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 ($P1_{acnA}$) and 50 bp ($P2_{acnA}$) upstream of the *acnA* coding region, and one *acnB* promoter (P_{acnB}) 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 σ^{38} from $P1_{acnA}$, and from $P2_{acnA}$ 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 P_{acnB} . Comparable studies with *fum-lacZ* fusions indicated that transcription of *fumC*, but not of *fumA* or *fumB*, is initiated by RNA polymerase containing σ^{38} . 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

Strain or plasmid	Relevant characteristic	Source or reference*
W3110	Prototroph	Laboratory collection
DH5 α	$\Delta(\arg F-lac)U169$ ($\phi 80\Delta lacZM15$) <i>recA</i>	Sambrook <i>et al.</i> (1989)
MC1000	$\Delta lacX74$ $\Delta(araA-leu)$	Silhavy <i>et al.</i> (1984)
JRG1999	MC1000 $\Delta crpT8$	S. T. Cole ¹
RK4353	$\Delta(\arg F-lac)U169$	V. Stewart ²
RK5279	RK4353 <i>fmr-250</i>	V. Stewart ²
RJ1802	RK4353 <i>fis-767</i>	Ball <i>et al.</i> (1992)
JRG3227	RK4353 <i>fruR</i>	Park <i>et al.</i> (1995)
MC4100	$\Delta(\arg F-lac)U169$ <i>rpsL</i>	Silhavy <i>et al.</i> (1984)
H1941	MC4100 Δfur	K. Hantke ³
LM1A	MC4100 <i>arcA1 zjj::Tn10</i>	McWalter <i>et al.</i> ⁴
RM315	MC4100 $\Delta fmr arcA1 zjj::Tn10$	Sawers & Suppmann (1992)
RH90	MC4100 <i>rpoS359::Tn10</i>	R. Henнге-Aronis ⁵
GC4468	$\Delta(\arg F-lac)U169$ <i>rpsL sup</i>	Greenberg <i>et al.</i> (1990)
DJ901	GC4468 $\Delta soxRS$	Greenberg <i>et al.</i> (1990)
pGS447	pUC119, <i>acnA</i> ⁺ , Ap ^R	Prodromou <i>et al.</i> (1991)
pGS782	pUC119, <i>acnB</i> ⁺ , Ap ^R	Bradbury <i>et al.</i> (1996)
pGS279	<i>crp</i> ⁺ , Ap ^R Cm ^R	Spiro <i>et al.</i> (1989)
pCH21	<i>fmr</i> ⁺ , Ap ^R Cm ^R	C. H. Higgins ⁶
pRB38	<i>arcA</i> ⁺ , Cm ^R	R. Buxton ⁷
pMH15	<i>fur</i> ⁺ , Cm ^R	K. Hantke ³
pGS824	<i>rpoS</i> ⁺ , Cm ^R	This work
λ G185	<i>fumA-lacZ</i> fusion	Woods & Guest (1987)
λ G186	<i>fumB-lacZ</i> fusion	Woods & Guest (1987)
λ G187	<i>fumC-lacZ</i> fusion	Woods & Guest (1987)
λ G244	<i>acnA-lacZ</i> fusion	Gruer & Guest (1994)
λ G259	<i>acnB-lacZ</i> fusion	Gruer <i>et al.</i> (1997b)

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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 *acnB*⁺ 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 expo-

mental 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 *lacnA-lacZ* translational fusion phage, λ G244 (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 *lacnB-lacZ* translational fusion phage (λ G259; Fig. 1b) was also constructed previously (Gruer *et al.*, 1997b). The *rpoS* multicopy plasmid (pGS824) was constructed by ligating a 4.3 kb *EcoRI-HindIII* fragment containing the *rpoS* gene from pMMkatF2 (Mulvey *et al.*, 1988) into pSU18 (Bartolomé *et al.*, 1991). Other plasmids and phages are listed in Table 1.

The *lacn-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 *λh80del9c* and monolysogens were identified by their sensitivity to *λcl90c17* and *λvir*. As described in previous studies with the *lacnA-lacZ* fusion phage (Gruer & Guest, 1994), sets of *lacnB-lacZ* monolysogens were assembled for each regulator, consisting of a parental strain, a regulator mutant, and a derivative of the latter transformed with a multicopy plasmid expressing the wild-type regulator gene, thus: CRP [MC1000, JRG1999, and JRG1999(pGS279)]; ArcA [MC4100, LM1A, and LM1A(pRB38)]; FNR [RK4353, RK5279, and RK5279(pCH21)]; FNR + ArcA [MC4100, RM315(*fnr arcA*), and RM315(pRB38) or RM315(pCH21)]; Fur [MC4100, H1941, and H1941(pMH15)] and σ^{38} [MC4100,

RH90, and RH90(pGS824)]. For Fis and FruR, *lacnB-lacZ* monolysogens of the parental strain (RK4353) and respective regulator mutants (RJ1802 and JRG3227) were used. Analogous sets of monolysogens were isolated for the FNR + ArcA, σ^{38} , Fis and FruR series with *lacnA-lacZ* (λ G244), and for the σ^{38} series with three *λfum-lacZ* fusion phages: *λfumA-lacZ* (λ G185); *λfumB-lacZ* (λ G186) and *λfumC-lacZ* (λ G187), as previously described (Gruer & Guest, 1994).

Microbiology and enzymology. The basic microbiological methods and media have been described previously (Gruer *et al.*, 1997b). β -Galactosidase was assayed according to Miller (1972) using monolysogens containing *lacZ* translational fusion phages grown to late exponential phase for *lacnB-lacZ* and *λfum-lacZ*, or early stationary phase for *lacnA-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 μ M, final concn) at OD₆₅₀ 0.6, 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* DH5 α 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.*, 1981) from bacteria grown in L broth to exponential phase (OD₆₅₀ 0.65) and early stationary phase (OD₆₅₀ 2.0): where stated, methyl viologen (0.2 mg ml⁻¹) was added 1 h before

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, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sal*I. The subscript (,) refers to a flanking vector site and engineered sites (Bradbury *et al.*, 1996) are enclosed in square brackets.

sampling. Bacteria equivalent to 50 ml exponential culture (OD_{650} 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% agarose-formaldehyde gels with MOPS buffer and transferred to nylon membranes using $10 \times$ SSC (Sambrook *et al.*, 1989). Hybridization with [α - 32 P]dCTP-labelled probes was as instructed in the Pharmacia 'Ready to Go' labelling kit. The probe for *acnA* transcripts was a 0.4 kb *EcoRI*–*Bam*HI 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 & Dürre (1992) but modified to allow continuous incorporation of [α - 32 P]dCTP. Total RNA (100 µg) was annealed with 10 pmol primer in 7 µl 10 mM Tris/HCl (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 placental 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 - 32 P]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 precipitation. 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 Biologicals. AMV reverse transcriptase was from Life Sciences. Radiolabelled [α - 32 P]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 stationary-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 methylviologen-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, *P*_{*acnA*} and *P*_{*acnA*}, 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 analysis with RNA from exponential-phase cultures indicated that *P*_{*acnA*} is active in both redox-stressed and unstressed cultures but no evidence was found for transcription from *P*_{*acnA*} 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 *P*_{*acnA*}. No other transcriptional start sites having the same clockwise polarity were detected in the long intergenic region upstream of *P*_{*acnA*}. When the sizes predicted for the corresponding monocistronic *acnA* transcripts, 3.130 and 3.126 kb from *P*_{*acnA*} and 2.773 kb from *P*_{*acnA*}, are compared with the relative activities of the promoters under different conditions (Table 2), it is concluded that *P*_{*acnA*} 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 *P*_{*acnA*} 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 corresponding 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 σ^{70} - or σ^{38} -dependent promoters. Instead, it contains sequences that more closely resemble a gearbox promoter (Vicente *et al.*, 1991), CTGgcA and CaGCAAGcc separated by 13 bp (Fig. 4a), compared to the gearbox consensus: CTGCAA and CGGC^A_TAGTA separated by 14 to 16 bp.

Fig. 2. Northern blot hybridization showing transcripts of the aconitase genes. (a) The *acnA* probe was used with RNA extracted from cultures of W3110 entering stationary phase in the absence or presence of methyl viologen (lanes 1 and 2, respectively). (b) The *acnB* probe was used with RNA extracted from exponential-phase or early-stationary-phase cultures of W3110 (lanes 3 and 4, respectively). The RNA standards (kb) provide calibrations for the hybridizing transcripts (arrowed).

Fig. 3. Primer extension mapping of the transcriptional start sites of the *acn* genes. The start sites (*) associated with specific promoters are shown with sequence ladders for their location in Fig. 4: (a) $P1_{acnA}$, (b) $P2_{acnA}$, and (c) P_{acnB} . In (a) there is a 0.5 bp discrepancy between the sequence ladder and the primer extension products, but the validity of the interpretation is supported by other experiments.

The second *acnA* promoter region ($P2_{acnA}$) contains a well-placed -10 hexamer which matches the σ^{70} 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 $P2_{acnA}$ promoter sequence contains -35 (TTATCA) and -10 (TGTTAT) 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 2.761 and 2.760 kb, in reasonable agreement with the 2.5 kb transcript detected by Northern blotting. The start sites are preceded by a potential promoter, P_{acnB} , having -35 and -10 hexamers differing at only one and two positions (respectively) from those of the canonical σ^{70} 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 : λ G244, *lacnA-lacZ* and λ G259, *lacnB-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⁺B⁺ Δ lac* (*lacn-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

Fig. 4. Nucleotide sequences upstream of the *acnA*- and *acnB*-coding regions. The transcriptional start sites and translational initiation sites (bold), the -35 and -10 hexamers of putative promoters (boxed), alternative hexamers for *P2_{acnA}* (dashed overlined), ribosome binding sites (RBS), and potential binding sites for FNR (black box), CRP (bold overlined), ArcA (underlined) and Fis (dashed underlined), are shown for (a) *acnA* (co-ordinates 701–1250; Prodromou *et al.*, 1992) and (b) *acnB* (co-ordinates 19951–20250; Fujita *et al.*, 1994).

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.

Table 2. Primer extension analysis of transcription from two *acnA* promoters

The results of primer extension analysis are summarized for *P1_{acnA}* and *P2_{acnA}* using RNA extracted from L-broth cultures harvested in exponential phase (OD₆₅₀ 0.65) or early stationary phase (OD₆₅₀ 2.0) without (–) and with (+MV) the addition of methyl viologen (0.2 mg ml⁻¹) 1 h before harvesting. The relative amounts of product formed (–/+ /+ /+ /+) refer to individual promoters, not to comparisons between promoters.

Promoter	Exponential phase		Stationary phase	
	–	+MV	–	+MV
<i>P1_{acnA}</i>	–	–	+	+
<i>P2_{acnA}</i>	+	+	+	+++

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 (to 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*

Fig. 5. Effects of CRP, SoxRS and Fur on *acnB-lacZ* expression. The β -galactosidase activities of aerobically grown late-exponential-phase L-broth cultures of *lacnB-lacZ* (λ G259) monolysogens having wild-type (■), regulator mutant (▨), and mutant plus multicopy regulator plasmid (□) backgrounds, were compared for the effects of: (a) *crp*; glucose (1%, w/v) was added to the growth medium where indicated; (b) *soxRS*; redox-stress was imposed by adding methyl viologen (0.2 mg ml⁻¹) 1 h before harvesting; and (c) *fur*; iron was depleted by adding bipyridyl (200 μ M) 1 h before harvesting where indicated.

Fig. 6. Effects of ArcA and FNR on *acnA-lacZ* and *acnB-lacZ* expression. β -Galactosidase activities of stationary-phase cultures of *lacnA-lacZ* (λ G244) monolysogens, and late-exponential-phase cultures of *lacnB-lacZ* (λ G259) monolysogens, were compared after aerobic and anaerobic growth in L broth. The fusion prophages were established in wild-type (■), *arcA* and *fnr* single or double mutant (▨), and mutant containing multicopy *arcA*⁺ (□) or *fnr*⁺ (▨) plasmids, as backgrounds for studying the effects of: (a) *arcA fnr* double mutation on *acnA-lacZ* expression; (b) *arcA* on *acnB-lacZ*; (c) *fnr* on *acnB-lacZ*; and (d) *arcA fnr* double mutation on *acnB-lacZ* expression.

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.

Fig. 7. Effects of σ^{38} on the expression of single-copy *acnA*-, *acnB*-, *fumA*- and *fumC*-*lacZ* fusions. The β -galactosidase activities were assayed after aerobic growth in L broth. Monoclonal derivatives of a parental strain (■), an *rpoS* mutant (▨) and an *rpoS* mutant transformed with a multicopy *rpoS*⁺ plasmid (□), contained the following fusion prophages: (a) λ *acnA*-*lacZ*; (b) λ *acnB*-*lacZ*; (c) λ *fumA*-*lacZ* and (d) λ *fumC*-*lacZ*.

Fig. 8. Effects of Fis and FruR on the expression of single-copy *acnA*- and *acnB*-*lacZ* fusions. The β -galactosidase activities were assayed after aerobic growth in L broth (*fis*) or in L broth with (+) or without (-) glucose (*fruR*). Monoclonal derivatives of parental (■) and mutant strains (▨) were compared for the effects of: (a) *fis* on *acnA*-*lacZ*; (b) *fis* on *acnB*-*lacZ*; (c) *fruR* on *acnA*-*lacZ*; and (d) *fruR* on *acnB*-*lacZ* expression.

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 σ^{38} -independent (Fig. 7c). The very low aerobic expression of the *fumB*-*lacZ* fusion was likewise unaffected by σ^{38} (data not shown). However, despite its apparent σ^{38} -independence, expression of the *fumC* fusion was activated ninefold by amplifying σ^{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 (*ndh*) 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 (Ramseier *et al.*, 1993; Saier & Ramseier, 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 σ^{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).

Fig. 9. Regulation of the aconitase genes of *E. coli*. The effects of global regulators on the activities of specific promoters, (a) *acnA* and (b) *acnB*, are indicated. The broken arrows denote that the involvement of the regulator may be indirect.

Although no transcript initiating at $P1_{acnA}$ 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, P_{acnB} (Figs 4b, 9).

Regulation of *acnA* gene transcription

The upstream *acnA* promoter ($P1_{acnA}$) resembles a so-called gearbox promoter, which often controls genes whose transcription is inversely proportional to growth rate and are positively regulated by σ^{38} (Vicente *et al.*, 1991). Furthermore, σ^{38} 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 σ^{38} -dependent activation of *acnA* transcription from $P1_{acnA}$ may thus form part of a more general stress-induced response.

The second promoter ($P2_{acnA}$) more typically resembles a factor-dependent σ^{70} 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 $P2_{acnA}$ 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 *arcA* 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 ≥ 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 fur* 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 $P2_{acnA}$ 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 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 (Ramseier *et al.*, 1993; Saier & Ramseier, 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 σ^{38} . Possible explanations are that: (i) free σ^{38} could bind directly to promoter DNA, as has been shown for the σ^{54} protein (Buck & Cannon, 1992); (ii) RNA polymerase core enzyme, no longer bound to σ^{38} in the *rpoS* mutant, may become available for use with σ^{70} and thus enhance transcription at *P*_{acnB} and other σ^{70} promoters; or (iii) σ^{38} polymerase may function indirectly by activating the synthesis of an *acnB* repressor. The reason why FruR 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*_{2_{acnA}} 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 & Gunsalus, 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 σ^{38} . 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 σ^{38}). 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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