Characterization of a regulatory gene essential for the production of the angucycline-like polyketide antibiotic auricin in *Streptomyces aureofaciens* CCM 3239

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A gene, *aur1P*, encoding a protein similar to the response regulators of bacterial two-component signal transduction systems, was identified upstream of the *aur1* polyketide gene cluster involved in biosynthesis of the angucycline-like antibiotic auricin in *Streptomyces aureofaciens* CCM 3239. Expression of the gene was directed by a single promoter, *aur1Pp*, which was transcribed at low levels during the exponential phase and induced just before the stationary phase. A divergently transcribed gene, *aur1R*, has been identified upstream of *aur1P*, encoding a protein homologous to transcriptional repressors of the TetR family. The *aur1P* gene was disrupted in the *S. aureofaciens* CCM 3239 chromosome by homologous recombination.

The mutation in the *aur1P* gene had no effect on growth and differentiation. However, biochromatographic analysis of culture extracts from the *S. aureofaciens aur1P*-disrupted strain revealed that auricin was not produced in the mutant. This indicated that *aur1P* is essential for auricin production. Transcription from the previously characterized *aur1Ap* promoter, directing expression of the first gene, *aur1A*, in the auricin gene cluster, was dramatically decreased in the *S. aureofaciens CCM 3239 aur1P* mutant strain. Moreover, the Aur1P protein, overproduced in *Escherichia coli*, was shown to bind specifically upstream of the *aur1Ap* promoter region. The results indicated that the Aur1P regulator activates expression of the auricin biosynthesis genes.

**INTRODUCTION**

Gram-positive soil bacteria of the genus *Streptomyces* undergo a complex process of morphological differentiation, accompanied by the production of biologically active secondary metabolites, including the majority of known antibiotics (Chater, 1998). Most of the antibiotics are produced by complex biosynthetic pathways encoded by physically clustered genes. The gene clusters are usually regulated by pathway-specific transcriptional activators that are located in these clusters. In addition, various global regulatory genes have been identified, which affect antibiotic production indirectly and have pleiotropic roles in stress response and morphological differentiation. Most of these pleiotropic regulatory genes have been shown to influence the activity of the pathway-specific regulatory genes. Expression of both types of regulatory gene is influenced by a variety of physiological and environmental factors, including growth rate, signalling molecules, imbalances in metabolism and various physiological stresses (Chater & Bibb, 1997). Investigation of the regulatory mechanisms of antibiotic production is of great interest, as these studies provide a potential platform for manipulating industrially important strains to increase production of their secondary metabolites. There are several examples of studies describing elevated levels of antibiotic production in streptomycetes as a result of overexpression of positive regulatory genes (Gramajo *et al*., 1993; Takano *et al*., 1992; Stratigopoulos *et al*., 2004).

In streptomycetes, several antibiotic regulatory genes have been identified that belong to the family of response regulators known as bacterial two-component signal transduction systems. These widespread regulatory systems transduce signals of external or internal conditions into the expression of particular genes. They consist of a sensor histidine kinase and a response regulator. The histidine kinase is autophosphorylated at a histidine residue in response to a specific signal, and this phosphoryl group is transferred to an aspartate residue of the response regulator. The resulting phosphorylated response regulator activates the transcription of target genes (Stock *et al*., 2000). The analysis of the

**Abbreviations:** SARP, *Streptomyces* antibiotic regulatory protein; TSP, transcription start point.

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Streptomyces coelicolor A3(2) genome sequence has revealed the presence of a high number (67) of gene pairs encoding two-component signal transduction systems (Hutchings et al., 2004). Some of the two-component systems (e.g. AbsA1/AbsA2 and CutR/CurS) belong to global regulators affecting antibiotic production in streptomycetes (Brian et al., 1996; Chang et al., 1996). However, some of the pathway-specific streptomycte antibiotic response-regulator genes (e.g. redZ, jadR1, dnrN and brpA) do not appear to be associated with a histidine kinase sensor gene and lack a conserved phosphorylated aspartate residue (Guthrie et al., 1998; Yang et al., 2001; Furuya & Hutchinson, 1996; Raibaud et al., 1991).

In *Streptomyces aureofaciens* CCM 3239, we have previously identified a type II polyketide synthase gene cluster, *aur1*, which is responsible for production of the angucycline-like polyketide antibiotic auricin (Novakova et al., 2002). Although auricin has been readily detected in the strain grown on solid Bennet medium (Novakova et al., 2002), its purification by HPLC for structural analysis has been hampered by very low yields (J. Kormanec, unpublished results). Similar problems were recently described for other homologous angucycline clusters (Lombo et al., 2004; Metsa-Ketela et al., 2004; Pang et al., 2004). It therefore seems that these polyketide synthase clusters are very tightly regulated. Investigation of this regulation may help to overcome the problem of low yield, as this knowledge could facilitate efforts to engineer strains that overproduce these secondary metabolites.

In a search for *S. aureofaciens* CCM 3239 promoters dependent on the homologue of the principal sigma factor HrdA (Kormanec et al., 1993), we identified a promoter directing expression of a gene, *aur1P*, encoding an unusual homologue of the family of response regulators of bacterial two-component systems, which proved to belong to the auricin cluster. Streptomycetes contain several close homologues of principal sigma factors. In *S. aureofaciens* CCM 3239, four genes, *hrdA*, *hrdB*, *hrdD* and *hrdE*, encoding homologues of principal sigma factors were identified (Kormanec et al., 1992). However, only the *hrdB* gene is essential for viability and has been suggested to encode a functional principal sigma factor. The function of other *hrd*-encoded homologues is unclear, although they are expressed at various stages during differentiation (Buttner & Lewis, 1992; Kormanec et al., 1993; Kormanec & Farkasovsky, 1993). In this paper, we provide evidence that *aur1P* is essential for auricin production in *S. aureofaciens* CCM 3239. We further describe its transcriptional regulation and show that Aur1P is the transcriptional activator that binds to the promoter that directs expression of the *aur1* cluster.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** *S. aureofaciens* CCM 3239 wild-type was from the Czechoslovak Collection of Microorganisms, Brno, Czech Republic. *S. aureofaciens* CCM 3239-A4, containing the *hrdA*-disrupted allele (Kormanec et al., 1993), was previously prepared in our laboratory. *Escherichia coli* XL1Blue, which was used as a host, and plasmid pBluescript II SK+ were from Stratagene. Plasmid pSB40 (Park et al., 1989) was kindly provided by Dr M. K. Winson, University of Nottingham, UK. pPM927 (Smokvina et al., 1990) was provided by Mark J. Buttner, John Innes Institute, Norwich, UK. The plasmids pAPHH1, containing the kanamycin resistance gene *aphII*, and pTSR1, containing the thiostrepton resistance gene *trs*, had been prepared previously (Kormanec et al., 1998). The *E. coli* expression plasmid pAC5mut2, containing a p15A origin of replication and a strong IPTG-inducible *Tet* expression, is described in Novakova et al. (1998). *E. coli* BL21(DE3) pLysS and the vector pET28a, used for *aur1P* overexpression, were obtained from Novagen. Growth and transformation of *S. aureofaciens* strains were carried out as described in Kormanec et al. (1993). The phenotype of the *S. aureofaciens* CCM 3239, *aur1P*: *trs* mutant was analysed after growth on solid minimal medium (MM) (Kieser et al., 2000) and rich Bennet medium (Horinouchi et al., 1983). For DNA isolation from *E. coli* cultures, strains were inoculated into 20 ml liquid LB medium (Ausubel et al., 1995) supplemented with ampicillin (50 µg ml⁻¹), chloramphenicol (40 µg ml⁻¹) and 1 mM IPTG, and grown at 37°C for 16 h. For RNA isolation from liquid-grown cultures, 10⁵ c.f.u. of the particular *S. aureofaciens* strain was inoculated into 50 ml NMP liquid medium (Kieser et al., 2000) containing mannitol (0.5%, w/v) as the carbon source, and grown at 30°C to different growth phases. For RNA isolation from surface cultures, 10⁶ c.f.u. of *S. aureofaciens* was spread on sterile cellophane membranes placed on Bennet medium (Horinouchi et al., 1983), and grown to an appropriate phase of development at 30°C. Conditions for *E. coli* growth and transformation are described by Ausubel et al. (1995).

**DNA manipulations.** 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* strains was isolated according to Kieser et al. (2000). Colony blot hybridization was performed as described in Ausubel et al. (1995). DNA fragments for S1-nuclease mapping and binding studies were isolated from agarose gels as described in Kormanec (2001). The DNA fragments and oligonucleotides were labelled at their 5'-ends with [α-³²P]cTPC (ICN, 1·665 × 10¹¹ Bq mmol⁻¹) and T4 polynucleotide kinase (Biolabs), as described in Ausubel et al. (1995). Nucleotide sequencing was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analysed on an Applied Biosystems model 373 DNA sequencer. DNA sequence ladders G + A and T + C for S1-nuclease mapping were performed by the chemical method (Maxam & Gilbert, 1980). Site-directed mutagenesis was done with the Chamelon mutagenesis kit from Promega.

**Detection of *E. coli* clones containing the *S. aureofaciens* hrda-dependent promoter fragment.** The *S. aureofaciens* CCM 3239 *hrda* gene (Kormanec et al., 1993) was mutagenized to introduce a single *Ndel* site in the start codon using a mutagenic primer MUT29 (5' - GAGGTCGCGCATATGCAACCGAGAC-3'). The gene was then cloned as a 1.750 bp *Ndel*-HindIII fragment in pAC5mut2, resulting in plasmid pAC-hrda1. An *S. aureofaciens* CCM 3239 genomic library was prepared by cloning partially Sau3AI-digested chromosomal DNA fragments (0·5–1·2 kb) into the BamHI site of pBS40. The library (about 80,000 clones) was transformed into *E. coli* XL1Blue containing the compatible plasmid pAC-hrda1. The positive fragments containing potential *hrda*-dependent promoters were screened on LBACIX plates by the procedure described by Novakova et al. (1998).

**RNA isolation and S1-nuclease mapping.** Total RNA from *E. coli* and from *S. aureofaciens* CCM 3239 was prepared as described by Kormanec (2001). The integrity of the RNA was indicated by sharp RNA bands after electrophoresis in agarose containing 2·2 M

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formaldehyde. High-resolution S1-nuclease mapping was done as in Kormanec (2001). Samples (40 μg) of RNA (estimated spectrophotometrically) were hybridized to approximately 0-02 pmol of suitable DNA probe labelled at the 5' end with [β-32P]ATP (~10^6 d.p.m. (pmol probe)^{-1}). The S1 probes used (Fig. 1a) were prepared as follows: probe 1 was prepared by PCR amplification from plasmid pHRA5 using the 5' end-labelled universal oligonucleotide primer 5'-CGCCAGGGTTTTCCCAGTCACGAC-3' from the lacZa coding region and the primer mut80 (5'-GGGTTCCGGCCACA-TTCCCCG-3') from the 5' region flanking the polylinker of pSB40; probe 2 was a 550 bp BamHI–NotI fragment uniquely labelled on the 5' end at the BamHI site; probe 3 was a 970 bp BsiWI–BamHI fragment uniquely labelled on the 5' end at the BsiWI site. The control hrdBP2 promoter probe has been described.

Fig. 1. (a) Genetic organization of the S. aureofaciens CCM 3239 aur1 cluster (Novakova et al., 2002). Genes are indicated by arrows, and the new regulatory genes are shown by darker arrows. The expansion shows the 3344 bp TaqI fragment cloned in the CiaI site of pBluescript II SK+, resulting in pJUP3. The hatched box marks the position of the tsr gene cloned between the Ncol and SacI sites of plasmid pJUP3, resulting in plasmid pJUP3H, which was used for the aur1P gene replacement experiments. The open box indicates the corresponding position of a 1046 bp Sau3AI positive fragment (comprising the aur1P promoter) of plasmid pHRA5. The thin lines below the map represent DNA fragments (5' labelled at the end marked with an asterisk) that were used as probes in S1-nuclease mapping experiments. Bent arrows indicate the positions and direction of transcription from the aur1Ap and aur1Pp promoters. Relevant restriction sites are indicated. (b) High-resolution S1-nuclease mapping of the transcription start point (TSP) of the aur1Pp promoter in the E. coli two-plasmid system. Total RNA isolation and high-resolution S1-nuclease mapping were performed as described in Methods. The 5' labelled DNA fragment corresponding to S1 probe 1 (Fig. 1a) was hybridized with 40 μg RNA isolated from stationary-phase-grown E. coli containing pHRDA5 and pAC5mut2 (lane 1), and pHRDA5 and pAC5-hrdA1 (lane 2). E. coli tRNA was used as control (lane C). 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 the RNA-protected fragment and the thick bent vertical arrow indicates the nucleotide corresponding to the TSP. Before assigning the TSP, 1-5 nt 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. All S1-nuclease mapping experiments were performed twice with independent sets of RNA with similar results.
in Kormanec & Farkasovsky (1993). 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).

**Disruption of the S. aureofaciens CCM 3239 aur1P gene cluster.** The plasmid pUP3 contained a 3344 bp TspI fragment in the Cia site of pBluescript II SK+ (Fig. 1a). A 1050 bp HindIII (blunt-ended)–SacI DNA fragment from pTSRI, containing the 5′ gene of Streptomyces aureus, was inserted between the Ncol (blunt-ended) and SacI sites of pUP3. A 1300 bp KpnI-Smal fragment of pAPHII1, containing the kanamycin-resistance gene of Tn5, was inserted between the KpnI and XhoI (blunt-ended) sites of the resulting plasmid pUP3G, to create pUP3H (Fig. 1a), which was finally used to transform *S. aureofaciens* CCM 3239 protoplasts to thiostrepton resistance, as described in Kormanec et al. (1993). Since pUP3H was unable to replicate in *S. aureofaciens* CCM 3239, thiostrepton-resistant transformants were expected to arise from homologous recombination between the *S. aureofaciens* insert in the plasmid and the corresponding region in the chromosome. The thiostrepton-resistant clones were further examined for kanamycin sensitivity that would indicate a double recombination event. Three kanamycin-resistant clones were further examined for kanamycin resistance, as described in Kormanec (1993). The plasmid pPMaur1P, used for complementation of the *S. aureofaciens* sensitivity that would indicate a double crossover event, was inserted between the *C. aureofaciens* gene of *Streptomyces azureus* (blunt-ended)–EcoRI fragment from pTSRI, containing the kanamycin-resistance gene of Tn5 (Maxam & Gilbert, 1980). The plasmid pJUP3 contained a 3344 bp KpnI–SmaI DNA fragment, as described by Ausubel et al. (1995). The 32P-labelled DNA fragment (0.8 ng, 1000 d.p.m.) was incubated with increasing amounts of purified Aur1P 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 DTG, 12% (v/v) glycerol], 2 μg sonicated salmon sperm DNA and 4.5 μg BSA. After incubation, protein-bound and free DNA were 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 X-ray film.

**DNase I footprinting.** Binding reactions were performed in 30 μl of binding buffer, essentially under the same conditions as for the gel mobility-shift assays with 2 ng of the 32P-labelled DNA fragment (8000 d.p.m.) and increasing amounts of purified Aur1P. After incubation for 15 min at 30 °C, 3 μl DNase I solution [5 U DNase I (Boehringer Mannheim) in 100 mM MgCl2, 100 mM DTG] 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 ml-1 t-RNA), and extracted with 30 μl of 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 Speed Vac 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.

**Protein analysis.** Protein concentrations were determined according to Bradford (1976) with BSA as standard. Denaturing SDS-PAGE of proteins was done as described by Laemmli (1970), and Protein analysis. Protein concentrations were determined according to Bradford (1976) with BSA as standard. Denaturing SDS-PAGE of proteins was done as described by Laemmli (1970), and gels were stained with Coomassie blue R250.

**RESULTS**

**Cloning of the S. aureofaciens CCM 3239 chromosomal fragment containing the putative hrdA-dependent promoter.**

In order to identify *S. aureofaciens* CCM 3239 promoters recognized by the principal sigma factor homologue HrdA (Kormanec et al., 1993), an *S. aureofaciens* CCM 3239 genomic library (Methods) was screened by the procedure previously established (Novakova et al., 1998) in *E. coli* containing the compatible plasmid pAC-hrdA1, having the *hrdA* gene under the control of the IPTG-inducible trc promoter. Sequence analysis of one of the identified positive clones (the plasmid pHrD5A5 carrying a 1046 bp Sau3AI DNA fragment; Fig. 1a) that was positive only in the background of the *hrdA*-encoded sigma factor (*E. coli* containing pAC-hrdA1) revealed a 3′-truncated gene encoding a protein with a high sequence similarity to response-regulator proteins of bacterial two-component signal transduction systems.

In order to identify a potential TSP for the putative
hrdA-dependent promoter in E. coli, high-resolution S1-nuclease mapping was performed using 5' labelled probe 1 and RNA isolated from E. coli containing the plasmids pHrDA5 and pAC-hrdA1 (Fig. 1a). A single RNA-protected fragment with a TSP at A, 35 bp upstream of the most likely translation initiation codon ATG of the putative response regulator gene, was identified (Fig. 1b, lane 2). This TSP was 4 bp downstream of the sequence TTGAC-N_{16-18}TATCCTT, which is similar to the consensus sequence (TTGACN-N_{16-18}TAGPuT) of the promoters recognized by the principal sigma factor HrdB of S. coelicolor (Brown et al., 1992). A much weaker RNA-protected fragment was identified with a control RNA from E. coli containing the plasmids pHrDA5 and pAC5mut2 (Fig. 1b, lane 1), which indicates partial recognition of the promoter by the E. coli RNA polymerase containing the principal sigma factor $^{70}$.

To clone the region downstream of the putative hrdA-dependent promoter, we used the chromosome-walking procedure. An S. aureofaciens CCM 3239 genomic library [TaqI partially digested chromosomal fragments (2-4 kb) cloned into the Ccl site of Bluescript II SK+] was hybridized with the 1050 bp EcoRI positive DNA fragment from pHrDA5. One positive clone, the plasmid pJUP3 (Fig. 1a) containing an overlapping 3-3 kb TaqI DNA fragment, was identified, and the fragment was sequenced on both strands. Interestingly, the sequence identified a complete gene encoding a putative response regulator which was immediately followed by the aur1O gene, previously identified at the beginning of the aur1 polyketide synthase gene cluster that is involved in the biosynthesis of an angucycline-like polyketide antibiotic, auricin (Novakova et al., 2002). Based on its role in auricin biosynthesis (see below), we named the response-regulator-like gene aur1R, and the putative hrdA-dependent promoter directing its expression as aur1Rp. Another divergently transcribed gene has been located upstream of the aur1P gene. This gene was named aur1R (Fig. 1a).

Characterization of the deduced protein products of aur1P and aur1R

Comparison of the deduced protein product of the aur1P gene with databases revealed significant end-to-end sequence similarity to response-regulator proteins of bacterial two-component signal transduction systems. Response regulators are characterized by an N-terminal response regulatory domain of the CheY family containing four highly conserved residues that are believed to compose the active site, with phosphorylation occurring at the second aspartate residue (amino acid 89 in Aur1P) (Stock et al., 2000). Almost all of these residues are conserved in Aur1P, except for the aspartic acid residue (amino acid 47) closest to the N-terminal end. This residue is conservatively replaced by a glutamate residue in Aur1P. However, the conservation of this aspartic residue does not seem to be so critical for the function of response regulators in streptomycetes (Hutchings et al., 2004). Moreover, all the conserved residues which make up the hydrophobic core in the response regulatory domain are also highly conserved in Aur1P (Fig. 2a).

Response regulators have been categorized into several subfamilies (OmpR, NarL and NtrC) on the basis of conservation of the C-terminal effector DNA-binding domain that generally controls the adaptive response (Stock et al., 2000). Aur1P is clearly a member of the OmpR subfamily, and shows significant sequence similarity in the whole region of the OmpR effector domain (Fig. 2a). The OmpR subfamily is characterized by a specific fold that contains several $\alpha$-helices and antiparallel $\beta$-sheets in the C-terminal region, leading to specific winged-helical structures. The two helices, $\alpha 2$ and $\alpha 3$, and a 10-residue loop between them (Fig. 2a), function in an analogous fashion to the canonical helix-turn-helix motif. Helix $\alpha 3$ and the loop connecting two C-terminal $\beta$-strands, $\beta 6$ and $\beta 7$, are DNA recognition sites, while the loop connecting helices $\alpha 2$ and $\alpha 3$ may interact with the $\alpha$-subunit of RNA polymerase in promoting the initiation of transcription. Amino acids that form part of $\alpha 1$ and $\alpha 2$, as well as hydrophobic core residues, are highly conserved. The most conserved residues form part of the recognition helix $\alpha 3$ and the recognition wing W1 (Martinez-Hackert & Stock, 1997). Aur1P contains all the conserved residues of the OmpR family signature motifs. Moreover, 18 of the 22 residues that constitute the hydrophobic core of the OmpR DNA-binding fold (Martinez-Hackert & Stock, 1997) are also highly conserved in Aur1P (Fig. 2a).

Interestingly, the proteins to which Aur1P exhibited the highest overall similarity were several recently characterized Streptomyces response-regulator-like proteins that act as positive regulators for angucycline-like biosynthesis gene clusters, including JadR1 from the Streptomyces venezuelae ISP5230 jadomycin B biosynthesis gene cluster (74 % identity) (Yang et al., 2001), and LndI and LanI from the Streptomyces globisporus 1912 and Streptomyces cyanogenus S136 landomycin E and landomycin A gene clusters, (66 and 62 % identity, respectively) (Rebets et al., 2003). Two further proposed Streptomyces antibiotic regulatory proteins have also been identified as being similar to Aur1P, and they both likely belong to the same family of proteins (Fig. 2a). One of them, SimReg1 (43 % identity), is a putative regulator from the simocyclinone cluster of Streptomyces antibioticus Tu 6040, which produces an angucycline class antibiotic (Trefzer et al., 2002), but interestingly, another homologue of this family, Med-ORF30 (56 % identity), has been found in the medermycin gene cluster of Streptomyces sp. AM-7161, which produces a benzoisochromanquinone class polyketide, not an angucycline (Ichinose et al., 2003). A comparison of the amino acid sequence of Aur1R encoded by the divergently transcribed gene with sequences in databases showed that the closest resemblance is to the potential repressor JadR2 of the S. venezuelae ISP5230 jadomycin B biosynthesis gene cluster (55 % identity) (Yang et al., 1995) and to a group of repressor proteins of the TetR family,
including γ-butyrolactone-binding repressor proteins from different Streptomyces species (Fig. 2b).

Transcriptional analysis of the aur1Pp promoter

In order to investigate the activity of the aur1Pp promoter in S. aureofaciens CCM 3239 and its proposed dependence upon hrdA, high-resolution S1-nuclease mapping was performed using probe 2 (Fig. 1a) and RNA isolated from S. aureofaciens CCM 3239 and its isogenic hrdA mutant, S. aureofaciens CCM 3239-A4 (Kormanec et al., 1993), during growth in liquid NMP medium with mannitol as the carbon source. As shown in Fig. 3a, a single RNA-protected fragment was identified that corresponded to the aur1Pp
promoter, with a TSP at A, which is at an identical position to that of the aur1P promoter in the E. coli two-plasmid system. This position is 35 nt upstream of the most likely translation initiation codon, ATG (Fig. 3b). No RNA-protected fragment was identified with tRNA as a control (Fig. 3a, lane C). The promoter was induced at the late exponential phase. However, when RNA from the same time points was prepared from the S. aureofaciens CCM 3239 hrdA mutant, RNA-protected fragments with similar time-course intensities were identified that corresponded to the aur1P promoter (Fig. 3a). These results indicated that this promoter is likely not dependent in vivo upon hrdA in S. aureofaciens CCM 3239. A possible explanation for this discrepancy may be that this putative hrdA-dependent promoter is recognized by the principal sigma factor HrdB or some of its homologues, such as HrdD and HrdE.

Disruption of the S. aureofaciens CCM 3239 aur1P gene

In order to investigate the function of the aur1P gene, a chromosomai copy of S. aureofaciens aur1P was inactivated by a double crossover using a method for disruption of S. aureofaciens CCM 3239 genes (Kormanec et al., 1993). The thiorstretan-resistance gene, tsr, was used to replace a 347 bp NcoI–SacI fragment, removing the 5’-coding region of the aur1P gene (Fig. 1a), and this construct was inserted into the chromosome of S. aureofaciens CCM 3239 by homologous recombination, resulting in the aur1P-disrupted strain S. aureofaciens CCM 3239, aur1P::tsr (Methods). The correct integration through a double crossover was confirmed by Southern blot hybridization (data not shown). The disruption did not affect growth and differentiation of the bacterium. S. aureofaciens CCM 3239, aur1P::tsr was investigated for production of auricin using the Gram-positive bacterium Bacillus subtilis. Ethyl acetate extracts from solid-grown wild-type and aur1P-disrupted strains were analysed by TLC followed by a bioassay against B. subtilis (Methods). The inhibition zones, including the spot for auricin, could be identified in the case of the wild-type S. aureofaciens CCM 3239 strain, as reported previously (Novakova et al., 2002). However, the extract from the aur1P-disrupted strain lacked the inhibition zone corresponding to auricin (Fig. 4). To verify that this phenotype was solely due to the deletion of aur1P, S. aureofaciens CCM 3239, aur1P::tsr was complemented in trans by transformation with the plasmid pPMaur1P, which contained the aur1P gene, including its promoter, cloned in the integrative plasmid pPM927 (Methods). As shown in Fig. 4, production of auricin was fully restored to the complemented strain, showing that the lack of auricin is indeed due to aur1P disruption. Thus, it indicated that the aur1P gene was essential for auricin production.

Transcriptional analysis of the aur1Ap promoter in the S. aureofaciens CCM 3239 aur1P mutant

We had previously characterized the aur1Ap promoter, which directs the first characterized gene of the auricin cluster, aur1A, which in turn encodes a putative oxygenase. Expression of the promoter is induced at the time of aerial mycelium formation (Novakova et al., 2002). To investigate whether the aur1P disruption has an effect on aur1P transcription, S1-nuclease mapping was performed using RNA isolated from S. aureofaciens CCM 3239 wild-type and aur1P-mutant strains during differentiation on solid Bennett medium. A single RNA-protected fragment corresponding to the aur1Ap promoter was identified using probe 3 with the RNA isolated from the wild-type strain; the time-course of expression was similar to that previously published (Fig. 5a). However, the level of aur1Ap mRNA from all developmental stages was dramatically decreased in the aur1P mutant (Fig. 5a). The results indicated that the aur1P mutation dramatically affected transcription from the aur1Ap promoter. Thus, the promoter is directly or indirectly dependent upon aur1P. 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 differentiation. RNA-protected fragments corresponding to the hrdBp2 promoter were identified with all RNA samples (Fig. 5b).
Binding of Aur1P to the *aur1Ap* promoter region

To check whether the dependence of the *aur1Ap* promoter upon *aur1P* is direct, the *aur1P* gene was overexpressed in the *E. coli* T7 RNA polymerase expression system (Methods), and the purified N-terminal His-tagged Aur1P was examined for its binding to the *aur1Ap* promoter region. Total protein extracts of *E. coli* transformed with the plasmid pET-aur1P and the cloning plasmid pET28a after induction with IPTG at 30 °C were examined by SDS-PAGE. A prominent band was clearly visible after induction with IPTG (Fig. 6a). This protein was partially present in the soluble fraction of the *E. coli* cell extracts, and was purified by native Ni²⁺-affinity chromatography, as described in Methods. The estimated molecular mass of the
purified His-tagged Aur1P on SDS-PAGE (33 kDa) corresponded closely to the theoretical $M_r$, 32 454, calculated by adding the $M_r$ of the His-tag fusion (2181) to the predicted $M_r$ from the Aur1P amino acid sequence (30 273). The purified protein was used in a gel mobility-shift assay with the 32P-labelled 200 bp aur1Ap promoter DNA fragment (positions -167 to +32 bp, in relation to the TSP of the aur1Ap promoter, Figs 1a and 6c). As shown in Fig. 6b, two retarded bands were clearly visible, which may correspond to two different complexes. The specificity of the interaction was demonstrated by the competitive binding of the unlabelled fragment (Fig. 6b, lane 5). These results indicate that Aur1P is capable of binding to the aur1Ap promoter region. To locate this Aur1P binding site in the aur1Ap promoter region, DNase I footprinting assays were carried out using the same 200 bp aur1Ap promoter fragment. As shown in Fig. 6c, the region from -134 to -46 bp upstream of the TSP was protected. The position of the binding site is indicated in Fig. 6d. The results of the binding studies indicated that the dependence of the aur1Ap promoter upon

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DISCUSSION

Owing to the exact timing of production and the potential toxicity to their producers, antibiotic biosynthesis in streptomycetes must be tightly controlled on several levels. Regulation at the lowest level is represented by the pathway-specific transcriptional activators which are genetically

Fig. 6. (a) Overproduction of Aur1P in E. coli. Samples were analysed by SDS-PAGE (12.5% acrylamide). Cell-free extracts were prepared from E. coli BL21(DE3) pLysS carrying the corresponding plasmid grown at 30 °C before induction (lane 2) and after 3 h induction with IPTG (lanes 1 and 3). Lane 1, E. coli containing pET28a; lanes 2 and 3, E. coli containing pET-aur1P; lane 4, purified His-tagged Aur1P protein after Ni2+-affinity chromatography. Lane S, molecular mass markers. (b) Gel-mobility shift-assays of 0.8 ng of a 32P-labelled 200 bp Smal–EcoRI fragment containing the aur1Ap promoter region (Fig. 6c) with increasing amounts of purified His-tagged Aur1P. Lane 1, labelled fragment in the absence of protein; lanes 2, 3 and 4, 180 ng, 1.8 μg and 3.6 μg, respectively, of the purified His-tagged Aur1P protein. Addition of 100 ng of the unlabelled 200 bp aur1Ap-promoter DNA fragment was used to demonstrate Aur1P binding specificity (lane 5). The arrows indicate the free DNA fragment and shifted fragments corresponding to the proposed complexes. (c) DNase I footprints of Aur1P binding to the 5' end-labelled 200 bp aur1Ap promoter DNA fragment (2 ng). The vertical bar indicates the position of the Aur1P binding site. The numbering is relative to the TSP of the aur1Ap promoter (Fig. 6c). Lane 1 is without the His-tagged Aur1P protein sample. Lanes 2 and 3 contain 5.6 and 16.8 μg, respectively, of the purified His-tagged Aur1P 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. (d) The nucleotide sequence of the S. aureofaciens CCM 3239 aur1Ap promoter region. The deduced protein products are given in the single-letter amino-acid code in the second position of each codon. The TSP of the aur1Ap promoter is indicated by a bent arrow. The −10 and −35 boxes of the promoter are in bold type and underlined. The nucleotides that were protected from DNase I by Aur1P binding are shaded. Arrows denote the positions of tandem repeat sequences in the protected region. Relevant restriction sites are indicated. The sequence numbers refer to the deposited nucleotide sequence in the GenBank/EMBL/DDBJ databases under accession number AY956334.
linked to biosynthetic gene clusters and activate transcription of the corresponding antibiotic biosynthesis genes. The genes encoding these pathway-specific regulators have been identified in many antibiotic gene clusters, and they appear to constitute several families of homologous transcriptional regulators. Most of them belong to the growing family of *Streptomyces* antibiotic regulatory proteins (SARPs) that are characterized by the N-terminal-located DNA binding domain of the OmpR family of winged-helix transcription factors (Wietzorreck & Bibb, 1997). In several cases, the activity of the pathway-specific genes has been shown in turn to be regulated by genes at a higher level in the regulatory hierarchy, including global regulatory genes mediating environmental, nutritional and growth-rate effects. Several of these global regulators that govern expression of the *Streptomyces* pathway-specific antibiotic regulators have been shown to belong to the widespread response-regulator family of bacterial two-component signal transduction systems (Hutchings et al., 2004; Stock et al., 2000). These homologues of response regulators include DnrN, which directly governs expression of the SARP gene *aur1* from the *Streptomyces puercetus* daunorubicin gene cluster (Furuya & Hutchinson, 1996), and RedZ, which affects the expression of the SARP gene from the *S. coelicolor* A3(2) undecylprodigiosin gene cluster, *redD* (Guthrie et al., 1998). Interestingly, these genes are not typically linked with genes encoding the corresponding sensor histidine kinases. Sequence analysis of their proposed C-terminal DNA-binding effector domains indicates that they both belong to the NarL subfamily of response regulators, which contain a fold with the typical helix–turn–helix motif (Stock et al., 2000). The NarL subfamily also includes the response regulator AbsA2, which, together with its sensor histidine kinase, AbsA1, has been shown to repress antibiotic biosynthetic genes in *S. coelicolor* A3(2) (Brian et al., 1996).

In the present paper, we have described a gene, *aur1P*, encoding a homologue of response regulators that, in contrast to the examples described above, directly and positively affected the expression of biosynthetic genes for the angucycline-like polyketide antibiotic auricin in *S. aureofaciens* CCM 3239, thus clearly representing the pathway-specific transcriptional activator for the *aur1* antibiotic gene cluster. This conclusion is supported by the following experimental data: (i) the *S. aureofaciens* CCM 3239, *aur1P::tsr* mutant did not produce auricin, and its production was restored by a copy of the *aur1P* gene including its promoter in trans in this mutant; (ii) transcription of the *aur1Ap* promoter, directing expression of the first gene, *aur1A*, encoding a putative oxygenase in the *aur1* cluster, was dramatically decreased in the *aur1P* mutant; (iii) this dependence was shown to be direct by confirmation of binding of Aur1P to the *aur1Ap* promoter by *in vitro* DNA-binding assays. Thus, this study revealed that the new transcriptional regulator Aur1P is essential for production of auricin and directly controls the activity of the *aur1Ap* promoter that governs transcription of the first gene *aur1A* of the auricin polyketide synthase gene cluster.

While the gene was identified using our previously established *E. coli* two-plasmid system (Novakova et al., 1998) as being dependent upon a homologue of the principal sigma factor gene, *hrdA* (Kormanec et al., 1993), and the position of the putative *hrdA*-dependent promoter, *aur1Pp*, located in the *E. coli* system was identical in *S. aureofaciens* CCM 3239, the transcriptional analysis in *S. aureofaciens* CCM 3239 wild-type and the *hrdA* mutant did not confirm this dependence. It is likely that the *aur1Pp* promoter is recognized by the principal sigma factor HrDB or one of its homologues, such as HrdD or HrDE. All Hrd sigma factors are highly similar in the regions 2-4 and 4-2 which are responsible for interaction with cognate promoters (Kormanec et al., 1992). Thus, they can all recognize very similar promoters. In fact, using our *E. coli* two-plasmid system (Novakova et al., 1998) with other *hrd* homologues, we have found that the putative *hrdA*-dependent promoter *aur1Pp* is also active with the other homologues, HrDB and HrDE, indicating some cross-recognition of the *aur1Pp* promoter with additional Hrd sigma factors. However, similar S1-mapping experiments in the *S. aureofaciens* *hrdD* mutant revealed that expression of the *aur1Pp* promoter is not affected in this mutant (J. Kormanec, unpublished results). The *aur1Pp* promoter has been found to be induced at the beginning of stationary phase, which is typical for expression of pathway-specific transcriptional activators (Chater & Bibb, 1997). Considering this time-course expression of *aur1Pp*, it is unlikely that *aur1Pp* could be controlled by HrDA or HrDE, as the *hrdE* gene is not expressed under these conditions and the *hrdA* gene is expressed later during sporulation (Kormanec & Farkasovsky, 1993). As only *hrdB* and *hrdB* are expressed in substrate mycelium (Kormanec & Farkasovsky, 1993), it is likely that the *aur1Pp* promoter is recognized by both HrDB and HrDE in *S. aureofaciens* CCM 3239.

The Aur1P protein was related to several recently characterized *Streptomyces* response-regulator-like proteins that act as pathway-specific transcriptional activators in several, mainly angucycline, biosynthetic gene clusters (Fig. 2a). They are all distinct from the members of the SARP family in that they belong to the family of response regulators of bacterial two-component signal transduction systems and contain a winged-helix DNA-binding domain of the OmpR family in their C-terminal region, while a similar DNA-binding domain is found in the N-terminal region in the SARP family (Wietzorreck & Bibb, 1997). Moreover, all these proteins are dissimilar to two previously characterized *Streptomyces* response regulators of the NarL family, DnrN (Furuya & Hutchinson, 1996) and RedZ (Guthrie et al., 1998), which have been shown to govern the expression of SARP genes (13 and 15 % identity to Aur1P, respectively). Thus, it seems that this specific group of mainly angucycline-related transcriptional regulators constitutes a new, separate branch of the SARP family. Interestingly, as for *redZ* and *dnn*, all the response-regulator-like genes of this family are also not typically linked with genes encoding the corresponding sensor histidine kinases.
In addition to Aur1P, only three members of this emerging family have been characterized in more detail and shown to be essential for antibiotic production. The first characterized, JadR1, from the S. venezuelae ISP5230 jadomycin B biosynthesis gene cluster, has been proved to be essential for biosynthesis of this antibiotic. Together with a deduced protein product, JadR2, encoded by a divergently expressed gene, this JadR1/JadR2 regulatory pair has been suggested to represent a novel two-component system linking antibiotic synthesis to stress. It has been suggested that jadR1 is not expressed under unstressed conditions, and that this absence is due to repression exerted by a proposed repressor, JadR2. However, this conclusion has not been corroborated by any transcriptional analysis (Yang et al., 2001). Interestingly, a gene highly homologous to jadR2 and similarly organized was identified upstream of aur1P (Figs 1a and 2b). Its product, Aur1R, may have a similar function to that of its homologue in the jadomycin B gene cluster. However, we could not detect any increase of auricin production after the application of different stress conditions (I. Kormanec, unpublished results). Therefore, further studies will be needed to investigate their potential stress dependence.

Two other members of this family have been identified in two landomycin gene clusters: Lndl and Lanl from S. globisporus 1912 and the S. cyanogenus S136 landomycin E and landomycin A gene clusters. Both have been shown to be essential for biosynthesis of the corresponding antibiotic and have been shown to be interchangeable (Rebets et al., 2003). In the course of writing our paper, Lndl was confirmed to be a DNA-binding protein. By gel mobility-shift assay, Lndl was shown to bind to its own promoter and to the promoter located upstream of the oxygenase gene lndEp (the homologue of aur1A in the auricin cluster: its position in the cluster is also similar). Using the EGFP reporter system, transcription of the lndEp promoter was similarly induced later in growth and spatially in the substrate mycelium (Rebets et al., 2005). However, comparison of the lndEp and aur1Ap promoters has not revealed any significant similarity (data not shown).

The in vitro binding experiments clearly indicated that Aur1P binds directly to the aur1Ap promoter region, upstream of the proposed −35 region of the promoter (Fig. 6c). This type of binding is typical for transcriptional activators. Interestingly, gel-retardation analysis of the aur1Ap promoter fragment with increasing concentrations of Aur1P resulted in two complexes (Fig. 6b). These results indicate that Aur1P may bind to two independent binding motifs present in the aur1Ap promoter region. Thus, at low concentration, Aur1P may randomly recognize and bind to one binding motif, and at saturated concentration, Aur1P may bind to two motifs. The members of the OmpR family for which DNA recognition sites have been determined appear to bind to direct repeat DNA sequences. However, there is variation in the arrangement of sites, both with respect to the number of recognition sites and the spacing between them (Martinez-Hackert & Stock, 1997). Inspection of the DNase I-protected region in the aur1Ap promoter has not revealed any similarity to the previously published binding motifs of several members of OmpR family, including Streptomyces SAR (data not shown). However, sequence analysis of this protected region has revealed a tandem repeat sequence TCCTTTG separated by a 24 bp spacer region. Moreover, both motifs were accompanied by partially similar sequence regions (CCTTG and CCT) in their vicinity (Fig. 6c). Thus, these regions might serve as binding sites for Aur1P. However, further experiments are needed to prove this hypothesis.

In conclusion, we have characterized a gene, aur1P, in the polyketide gene cluster aur1 for the angucycline-like antibiotic auricin of S. aureofaciens CCM 3239, which is essential for biosynthesis of this antibiotic. Its deduced protein product, Aur1P, strongly resembles members of the OmpR subfamily of response regulators of bacterial two-component signal transduction systems. Transcription of aur1P is induced at the onset of stationary phase. Aur1P is essential for expression of the first promoter, aur1Ap, in the aur1 gene cluster governing expression of the oxygenase gene aur1A, and it specifically binds to the promoter. DNase I footprinting analysis indicates an Aur1P-binding region from −134 to −46 bp upstream of the TSP of aur1Ap. The results indicate that Aur1P is a pathway-specific transcriptional activator for the auricin gene cluster in S. aureofaciens CCM 3239.

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