Transcriptional regulation of the \textit{macs1-fadD1} operon encoding two acyl-CoA synthases involved in the physiological differentiation of \textit{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 \textit{Streptomyces coelicolor}. \textit{fadD1} and \textit{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 \textit{macs1-fadD1} operon is positively regulated by AcsR, a LuxR-type transcriptional regulator. In an \textit{acsR} mutant, expression of the \textit{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 \textit{fadD1} null mutant. The absence of \textit{macs1-fadD1} induction in the \textit{acsR} mutant was restored by complementation with a wild-type copy of the \textit{acsR} gene, showing a strict link between AcsR and induction of the \textit{macs1-fadD1} operon. Gel mobility shift assays and DNase I footprinting indicated that AcsR binds to specific sequences about +162 nucleotides downstream of the \textit{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 \textit{pacsR} and \textit{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 \textit{macs1-fadD1} operon.

INTRODUCTION

Fatty acids serve a number of essential and regulatory functions. Many cells, although capable of \textit{de novo} synthesis, scavenge fatty acids from the extracellular environment, thereby bypassing energy-expensive synthetic reactions. In \textit{Streptomyces coelicolor}, fatty acids with different chain lengths (C4–C18) are efficiently degraded through the \textit{c}atabolic pathway appear to be constitutively synthesized (Banchio & Gramajo, 1997), in sharp contrast to \textit{Escherichia coli} whose \textit{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 \textit{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 \textit{Streptomyces} species, including \textit{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 \textit{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.
**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_coeleicolor/) 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 *macl*, 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 ACSs, 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. Actionorhodin (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 *macl-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 *macl-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. They were added and the labelled cDNA was run on a 6% acrylamide-urea sequencing gel alongside a reference sequencing ladder generated using a Wizard kit (Promega). 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 μm; 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 mycelia were disrupted by sonication with a Vibramel ultrasonic processor (VCX600 sonicator; Sonic & Materials) for 10 s at 4°C. After centrifugation for 15 min at 4°C and 15000 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.

**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). Streptomycetes DNA and protoplasts were prepared and transformed as described by Kieser et al. (2000).

**Primer extension analysis.** Primer extension was used to determine the 5′ end of mRNA transcripts. One microlitre primer Pro2 (5′-GGTGTAAGCTCCTCCGCGTGTTCCAG-3′) was annealed to 20 μg heat-denatured RNA from cultures grown to stationary or exponential phase. The annealed primer/RNA solution was then dissolved in 20 μl reverse transcription mix [50 mM Tris/HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, [γ-32P]dATP (3000 Ci mmol−1 = 1·1×106 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-potentiated and redissolved in 4 μl TE buffer. Sequencing stop solution (6 μl) 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 μg each RNA sample was treated with formaldehyde (6.5%, v/v), formaldehyde (6.5%, v/v), formamide (6.5%, v/v), formamide (6.5%, v/v), formaldehyde (6.5%, v/v), formaldehyde (6.5%, v/v), formaldehyde (6.5%, v/v), formaldehyde (6.5%, v/v), and SSC (1×) 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′-AGCCCGGACCGGGAACACCGG- AAGGTGCT-3′) and RTdn (5′-GGTGTCGGCGGAGCGGGGCTG- GT-3′). As a control, we followed the expression of accB, the gene that encodes the essential carboxyltransferase subunit of the acyl-CoA carboxylase of *S. coelicolor* (Rodriguez et al., 2001). The PCR product employed as accB probe was generated with the oligonucleotide pair accBup (5′-GGAGGGCATTGGCGGCGAGCTGTTTCAGAGG-3′) and accBdn (5′-GGAGGGCATTGGCGGCGAGCTGTTTCAGAGG-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. pmacl 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-macl intergenic region was also assessed (Table 1, Fig. 2a). Plasmids were introduced into S. coelicolor by conjugation from E. coli ET 12567/pUL8002 (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-macl 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’-TGTGGGCTCTAGAAGATCAG-GAC-3’; Dis4, 5’-CTCTGGATATCTAGAAGGACGC-3’), and the Froup (5’-GTCGCGCTGTCGGGACGACGCAGC-3’), and the Pro2 (5’-GGTTGATCTCAGGATGTCACGGCCAG-3’) primers (Ho et al., 1989).

The phrdB–luxAB transcriptional fusion harboured in pIJ5985 (Aigle et al., 2000) was introduced into M145 by conjugation from E. coli ET 12567/pUL8002, yielding the strain MC8500.

Assay of catechol 2,3-dioxygenase in cell-free extracts. maecl-fadD1 and acsR promoter activities were assessed using pX648 and pX292 derivatives of the low copy number SCP2-based vector pX4 (Gonzalez-Ceron et al., 2001). pX648 contains the promoterless xylTE genes under control of the maecl-fadD1 promoter. pX292 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 maecl-xylTE transcriptional fusion contained in pX559. For the construction of this fusion the acsB promoter sequence was amplified by PCR using the oligonucleotides AccBup (5’-CGAGCGTTAATCAG-3’) and AccBdn (5’-GTACCGATATCTTAGAAGGACGC-3’), and cloned in front of the xylTE genes on pX4. 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 A375 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 A640 of the supernatant was determined and the Act concentration was calculated using the molar absorption coefficient at 640 nm of 25 320 (Bystricky et al., 1996).

Expression and purification of AcsR in E. coli. pET containing the translational fusion hist–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 Ndel and HindIII and used as a backbone. The acsR gene was amplified by PCR using the 25S cosmid (Redenbach et al., 1996) as a template with Regup (5’-GCGGGAATTCGACACGCGCCGCTG-GGAGC-3’) and Regdn (5’-GGCGGGAATTCGACACGCGCCGCTG-GGAGC-3’) primers. The forward and reverse primers contained Ndel 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 Ndel/HindIII and the acsR-containing fragment was cloned into Ndel/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 000g 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 000g. 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 NaH2PO4 (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 Smal-digested Am cassette. The final construct, named pCB003, was introduced by transformation into E. coli ET12567/pUL28002 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>S. coelicolor</em></td>
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</tr>
<tr>
<td>M145</td>
<td>Parental strain SCP1- SCP2-</td>
<td>Kieser <em>et al.</em> (2000)</td>
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<tr>
<td>MCB648</td>
<td><em>S. coelicolor</em> M145 with pCB648, Amr</td>
<td>This work</td>
</tr>
<tr>
<td>MBC226</td>
<td><em>S. coelicolor</em> M145 with pCB226, Amr</td>
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<td>MCB846</td>
<td><em>S. coelicolor</em> M145 with pCB846, Amr</td>
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<td>Mutant of <em>S. coelicolor</em> M145 with insertional inactivation of acsR, Amr</td>
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<td>MCB648*</td>
<td><em>S. coelicolor</em> M145 with pCB648*, Amr</td>
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<tr>
<td>MCB846*</td>
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<td>MXE648</td>
<td><em>S. coelicolor</em> M145 with pXE648, Th'</td>
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<td>MXE292</td>
<td><em>S. coelicolor</em> M145 with pXE292, Th'</td>
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<td>CBR244</td>
<td>Mutant of <em>S. coelicolor</em> M145 with insertional inactivation of acsR, Amr, carrying the pXE4648 plasmid, Th', and pCB244 ectopic integrative derivative, Km'</td>
<td>This work</td>
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<td>MCB500</td>
<td><em>S. coelicolor</em> M145 with pIJ5985, Amr</td>
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<td>MCB40</td>
<td>Mutant of <em>S. coelicolor</em> M145 with an in-frame deletion of fadD1</td>
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</tr>
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<td><strong>E. coli</strong></td>
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<tr>
<td>DH5x</td>
<td>lacU169 (80lacZAM15) endA1 recA1 hsdR17 deoR supE44 thi-1 12 gyrA96 relax</td>
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</tr>
<tr>
<td>BL21(DE3)</td>
<td>BL21(DE3) ompT(DE3)</td>
<td>Studier &amp; Moffatt (1986)</td>
</tr>
<tr>
<td>ET 12567</td>
<td>supE44 hsdS20 ara-14 proA2 lacY galK2 rpsL20 xyl-5 mtl-1 Ddcm Ddcm DhsDM Cm'</td>
<td>Paget <em>et al.</em> (1999)</td>
</tr>
<tr>
<td>GM33</td>
<td><em>E. coli</em> K-12 dam-3 sup-85</td>
<td>Marinus &amp; Morris (1974)</td>
</tr>
<tr>
<td><strong>Vectors</strong></td>
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<tr>
<td>pBluescript SK(1)</td>
<td>Phagemid vector (Ap^ lacZ9)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>PCR-Blunt Easy</td>
<td>Used for cloning PCR products</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Used for cloning PCR products</td>
<td>Promega</td>
</tr>
<tr>
<td>pSET151</td>
<td>Used for the conjugal transfer of DNA from <em>E. coli</em> to <em>Streptomyces</em> spp. (Ap^ Th' lacZ9)</td>
<td>Hilleman <em>et al.</em> (1991)</td>
</tr>
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<td>pET28a</td>
<td>Phagemid vector for expression of recombinant proteins under control of strong T7 transcription and translation signals (Km')</td>
<td>Novagen</td>
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<td><strong>Recombinant plasmids</strong></td>
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<tr>
<td>pETR</td>
<td>pET28a with acsR under the control of strong T7 transcription and translation signals</td>
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<td>pCB226</td>
<td>pIJ5971 derivative vector containing 226 bp fragment of macs1-acsR intergenic region</td>
<td>This work</td>
</tr>
<tr>
<td>pCB648</td>
<td>pIJ5971 derivative vector containing 648 bp fragment of macs1-acsR intergenic region</td>
<td>This work</td>
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<tr>
<td>pCB846</td>
<td>pIJ5971 derivative vector containing the same 648 bp insert as pCB648 of acsR- macs1 intergenic region but cloned in the opposite orientation</td>
<td>This work</td>
</tr>
<tr>
<td>pCB648*</td>
<td>pIJ5971 derivative vector containing 648 bp mutant fragment of macs1-acsR intergenic region</td>
<td>This work</td>
</tr>
<tr>
<td>pCB846*</td>
<td>pIJ5971 derivative vector containing the same 648 bp mutant insert as pCB648* but cloned in the opposite orientation</td>
<td>This work</td>
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<tr>
<td>pXE468</td>
<td>pXE4 derivative vector containing 648 bp fragment of macs1-acsR intergenic region</td>
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</table>
Vector pKOS244-11 was used to complement the acsR mutation (Rodriguez 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′-GTGCAAGGCTTCCAGGGCCCTAATGGA-3′ and 5′-GTCGCA- CGGTGACCGCTCCCTCCGTTG-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 TE 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 TE 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′-TGATCTGCCCAGGAGACGGAGTGTGGC-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 μl Tag 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′-GACTCGACGACGCCGCAAG-3′)/Pro4 (5′-TGTGCGTCGCCGAAAGATCGAGG-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, TTTGCC, highly similar to those for Streptomyces sp., TTGACR (R = A or G), and 17 nt downstream, a putative −10 sequence, GCCTGAG, with a poor match to the −10 consensus sequence TAGART (R = A or G) recognized by the principal sigma factor σ²ACR (Strohl, 1992). To confirm the promoter activity within the

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**Table 1. cont.**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pXE292</td>
<td>pXE4 derivative vector containing 292 bp fragment of <em>macs1-acsR</em> intergenic region</td>
<td>This work</td>
</tr>
<tr>
<td>pXE559</td>
<td>pXE4 derivative vector containing 559 bp fragment of <em>acsR</em> promoter region</td>
<td>This work</td>
</tr>
<tr>
<td>pCB244</td>
<td>pTR244 derivative vector containing 1265 bp fragment of <em>acsR</em> gene coding region</td>
<td>This work</td>
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<tr>
<td>pCB003</td>
<td>pSET151 with <em>aa(3)IV</em> (Am') gene inserted in the <em>acsR</em> coding region</td>
<td>This work</td>
</tr>
<tr>
<td>pCB001</td>
<td>pGEM-T Easy derivative vector containing the <em>acsR</em> coding sequence</td>
<td>This work</td>
</tr>
<tr>
<td>pCB002</td>
<td>pSET151 derivative vector containing the <em>acsR</em> coding sequence</td>
<td>This work</td>
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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.
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 or SMM-glucose as 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 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 carboxylase 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 p*macs1-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 (TTGCGG) 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.*
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 +210 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
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 pmacluxAB 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

![Diagram](image-url)

**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, 7.5, 12-fold competitor excess, respectively.

![Image](image-url)

**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.
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

**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 heptamer 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 heptamer 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* σ70 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 \(\text{pstS}\) operon and the \(\text{phoA}\) gene of \(\text{Bacillus subtilis}\). 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 & Hulett, 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 \(\text{E. coli}\) 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 \(\text{macs1-fadD1}\) 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 \(\text{macs1-fadD1}\) transcript. A search for additional AcsR binding sites upstream of \(\text{pmacs1}\) 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 \(\text{pmacs1}\) 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 \(\text{pmacs1}\) 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 \(\text{luxAB}\) reporter cassette in MCB226, Fig. 2b).

An additional complexity for the mechanism of regulation of these promoters could be that the TSS of the \(\text{macs1-fadD1}\) 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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Regulation of acyl-CoA synthases in S. coelicolor


