Transcriptional regulation of the aconitase genes (acnA and acnB) of Escherichia coli

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Escherichia coli contains two differentially regulated aconitase genes, acnA and acnB. Two acnA promoters transcribing from start points located 407 bp (P1acnA) and 50 bp (P2acnA) upstream of the acnA coding region, and one acnB promoter (PacnB) with a start point 95 bp upstream of the acnB coding region, were identified by primer extension analysis. A 2.8 kb acnA monocistronic transcript was detected by Northern blot hybridization, but only in redox-stressed (methyl-viologen-treated) cultures, and a 2.5 kb acnB monocistronic transcript was detected in exponential- but not stationary-phase cultures. These findings are consistent with previous observations that acnA is specifically subject to SoxRS-mediated activation, whereas acnB encodes the major aconitase that is synthesized earlier in the growth cycle than AcnA. Further studies with acn-lacZ gene fusions and a wider range of transcription regulators indicated that acnA expression is initiated by σE from P1acnA, and from P2acnA it is activated directly or indirectly by CRP, FruR, Fur and SoxRS, and repressed by ArcA and FNR. In contrast, acnB expression is activated by CRP and repressed by ArcA, FruR and Fis from PacnB. Comparable studies with fum-lacz fusions indicated that transcription of fumC, but not of fumA or fumB, is initiated by RNA polymerase containing σE. It is concluded that AcnB is the major citric acid cycle enzyme, whereas AcnA is an aerobic stationary-phase enzyme that is specifically induced by iron and redox-stress.

Keywords: aconitase, fumarase, citric acid cycle genes, transcription regulation, global regulators

INTRODUCTION

Aconitase (EC 4.2.1.3) catalyses the reversible isomerization of citrate and isocitrate via cis-aconitate in the citric acid cycle. It is a monomeric enzyme containing an unstable [4Fe-4S] cluster which is reversibly converted to an inactive [3Fe-4S] form (Beinert et al., 1996). The aconitase protein family contains aconitases, isopropylmalate isomerases and homoaconitases from diverse sources, and the iron-dependent regulatory proteins (IRP) which regulate either the translation or the stabilities of specific vertebrate mRNAs (Klausner & Rouault, 1993; Frishman & Hentze, 1996; Hentze & Kuhn, 1996; Gruer et al., 1997a). The bifunctional IRP1 proteins, which operate either as cytoplasmic aconitases or as site-specific RNA-binding proteins depending on the reversible incorporation or disruption of the iron-sulphur clusters, are particularly interesting. A structural archetype for all members of this family is provided by the porcine mitochondrial aconitase (Robbins & Stout, 1989). The basic structure contains three structural domains, tightly packed around the iron-sulphur cluster, that are located beneath a deep active-site cleft and the upper (fourth) domain. However, three different domain arrangements have now been recognized (Gruer et al., 1997a). In most cases domain four is at the C-terminal end, connected to the rest of the molecule by a long linker, but in some bacterial aconitases (notably AcnB of Escherichia coli, see below) domain four is located at the N-terminus, and in the heterodimeric bacterial isopropylmalate isomerases the fourth domain is provided by an independent subunit.

Biochemical and genetic studies have established that E. coli contains two genetically distinct aconitases (designated AcnA and AcnB) encoded by the acnA and acnB genes (Prodromou et al., 1991; Bradbury et al., 1996). There may even be a third aconitase (AcnC) responsible for the low residual aconitase activity detected in a genetically engineered acnA acnB double mutant (Gruer et al., 1997b). The acnA gene is located at 28 min
Table 1. E. coli strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic</th>
<th>Source or reference*</th>
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<tr>
<td>W3110</td>
<td>Prototroph</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>DH5α</td>
<td>ΔargF–lacU169 Δ80lacZM15 recA</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>MC1000</td>
<td>ΔlacX74 ΔaraA–leu</td>
<td>Silhavy et al. (1984)</td>
</tr>
<tr>
<td>JRG1999</td>
<td>MC1000 ΔacrT8</td>
<td>S. T. Cole^1</td>
</tr>
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<td>ΔargF–lacU169</td>
<td>V. Stewart^2</td>
</tr>
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<td>ΔargF–lacU169 fmr-250</td>
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</tr>
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<td>RK4353 fts-767</td>
<td>Ball et al. (1992)</td>
</tr>
<tr>
<td>JRG3227</td>
<td>RK4353 fruR</td>
<td>Park et al. (1995)</td>
</tr>
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<td>Silhavy et al. (1984)</td>
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<td>H1941</td>
<td>MC4100 Δfur</td>
<td>K. Hanitke^3</td>
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<tr>
<td>LM1A</td>
<td>MC4100 arcA1 zjj::Tn10</td>
<td>McWalter et al.^4</td>
</tr>
<tr>
<td>RM315</td>
<td>MC4100 Δfur arcA1 zjj::Tn10</td>
<td>Sawers &amp; Suppmann (1992)</td>
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<td>RH90</td>
<td>MC4100 rpoS359::Tn10</td>
<td>R. Hengge-Aronis^5</td>
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<td>ΔargF–lacU169 rpsL sup</td>
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<tr>
<td>pRB38</td>
<td>arcA^+, Cm^R</td>
<td>R. Buxton^7</td>
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<td>K. Hanitke^a</td>
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<td>pGS824</td>
<td>rpoS^+, Cm^R</td>
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<td>fumA–lacZ fusion</td>
<td>Woods &amp; Guest (1987)</td>
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<td>ΔG259</td>
<td>acnB–lacZ fusion</td>
<td>Gruer et al. (1997b)</td>
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(1350 kb) in the E. coli chromosome (see Fig. 1), whereas the acnB gene is situated at 2-85 min (131 kb). The AcnA protein exhibits a remarkable 53 % sequence identity to the bifunctional IRP1 proteins, compared to 29 % identity to the mitochondrial aconitases, and only 17 % to AcnB, the most remote member of the aconitase family. Growth tests have suggested that an acnA mutation is complemented by the resident acnA^+ gene (because it fails to generate a distinct phenotype) whereas an acnB mutation produces a mutant phenotype which is only partially complemented by the acnA^+ gene (Gruer et al., 1997b). Enzymological and regulatory studies with acn–lacZ fusions have further indicated that the synthesis of both enzymes is subject to catabolite and anaerobic repression, and that AcnB is the major citric acid cycle enzyme, whereas AcnA is more important in stationary phase (Gruer & Guest, 1994; Gruer et al., 1997b). Thus it was observed that the aconitase activity of an acnB mutant is reduced to one-third of the wild-type level whereas that of an acnA mutant is little affected. It was also observed that an acnB–lacZ fusion is strongly expressed in the exponential phase whereas a comparable acnA–lacZ fusion is not expressed until early stationary phase (and then only weakly). Furthermore, the acnA–lacZ fusion was specifically induced by redox-stress and by iron via SoxRS- and Fur-activated regulatory mechanisms. Despite its close similarity to IRP1, there is as yet no evidence to suggest that AcnA performs a redox- or iron-dependent regulatory function.

To better understand the relative roles of AcnA and AcnB, the transcriptional organization of the corresponding acn genes was investigated by Northern blot analysis and primer extension mapping. The responses of an acnB–lacZ translational fusion to a variety of transcriptional regulators were also investigated and compared to those of an analogous acnA–lacZ fusion. This showed that the acnB gene is subject to repression by ArcA, Fis and FruR and activation by CRP, whereas the acnA gene is repressed by ArcA and FNR, activated by FruR, CRP, Fur and SoxRS, and initiated by σ^38 in stationary phase.
METHODS

Bacterial strains, plasmids and bacteriophages. The strains of E. coli K-12 are listed in Table 1. The source of acnA DNA was pGS447 (Prodromou et al., 1991) and the corresponding lacA–lacZ translational fusion phage, λG244 (Fig. 1a), was constructed previously (Gruer & Guest, 1994). The plasmid containing the entire acnB coding region (pGS782; Fig. 1b) was constructed previously by ligating a PCR-amplified fragment into pUC119 (Bradbury et al., 1996) and the lacB–lacZ translational fusion phage (λG259; Fig. 1b) was also constructed previously (Gruer et al., 1997b). The proS multicopy plasmid (pGS824) was constructed by ligating a 4.3 kb EcoRI-HindIII fragment containing the proS gene from pMMkatF2 (Mulvey et al., 1988) into pSU18 (Bartolome et al., 1991). Other plasmids and phages are listed in Table 1.

The acn–lacZ fusion phages were established as single-copy prophages in different sets of host strains for investigating the roles of global regulators in acn gene expression. Lysogens were selected initially with ϕ80del9c and monolysogens were identified by their sensitivity to methyl viologen (0.2 mg ml−1) was added 1 h before sampling. Activities (Miller units) were averaged from at least four independent cultures, the variation between samples being <10%.

DNA manipulation, RNA extraction, Northern hybridization and primer extension analysis. E. coli DH5α was the routine transformation host (Table 1) and DNA was prepared and manipulated by standard procedures (Sambrook et al., 1989). RNA was extracted by the hot acid phenol method (Aiba et al., 1989) from bacteria grown in L broth to exponential phase (OD600 0.65) and early stationary phase for acnA–lacZ, in unsupplemented L broth or L broth containing glucose (1%). In experiments involving SoxRS regulation, redox-stress was imposed by adding methyl viologen (0-2 mg ml−1, final concn) 1 h before the cultures were sampled, and in Fur studies, available iron was depleted by adding bipyridyl (200 μM, final concn) at OD660 0.6, 1 h before sampling. Activities (Miller units) were averaged from at least four independent cultures, the variation between samples being <10%.

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![Fig. 1. Restriction maps of the acnA and acnB regions showing the segments of DNA cloned in specific plasmids, the probes used for Northern blot analysis, the oligonucleotides used in primer extension analysis and the structures of the acnA–lacZ and acnB–lacZ fusion phages. Relevant restriction sites are indicated: B, BamHI; E, EcoRI; K, KpnI; P, PstI; S, Sall. The subscript (.) refers to a flanking vector site and engineered sites (Bradbury et al., 1996) are enclosed in square brackets.](image-url)
sampling. Bacteria equivalent to 50 ml exponential culture (OD
600 0.65) were resuspended in 3 ml 20 mM sodium acetate (pH
5.5) containing 0.5% SDS and 1 mM EDTA, and extracted at
60 °C with phenol (equilibrated with 20 mM sodium
acetate, pH 5.5). The phenol extraction of the aqueous extract
was repeated, and the RNA recovered after three successive
ethanol precipitations was dissolved in diethylpyrocarbonate-
treated water.

Samples of RNA (20 μg) denatured with formamide and
formaldehyde, were fractionated electrophoretically in 1%
arose–formaldehyde gels with MOPS buffer and transferred
to nylon membranes using 10 x SSC (Sambrook et al., 1989).
Hybridization with [α-32P]dCTP-labelled probes was as in-
structed in the Pharmacia 'Ready to Go' labelling kit. The probe
for acnA transcripts was a 0.4 kb EcoRI–BamHI fragment of
pGS447 (probe A in Fig. 1a) and the acnB probe was a
0.1 kb EcoRI fragment of pGS782 (probe B in Fig. 1b).

Primer extension analysis was performed according to
Gerischer & Diirre (1992) but modified to allow continuous
incorporation of [α-32P]dCTP. Total RNA (100 μg) was
annealed with 10 pmol primer in 7 μl 10 mM Tris/HC (pH
8.3) plus 5 mM KCl (80 °C for 5 min and cooled slowly,
before incubating for 10 min at 37 °C with 1.5 μl reverse
transcriptase buffer (Life Sciences), 0.5 μl actinomycin D
(500 μg ml-1), 0.5 μl placentale ribonuclease inhibitor
(Promega), 0.5 μl sodium pyrophosphate (80 mM), 3 μl of
a labelling mix containing dATP, dGTP and TTP (each at
5 μM) plus [α-32P]dCTP (9 μM), and 2 μl AMV reverse
transcriptase (50 U; Life Sciences). Thereafter, 3 μl of a
solution containing the four dNTPs (each at 5 mM) was added
and incubation continued for a further 90 min at 37 °C. The
reactions were stopped with 1 μl EDTA (0.5 M), treated with
RNase A (50 μg ml-1) for 5 min, and 150 μl TE was added
prior to phenol/chloroform extraction and ethanol precipi-
tation.) Samples in 4 μl TE plus 3 μl Sequenase Stop Solution
were fractionated by electrophoresis in 6% acrylamide/7 M
urea gels alongside a sequence ladder derived from the
corresponding DNA and primer. The oligonucleotide primers
(see Fig. 1) were: S400 (588–560); S401 (990–961) and S402
(1319–1288) for acnA (numbered according to Prodromou
et al., 1992) and S405 (20324–20295); S404 (19576–19551) and
S403 (19950–19925) for acnB (numbered according to Fujita
et al., 1994).

Materials. Restriction enzymes were from Northumbria Bio-
logicals. AMV reverse transcriptase was from Life Sciences.
Radiolabelled [α-32P]dCTP (110 TBq mmol-1) and nylon
membranes were from Amersham and the RNA calibration
ladder was from Life Technologies.

RESULTS
Transcript analysis for the acnA gene

Northern blot hybridization was used to detect acnA
transcripts in RNA from W3110 (wild-type) using a
400 bp segment of the acnA coding region as the probe
(Fig. 1a). A transcript of approximately 2.8 kb was
detected in RNA from cultures that had been redox-
stressed by treating with methyl viologen 1 h before
reaching stationary phase, but not in untreated sta-
tionary-phase cultures (Fig. 2a). A transcript of the same
size was just detectable in cultures that had been
similarly stressed and sampled whilst still in exponential
phase, but again no transcript could be detected in
comparable untreated cultures (data not shown), nor in
stationary-phase cultures grown with extra iron, 83 μM
(data not shown). These observations are consistent
with those made previously with an acnA–lacZ fusion
which indicated that acnA gene expression is low, even
at its highest level in stationary phase, but can be
increased in a soxRS-dependent manner by methyl-
viologen-induced redox-stress (Gruer & Guest, 1994).

Transcriptional start sites for the acnA gene were
defined by primer extension analysis using RNA from
early-stationary-phase cultures of W3110 and three
primers spanning the 1003 bp cysB–acnA intergenic
region (Fig. 1a). Two promoters, P1acnA and P2acnA,
were identified by a pair of start sites 407 and 403 bp
upstream of the acnA coding region and a single start
site 50 bp upstream of the coding region, respectively
(Fig. 3a, b; Fig. 4a). The same set of start sites was
observed with RNA samples from both redox-stressed
and unstressed stationary-phase cultures, despite the
failure to detect transcripts in the latter samples by
hybridization (Table 2). It is presumed that transcripts
of low abundance are more readily detected by primer
extension analysis. In contrast, primer extension analy-
sis with RNA from exponential-phase cultures indicated
that P2acnA is active in both redox-stressed and un-
stressed cultures but no evidence was found for tran-
scription from P1acnA in either type of exponential-phase
culture (Table 2). This suggests that the higher level
of acnA–lacZ expression observed in stationary phase may
be generated from P1acnA. No other transcriptional start
sites having the same clockwise polarity were detected in
the long intergenic region upstream of P1acnA. When the
sizes predicted for the corresponding monocistronic
acnA transcripts, 3–130 and 3–126 kb from P1acnA and
2–773 kb from P2acnA were compared with the relative
activities of the promoters under different conditions
(Table 2), it is concluded that P2acnA is the source of the
major transcript (2.8 kb approx.) detected in redox-
stressed cultures.

The slightly shorter and least abundant transcript
generated by P1acnA probably arises by primer slippage
during RNA chain initiation. This is because initiation
normally involves the formation of many short, loosely
bound, abortive transcripts (< 9 nucleotides), which can
be translocated before a productive transcript escapes
the promoter (Chamberlin & Hsu, 1996). As can be seen
in Fig. 4(a), a 5-nucleotide transcript initiating at the
upstream site (407 bp from the coding region) and
having TG(UG) at its 3' end, might translocate 4 bp
downstream to a position where the TG motif is
repeated. This would generate a transcript correspond-
ing to the minor transcript which appears to have
initiated 4 bp downstream from the major site.
The corresponding promoter region lacks −35 and −10
hexamers resembling those of σ70 or σ87-dependent
promoters. Instead, it contains sequences that more
closely resemble a gearbox promoter (Vicente et al.,
1991), CTGcaaA and CAGCGAAGC separately by 13 bp
(Fig. 4a), compared to the gearbox consensus: CTGGCA
and CAGCAGTA separated by 14 to 16 bp.
The second acnA promoter region (P2<sub>acnA</sub>) contains a well-placed −10 hexamer which matches the σ<sup>70</sup> consensus at four positions, but there is no corresponding −35 hexamer (Fig. 4a). However, it resembles an 'extended −10 promoter' because the −10 hexamer is preceded by a TGn motif, which is known to increase promoter strength and even to allow transcription initiation in the absence of a −35 hexamer (Kumar et al., 1993). Another potential P2<sub>acnA</sub> promoter sequence contains −35 (TTATCA) and −10 (TGTAT) hexamers (marked by dashed overlines in Fig. 4a), but these are sub-optimally separated from each other (19 bp) and from the transcription start site (9 bp).

Transcript analysis for the acnB gene

Northern blot hybridization was used with a probe from the 5' end of the acnB coding region (Fig. 1b) for analysing acnB transcription. A single transcript of approximately 2.5 kb was detected in samples of RNA from exponential-phase cultures but no transcripts could be found in early-stationary-phase samples (Fig. 2b). This pattern of expression was unaffected by redox-stress (data not shown), and it is consistent with previous observations that AcnB activity and acnB expression increase early in the growth cycle but decline in stationary phase (Gruer et al., 1997b).

Primer extension analysis with RNA from exponential-phase cultures and three different primers designed to span the yacH-acnB intergenic region (Fig. 1b) indicated that acnB transcription is initiated 96 and 95 bp up-stream of the coding region (Figs 3c, 4b). The sizes predicted for the monocistronic acnB transcripts extending to a putative rho-independent acnB terminator are 2761 and 2760 kb, in reasonable agreement with the 2.5 kb transcript detected by Northern blotting. The start sites are preceded by a potential promoter, P<sub>acnB</sub>, having −35 and −10 hexamers differing at only one and two positions (respectively) from those of the canonical σ<sup>70</sup> sequences (Fig. 4b). The −10 region also resembles that of an 'extended −10 promoter' in which the −10 hexamer is preceded by a TGn motif (Kumar et al., 1993).

Studies with acn-lacZ translational fusions

Studies on the expression of individual acn genes were made with single-copy acn-lacZ translational fusions in which the acnA or acnB promoters accompanied by parts of the corresponding coding regions are linked 'in phase' to the lacZ coding region and transferred to phage λ: λG244, λacnA-lacZ and λG259, λacnB-lacZ (Table 1, Fig. 1). The fusion phages were established as prophages in a variety of lacZ deletion hosts such that the β-galactosidase activities reflect the overall changes in the transcription and translation of the acn genes. The effects of global regulators were generally assessed by comparing a parental monolysogen, acnA<sup>+</sup>B<sup>+</sup>Δlac (λacn−lacZ), with derivatives lacking the regulator, and others containing multiple copies of the regulatory gene.

Regulation of acn gene expression by CRP. Previous studies indicated that AcnA and AcnB are both subject to...
Table 2. Primer extension analysis of transcription from two acnA promoters

<table>
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<tr>
<th>Promoter</th>
<th>Exponential phase</th>
<th>Stationary phase</th>
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<tbody>
<tr>
<td></td>
<td>− + MV</td>
<td>− + MV</td>
</tr>
<tr>
<td>P1acnA</td>
<td></td>
<td>− + MV</td>
</tr>
<tr>
<td>P2acnA</td>
<td>+ + + + + +</td>
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Regulation of acn gene expression by SoxRS and Fur. The acnA gene was previously shown to be induced by redox-stress via the SoxRS system, repressed by iron-depletion, and activated by the ferric uptake regulator, Fur (Gruer & Guest, 1994). In contrast, expression of the acnB gene appeared to be essentially independent of these regulatory mechanisms. The activity of the acnB-lacZ fusion was little affected by soxRS deletion (Fig. 5b); the inducing effects of methyl viologen, particularly apparent in the mutant background, could be due to an indirect compensatory mechanism that enhances AcnB synthesis during severe oxidative stress. Expression of the acnB-lacZ fusion was lowered to a limited extent during iron-depletion with bipyridyl (Fig. 5c). However, the failure of the multicopy fur+ plasmid to reverse the small derepression observed in the fur mutant indicated that acnB is neither activated nor repressed by Fur.

Regulation of acn gene expression by ArcA and FNR. Studies with an acnA-lacZ fusion previously showed that the acnA gene is anaerobically repressed by ArcA (Gruer & Guest, 1994; Gruer et al., 1997b). However, this repression was only partially reversed (38%) by deleting the arcA gene, suggesting that some other mechanism of anaerobic repression might be operating. It was also concluded that any involvement of FNR must be indirect (or operational only in the absence of ArcA), because acnA expression was not enhanced in an fnr mutant, but it was partially repressed under aerobic conditions by fnr+ amplification in an arcA+ strain (Gruer & Guest, 1994). When further investigated with an arcA fnr double mutant, it was found that the aerobic expression of the acnA-lacZ fusion is partially and equally repressed (42% of the parental level) by multiple copies of either regulatory gene, arcA+ or fnr+ (Fig. 6a). However, the severe anaerobic repression in the parental strain was almost fully (92%) reversed in the double mutant but reimposed by multiple copies of each regulatory gene (Fig. 6a). This indicates that acnA expression is anaerobically repressible by ArcA and FNR, the effect of FNR being masked by ArcA in an fnr

catabolite repression, and evidence for the CRP-dependent activation of the acnA gene came from the 3.5-fold lowering of acnA-lacZ expression in a crp mutant and the enhanced activity after transformation with a crp+ multicopy plasmid (Gruer & Guest, 1994; Gruer et al., 1997b). The activity of the single-copy acnB-lacZ fusion was likewise shown to be reduced 2.7-fold in a crp deletion strain and restored (but not enhanced) by the multicopy crp+ plasmid (Fig. 5a). The activities of the crp+ strains were also reduced approximately twofold by adding glucose (1%). Thus it was concluded that the cAMP-CRP complex is required to activate transcription of both the acnA and acnB genes.
mutant but more apparent in an arcA mutant. As concluded previously by Gruer & Guest (1994), the effect of FNR is not simply due to its known ability to activate arcA transcription (Compan & Touati, 1994).

The responses obtained with the acnB–lacZ fusion indicated that ArcA functions as an anaerobic repressor of acnB transcription and that the acnB gene is not regulated by FNR. The aerobic expression was seemingly poised in the working range of ArcA because the activity was enhanced 1.25-fold in the arcA deletion strain but repressed 2-fold in the multicopy arcA host (Fig. 6b). The parental activity was anaerobically repressed 4.3-fold and further repressed by multicopy arcA+, but this repression was abolished in the arcA deletion strain (Fig. 6b). In contrast, the effects of inactivating or amplifying the fnr+ gene were insignificant under aerobic and anaerobic conditions (Fig. 6c). However, this simple picture of ArcA-mediated anaerobic repression was complicated by the behaviour of the acnB–lacZ fusion in the arcA fnr double mutant (Fig. 6d). First, there was no significant anaerobic derepression of acnB–lacZ activity in the double mutant, suggesting that in the absence of both regulators, some other repressor might invade the acnB promoter. Second, multiple copies of the fnr+ gene lowered aerobic and anaerobic expression to 68% and 58% of the respective parental levels, suggesting that in the absence of ArcA, amplified FNR can interact negatively at the acnB promoter.

**Regulation of the acn and fum gene expression by σ^{38}.** The role of σ^{38} in aconitase gene expression was investigated because acnA is not maximally expressed until stationary phase (Gruer et al., 1997). Accordingly, it was found that acnA–lacZ expression is lowered fivefold by disrupting the rpoS gene and restored by a multicopy rpoS+ plasmid (Fig. 7a), indicating that the acnA gene is subject to σ^{38}-mediated transcription initiation. In contrast, expression of the acnB–lacZ fusion was enhanced 2.4-fold in the rpoS mutant and restored to the parental level by the multicopy rpoS+ plasmid (Fig. 7b). The apparent negative control of acnB by σ^{38} will be discussed below.
The present studies were extended to include the *fumA* and *fumB* genes which encode analogous [4Fe-4S]-containing aerobic and anaerobic fumarases (*FumA* and *FumB*, respectively), and the *fumC* gene, which encodes an oxygen-stable enzyme (*FumC*) that closely resembles the mitochondrial fumarases (Woods *et al.*, 1986; Bell *et al.*, 1989). The patterns of activity observed with the *fum–lacZ* fusions indicated that *fumA* expression is $\sigma^{38}$-independent (Fig. 7c). The very low aerobic expression of the *fumB–lacZ* fusion was likewise unaffected by $\sigma^{38}$ (data not shown). However, despite its apparent $\sigma^{38}$-independence, expression of the *fumC* fusion was activated ninefold by amplifying $\sigma^{38}$ (Fig. 7d).

**Regulation of acn gene expression by Fis.** The effect of Fis on *acn* gene expression was examined because Fis-regulated genes are, like *acnB*, strongly expressed in mid-exponential phase, and also because Fis has been shown to repress the succinate dehydrogenase (*sdh*) and NADH dehydrogenase II (*nbd*) genes (Finkel & Johnson, 1992; Xu & Johnson, 1995; Green *et al.*, 1996). Deleting the *fis* gene had no effect on *acnA–lacZ* expression (Fig. 8a) but it was accompanied by a twofold increase in *acnB–lacZ* expression (Fig. 8b), indicating that *acnB* is repressed by Fis.

**Regulation of acn gene expression by FruR.** FruR (also known as Cra) regulates the expression of many genes involved in carbon metabolism. Thus, it activates the isocitrate lyase, malate synthase and isocitrate dehydrogenase genes, and represses those encoding glycolytic enzymes (Rameier *et al.*, 1993; Saier & Rameier, 1996). Studies with the *acn–lacZ* fusions indicated that *acnA* is subject to Fru-mediated activation (Fig. 8c), whereas the *acnB* gene is repressed by FruR (Fig. 8d). Furthermore, these effects were still observed in the presence of glucose, which is presumed to abolish CRP-mediated activation. Indeed, the combined effect of eliminating FruR- and CRP-mediated activation was a 10-fold lowering of *acnA* expression (Fig. 8c). Conversely, *acnB* expression was enhanced eightfold by abolishing FruR- and glucose-mediated repression (Fig. 8d).

**DISCUSSION**

The factors controlling the expression of the *acnA* and *acnB* genes of *E. coli* are summarized in Fig. 9. Both genes are subject to ArcA-mediated anaerobic repression and CRP-mediated catabolite repression, but they differ insofar as *acnA* gene transcription is activated by Fur, SoxRS and FruR, can be initiated by $\sigma^{38}$, and is repressed by FNR, whereas the *acnB* gene is repressed by Fis and FruR but unaffected by the other regulators. The *acnA* gene is only weakly expressed compared to the *acnB* gene, even after redox-stress induction. This is reflected in the relatively small effect of *acnA* mutation on aconitase specific activity compared with that inflicted by *acnB* mutation (Gruer *et al.*, 1997b). It is also consistent with the observation that using Northern blotting, *acnA* transcripts (2.8 kb) could only be detected in redox-stressed cultures. These transcripts were correlated with one of the two *acnA* promoters ($P_2^{acnA}$) which is responsible for the basal level of *acnA* transcription that occurs throughout the growth cycle as well as that induced by redox-stress (Table 2; Figs 4a, 9).
Although no transcript initiating at P1acnA could be detected by Northern blotting, this upstream promoter is apparently responsible for the increase in acnA expression that occurs as cultures enter stationary phase (Table 2). In contrast, acnB gene expression was solely dependent on transcription from a single promoter, P1acnB (Figs 4b, 9).

Regulation of acnA gene transcription

The upstream acnA promoter (P1acnA) resembles a so-called gearbox promoter, which often controls genes whose transcription is inversely proportional to growth rate and are positively regulated by $\sigma^m$ (Vicente et al., 1991). Furthermore, $\sigma^m$ is an alternative sigma factor used for the activation of genes concerned with general stress conditions as well as stationary-phase survival (Hengge-Aronis, 1993, 1996). The $\sigma^m$-dependent activation of acnA transcription from P1acnA may thus form part of a more general stress-induced response.

The second promoter (P2acnA) more typically resembles a factor-dependent $\sigma^p$ promoter, by virtue of its poor -35 region (Fig. 4a). This promoter is the site of SoxRS-mediated activation, but the absence of a convincing SoxS binding site (Li & Demple, 1996) upstream of P2acnA suggests that the mode of activation might be indirect. There is a potential CRP site centred at -40.5, resembling a CRP (class II) activated promoter (Busby & Kolb, 1996; Fig. 4a), which could account for the observed CRP-dependence of acnA expression. This site is overlapped by potential ArcA and FNR binding sites (centred at -37.5 and -40.5, respectively). Attention has previously been drawn to this ArcA site by Lynch & Lin (1996) even though there are a further 10 potential ArcA sites matching the consensus at $\geq$7 out of 10 positions, in the untranscribed intergenic region. It is not known which are responsible for the well-established ArcA-mediated anaerobic repression of acnA (Gruer & Guest, 1994). The repressing role of FNR that operates in the absence of ArcA was here confirmed with an arcA fnr double mutant. It might be mediated by the overlapping arrangement of the two binding sites, if ArcA has the higher affinity for this region and thus normally displaces FNR (Fig. 4a). However, the situation may be more complex, because FNR centred at -40.5 might be expected to activate rather than repress, and because FNR-mediated repression often involves FNR interactions at two or more sites (Guest et al., 1996). It is therefore possible that other potential FNR sites centred at -76.5 and -247.5 (Fig. 4a) may be associated with the observed repression. Whether FNR performs a modulating or other role in the regulation of acnA expression in an arcA+ strain background has yet to be determined.

The presence of potential Fur sites (centred at -321, -318, -315 and -229 relative to the P2acnA transcriptional start site) seemed to be consistent with the established Fur- and iron-dependence of acnA gene expression (Gruer & Guest, 1994). However, despite matching the Fur site consensus at 12–14 out of 19 positions, recent searches using a scoring matrix as established ArcA-mediated anaerobic repression of acnA (Gruer et al., 1993). The repressing role of FNR that is normally displaced FNR (Fig. 4a). However, despite matching the Fur site consensus at 12–14 out of 19 positions, recent searches using a scoring matrix assembled from 16 E. coli Fur sites revealed no significant matches. It is therefore concluded that the regulation of acnA by Fur is indirect. FruR (Cra) is a transcriptional regulator which, together with CRP, controls the expression of genes involved in C metabolism, particularly with respect to the direction of metabolic flow, glycolysis being repressed whereas gluconeogenesis is activated (Ramsier et al., 1993; Saier & Ramsier, 1996). The acnA gene, like genes encoding some other citric acid and glyoxylate cycle enzymes, is activated by FruR. However, it is significant that acnB is repressed by FruR (see below): no potential FruR binding sites were detected in either promoter region.

Regulation of acnB gene transcription

Apart from being activated by CRP and repressed by ArcA, the acnB gene exhibited a very different pattern of regulation to that of acnA. The promoter region contains only one potential CRP binding site, centred at a position (-37.5) that is normally associated with repression rather than activation (Fig. 4b; Busby & Kolb, 1996). In contrast, there are many putative ArcA sites, which match the binding site consensus at 7–8 out of 10 positions (Lynch & Lin, 1996) and could thus be associated with the ArcA-mediated anaerobic repression of acnB. Seven such sites are shown in Fig. 4(b) and there are three more located further upstream. The observed decline in acnB expression and AcnB activity observed in stationary phase (Gruer et al., 1997b) is interesting because it could be related to the ArcA-mediated repression of citric acid cycle enzymes during carbon starvation, as part of a general survival strategy aimed at
conserving endogenous reserves and limiting the production of oxygen radicals by continued aerobic respiratory activity (Nyström et al., 1996).

The observed derepression of acnB in the rpoS mutant and reversal by the rpoS+ plasmid (Fig. 7b) suggests that the acnB gene is repressed by σ88. Possible explanations are that: (i) free σ88 could bind directly to promoter DNA, as has been shown for the σ38 protein (Buck & Cannon, 1992); (ii) RNA polymerase core enzyme, no longer bound to σ38 in the rpoS mutant, may become available for use with σ70 and thus enhance transcription at P_acnB and other σ70 promoters; or (iii) σ38 polymerase may function indirectly by activating the synthesis of an acnB repressor. The reason why FurR represses acnB but activates acnA is unclear. It may be related to the preferential use of one of the two enzymes in the citric acid and glyoxylate cycles or in stress responses. Fis (the factor for inversion stimulation) was originally identified by virtue of its role in site-specific DNA inversion, and its function indirectly by activating the synthesis of an acnB repressor. The reason why FurR represses acnB but activates acnA is unclear. It may be related to the preferential use of one of the two enzymes in the citric acid and glyoxylate cycles or in stress responses. Fis (the factor for inversion stimulation) was originally identified by virtue of its role in site-specific DNA inversion, but is now increasingly associated with the activation and repression of gene expression, presumably via its ability to induce DNA-bending (Xu & Johnson, 1995). The acnB promoter region contains three potential Fis binding sites (Fig. 4b). One of the sites overlaps the −35 hexamer, but occluding RNA polymerase is not the only mechanism that has been proposed for Fis-mediated repression.

The existence of two aerobic but differentially regulated aconitases parallels that of the fumarases, where there are major (FumA) and minor (FumC) aerobic enzymes, encoded by adjacent genes (fumA-fumC), and an anaerobic enzyme (FumB). It is now apparent from studies with the corresponding lacZ fusions that the fumA promoter resembles P_acnB in being anaerobically repressed by ArcA, whereas the fumC promoter resembles P_acnC in its dual regulation by ArcA and FNR, although there is evidence that fumC transcription is partially facilitated by readthrough from the upstream fumA gene promoter (Woods & Guest, 1987; Guest & Russell, 1992; Gruer & Guest, 1994; Park & Gunalsus, 1995). The relationship is further strengthened by showing that the genes (fumC and acnA) encoding the minor aerobic enzymes are alone activated by SoxRS and σ88. However, it breaks down with respect to Fur, which activates the minor aconitase gene (acnA) but not the minor fumarase gene (fumC) except by readthrough transcription from its primary activation target, the gene encoding the major fumarase (fumA). Nevertheless, it is clear that for aconitase and fumarase, there are major aerobic enzymes (AcnB and FumA) whose transcriptional regulation is typical of citric acid cycle enzymes, whereas the minor enzymes (AcnA and FumC) are adapted for stress-related responses (activation by SoxRS and transcription by σ88). Current work is aimed at characterizing the putative third aconitase (AcnC), the minor residual aconitase activity of an acnA acnB double mutant, and ascertaining whether AcnA (or AcnB) perform iron-dependent regulatory functions analogous to mammalian IRP1.

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**REFERENCES**


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