to the calculated *M*_r of the 6 × His-tagged Aur1R protein (24 012). The concentration of protein was determined according to Bradford (1976) with BSA as a standard.

Analysis of secondary metabolites. Spores (5 × 10⁸ c.f.u.) of *S. aureofaciens* CCM 3239 and the corresponding *S. aureofaciens* Δ aur1R1 mutant strains were inoculated into 50 ml Bennet medium in 250 ml Erlenmeyer flasks, and the cultures were grown on an orbital shaker at 270 r.p.m. and 28 °C. Aliquots (4 ml) were taken from the cultures at particular time points and pelleted by centrifugation [8000 m s⁻¹ (820 g), 5 min]. Supernatants were extracted twice with the same volume of ethyl acetate. Residual water was removed with sodium sulphate and sodium chloride, and extracts were evaporated under vacuum. The pellets were dissolved in 30 µl methanol, and 5 µl aliquots were subjected to TLC analysis on silica gel 60 F₂₅₄ plates (Merck) with *n*-butanol saturated with water. Dried TLC plates were analysed by biochromatography by placing them on LB agar plates and overlaying with soft nutrient agar (Kieser *et al.*, 2000) containing a fresh culture of *Bacillus subtilis*. The agar was allowed to solidify and plates were incubated for 16 h at 37 °C and screened visually for growth-inhibition zones. Extracts used for electrophoretic mobility shift assays (EMSAs) were prepared from *S. aureofaciens* CCM 3239 and *S. aureofaciens*, *aur1::tsr* (Novakova *et al.* 2002) grown in 50 ml of medium as described above, although the whole culture medium was extracted twice with ethyl acetate and finally dissolved in 30 µl methanol.

EMSA. A 200 bp *Sma*I-*Eco*RI DNA fragment encompassing the *aur1Ap* promoter uniquely labelled on the 5' end at the *Eco*RI site has been described previously (Novakova *et al.*, 2005). A 406 bp fragment (probe 2) comprising the *aur1R-aur1P* intergenic region [nucleotide positions -291 to +115 with respect to the transcription start point (TSP) for the *aur1Pp* promoter] was prepared by PCR amplification from pJUP3 using the 5' end-labelled primer Aur1PS1 and unlabelled primer Aur1RS1. EMSA was done essentially as described by Ausubel *et al.* (1995). The ³²P-labelled DNA fragments [0.04 ng, ~2000 d.p.m. (120 kBq)] were incubated with increasing amounts of purified Aur1R protein for 15 min at 30 °C in a 15 µl total volume of the binding buffer [12.5 mM Tris, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 12 % (v/v) glycerol], 2 µg sonicated salmon sperm DNA and 4.5 µg BSA. After incubation, protein-bound and free DNA was resolved on a non-denaturing polyacrylamide gel containing 4 % (w/v) acrylamide, 0.05 % (w/v) bisacrylamide and 2.5 % (w/v) glycerol,

running at 4 °C (after a 1 h prerun at 30 mA) in a high-ionic-strength buffer containing 50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5, at the same current. The gels were dried and exposed to an X-ray film.

The effect of auricin on the ability of Aur1R to bind the DNA probe was tested by adding 1 µl of the undiluted extracts or a series of twofold-diluted extracts (with methanol) to the 15 µl EMSA binding reaction.

DNase I footprinting. Binding reactions were performed in 30 µl binding buffer at the same conditions as for the EMSA with 0.3 ng [~12 000 d.p.m. (720 kBq)] of either the coding or non-coding strand 5' end-labelled 406 bp DNA fragment encompassing the *aur1R-aur1P* intergenic region (probe 1 and probe 2, respectively) and increasing amounts of purified Aur1R. After incubation for 15 min at 30 °C, 3 µl DNase I solution [5 U DNase I ml⁻¹ (Roche) in 100 mM MgCl₂, 100 mM DTT] was added to the binding reaction. The reaction was incubated for 40 s at 37 °C, stopped by adding 7.5 µl DNase I stop buffer (3 M ammonium acetate, 0.25 M EDTA, 0.1 mg tRNA ml⁻¹) and extracted with 30 µl alkaline phenol/chloroform. The aqueous phase was precipitated by adding three volumes of ethanol. The resulting pellet, after washing with 70 % (v/v) ethanol and SpeedVac drying, was suspended in 5 µl Maxam loading buffer [80 % (v/v) formamide, 1 mM EDTA, 10 mM NaOH, 0.05 % (w/v) bromophenol blue, 0.05 % (w/v) xylene cyanol FF]. The DNA fragments were analysed on 6 % DNA sequencing gels together with G+A and T+C sequencing ladders derived from the end-labelled fragment (Maxam & Gilbert, 1980). After electrophoresis, the gels were dried and exposed to an X-ray film.

RESULTS

Disruption of the *aur1R* gene positively affects auricin production

Expression of the auricin biosynthetic genes in *S. aureofaciens* CCM 3239 has been previously shown to be under the control of the pathway-specific positive regulator Aur1P, which belongs to the family of response regulators of bacterial two-component signal transduction systems. A divergently transcribed gene, *aur1R*, has been identified upstream of *aur1P* and shown to encode a repressor belonging to the TetR family (Novakova *et al.*, 2005). To investigate the role of the *aur1R* gene in auricin biosynthesis and regulation, a chromosomal copy of *S. aureofaciens* *aur1R* was inactivated using a PCR targeting system (REDIRECT method) for disruption of *Streptomyces* genes (Gust *et al.*, 2003). The mutation did not affect growth and differentiation (data not shown), and the mutant strain *S. aureofaciens* Δ aur1R1 grew at a similar rate to the wild-type strain (Fig. 1a). Wild-type *S. aureofaciens* CCM 3239 and the *S. aureofaciens* Δ aur1R1 mutant were investigated for production of auricin as described previously (Novakova *et al.*, 2002). Ethyl acetate extracts from the wild-type and the *aur1R*-disrupted strains were prepared from liquid-grown strains cultivated in the best auricin-production medium, Bennet medium, and analysed by TLC, followed by a bioassay against *B. subtilis*. As for many other antibiotics, production of auricin begins at the beginning of the stationary phase (12 h). No auricin was detected during exponential phase (9 h) in both strains (data not shown). At

Fig. 1. (a) Growth curve of *S. aureofaciens* CCM 3239 wild-type (WT) and *S. aureofaciens* *aur1R*-disrupted ($\Delta aur1R$) strains in liquid Bennet medium. Each point represents the mean of three assays; error bars, SD. (b) Analysis of auricin production in the wild-type, *aur1R*-disrupted and complemented *S. aureofaciens* strains by biochromatography assay. Ethyl acetate extracts from *S. aureofaciens* CCM 3239 wild-type (WT), *aur1R*-disrupted ($\Delta aur1R$) and complemented ($\Delta aur1R$ + pPM-*aur1R*) strains grown to the indicated time points in Bennet medium were resolved by TLC and overlaid with *B. subtilis*, as described in Methods. The inhibition zone corresponds to auricin (Novakova *et al.*, 2002).

all time points, the inhibition zones corresponding to auricin in the *aur1R*-deletion mutant were clearly and reproducibly larger than those of the wild-type *S. aureofaciens* CCM 3239 strain (Fig. 1b). This inhibition zone was absent in the ethyl acetate extracts prepared either from the *S. aureofaciens*, *aur1::tsr* strain with deleted critical PKS genes (Novakova *et al.*, 2002) or from the *S. aureofaciens*, *aur1P::tsr* strain with a deleted auricin pathway-specific activator (Novakova *et al.*, 2005), which were grown under identical conditions (results not shown). Therefore, it was obvious that this inhibition zone corresponded to auricin.

To verify that this phenotype was solely due to the deletion of *aur1R*, *S. aureofaciens* $\Delta aur1R1$ was complemented *in trans* by transformation with plasmid pPM-*aur1R*, containing the *aur1R* gene including its promoter cloned in the integrative plasmid pPM927. The level of auricin decreased in the complemented strain to a level even lower than in the wild-type strain (Fig. 1b), showing that the increase of auricin in *S. aureofaciens* $\Delta aur1R1$ was indeed due to the *aur1R* disruption. These results therefore indicated that the *aur1R* gene likely encodes a negative regulator of auricin production.

Transcriptional analysis of *aur1R*

Expression of *aur1R* was investigated by S1 nuclease mapping using probe 1 and RNA isolated from *S. aureofaciens* CCM 3239 at various stages of growth in liquid Bennet medium. As shown in Fig. 2(a), a single RNA-protected fragment was identified that corresponded to the *aur1Rp* promoter with a TSP at A, 24 nucleotides upstream of the most likely translation initiation codon GTG (Fig. 4b). No RNA-protected fragment was identified with tRNA as a control (Fig. 2a, lane C). The promoter was regulated in a growth stage-dependent manner in Bennet medium and induced at the beginning of stationary phase, correlating with auricin production. The sequence of the *aur1Rp* promoter and flanking regions is shown in Fig. 4(b). The putative -10 region of *aur1Rp* shows quite high similarity (five out of six matches) to the -10 region of the consensus sequence recognized by the major *Streptomyces* sigma factor σ^{HrdB} (Brown *et al.*, 1992), although the sequence around the -35 region shows only limited similarity (three out of six matches). The two regions are separated by an optimal spacer of 17 bp. Therefore, as for the divergent *aur1Pp* promoter (Novakova *et al.*, 2005), it

Fig. 2. High-resolution S1 nuclease mapping of the TSP for the *aur1Rp* promoter in *S. aureofaciens* CCM 3239 wild-type strain (a), and for the *aur1Pp* and *aur1Rp* promoters in *S. aureofaciens* CCM 3239 wild-type (wt) and *S. aureofaciens* *aur1R*-disrupted ($\Delta aur1R$) strains (b, c). The 5'-labelled DNA fragments were hybridized with 40 μ g RNA and treated with 120 U S1 nuclease. RNA was isolated from the cultures grown in liquid Bennet medium at the time points indicated, which corresponded to the different growth phases (Fig. 1a): the exponential phase (7 and 9 h), the end of exponential phase (12 h) and the stationary phase (14 and 16 h). *E. coli* tRNA was used as a control (lane C). (d) Control S1 nuclease mapping experiments with the same RNA samples and a DNA probe for the *hrdBp2* promoter (Kormanec & Farkasovsky, 1993). The RNA-protected DNA fragments were analysed on DNA sequencing gels together with G+A (lane A) and T+C (lane T) sequencing ladders derived from the end-labelled fragments (Maxam & Gilbert, 1980). The thin horizontal arrow indicates the position of an RNA-protected fragment and thick bent vertical arrows indicate the nucleotide corresponding to the TSP. Before assigning the TSP, 1.5 nucleotides was subtracted from the length of the protected fragment to account for the difference in the 3' ends resulting from S1 nuclease digestion and the chemical sequencing reactions. In every experiment, the same RNA preparations were hybridized in parallel with all of the probes. All S1 nuclease mapping experiments were performed twice with independent sets of RNA with similar results.

putative oxygenase (Novakova *et al.*, 2002). Transcription of the *aur1Ap* promoter was directly activated by the auricin pathway-specific activator Aur1P (Novakova *et al.*, 2005). Therefore, to investigate whether *aur1R* disruption has an effect on transcription of both *aur1A* and *aur1P* genes, S1 nuclease mapping was performed using probes 2 and 3 and RNA isolated from *S. aureofaciens* CCM 3239 wild-type and the *aur1R* mutant strains cultivated in liquid Bennet medium, as for analysis of *aur1R* transcription. As shown in Fig. 2(b, c), with the RNA isolated from the wild-type strain, single RNA-protected fragments were identified with TSPs that corresponded to the *aur1Ap* and *aur1Pp* promoters, as published previously (Novakova *et al.*, 2005). As for *aur1Rp*, both promoters were inactive in the exponential growth phase (7 and 9 h) and induced at the beginning of the stationary phase (12 h), which correlates with auricin production. However, the levels of *aur1Ap* and *aur1Pp* mRNA from all the time points were reproducibly increased in the *aur1R* mutant. As an internal control, S1 nuclease mapping was performed with the same RNA samples using a probe fragment specific for the *S. aureofaciens* *hrdBp2* promoter, which is expressed fairly constantly during growth and differentiation (Kormanec & Farkasovsky, 1993). RNA-protected fragments of similar intensities corresponding to the *hrdBp2* promoter were identified with all RNA samples (Fig. 2d). The results indicated that the *aur1R* mutation positively affects transcription from both promoters. Therefore, increased transcription from the *aur1Pp* and the *aur1Ap* promoters is responsible for the increase in auricin production in the *aur1R* mutant. The results are also consistent with the

is likely that the *aur1Rp* promoter is transcribed by an RNA polymerase holoenzyme containing the principal sigma factor σ^{HrdB} .

Dependence of *aur1A* and *aur1P* transcription on *aur1R*

Previously, we had characterized the *aur1Ap* promoter, which directs expression of the first characterized biosynthetic gene of the auricin cluster, *aur1A*, which encodes a

putative role of Aur1R as a negative repressor of auricin expression.

The Aur1R protein binds specifically to the *aur1Pp* and *aur1Rp* promoters and its binding is affected by auricin or its intermediate(s)

To investigate whether Aur1R directly regulates *aur1Ap* and *aur1Pp* transcription, the *aur1R* gene was over-expressed in the *E. coli* T7 RNA polymerase expression system, and the purified N-terminal His-tagged Aur1R was examined for its binding to the *aur1Ap* and *aur1Pp* promoter regions by EMSA. Two 32 P-labelled DNA fragments were used for the binding studies: a 200 bp fragment encompassing the *aur1Ap* promoter (nucleotide positions -167 to $+32$ with respect to the TSP) has been described previously for binding of Aur1P (Novakova *et al.*, 2005); and a 406 bp fragment comprising the *aur1R*–*aur1P* intergenic region (nucleotide positions -291 to

$+115$ with respect to the TSP for the *aur1Pp* promoter). No binding was identified with the *aur1Ap* promoter DNA fragment (data not shown). However, using the 406 bp *aur1R*–*aur1P* intergenic DNA fragment, four retarded bands were clearly visible with an increasing concentration of Aur1R, which may correspond to four different complexes (Fig. 3). The specificity of the interaction was demonstrated by the competitive binding of the unlabelled fragment (Fig. 3, lane 6). These results indicated that Aur1R is capable of binding to the *aur1R*–*aur1P* intergenic region.

To investigate the possibility that auricin or its intermediate is an effector ligand for Aur1R, the culture supernatants of *S. aureofaciens* CCM 3239 and its isogenic *S. aureofaciens*, *aur1::tsr* mutant with a deletion of critical PKS genes (Novakova *et al.*, 2002), grown for 14 h in Bennet medium, were extracted with ethyl acetate, dried and dissolved in methanol. Decreasing amounts of extracts were used in a series of twofold dilutions in binding

Fig. 3. (a) Detection of protein–DNA complexes by EMSA using 0.04 ng of a 32 P-labelled 406 bp DNA fragment comprising the *aur1R*–*aur1P* intergenic region (probe 2) with increasing amounts of purified His-tagged Aur1R. Lane 1, labelled fragment in the absence of protein; lanes 2–5, 2, 5, 10 and 15 μg, respectively, of the purified His-tagged Aur1R protein. Addition of 200 ng unlabelled 406 bp *aur1R*–*aur1P* intergenic DNA fragment was used to demonstrate Aur1R binding specificity (lane 6). The arrows indicate the free DNA fragment and shifted fragments corresponding to the proposed complexes. (b) Effect of ligand(s) on the Aur1R binding activity. EMSA was done with 0.01 ng 32 P-labelled probe 2 and 4 μg Aur1R with increasing amounts of culture extracts from the wild-type (WT) and the *S. aureofaciens aur1::tsr* mutant ($\Delta aur1$; Novakova *et al.*, 2002) in a series of twofold dilution samples. Methanol was used as a solvent. A 1 μl volume of methanol or the particular extract was used in each 15 μl binding reaction. A 1 μl volume of the extract corresponded to the amount of auricin present in 1.67 ml liquid culture prepared as described in Methods.

Fig. 4. (a) DNase I footprints of Aur1R binding to 0.3 ng of a 5' end-labelled, either coding or non-coding strand, 406 bp DNA fragment encompassing the *aur1R*–*aur1P* intergenic region (probe 1 and probe 2, respectively). The vertical bars indicate the positions of the Aur1R binding sites. The numbering is relative to the TSP of the *aur1Pp* and *aur1Rp* promoters. The TSPs of the promoters are indicated by bent arrows. Lane 1 is without the His-tagged Aur1R protein sample. Lanes 2 and 3 contain 30 and 75 µg, respectively, of the purified His-tagged Aur1R protein. Lanes A and T represent G+A and C+T sequencing ladders, respectively (Maxam & Gilbert, 1980). All binding experiments were performed twice with independent sets of protein samples, giving similar results. (b) The nucleotide sequence of the *S. aureofaciens* CCM 3239 *aur1R*–*aur1P* intergenic region. The deduced protein products are given using the single-letter amino acid code at the second position of each codon. The TSPs of the *aur1Pp* and *aur1Rp* promoters are indicated by bent arrows. The putative –10 and –35 boxes of the promoters are in bold type and underlined. The nucleotides that were protected from DNase I by Aur1R binding are shaded. The nucleotide sequence shown is a part of the *aur1* cluster that has been deposited in GenBank/EMBL/DBJ databases under accession number AY956334.

reactions of the 406 bp *aur1R*–*aur1P* intergenic DNA fragment with a constant concentration of Aur1R. An increasing concentration of extract from the wild-type *S. aureofaciens* dissociated Aur1R from the promoter regions, but similar amounts of extract from the *aur1*-disrupted *S. aureofaciens* strain had no effect on Aur1R binding (Fig. 3b).

To map the binding sites for Aur1R within the region, a DNase I footprinting assay was carried out using the same 406 bp *aur1R*–*aur1P* intergenic DNA fragment, 5' end-labelled at either end. Using both coding and non-coding strands, two clearly separated regions were protected. Interestingly, both protected regions covered the *aur1Rp* and *aur1Pp* promoters, respectively, in almost identical positions with respect to the TSPs for the promoters, occupying both putative –10 and –35 promoter elements (Fig. 4). This repressor mode of binding to the promoters is consistent with the proposed function of Aur1R as a negative transcriptional regulator belonging to the TetR family. Inspection of both DNase I-protected regions in the *aur1Rp* and *aur1Pp* promoters revealed that both binding regions are similar and contain a partial inverted repeat that is partially similar to the autoregulatory element (ARE) consensus sequence of the *Streptomyces* γ -butyrolactone autoregulator receptor proteins (Fig. 5) (Folcher *et al.*, 2001). The results of the binding studies indicated that the effect of *aur1R* upon *aur1P* expression is direct, and that the effect of *aur1R* upon *aur1A* expression is likely indirect, through the activation of Aur1P, which has been shown to directly bind to the *aur1Ap* promoter (Novakova *et al.*, 2005). The results suggested a cascade regulation of the *aur1* cluster via negative and positive modes. In addition to *aur1Pp*, Aur1R also bound to its own *aur1Rp* promoter, indicating negative feedback control.

DISCUSSION

Antibiotic biosynthesis is very often tightly regulated at several levels to ensure exact timing of production and to avoid the potential toxicity of an antibiotic to the producer. The activity of the pathway-specific regulatory genes is often regulated by genes at a higher level, including global regulatory genes mediating environmental,

nutritional and growth-rate effects (Bibb, 2005). The results of the present study indicate a similar regulatory cascade for the angucycline-like auricin gene cluster in *S. aureofaciens* CCM 3239. As for other similar angucycline gene clusters, auricin is produced with very low yields that indicate tight regulation. Two regulatory genes, *aur1P* and *aur1R*, have been identified in the auricin polyketide gene cluster. The *aur1P* gene, encoding a homologue of response regulators, has been shown to represent a pathway-specific transcriptional activator that directly controls the activity of the *aur1Ap* promoter, which governs transcription of the first biosynthetic gene, *aur1A*, in the auricin gene cluster in *S. aureofaciens* CCM 3239 (Novakova *et al.*, 2005). The present results demonstrate that the divergently located *aur1R* gene has a negative role in regulation of auricin production through a direct inhibition of *aur1P* expression. This repressor mode of Aur1R binding to the *aur1Pp* promoter is likely relieved by an interaction of Aur1R with auricin or its intermediate(s). Moreover, Aur1R also bound its own promoter in an almost identical way, which indicates a negative feedback autoregulatory control of *aur1R* expression.

The *aur1R* gene encodes a protein homologous to members of the TetR family of transcriptional regulators, which function mainly as repressors. Like many other TetR-family repressors, Aur1R consists of an N-terminal conserved HTH DNA-binding domain and a variable C-terminal effector-binding domain. (Ramos *et al.*, 2005). The highest similarity of the Aur1R DNA-binding domain was to members of the subfamily of γ -butyrolactone autoregulator pseudo-receptors, e.g. JadR2 (Yang *et al.*, 1995), TylQ (Stratigopoulos & Cundliffe, 2002), BarB (Matsuno *et al.*, 2004) and AlpW (Bunet *et al.*, 2008), which all constitute an independent branch in the phylogenetic tree of γ -butyrolactone autoregulator receptors and likely do not bind γ -butyrolactone autoregulators (Nishida *et al.*, 2007). Like Aur1R, almost all the pseudo-receptors act as negative regulators of antibiotic production and play a role in the specific repression of pathway-specific transcription activator genes of the particular secondary metabolite gene cluster. Many of them have also been shown to be under the direct control of bona fide

Fig. 5. Comparison of the nucleotide sequences of the *aur1Pp* and *aur1Rp* promoters protected by Aur1R with the autoregulatory element (ARE) consensus sequence of the *Streptomyces* γ -butyrolactone autoregulator receptor proteins (Folcher *et al.*, 2001). Identical residues are highlighted in black. Arrows indicate the position of the 22 bp inverted repeat sequence identified as a consensus sequence (ARE box) of the γ -butyrolactone autoregulator receptor protein ArpA (Onaka & Horinouchi, 1997).

γ -butyrolactone autoregulator receptors (Matsuno *et al.*, 2004; Stratigopoulos *et al.*, 2002). Therefore, it seems likely that pseudo-receptor genes play a common role in the negative regulation of secondary metabolite biosynthesis as target genes of autoregulator receptors in the autoregulator signalling cascade. Our preliminary experiments revealed a likely true γ -butyrolactone autoregulator receptor which bound the *aur1Rp* promoter in *S. aureofaciens* CCM 3239, thus indicating a similar regulation of auricin biosynthesis (J. Kormanec, unpublished results).

The members of the TetR family are controlled by small effector ligands, which after interaction with the protein inhibit their binding to the cognate operator sites (Ramos *et al.*, 2005). Several *Streptomyces* antibiotic pathway TetR family regulators (DnrO, SimR, LanK and ActR) have been shown to respond to the mature antibiotic and/or pathway intermediates as effector ligand (Jiang & Hutchinson, 2006; Le *et al.*, 2009; Ostash *et al.*, 2008; Tahlan *et al.*, 2007). However, no effector ligand has yet been identified for this subfamily of γ -butyrolactone autoregulator pseudo-receptors. Aur1R is the first example of a member of this subfamily regulated by an antibiotic or its intermediate(s).

An interesting feature of Aur1R is its manner of binding to *aur1Rp* and *aur1Pp* promoters. While DNase I footprinting analysis revealed two binding regions that overlapped both promoters (Fig. 4), four different complexes have been identified by EMSA using a DNA fragment encompassing both promoters with increasing amounts of Aur1R (Fig. 3). One explanation might be that one molecule of Aur1R binds one half of the palindromic operator site (Fig. 5). Assuming different Aur1R affinities to particular parts of the operators, we could explain four different complexes with increasing Aur1R concentration in EMSA analysis. However, this scenario is rather unlikely, as members of the TetR family in general bind their recognition sequences as dimers (Ramos *et al.*, 2005). The other possibility is that two Aur1R dimers bind a single operator comprising a 22 bp inverted repeat (Fig. 5) cooperatively, as has been described for another TetR homologue, QacR. In contrast to TetR, which binds a 15 bp inverted repeat operator as a single dimer, two dimers of QacR bind a longer operator site (a single 28 bp inverted repeat) cooperatively from the opposite sites of the DNA helix (Schumacher *et al.*, 2002). The longer Aur1R operator inverted repeat (22 bp) might indicate this mechanism. However, a detailed structural analysis would be needed to explain more clearly how Aur1R binds to its operator site.

Based on the present data we can conclude that Aur1R is a negative regulator of auricin biosynthesis through inhibition of transcription of the gene encoding the pathway-specific activator Aur1P. We suggest that the role of Aur1R is to repress transcription of *aur1P* until the bacterial cells are fully prepared for auricin production, by for example permitting earlier expression of auricin resistance. Auricin or its intermediates likely represent ligand(s) that after binding to Aur1R relieve its binding to the *aur1Pp*

promoter and consequently induce its transcription, resulting in accumulation of threshold amounts of the pathway-specific activator Aur1P to induce expression of auricin biosynthetic genes. Recent results concerning an Aur1P homologue, JadR1, confirm that jadomycin and its intermediate bind to JadR1 and that this binding constitutes a feedback control of jadomycin biosynthesis in *Streptomyces venezuelae* (Wang *et al.*, 2009). It is likely that a similar feedback mechanism also exists for the auricin cluster. The results of the transcriptional analysis of the *aur1A* and *aur1P* genes (Fig. 2), where the level of both transcripts decreased in the later stages, are consistent with this hypothesis. Therefore, after successful purification and structural analysis of auricin, similar experiments to investigate auricin activation will be pursued.

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