Transcriptional regulation of the *macs1-fadD1* operon encoding two acyl-CoA synthases involved in the physiological differentiation of *Streptomyces coelicolor*

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The long-chain acyl-CoA synthase (ACS) FadD1 plays an important role in timing the levels of antibiotic production in *Streptomyces coelicolor*. *fadD1* and *macs1*, encoding a putative medium-chain ACS, are part of a two-gene operon, whose expression is induced during the stationary phase of growth. Here it is reported that transcription of the *macs1-fadD1* operon is positively regulated by AcsR, a LuxR-type transcriptional regulator. In an *acsR* mutant, expression of the *macs1-fadD1* genes loses its normal up-regulation and the mutant becomes deficient in antibiotic production, in a clear correlation with the phenotype shown by a *fadD1* null mutant. The absence of *macs1-fadD1* induction in the *acsR* mutant was restored by complementation with a wild-type copy of the *acsR* gene, showing a strict link between AcsR and induction of the *macs1-fadD1* operon. Gel mobility shift assays and DNase I footprinting indicated that AcsR binds to specific sequences about +162 nucleotides downstream of the *macs1* transcriptional start site. In the putative operator sequence three almost identical direct tandem repeats of seven nucleotides were identified where the central sequence is essential for AcsR recognition and binding. Transcriptional fusions of the divergent *pacsR* and *pmacs1* promoters indicated that AcsR does not regulate its own transcription, and that it binds to the operator region to control exclusively the growth-phase induction of the *macs1-fadD1* operon.

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INTRODUCTION

Fatty acids serve a number of essential and regulatory functions. Many cells, although capable of *de novo* synthesis, scavenge fatty acids from the extracellular environment, thereby bypassing energy-expensive synthetic reactions. In *Streptomyces coelicolor*, fatty acids with different chain lengths (C4–C18) are efficiently degraded through the β -oxidation cycle and the enzymes responsible for this catabolic pathway appear to be constitutively synthesized (Banchio & Gramajo, 1997), in sharp contrast to *Escherichia coli* whose β -oxidation enzymes are induced by long-chain fatty acids (Black & Dirusso, 1994; Overath *et al.*, 1969). The first step in any further utilization of the fatty acids is biosynthesis of their CoA derivatives. This step is catalysed by acyl-CoA synthases (ACSs), which produce fatty acyl-CoAs from fatty acids, ATP and CoA (Watkins, 1997). Fatty

acyl-CoAs are bioactive compounds involved in protein transport, enzyme activation or deactivation, protein acylation, cell signalling and transcriptional control, in addition to serving as substrates for β -oxidation and triacylglyceride (TAG) and phospholipid biosynthesis (Brofman *et al.*, 2005; Dirusso *et al.*, 1992; Gordon, 1990; Korchak *et al.*, 1994; Pfanner *et al.*, 1989). Given the multiple roles of fatty acyl-CoAs, it is clear that fatty ACS occupy a pivotal role in cellular homeostasis, particularly in lipid metabolism.

Interestingly, in several *Streptomyces* species, including *S. coelicolor*, drops of TAG have been found in the cytoplasm as storage compounds (Olukoshi & Packter, 1994; Packter & Olukoshi, 1995). Degradation of these neutral lipids by endogenous lipases is an internal source of fatty acids. It has been proposed that β -oxidation of the free fatty acids to acetyl-CoA could be the source of carbon units for the biosynthesis of many polyketide compounds (Olukoshi & Packter, 1994). Consequently, the ACS activity involved in activation of the TAG-derived fatty acids to their CoA thioesters would play a key role in antibiotic production.

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Abbreviations: ACS, acyl-CoA synthase; Act, actinorhodin; Am, apramycin; Km, kanamycin; Th, thiostrepton; TSS, transcriptional start site; wt, wild-type.

E. coli contains only one ACS (FadD) with a broad substrate specificity (Kameda & Nunn, 1981). At the other extreme, the actinomycete *Mycobacterium tuberculosis* contains 36 genes annotated as putative ACSs (Cole *et al.*, 1998). In the *S. coelicolor* genome database (http://www.sanger.ac.uk/Projects/S_coelicolor/) at least 15 ORFs have been annotated as putative fatty acid-CoA synthases, but so far only one of the gene products, FadD1, has been characterized (Banchio & Gramajo, 2002).

Our previous studies on the ACS FadD1 of *S. coelicolor* demonstrated that this enzyme has broad substrate specificity, although saturated long-chain fatty acids appear to be preferred. We also determined that *fadD1* was part of a two-gene operon together with *macs1*, which also encodes a putative ACS with closest homology to medium-chain ACSs, broadly known as SA proteins (Fujino *et al.*, 2001). A mutation in *fadD1* appeared to alter the levels of other ACS(s), suggesting a possible regulatory role for the acyl-CoAs synthesized by this enzyme (Banchio & Gramajo, 2002). An interesting correlation with this hypothesis was the observation that the *fadD* mutant had a severe deficiency in antibiotic production. Actinorhodin (Act) biosynthesis in this mutant was remarkably reduced compared with the wild-type (wt) strain, independently of the growth media used. This deficiency was related to delayed expression of the Act biosynthetic genes and most probably due to the lack of induction of the pathway-specific activator *actII*-ORF4 (Banchio & Gramajo, 2002). Our previous studies, based on RT-PCR and Northern blotting, indicated that expression of the *macs1-fadD1* genes was regulated at the transcriptional level in a growth-phase-dependent manner, in a tight correlation with the physiological differentiation of this organism at the late stages of growth (Banchio & Gramajo, 2002).

Here we investigated the components and possible mechanisms involved in transcriptional regulation of *macs1-fadD1*. Gene expression assays and *in vitro* DNA-binding studies led to the identification and characterization of a transcriptional activator of this operon, AcsR, whose main role is to control the growth-phase-dependent induction of the *macs1-fadD1* operon by binding to a very unusual operator.

METHODS

Strains, media and growth conditions. *E. coli* DH5 α (Hanahan, 1983) and BL21(DE3) (Studier & Moffatt, 1986) were used as hosts for cloning experiments. They were grown either on solid or in liquid Luria-Bertani medium at 37 °C. The following antibiotics were used to select recombinant *E. coli* strains: 100 μ g ampicillin ml⁻¹, 50 μ g kanamycin (Km) ml⁻¹, 20 μ g chloramphenicol ml⁻¹ or 100 μ g apramycin (Am) ml⁻¹.

S. coelicolor M145 (Kieser *et al.*, 2000) was used as a wt recipient for construction of the isogenic mutants. M145 or derivatives were grown at 30 °C on soya flour mannitol (MS) agar (Kieser *et al.*, 2000) or in SMM medium (Takano *et al.*, 1992) supplemented with oleate (0.1 % w/v) (SMM-oleate) or glucose (1 % w/v) (SMM-glucose) as carbon

source. The antibiotics Am, thiostrepton (Th) and Km were added at final concentrations of 10, 50 and 200 μ g ml⁻¹ to solid medium, and at 10, 5 and 25 μ g ml⁻¹ to liquid medium, respectively.

Strains and plasmids constructed and used in this study are listed in Table 1.

DNA manipulation and transformation procedures. Plasmid DNA was extracted from *E. coli* using a Wizard kit (Promega). DNA fragments were purified from agarose gels with a GFX kit (Amersham). Restriction enzymes were used as recommended by the manufacturers. DNA fragments were amplified by PCR (Mullis & Faloona, 1987; Saiki *et al.*, 1988). Competent *E. coli* cells were prepared and transformed according to standard methods described by Sambrook *et al.* (1989). *Streptomyces* DNA and protoplasts were prepared and transformed as described by Kieser *et al.* (2000).

RNA extraction. A volume of 30–50 ml *S. coelicolor* M145 wt or *S. coelicolor* CBR4 culture was pelleted, and the mycelium was washed, resuspended in 0.3 ml cold TE buffer and added to 0.5 g glass beads (212–300 μ m; Sigma), 0.1 ml Macaloid (2 %, w/v), 0.05 ml SDS (10 %, w/v) and 0.25 ml Tris-EDTA saturated phenol/chloroform (1:1, v/v). The hyphae were disrupted by sonication with a Vibracell ultrasonic processor (VCX600 sonicator; Sonics & Materials) for 10 s at 4 °C. After centrifugation for 15 min at 4 °C and 15 000 g, supernatants were collected and treated with phenol/chloroform (1:1, v/v). RNA was precipitated at –20 °C with 1/10 volume of 3 M sodium acetate (pH 7) and 3 volumes of absolute ethanol, rinsed with cold 70 % (v/v) ethanol and resuspended in water. RNA concentration was determined by measuring absorbance at 260 nm.

Primer extension analysis. Primer extension was used to determine the 5' end of mRNA transcripts. One microlitre primer Pro2 (5'-GGTGTAGTCCTCGCGGTGTTCCAG-3') was annealed to 20 μ g heat-denatured RNA from cultures grown to stationary or exponential phase. The annealed primer/RNA solution was then dissolved in 20 μ l reverse transcription mix [50 mM Tris/HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, [α -³²P]dATP (3000 Ci mmol⁻¹ = 1.1 $\times$ 10⁵ GBq mmol⁻¹) and 1.3 mM each of the dNTPs, 20 U RNasin inhibitor, 10 U AMV reverse transcriptase] and incubated at 37 °C for 2 h. cDNA synthesis was stopped and the reaction mixture was extracted with phenol/chloroform, ethanol-precipitated and redissolved in 4 μ l TE buffer. Sequencing stop solution (6 μ l) was added and the labelled cDNA was run on a 6 % acrylamide-urea sequencing gel alongside a reference sequencing ladder generated from M13 phage DNA.

Slot blot hybridization. Analysis of *macs1-fadD1* and *accB* expression was carried out by slot blot assay. Before blotting, 20 μ g each RNA sample was treated with formamide (50 %, v/v), formaldehyde (6.5 %, v/v) and SSC (1 $\times$) for 15 min at 68 °C. RNAs were then filtered through nitrocellulose filters using a slot blotter (Bio-Rad). After 2 h at 80 °C each membrane with immobilized RNA was hybridized with the corresponding probe generated by PCR.

The PCR product employed as *macs1-fadD1* probe was generated with the oligonucleotide pair, RTup (5'-AGCCCGACCGGACACCGCG-AAGGTGCT-3') and RTdn (5'-GGTGTGCGCCGAGCAGCGGGGTG-GT-3'). As a control, we followed the expression of *accB*, the gene that encodes the essential carboxyltransferase subunit of the acetyl-CoA carboxylase of *S. coelicolor* (Rodríguez *et al.*, 2001). The PCR product employed as *accB* probe was generated with the oligonucleotide pair accBEup (5'-CGAGGCCATGCGGGCGCGCATGGTCAAGGAG-3') and accBEdn (5'-GGAGGGCCGTGATGGCGCGACITCTCTCGGG-3').

The PCR products were checked on agarose gels, purified using a GFX kit (Amersham) and labelled with [α -³²P]dATP using the

Prime-a-Gene labelling system (Promega). Probe purification, denaturation, hybridization and washes were carried out as described by Sambrook *et al.* (1989).

Luciferase activity tests. *pmacs1* activity was assessed using pCB648, a derivative of the integrative *luxAB*-based reporter plasmid pIJ5971 (M. S. B. Paget, personal communication) (Table 1, Fig. 2a). pCB226 containing *luxAB* under the control of a 226 bp segment of the *acsR-macs1* intergenic region was also assessed (Table 1, Fig. 2a). Plasmids were introduced into *S. coelicolor* by conjugation from *E. coli* ET 12567/pUZ8002 (Bierman *et al.*, 1992; Paget *et al.*, 1999). Plasmid-containing strains were grown in 50 ml SMM-glucose or SMM-oleate, and culture samples were harvested at different time points. Luciferase activity was determined by adding 100 µl 1% (v/v) *n*-decylaldehyde in 9% (v/v) ethanol to 100 µl culture, and measuring light production over 30 s using a scintillation counter. The value for each sample corresponds to the mean of three different measurements; light production was standardized to protein concentration.

For the construction of plasmids pCB648* and pCB846*, a 648 bp DNA fragment was cloned in pIJ5971 in both orientations. These DNA fragments correspond to the *acsR-macs1* intergenic region (+337 to -311) where the central heptameric sequence CGTTCCG remained wt (pCB648 and pCB846) or was replaced by the CTCTAGA sequence (pCB648* and pCB846*), which includes a *XbaI* restriction site (underlined). The mutation was introduced by a PCR-based method with two rounds of PCR using specific oligonucleotides containing the appropriate mutations (Dis3, 5'-TGTGCGTCTCTAGAGATCACGAC-3'; Dis4, 5'-CCTCGTGATCTCTAGAGGACGAC-3'), and the Froup (5'-GTCCGCCTCGTCCGCGACACAGCCT-3') and Pro2 (5'-GGTGTAGTCTCGCGGTGTCCAGC-3') external primers (Ho *et al.*, 1989).

The *phrB-luxAB* transcriptional fusion harboured in pIJ5985 (Aigle *et al.*, 2000) was introduced into M145 by conjugation from *E. coli* ET 12567/pUZ8002, yielding the strain MCB500.

Assay of catechol 2,3-dioxygenase in cell-free extracts. *macs1-fadD1* and *acsR* promoter activities were assessed using pXE648 and pXE292 derivatives of the low copy number SCP2-based vector pXE4 (Gonzalez-Ceron *et al.*, 2001). pXE648 contains the promoterless *xylTE* genes under control of the *macs1-fadD1* promoter. pXE292 contains the promoterless *xylTE* genes under control of the *acsR* promoter (Table 1).

As a control of the *xylTE* reporter system we analysed the expression profile of the *paccB-xylTE* transcriptional fusion contained in pXE559. For the construction of this fusion the *accB* promoter sequence was amplified by PCR using the oligonucleotides AccBup (5'-CGAAGCTTCTACGCTCCGGGTGAGCGAA-3') and AccBdn (5'-CGGGATCCCGTCAGCTTGCCCTTGGCGTG-3'), and cloned in front of the *xylTE* genes on pXE4. Plasmids containing the three different fusions were independently introduced into *E. coli* GM33 (Marinus & Morris, 1974), and then purified and transformed into *S. coelicolor*. Transformants were grown in 50 ml SMM-glucose or SMM-oleate, and samples of the cultures were harvested at different time points. The catechol 2,3-dioxygenase activity was measured as described by Kieser *et al.* (2000). The mycelium was resuspended in buffer (100 mM phosphate pH 7.5, 20 mM EDTA pH 8.0, 10% v/v acetone) and disrupted by sonication. Triton X-100 was added to a final concentration of 0.1% (v/v) and centrifuged for 5 min. To 0.5 ml preincubated (30 °C) assay buffer (10 mM phosphate buffer pH 7.5, 0.2 mM catechol) a defined volume of the supernatant sample was added and the change in A_{375} was followed. Using the slope of the linear part of the spectrophotometric output the specific activity was

calculated as described by Kieser *et al.* (2000). Protein concentration was determined with Bradford reagent (Bradford, 1976). The catechol 2,3-dioxygenase activities were standardized to protein concentration.

Spectrophotometric analysis of Act. One millilitre whole broth was added to KOH to give a final concentration of 1 M; the solution was mixed vigorously and centrifuged at 4000 g for 5 min. The A_{640} of the supernatant was determined and the Act concentration was calculated using the molar absorption coefficient at 640 nm of 25 320 (Bystrykh *et al.*, 1996).

Expression and purification of AcsR in *E. coli*. pETR containing the translational fusion *his6-acSR* under control of the T7 promoter was introduced into *E. coli* BL21(DE3) carrying pTF16, which contains the *tig* gene, encoding a chaperone that helps to prevent aggregate formation (Nishihara *et al.*, 2000; Thomas *et al.*, 1997). For construction of pETR, pET-28a (Novagen), harbouring a Km resistance gene as a selection marker, was digested with *NdeI*/*HindIII* and used as a backbone. The *acsR* gene was amplified by PCR using the 2G5 cosmid (Redenbach *et al.*, 1996) as a template with Regup (5'-GGACATATGGCGGTGGACGCGGTGGAC-3') and Regdn (5'-GCGGGAATTTCGAGCAGGCGCTGGTGCTG-3') primers. The forward and reverse primers contain *NdeI* and *HindIII* sites (underlined), respectively. The PCR-amplified fragment was cloned in pGEM-T easy (Promega) and the resulting plasmid named pCB001. pCB001 was digested with *NdeI*/*HindIII* and the *acsR*-containing fragment was cloned into *NdeI*/*HindIII*-digested pET28a to generate pETR.

The resulting strain carrying pETR and pTF16 was grown at 30 °C in Luria-Bertani medium containing 25 µg Km ml⁻¹, 50 µg chloramphenicol ml⁻¹ and 0.1 mg arabinose ml⁻¹, until the culture reached OD₆₀₀ 0.6; IPTG was then added to a final concentration of 1 mM. Incubation was continued for 6 h at 22 °C. The cells were centrifuged for 10 min at 10 000 g and the pellet resuspended in 1/50 of the culture volume of buffer 1 (0.1 M NaH₂PO₄, 0.01 M Tris/HCl pH 8.0, 8 M urea). The mycelium was disrupted by stirring for 60 min and the cell debris was pelleted by centrifugation for 20 min at 10 000 g. The cell-free extract was loaded on a 100 µl nickel-nitrilotriacetic acid agarose (Ni-NTA; Qiagen) column previously equilibrated with buffer 1. The column was washed with buffer 2 (0.1 M NaH₂PO₄, 0.01 M Tris/HCl, 8 M urea, pH 6.3), and the AcsR protein was eluted with buffers at two pH values (0.1 M NaH₂PO₄, 0.01 M Tris/HCl, 8 M urea, pH 5.9 and pH 4.5) and analysed by SDS-PAGE with a 12.5% acrylamide gel. The eluted product was refolded while immobilized on Ni-NTA matrix using a linear 6–1 M urea gradient in 500 mM NaCl, 20% (v/v) glycerol, 20 mM Tris/HCl pH 7.5, containing protease inhibitors (Sigma). After renaturation, N-His₆-AcsR (H-AcsR) protein was eluted by adding 250 mM imidazole. Finally the purified protein was dialysed overnight at 4 °C in 20% (v/v) glycerol, 50 mM NaHPO₄ (pH 7.5), 300 mM NaCl, 1 mM EDTA.

Construction and complementation of *acsR* insertion mutants. An *acsR* mutant allele was constructed in which the *acsR* gene was interrupted by an Am-resistance cassette (Blondelet-Rouault *et al.*, 1997). For this, a 1.7 kb *EcoRI*-*HindIII* fragment containing the complete ORF was obtained from plasmid pCB001 and subcloned into digested pSET151 (Hillemann *et al.*, 1991), yielding pCB002. This plasmid was digested with *NotI*, filled to give blunt ends and ligated with *SmaI*-digested Am cassette. The final construct, named pCB003, was introduced by transformation into *E. coli* ET12567/pUZ8002 and then transferred into *S. coelicolor* by conjugation. Single-crossover exconjugants were selected on MS medium containing Th. Three such colonies were taken through three rounds of non-selective growth on MS medium, and spores were plated for single colonies scored for Th sensitivity. Deletions within *acsR* were confirmed by Southern blot hybridization. This mutant was named CBR4.

Table 1. Strains and plasmids

Strain or plasmid	Description	Reference
Strains		
<i>S. coelicolor</i>		
M145	Parental strain SCP1 [−] SCP2 [−]	Kieser <i>et al.</i> (2000)
MCB648	<i>S. coelicolor</i> M145 with pCB648, Am ^r	This work
MBC226	<i>S. coelicolor</i> M145 with pCB226, Am ^r	This work
MCB846	<i>S. coelicolor</i> M145 with pCB846, Am ^r	This work
CBR4	Mutant of <i>S. coelicolor</i> M145 with insertional inactivation of <i>acsR</i> , Am ^r	This work
MCB648*	<i>S. coelicolor</i> M145 with pCB648*, Am ^r	This work
MCB846*	<i>S. coelicolor</i> M145 with pCB846*, Am ^r	This work
MXE648	<i>S. coelicolor</i> M145 with pXE648, Th ^r	This work
MXE292	<i>S. coelicolor</i> M145 with pXE292, Th ^r	This work
CBR244	Mutant of <i>S. coelicolor</i> M145 with insertional inactivation of <i>acsR</i> , Am ^r , carrying the pXE4648 plasmid, Th ^r , and pCB244 ectopic integrative derivative, Km ^r	This work
MCB500	<i>S. coelicolor</i> M145 with pIJ5985, Am ^r	This work
MCB40	Mutant of <i>S. coelicolor</i> M145 with an in-frame deletion of <i>fadD1</i>	Banchio & Gramajo (2002)
<i>E. coli</i>		
DH5 α	<i>lacU169</i> (f80 <i>lacZ</i> Δ <i>M15</i>) <i>endA1 recA1 hsdR17 deoR supE44 thi-1 l2 gyrA96 relA1</i>	Hanahan (1983)
BL21(DE3)	BL21(DE3) <i>ompT</i> (DE3)	Studier & Moffatt (1986)
ET 12567	<i>supE44 hsdS20 ara-14 proA2 lacY galK2 rpsL20 xyl-5 mtl-1 Ddam Ddcm DhdsM Cm^r</i>	Paget <i>et al.</i> (1999)
GM33	<i>E. coli</i> K-12 <i>dam-3 sup-85</i>	Marinus & Morris (1974)
Vectors		
pBluescript SK(1)	Phagemid vector (Ap ^r <i>lacZ</i> 9)	Stratagene
PCR-Blunt	Used for cloning PCR products	Invitrogen
pGEM-T Easy	Used for cloning PCR products	Promega
pSET151	Used for the conjugal transfer of DNA from <i>E. coli</i> to <i>Streptomyces</i> spp. (Ap ^r Th ^r <i>lacZ</i> 9)	Hillemann <i>et al.</i> (1991)
pET28a	Phagemid vector for expression of recombinant proteins under control of strong T7 transcription and translation signals (Km ^r)	Novagen
pXE4	Promoter probe SCP2 derivative carrying 2 kb <i>xylE</i> region (Th ^r)	Gonzalez-Ceron <i>et al.</i> (2001)
pSET152	<i>Streptomyces</i> spp. integrating vector (Am ^r <i>lacZ</i> <i>oriT</i> _{RK2})	Bierman <i>et al.</i> (1992)
pIJ5971	Promoter probe vector pSET152 derivative carrying <i>luxAB</i> genes (Am ^r)	Kieser <i>et al.</i> (2000)
pUZ8002	RK2 derivative with defective <i>oriT</i> (Km ^r)	Paget <i>et al.</i> (1999)
pKOS244-11	pSAM2-derived integrating vector (Km ^r)	Rodriguez <i>et al.</i> (2004)
pTF16	pAR3 derivative containing the TF coding region (<i>tig</i> gene) (Cm ^r)	Nishihara <i>et al.</i> (2000)
pIJ5985	pIJ5971 derivative containing the <i>hrdB</i> promoter fragment	Aigle <i>et al.</i> (2000)
Recombinant plasmids		
pETR	pET28a with <i>acsR</i> under the control of strong T7 transcription and translation signals	This work
pCB226	pIJ5971 derivative vector containing 226 bp fragment of <i>macs1-acsR</i> intergenic region	This work
pCB648	pIJ5971 derivative vector containing 648 bp fragment of <i>macs1-acsR</i> intergenic region	This work
pCB846	pIJ5971 derivative vector containing the same 648 bp insert as pCB648 of <i>acsR- macs1</i> intergenic region but cloned in the opposite orientation	This work
pCB648*	pIJ5971 derivative vector containing 648 bp mutant fragment of <i>macs1-acsR</i> intergenic region	This work
pCB846*	pIJ5971 derivative vector containing the same 648 bp mutant insert as pCB648* but cloned in the opposite orientation	This work
pXE648	pXE4 derivative vector containing 648 bp fragment of <i>macs1-acsR</i> intergenic region	This work

Table 1. cont.

Strain or plasmid	Description	Reference
pXE292	pXE4 derivative vector containing 292 bp fragment of <i>macs1-acsR</i> intergenic region	This work
pXE559	pXE4 derivative vector containing 559 bp fragment of <i>accB</i> promoter region	This work
pCB244	pTR244 derivative vector containing 1265 bp fragment of <i>acsR</i> gene coding region	This work
pCB003	pSET151 with <i>aa(3)IV</i> (Am ^r) gene inserted in the <i>acsR</i> coding region	This work
pCB001	pGEM-T Easy derivative vector containing the <i>acsR</i> coding sequence	This work
pCB002	pSET151 derivative vector containing the <i>acsR</i> coding sequence	This work

Vector pKOS244-11 was used to complement the *acsR* mutation (Rodríguez *et al.*, 2004). For this, the *acsR* gene and its promoter region were amplified by PCR using the 2G5 cosmid as a template with 5'-GTCTGAAGCTTTCCAGCGCCAGTTGAAG-3' and 5'-GTGGC-ACGGTGAACCGTCCCCTCCGTGT-3' primers, and the PCR product was cloned into pKOS244-11. The resulting plasmid, pCB244, was introduced by transformation into *E. coli* GM33 (Marinus & Morris, 1974) and then into *S. coelicolor* CBR4 by protoplast transformation, selecting for Km-resistant exconjugants.

Gel mobility shift assays. H-AcsR at different concentrations was mixed with ³²P-labelled probe (20 000 c.p.m.) in a total volume of 20 µl TGED buffer containing 50 mM Tris/HCl pH 8, 1 mM DTT, 1 mM EDTA, 5% (v/v) glycerol, 100 mM NaCl and 1 µg competitor DNA [poly(dI-dC).poly(dI-dC)] for 30 min at 30 °C. The reaction mixtures were then resolved on a non-denaturing 5% (w/v) polyacrylamide gel in TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8) run at 4 °C with constant voltage (7 V cm⁻¹) for 2 h. After migration, gels were dried and the bands visualized by autoradiography.

The DNA fragments used as probes for mobility shift assays (fragments 1, 2 and 3) correspond to the positions +111 to +403, -106 to +151 and -311 to +151, respectively, relative to the *macs1* transcriptional start site (TSS) (Fig. 5a).

DNase I footprinting. The fragment 1 radiolabelled probe (40 000 c.p.m.) (Fig. 5a) was incubated for 30 min at 30 °C with different quantities of H-AcsR (purified from *E. coli*) in 20 µl TGED buffer containing 1 µg competitor DNA [poly(dI-dC).poly(dI-dC)]. The DNA was partially digested with DNase I (Promega) for 1 min at room temperature, digestion stopped by adding 100 µl stop solution [100 mM Tris/HCl pH 8.0, 100 mM NaCl, 1% (w/v) sodium *N*-lauroylsarcosinate, 10 mM EDTA/NaOH pH 8.0, 25 µg salmon sperm DNA ml⁻¹] and 300 µl phenol/chloroform (1:1). After ethanol precipitation, the pellet was washed with 70% (v/v) ethanol, dissolved in 6 µl formamide-dye mixture and heat denatured (94 °C for 5 min), then immediately placed on ice. Digestion products were resolved on an 8% (w/v) polyacrylamide gel together with a dideoxynucleotide termination sequencing reaction derived from M13 phage DNA included as size marker. After the electrophoresis, the gel was dried and exposed to an X-ray film.

RT-PCR analyses. RNA was isolated from M145 liquid cultures grown on SMM-oleate for 48 h. For each RT-PCR reaction, 15 µg RNA was hybridized for 5 min at 65 °C with 2 pmol Per1 primer (5'-TGATCTGCCGGGAGACGGAGTAGTCCG-3') and 0.66 µl RNase inhibitor (Promega) in enzyme buffer. The first strand synthesis of the cDNA from RNA was performed adding 20 U M-MVL reverse transcriptase (Promega) and 10 µM each dNTP, and incubating for 60 min at 42 °C. A 10 µl quantity of the mixture was used as a

template for the nested-PCR. The PCR reaction mixture contained 10 mM Tris/HCl (pH 8.3), 2 mM MgCl₂, 1.25 mM each of the four dNTPs, 10% (v/v) DMSO, 2 U *Taq* DNA polymerase, 20 pmol each primer in a final volume of 50 µl. Samples were subjected to 30 cycles of denaturation (95 °C, 1 min), annealing (58 °C, 1 min) and extension (72 °C, 1 min). PCR products were analysed by agarose gel electrophoresis. The following combinations of oligonucleotides were used: Per3 (5'-GACTCGACGAGACCGCCGAAG-3')/Pro4 (5'-TGTGCGTCCCGGAACGATCACGAG-3') and Per3/Pro2. The Per3 primer hybridizes 100 bp downstream of the most likely translation initiation codon of *acsR* (GTG), Pro4 hybridizes 38 bp and Pro2 193 bp upstream of the GTG, respectively (Fig. 1a). RT-PCR experiments without prior reverse transcription were performed on all RNA samples to assure exclusion of DNA contamination. Southern blotting of the RT-PCR products was performed to confirm the specificity of the assay. As a probe we used a radiolabelled PCR fragment corresponding to the amplification product obtained with the oligonucleotides Per3 and Pro4.

RESULTS

Identification of the *macs1-fadD1* promoter region

Previous studies suggested that *macs1-fadD1* is a growth-phase-regulated operon, i.e. its transcription is induced during the stationary phase of growth (Banchio & Gramajo, 2002). In order to understand the regulatory mechanisms controlling expression of these genes, we first identified the promoter region driving transcription of the operon. For this, we mapped the TSS of the *macs1-fadD1* mRNA by primer extension. Primer Pro2 (Fig. 1a) was hybridized to *S. coelicolor* RNA isolated at two time points from M145 liquid cultures. The reverse transcriptase reaction showed a single band of 337 nt when stationary phase RNA was used as a template (Fig. 1b), suggesting that the TSS of the operon occurs 269 bp upstream of the most likely translation initiation codon of *macs1* (ATG) (Fig. 1a). No products were detected when exponential phase RNA was used for the reverse transcriptase reaction. The TSS defined a putative promoter with a -35 sequence, TTGCCC, highly similar to those for *Streptomyces* sp., TTGACR (R = A or G), and 17 nt downstream, a putative -10 sequence, GCTGAG, with a poor match to the -10 consensus sequence TAGART (R = A or G) recognized by the principal sigma factor σ^{hrdB} (Strohl, 1992). To confirm the promoter activity within the

Fig. 1. *macs1-fadD1/acsR* intergenic region and mapping of the TSS of the *macs1-fadD1* operon. (a) Nucleotide sequence. Consensus -35 and -10 sequences, and the TSS of the *macs1-fadD1* mRNA are indicated; the regions protected in DNase I footprinting experiments by AcsR are represented by discontinuous lines and the shaded box indicates the putative operator site. The primers used in the RT-PCR and primer extension assays (Per1, Pre3, Pro4 and Pro2) are depicted by arrows under the sequence. The N-terminal sequences of the Macs1 and AcsR proteins are also shown. (b) Primer extension analysis of the *macs1-fadD1* transcript. Total RNA (20 µg) isolated from *S. coelicolor* M145 during stationary (lane 1) and exponential phase (lane 2) was used as a template for the reverse transcriptase reaction. The dideoxy sequencing reactions shown on the left of the figure correspond to M13 phage DNA and were carried out with M13 primer.

nucleotide region suggested by the primer extension experiments and to study its growth-phase-dependent transcription, we analysed the expression profile of this promoter throughout growth using transcriptional fusions to the *luxAB*-reporter system (Kieser *et al.*, 2000). Two DNA fragments, corresponding to a 226 bp *acsR/macs1-fadD1* intergenic region (positions +111 to +337 relative to the *macs1* TSS), not including the putative promoter sequences, and a 648 bp fragment (positions +337 to -311 relative to the *macs1* TSS) containing the promoter region predicted from sequence analysis, were cloned into the luciferase-based

reporter plasmid pIJ5971 (M. S. B. Paget, personal communication) (Fig. 2a, Table 1). The two plasmids, pCB226 (containing the 226 bp DNA fragment) and pCB648 (containing the 648 bp fragment) were introduced by conjugation into M145, yielding MCB226 and MCB648, respectively. Very low levels of luciferase activity were detected in both strains during exponential growth, either in SMM-oleate or in SMM-glucose medium. However, after the cells entered stationary phase, luciferase activity remained very low in MCB226 while MCB648 showed increasing values up to 60 h, independently of the carbon source used

Fig. 2. Growth-phase and carbon source-dependent expression of the *macs1-fadD1* operon. (a) Genetic map of the *macs1-fadD1* locus from *S. coelicolor* and construction of *pmacs1* transcriptional fusions to the *luxAB* reporter system. Grey arrows indicate the positions, relative lengths and directions of transcription of the various genes. Recombinant plasmids are represented as linear maps where the flanking lines correspond to plasmid DNA, grey boxes correspond to the *acsR-macs1* regions used for generating the fusions, and the *luxAB* genes are represented by white arrows. (b) Luciferase levels during growth of MCB648 (circles) and MCB226 (triangles). The *pmacs1* luciferase-driven expression profiles of cultures growing in SMM-oleate or SMM-glucose are indicated by black and white symbols, respectively. The values obtained are the means of three independent experiments. As a control we followed the expression of a *phrdb-luxAB* transcriptional fusion (strain MCB500, black squares). Growth of all the strains was almost identical to wt. EX, exponential phase; SP, stationary phase.

(Fig. 2b). Expression of *macs1-fadD1* during stationary phase was significantly lower in glucose-grown cultures compared with oleate-grown cultures (Fig. 2b), in correlation with the lower levels of ACS activity observed during the stationary phase of growth in cultures grown with glucose as the main carbon source (Banchio & Gramajo, 2002). As a control we followed the expression of a constitutive promoter using pIJ5985, a derivative of the integrative *luxAB*-based reporter plasmid pIJ5971, containing a 0.5 kb *hrdB* promoter fragment (Aigle *et al.*, 2000). As expected the control fusion, *phrdb-luxAB*, showed constant levels of expression up to 50 h.

Genetic organization of the *macs1-fadD1* DNA region

Interestingly, the putative *macs1-fadD1* promoter region maps within the SCO6194 (SC2G5.15c) ORF, a putative regulatory gene 125 bp upstream of the *macs1* translational start codon and oriented in the opposite direction. The close proximity of this possible regulator to the *macs1-fadD1* operon suggested a probable function for this protein in regulating the operon; therefore, we tentatively named it AcsR (for *acyl*-CoA *synthase* *regulator*).

The *acsR* gene encodes a putative DNA-binding protein of 279 aa with a calculated molecular mass of 28.6 kDa. AcsR showed extensive similarity to the putative LuxR-family response regulator NP_823208.1 of *Streptomyces avermitilis* MA-4680 and to a regulatory-like protein, NP_601759.1 of *Corynebacterium glutamicum* ATCC 13032 (73 and 46 %

end-to-end identity, respectively). In the C-terminal region, AcsR shows 47 % identity in a 63 aa overlap with the *S. coelicolor* transcriptional activator *redZ* (T42053) (Guthrie *et al.*, 1998). A putative helix–turn–helix motif lies within the 215–272 amino acid sequence (Brennan & Matthews, 1989). This probable DNA-binding domain resembles the helix–turn–helix motif in several LuxR-type transcriptional activators such as NarL of *E. coli* (36.4 % similarity), TraR of *Agrobacterium tumefaciens* (34.4 % similarity) and GacA of *Pseudomonas fluorescens* (22.7 % similarity). A BLAST search using the N-terminal portion of AcsR showed no significant homology with proteins of known function.

AcsR is necessary for growth-phase induction of the *macs1-fadD1* operon

To study the regulatory role of AcsR in the expression of the *macs1-fadD1* operon, an *acsR* mutant was generated by replacing the original wt gene by an Am-resistance allele. The appropriate disruption of *acsR* was confirmed by Southern blot hybridization, and the mutant strain named CBR4. Slot blot assays were performed using RNA from M145 and CBR4 grown in SMM-oleate medium isolated at different time points. In M145 the *macs1-fadD1* transcript was detected at low levels during exponential growth while a burst of expression was clearly observed after the cultures reached stationary phase (Fig. 3a). However, in the CBR4 mutant the intensity of the *macs1-fadD1* transcription signals remained very low throughout growth, suggesting that AcsR is necessary to enhance stationary phase expression of *macs1-fadD1*. To confirm that the change in the

Fig. 3. AcsR mediates growth-phase-dependent expression of *macs1-fadD1* genes. (a) Slot blot hybridization for the *macs1-fadD1* transcript. Total RNA (20 µg) from *S. coelicolor* cells grown in SMM-oleate liquid medium was blotted onto nitrocellulose filters and hybridized with the corresponding probe. The RNA was extracted from 24, 36 and 48 h old cultures; the growth curves for M145 and CBR4 were almost identical. As a control, we followed the expression of *accB* in both *S. coelicolor* strains. (b) Expression of the *macs1-fadD1* operon was measured as a function of catechol 2,3-dioxygenase activity. The activity of the enzyme at different time points is indicated by black circles for M145, white circles for CBR4 and black triangles for the complemented strain CBR244. As a control the *paccB-xylTE* dependent catechol 2,3-dioxygenase activity in the M145 (black squares) and CBR4 mutant (white triangles) was monitored throughout growth. EX, exponential phase; SP, stationary phase. (c) Act production in M145, CBR4 and *fadD1* mutant MCB40.

expression profile of *macs1-fadD1* was specific and not due to a pleiotropic effect caused by the *acsR* mutation, we also analysed the expression of the essential acyl-CoA carboxyl-transferase gene *accB* (Rodriguez *et al.*, 2001) in both strains. As shown in Fig. 3(a) the transcription profile of this gene was the same regardless of the genetic background of the strains.

To confirm that this effect was exclusively related to the absence of AcsR, we complemented the CBR4 mutant with pCB244, an integrative plasmid (Table 1) containing a wt copy of the *acsR* gene. This new strain was designated CBR244. To follow the time-dependent expression of the operon in the different genetic backgrounds we introduced the *pmacs1-xylTE* (pXE648) transcriptional fusion into M145, CBR4 and CBR244. While the levels of catechol 2,3-dioxygenase activity showed a clear growth-phase-dependent regulation of the operon in the wt and in the *acsR* complemented strains (CBR244), the mutant strain CBR4 showed very low levels of expression throughout growth (Fig. 3b). From these results it can be inferred that AcsR is required for growth-phase-dependent induction of *macs1-fadD1* transcription. As a control we studied a transcriptional fusion containing the promoter of *accB* (pXE559).

Since FadD1 is involved in the physiological differentiation of *S. coelicolor* (Banchio & Gramajo, 2002) and AcsR is an activator of *fadD1*, we analysed the effect of the *acsR* mutation on Act production. When grown in SMM-oleate the CBR4 mutant showed decreased levels of Act production after 72 h of growth, comparable with the low levels of production for the *fadD1* mutant MCB40 (Fig. 3c). The relationship between *acsR*, *fadD1* and antibiotic production was not restricted to a particular medium, since a severe reduction in the production of Act was observed in cultures of CBR4 and MCB40 grown for 90 h either in YEME or in liquid R5 (data not shown). These observations, together with the improved production of Act in a strain that expresses *fadD1* constitutively from the *ermE** promoter (Banchio & Gramajo, 2002), highlight the importance of FadD1 in the production of the secondary metabolite Act.

Transcriptional analysis of *acsR*

To study the growth-phase-dependent expression of *acsR*, a *pacsR-xylTE* transcriptional fusion was constructed. Since we were unable to map the TSS of *acsR* either by S1 mapping or by primer extension experiments, the DNA fragment (from +33 to -259 relative to the *acsR* translational start site) used for constructing the gene fusion was selected on the basis of three criteria: (1) the presence of putative -10 (TGTAAT) and -35 (TTGCCG) consensus sequences in the *acsR-macs1* intergenic region (Fig. 1a), (2) the fact that this DNA fragment cloned in pXE4 (giving rise to pXE292) showed promoter activity when introduced in M145, and (3) RT-PCR placed the *acsR* promoter region between the nucleotides -38 and -193 (relative to the *acsR* translational start site). As shown in Fig. 4(a) only the oligonucleotides Per3 and Pro4 (Fig. 1a) gave a PCR product of the expected size and specificity, suggesting that the TSS for *acsR* was most probably located in the DNA region flanked by the oligonucleotides Pro4 and Pro2. Incidentally, a putative -10 and -35 promoter sequence was found in this region. All this evidence indicates the existence of a promoter driving expression of *acsR* in the intergenic region of *acsR-macs1*.

Fig. 4. Identification of the *acsR-macs1* intergenic region containing the *acsR* promoter. (a) Southern blot hybridization of the RT-PCR products to localize the TSS of *acsR*. The primer used in the reverse transcriptase reaction (Per1) and the primer pair (Per3/Pro4, Per3/Pro2) used in the nested-PCR are indicated in Fig. 1(c). As a probe a radiolabelled PCR fragment corresponding to the oligonucleotide pair Per3/Pro4 amplification product was used. Lane 1, size marker; lane 2, primer pair Per3/Pro2; lane 3, primer pair Per3/Pro4. No specific amplification occurred when reverse transcriptase was omitted from the reaction mixture for the nested-PCR (lane 4, primer pair Per3/Pro2; lane 5 primer pair Per3/Pro4). (b) Expression of *acsR* was measured as a function of catechol 2,3-dioxygenase activity (pXE292). The activity of the enzyme at different time points is indicated by white symbols for M145, and black symbols for CBR4 grown in SMM-oleate (triangles) and SMM-glucose (circles) medium. EX, exponential phase; SP, stationary phase. Each time point represents data from three independent experiments. As a control the *paccB-xy/TE* dependent catechol 2,3-dioxygenase activity in the M145 (black squares) and CBR4 mutant (white squares) was monitored during growth in SMM-oleate.

In a wt background, expression of the *pacsR-xy/TE* (pXE292) transcriptional fusion increased steadily with cell density and reached a plateau as the culture entered stationary phase, in both SMM-oleate and SMM-glucose medium (Fig. 4b). Expression of this transcriptional fusion in the *acsR* mutant displayed a very similar cell density-dependent pattern of expression, although the final levels of catechol 2,3-dioxygenase activity were slightly lower in CBR4 (Fig. 4b). These results demonstrate that expression of *acsR* is also growth-phase dependent and that AcsR does not regulate its own expression.

AcsR specifically binds to the *acsR-macs1* intergenic region

To prove that AcsR exerted its regulatory function by binding to the promoter region in the *acsR-macs1* intergenic sequence, AcsR DNA-binding studies were performed by electrophoretic mobility shift assays. AcsR was overproduced and purified as H-AcsR fusion protein from *E. coli*/pETR (Table 1). Cell-free extracts prepared from IPTG-induced cultures showed a prominent protein band in Coomassie-stained SDS-PAGE gels in the region corresponding to the expected molecular mass of the fusion protein. Employing various binding conditions, the H-AcsR fusion protein did not interact with the Ni^{2+} matrix. However, we succeeded on purifying soluble H-AcsR under denaturing conditions (8 M urea) and then obtained a native form by refolding the protein using a linear decreasing (6–1 M) urea gradient in the presence of Ni^{2+} matrix. Immobilizing one end of the protein during renaturation appears to prevent intermolecular interactions that lead to aggregate formation (Holzinger *et al.*, 1996). Refolded H-AcsR was used in gel mobility shift DNA-binding assays with radiolabelled DNA probes (Fig. 5a, fragments 1, 2 and 3) corresponding to the intergenic and promoter regions of the *macs1-fadD1/acsR* genes. As shown in Fig. 5(b), AcsR binds specifically to the intergenic region (fragment 1), forming a single AcsR-DNA complex. The specificity of AcsR binding was partially determined by competing the radiolabelled probe with a 1.5- to 12-fold excess of unlabelled fragment 1 (Fig. 5c). No difference in probe migration was observed when the DNA fragments corresponding to sequences upstream of the *macs1-fadD1* TSS (Fig. 5a, fragments 2 and 3) were incubated with the same quantity of H-AcsR protein (data not shown).

DNase I footprint assays were performed to determine precisely the locations and sequences of the AcsR binding sites. As shown in Fig. 6, AcsR protected a region from +162 to +197 in the template strand and from +186 to +212 on the non-template strand (positions relative to the TSS of *macs1-fadD1*). DNase I treatment of the AcsR-DNA complex revealed two protected regions for both strands. The intergenic region recognized by AcsR contains three partially identical direct heptanucleotide repeats CGTTC(C/G)G separated by 14 and 13 nucleotides (Fig. 1a). In addition to the protected regions, several hypersensitive bands were detected, indicative of local deformation, presumably caused by bending of the DNA helix. These results, together with those obtained from the expression studies carried out in M145 and the CBR4 mutant (Fig. 3), indicate that AcsR is a transcriptional activator of the *macs1-fadD1* operon.

The central heptanucleotide motif is essential for binding and activation by AcsR

To confirm the role of the central heptanucleotide sequence as part of the recognition site for binding and biological activity of AcsR, a DNA fragment spanning nucleotides +337 to –311, relative to the *macs1* TSS, was chosen to

Fig. 5. Binding of AcsR to the *macs1-fadD1/acsR* intergenic region. (a) Schematic representation of the DNA fragments used as probes for mobility shift assays. The numbers flanking fragments 1, 2 and 3 indicate the base pairs upstream and downstream of the *macs1* TSS. (b) Gel mobility shift experiments were performed by incubating increasing concentrations of purified AcsR protein with a radiolabelled DNA fragment (10 000 c.p.m.) corresponding to the *acsR-fadD1* operon intergenic region (fragment 1). The amount of AcsR protein in each reaction was: lanes 1–6, 0, 0.5, 0.75, 1, 1.5 and 2 µg. (c) The shifts observed for the intergenic region were shown to be specific by a competition assay with unlabelled fragment 1. A fixed concentration of AcsR (1.5 µg) was incubated in the presence of increasing amounts of unlabelled fragment 1 (competitor). Lane 1, no protein-no competitor, lanes 2–7: 0, 1.5-, 3-, 5-, 9-, 12-fold competitor excess, respectively.

generate fusions to the *luxAB* reporter system. The DNA fragments containing the native or mutated intergenic sequence in the central heptameric repetition were cloned in pIJ5971 in both orientations to give the four different

plasmids (Table 1). Each plasmid was then introduced by conjugation into M145.

The effect of the mutation on expression of the *macs1-fadD1* operon was studied by measuring luciferase activity in M145 harbouring either pCB648 or pCB648* (wt and mutated heptanucleotide sequence, respectively). We also analysed the effect of this mutation on *acsR* transcription by assaying the luciferase activity in M145 derivatives carrying either pCB846 or pCB846* (wt and mutated heptanucleotide sequence, respectively). The *pmacs1-luxAB* reporter fusions assayed at different time points reflected a clear loss of regulation in the construct containing the mutated motif (Fig. 7a), indicating the importance of this sequence in transcriptional activation of the operon. In contrast, the expression pattern of *acsR* was not significantly modified when the central repeated sequence was mutated (Fig. 7b). To see if the mutation in the central heptanucleotide affected binding of AcsR, gel mobility shift assays were performed

Fig. 6. DNase I footprinting of AcsR binding to the *macs1-fadD1* operon and *acsR* intergenic region. Radiolabelled DNA fragments corresponding to the template (a) and non-template (b) strand of the intergenic region were incubated with increasing amounts of purified AcsR. Lanes A, C, G and T show a dideoxynucleotide termination sequencing reaction derived from M13 phage DNA included as a size marker. The square brackets on the right of the panels indicate regions protected by AcsR and arrows indicate hypersensitive sites.

Fig. 7. The central heptanucleotide box is essential for the binding and activation functions of AcsR. (a) Growth-phase-dependent expression of the *macs1-fadD1* operon in the presence of a wt and mutant AcsR binding region (pCB648, black bars; pCB648*, white bars). The *macs1-fadD1/acsR* intergenic region (−311 to +337 relative to the *macs1* TSS) was cloned in its wt and mutated version, in which the native central heptanucleotide sequence CGTCCG was replaced by CTCTAGA, into pJ5971, immediately upstream of a promoterless *luxAB* reporter gene. Luciferase activity was measured during growth in SMM-oleate. (b) Growth-phase-dependent expression of *acsR* in the presence of a wt and mutant AcsR binding region (pCB846, black bars; pCB846*, white bars). The *macs1-fadD1/acsR* intergenic region (+337 to −311 relative to the *macs1* TSS) was cloned in its wt and mutated version and luciferase activity was measured during growth in SMM-oleate. (c) Gel retardation analysis with AcsR protein and the intergenic region. A sample of 5 and 10 μ M purified AcsR was incubated with radio-labelled DNA fragments. Fragment I contains the wt sequence of the putative AcsR binding motif and fragment II has an *Xba*I recognition site replacing the central heptanucleotide repeat.

with the native and mutated DNA fragments and refolded H-AcsR. A retarded band was clearly visible when the native intergenic region was assayed but not with the mutant fragment (Fig. 7c). Therefore, the *in vivo* fusions and the

in vitro binding studies consistently indicated that at least the central heptanucleotide sequence is essential for AcsR recognition and binding, and for its role as an activator of the *macs1-fadD1* operon. Furthermore, AcsR binding to the intergenic region exclusively mediates induction of the operon and does not influence its own expression.

DISCUSSION

By using genetic and biochemical tools we have characterized the transcriptional regulation of the *macs1-fadD1* operon of *S. coelicolor*. Mutation and complementation studies of the *acsR* gene, which encodes a protein with homology to several members of the LuxR family of response regulators, indicated that AcsR is responsible for the growth-phase-dependent induction of the *macs1-fadD1* operon (Fig. 3a, b). Expression of *acsR* itself was also found to reach maximum levels during stationary phase (Fig. 4b).

In vitro, AcsR binds to the intergenic *acsR-macs1* region (Fig. 5). Considering the results of the DNase I footprinting analysis, the proposed binding sites of AcsR span a region from +162 to +197 for the coding strand, and +186 to +212 for the non-coding strand, relative to the *macs1* TSS. These experiments also showed that binding of AcsR may induce bending of the DNA, since several enhanced DNase I cleavage sites appeared within the protected regions of the promoter (Fig. 6). Analysis of the DNA sequence of this region revealed three almost identical direct tandem repeats of seven nucleotides that could act as the AcsR-binding sequences. We demonstrated the relevance of the central heptameric sequence by mutating the motif, which completely abolished the binding of AcsR to the DNA (Fig. 7c) and prevented the *in vivo* activation of the *macs1-fadD1* genes (Fig. 7a). Gel mobility shift experiments showed a single shifted complex formed at different concentrations of AcsR; given that the footprint (Fig. 6) extends over a region comprising two of the heptanucleotide repeats it may be that the single complex comprises an AcsR dimer, as opposed to a monomer. The potential role of the flanking heptameric sequences in AcsR binding, and therefore on transcriptional regulation of *pmacs1-fadD1*, remains to be established.

The location of the AcsR binding region downstream of the *pmacs1-fadD1* promoter is very unusual. Most classic activators have binding sites 30–80 nt upstream of the TSS (Collado-Vides *et al.*, 1991) and none of the general mechanisms based on the location of the recognition sites proposed for the ‘simple activation of gene transcription’ (Browning & Busby, 2004) would fit for AcsR. Of the few activators that have binding sites downstream of the TSS nearly all have additional binding sites closer to the promoter (Munson *et al.*, 2001). Rns, a virulence regulator in the AraC family of enterotoxigenic *E. coli*, requires binding sites upstream and downstream of its own promoter to function as an activator (Munson & Scott, 2000). Rns appears to increase the affinity of *E. coli* σ^{70} RNA polymerase (RNAP) for *prns* and facilitate formation of an open

complex (Munson & Scott, 2000). Another example is the phosphorylated PhoP (PhoP-P), which activates the weak promoter for the *pstS* operon and the *phoA* gene of *Bacillus subtilis*. The phosphorylated form of the activator binds to the upstream region and also protects sequences internal to the coding region of these two genes (Liu *et al.*, 1998). The PhoP-P binding sites in the coding region are necessary for full induction from either promoter during phosphate starvation (Qi & Hulet, 1998). A case of an activator that binds to a single site downstream of the promoters is DnaA, which activates the lambda pR promoter by binding to a DNA sequence several base pairs downstream of pR, where it may contact the β -subunit of the *E. coli* RNAP (Herman-Antosiewicz *et al.*, 1998). This may allow RNAP to overcome an intrinsic pause site at position +16 (Ellinger *et al.*, 1994).

The mechanism of this kind of transcription activation is not well established. Even though we cannot propose a model for AcsR transcriptional activation of the *macs1-fadD1* operon, it is tempting to speculate that this activator may contribute to the RNAP elongation complex to extend transcription beyond a sequence that would form tight hairpin secondary structures in the untranslated sequence of the *macs1-fadD1* transcript. A search for additional AcsR binding sites upstream of *pmacs1* by electrophoretic mobility shift assay, using a DNA fragment extending from nucleotide -310 to the TSS, gave negative results, indicating that the binding motifs downstream of the *pmacs1* promoter are the only ones involved in the activating mechanism of AcsR. Considering that activators rarely bind exclusively downstream of the -10 hexamer, we also attempted to identify alternative AcsR-dependent promoters within or downstream of the AcsR binding sites. Analysis of this region by primer extension assays did not reveal any TSSs within or downstream of the AcsR binding sites. We also could not show any other promoter activity downstream of *pmacs1* by using a DNA fragment containing the AcsR binding site (+162 to +212), but excluding the promoter region previously identified by primer extension (DNA fragment from +111 to +337 fused to the *luxAB* reporter cassette in MCB226, Fig. 2b).

An additional complexity for the mechanism of regulation of these promoters could be that the TSS of the *macs1-fadD1* operon maps 144 bp within the coding region of AcsR, generating at least 144 nt of complementary sequence during active transcription from the divergent promoters. Therefore, the potential formation of anti-sense mRNAs could also have a possible role in regulating this operon by directly interfering with ribosome binding or facilitating premature transcription termination.

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REFERENCES

- Aigle, B., Wietzorrek, A., Takano, E. & Bibb, M. J. (2000). A single amino acid substitution in region 1.2 of the principal sigma factor of *Streptomyces coelicolor* A3(2) results in pleiotropic loss of antibiotic production. *Mol Microbiol* **37**, 995–1004.
- Banchio, C. & Gramajo, H. C. (1997). Medium- and long-chain fatty acid uptake and utilization by *Streptomyces coelicolor* A3(2): first characterization of a Gram-positive bacterial system. *Microbiology* **143**, 2439–2447.
- Banchio, C. & Gramajo, H. C. (2002). A stationary-phase acyl-coenzyme A synthetase of *Streptomyces coelicolor* A3(2) is necessary for the normal onset of antibiotic production. *Appl Environ Microbiol* **68**, 4240–4246.
- Bierman, M., Logan, R., O'Brien, K., Seno, E. T., Rao, R. N. & Schoner, B. E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116**, 43–49.
- Black, P. N. & Dirusso, C. (1994). Molecular and biochemical analyses of fatty acid transport, metabolism, and gene regulation in *Escherichia coli*. *Biochim Biophys Acta* **1210**, 123–145.
- Blondelet-Rouault, M. H., Weiser, J., Lebrihi, A., Branny, P. & Pernodet, J.-L. (1997). Antibiotic resistance gene cassettes derived from the V interposon for use in *E. coli* and *Streptomyces*. *Gene* **190**, 315–317.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brennan, R. G. & Matthews, B. W. (1989). The helix-turn-helix DNA binding motif. *J Biol Chem* **264**, 1903–1906.
- Brofman, M., Orenella, A., Morales, M. N., Bieri, F., Waechter, F., Staubli, W. & Bently, P. (2005). Potentiation of diacylglycerol-activated protein kinase C by acyl-coenzyme A thioesters of hypolipidaemic drugs. *Biochem Biophys Res Commun* **159**, 1026–1031.
- Browning, D. F. & Busby, S. J. (2004). The regulation of bacterial transcription initiation. *Nat Rev Microbiol* **2**, 57–65.
- Bystrykh, L. V., Fernandez-Moreno, M. A., Herrema, J. K., Malpartida, F., Hopwood, D. A. & Dijkhuizen, L. (1996). Production of actinorhodin-related 'blue pigments' by *Streptomyces coelicolor* A3(2). *J Bacteriol* **178**, 2238–2244.
- Cole, S. T., Brosch, R., Parkhill, J. & 39 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.
- Collado-Vides, J., Magasanik, B. & Gralla, B. (1991). Control site location and transcriptional regulation in *Escherichia coli*. *Microbiol Rev* **55**, 371–394.
- Dirusso, C., Heimert, T. L. & Metzger, A. K. (1992). Characterization of FadR, a global transcriptional regulator of fatty acid metabolism in *Escherichia coli*. Interaction with the *fadB* promoter is prevented by long chain fatty acyl coenzyme A. *J Biol Chem* **267**, 8685–8691.
- Ellinger, T., Behnke, D., Bujard, H. & Gralla, J. D. (1994). Stalling of *Escherichia coli* RNA polymerase in the +6 to +12 region *in vivo* is associated with tight binding to consensus promoter elements. *J Mol Biol* **239**, 455–465.
- Fujino, T., Takei, Y. A., Sone, H., Ioka, R. X., Kamataki, A., Magoori, K., Takahashi, S., Sakai, J. & Yamamoto, T. T. (2001). Molecular identification and characterization of two medium-chain acyl-CoA synthetases, MACS1 and the *Sa* gene product. *J Biol Chem* **276**, 35961–35966.
- Gonzalez-Ceron, G., Licon, P. & Servin-Gonzalez, L. (2001). Modified *xylE* and *xylTE* reporter genes for use in *Streptomyces*: analysis of the effect of *xylT*. *FEMS Microbiol Lett* **196**, 229–234.

- Gordon, J. I. (1990). Protein *N*-myristoylation: simple questions, unexpected answers. *Clin Res* **38**, 517–528.
- Guthrie, E. P., Flaxman, C. S., White, J., Hodgson, A. D., Bibb, M. J. & Chater, F. K. (1998). A response-regulator-like activator of antibiotic synthesis from *Streptomyces coelicolor* A3(2) with an amino-terminal domain that lacks a phosphorylation pocket. *Microbiology* **144**, 727–738.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Herman-Antosiewicz, A., Wegrzyn, A., Taylor, K. & Wegrzyn, G. (1998). DnaA-mediated regulation of phage lambda-derived replicons in the absence of pR and Cro function. *Virology* **249**, 98–107.
- Hillemann, D., Puhler, A. & Wohlleben, W. (1991). Gene disruption and gene replacement in *Streptomyces* via single stranded DNA transformation of integration vectors. *Nucleic Acids Res* **19**, 727–731.
- Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51–59.
- Holzinger, A., Phillips, K. S. & Weaver, T. E. (1996). Single-step purification/solubilization of recombinant proteins: application to surfactant protein B. *Biotechniques* **20**, 804–808.
- Kameda, K. & Nunn, W. D. (1981). Purification and characterization of acyl coenzyme A synthetase from *Escherichia coli*. *J Biol Chem* **256**, 5702–5707.
- Kieser, T., Bibb, W. D., Buttner, M. J., Chater, K. F. & Hopwood, D. A. (2000). *Practical Streptomyces Genetics*. Norwich: John Innes Centre.
- Korchak, H. M., Kane, L. H., Rossi, M. W. & Corkey, B. E. (1994). Long chain acyl coenzyme A and signaling in neutrophils. An inhibitor of acyl coenzyme A synthetase, triacsin C, inhibits superoxide anion generation and degranulation by human neutrophils. *J Biol Chem* **269**, 30281–30287.
- Liu, W., Qi, Y. & Hulett, F. M. (1998). Sites internal to the coding regions of *phoA* and *pstS* bind PhoP and are required for full promoter activity. *Mol Microbiol* **28**, 119–130.
- Marinus, M. G. & Morris, N. R. (1974). Biological function for 6-methyladenine residues in the DNA of *Escherichia coli* K-12. *J Mol Biol* **85**, 309–322.
- Mullis, K. B. & Faloona, F. A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol* **155**, 335–350.
- Munson, G. P. & Scott, J. R. (2000). Rns, a virulence regulator within the AraC family, requires binding sites upstream and downstream of its own promoter to function as an activator. *Mol Microbiol* **36**, 1391–1402.
- Munson, G. P., Holcomb, L. G. & Scott, J. R. (2001). Novel group of virulence activators within the AraC family that are not restricted to upstream binding sites. *Infect Immun* **69**, 186–193.
- Nishihara, K., Kanemori, M., Yanagi, H. & Yura, T. (2000). Overexpression of trigger factor prevents aggregation of recombinant proteins in *Escherichia coli*. *Appl Environ Microbiol* **66**, 884–889.
- Olukoshi, E. R. & Packter, N. M. (1994). Importance of stored triacylglycerols in *Streptomyces*: possible carbon source for antibiotics. *Microbiology* **140**, 931–943.
- Overath, P., Pauli, G. & Schairer, H. U. (1969). Fatty acid degradation in *Escherichia coli*. An inducible acyl-CoA synthetase, the mapping of old-mutations, and the isolation of regulatory mutants. *Eur J Biochem* **7**, 559–574.
- Packter, N. M. & Olukoshi, E. R. (1995). Ultrastructural studies of neutral lipid localisation in *Streptomyces*. *Arch Microbiol* **164**, 420–427.
- Paget, M. S. B., Leibovitz, E. & Buttner, M. J. (1999). A putative two-component signal transduction system regulates σ^E , a sigma factor required for normal cell wall integrity in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **33**, 97–107.
- Pfanner, N., Orci, L., Glick, B. S., Amherdt, M., Arden, S. R., Malhotra, V. & Rothman, J. E. (1989). Fatty acyl-coenzyme A is required for budding of transport vesicles from Golgi cisternae. *Cell* **59**, 95–102.
- Qi, Y. & Hulett, F. M. (1998). PhoP~P and RNA polymerase σ^A holoenzyme are sufficient for transcription of Pho regulon promoters in *Bacillus subtilis*: PhoP~P activator sites within the coding region stimulate transcription *in vitro*. *Mol Microbiol* **28**, 1187–1197.
- Redenbach, M., Kieser, H. M., Denapate, D., Eichner, A., Cullum, J., Kinashi, H. & Hopwood, D. A. (1996). A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. *Mol Microbiol* **21**, 77–96.
- Rodriguez, E., Banchio, C., Diacovich, L., Bibb, M. J. & Gramajo, H. C. (2001). Role of an essential acyl coenzyme A carboxylase in the primary and secondary metabolism of *Streptomyces coelicolor* A3(2). *Appl Environ Microbiol* **67**, 4166–4176.
- Rodriguez, E., Ward, S., Fu, H., Revill, W. P., McDaniel, R. & Katz, L. (2004). Engineered biosynthesis of 16-membered macrolides that require methoxymalonyl-ACP precursors in *Streptomyces fradiae*. *Appl Microbiol Biotechnol* **66**, 85–91.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Strohl, W. R. (1992). Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic Acids Res* **20**, 961–974.
- Studier, F. W. & Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189**, 113–130.
- Takano, E., Gramajo, H. C., Strauch, E., Andres, N., White, J. & Bibb, M. J. (1992). Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **6**, 2797–2804.
- Thomas, J. G., Ayling, A. & Baneyx, F. (1997). Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from *E. coli*. To fold or to refold. *Appl Biochem Biotechnol* **66**, 197–238.
- Watkins, P. A. (1997). Fatty acid activation. *Prog Lipid Res* **36**, 55–83.