Two genetically-distinct and differentially-regulated aconitases (AcnA and AcnB) in *Escherichia coli*

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An *acnA* mutant of *Escherichia coli* was constructed by replacing the chromosomal *acnA* gene by an internally deleted derivative containing a *kan*^R cassette. Southern and Western blotting confirmed that the *acnA* gene had been replaced by the disrupted gene and that the aconitase A protein was no longer expressed. However, the mutant failed to exhibit the anticipated glutamate auxotrophy and it retained a residual aconitase activity. This activity was due to an analogous unstable enzyme(s) designated aconitase B. Studies on the regulation of aconitase A synthesis using an *acnA-lacZ* translational fusion showed that the *acnA* gene resembles other citric acid cycle genes in being subject to CRP-mediated catabolite repression and ArcA-mediated anaerobic repression. In addition to being activated by the SoxRS oxidative stress regulatory system, the *acnA* gene appeared to be activated by the ferric uptake regulator (Fur). It was concluded that the *acnA* gene belongs to at least four global regulatory networks, *crp*, *arcA*, *fur* and *soxRS*. In contrast, the aconitase B activity decreased after exposure to oxidative stress and was less affected by anaerobiosis. Comparable studies with the fumarase genes (*fumA*, *B* and *C*) indicated that *fumA* (encoding the unstable aerobic iron-sulphur-containing fumarase) is activated by the ferric uptake regulator (Fur) and *fumC* (encoding the stable fumarase) is activated by the SoxRS oxidative stress regulatory system.

Keywords: aconitase, *Escherichia coli*, citric acid cycle, fumarase, Fur-mediated transcriptional regulation

INTRODUCTION

Aconitase (EC 4.2.1.3) is a dehydratase-hydratase which catalyses the reversible isomerization of citrate and isocitrate via *cis*-aconitate in the citric acid and glyoxylate cycles. It is a monomeric enzyme with an essential but redox-inactive [4Fe-4S] centre. Until recently, very little was known about the aconitase of *Escherichia coli* and no aconitase-deficient mutants of *E. coli* had been isolated. However, sufficient enzyme was purified to raise a specific antiserum for use in screening λ -*E. coli* gene libraries for phages expressing the aconitase gene, *acnA* (Prodromou *et al.*, 1991). The *acnA* gene was located at 28 min (1350 kb) in the *E. coli* linkage map and the enzyme activity could be amplified up to 200-fold in plasmid-containing strains. The primary structure deduced from the nucleotide sequence of the *acnA* gene (Prodromou *et al.*, 1992) predicted that the *E. coli* enzyme has the same underlying structure as the porcine mitochondrial

aconitase (Robbins & Stout, 1989), with 29% amino acid sequence identity and conservation of 19 out of 20 active-site residues, including three cysteine residues involved in ligand binding to the [4Fe-4S] centre. Interestingly, the *E. coli* enzyme exhibited 53% sequence identity with the iron-responsive element binding protein (IRE-BP) or iron regulatory factor (IRF), the mammalian iron-responsive translational regulator which has since been identified as a cytoplasmic aconitase (Kaptain *et al.*, 1991; Kennedy *et al.*, 1992). It has been shown that mammalian tissues can interconvert the cytoplasmic aconitase and RNA-binding activities according to iron availability (Constable *et al.*, 1992; Haile *et al.*, 1992a; Emery-Goodman *et al.*, 1993). However, this functional switch is not associated with a simple [4Fe-4S] to [3Fe-4S] interconversion, but seemingly with a more extensive degradation of the iron-sulphur clusters, in order to accommodate the iron-responsive elements of relevant transcripts in the active-site cleft (Haile *et al.*, 1992b).

Several *E. coli* (de)hydratases which contain [4Fe-4S] clusters are particularly susceptible to inactivation by superoxide, including aconitase, fumarase A, and 6-phosphogluconate dehydratase (Gardner & Fridovich, 1991; Liochev & Fridovich, 1992). It is envisaged that the inactivation of these iron-sulphur enzymes occurs at an early stage of oxidative stress, so that they function as circuit breakers, halting the production of damaging superoxide radicals by temporarily shutting down oxidative metabolism (Gardner & Fridovich, 1991, 1992). Once the stress has passed, the (de)hydratases can be reactivated by intracellular Fe^{2+} and thiols, rather than having to be synthesized *de novo*.

The aim of the present work was to construct an *acnA* mutant of *E. coli*, by replacing the wild-type chromosomal gene with an *in vitro*-inactivated *acnA* gene, in order to investigate the Acn^- phenotype and to study the regulation of AcnA synthesis using an *acnA-lacZ* gene fusion. This revealed a residual aconitase activity, designated AcnB . It was found that *acnA* resembles other citric acid cycle genes in being subject to CRP-mediated catabolite repression and ArcA -mediated anaerobic repression, and that it is positively regulated by the ferric uptake regulator (Fur), which normally functions as a repressor, and by the oxidative stress regulatory system (SoxRS).

METHODS

Bacterial strains, plasmids, phagemids and bacteriophages.

The strains of *Escherichia coli* K12, with relevant genotypes and sources, are listed in Table 1. The phagemid containing the intact *acnA* gene, pGS447 (Fig. 1), had been assembled previously by ligating a 3.2 kb *Bam*HI fragment and a 1.02 kb *Bam*HI-*Pst*I fragment in pUC119 (Prodromou *et al.*, 1991). The source of the 1.3 kb *Nco*I-*Sa*I fragment containing the *kan*^R cassette was pMTL24-Kan, which was constructed by inserting *Pst*I-treated Km^R GenBlock (Pharmacia) into the multicloning site of pMTL24 (Chambers *et al.*, 1988), in order to provide the flanking *Nco*I and *Sa*I sites. Other plasmids are shown in Table 1 or Fig. 1.

The *acnA-lacZ* translational fusion was created by ligating the 1.6 kb *Bam*HI-*Sa*I fragment containing parts of the *cysB-acnA* intergenic and *acnA* coding regions into pNM482 to generate pGS563 (Fig. 1). The fusion was then transferred to λ RZ5 (M. Berman) by *in vivo* recombination in strain RK4353(pGS563) according to Spiro & Guest (1987). The corresponding *lacnA-lacZ* fusion phage (λ G244) was established as a single-copy prophage in different sets of host strains for investigating the roles of global regulators in *acnA* expression. Lysogens were selected initially with *lb80del9c* and monolysogens were identified by their sensitivity to *lc190c17*. Each set of *lacnA-lacZ* monolysogens consisted of a parental strain, a regulator mutant, and the mutant containing a multicopy plasmid expressing the wild-type regulator gene, respectively for the following regulators: CRP [MC1000, JRG1999, and JRG1999(pGS279)]; ArcA [MC4100, BW3218, and BW3218(pRB38)]; FNR [RK4353, RK5279, and RK5279(pCH21)]; and Fur [MC4100, H1941, and H1941(pMH15)]; see Table 1. Analogous *lfum-lacZ* monolysogens were isolated with the Fur set of hosts using the fusion phages described previously by Woods & Guest (1987): λ G185 (*lfumA-lacZ*); λ G186 (*lfumB-lacZ*); and λ G187 (*lfumC-lacZ*); see Fig. 1. In the case of the SoxRS regulator, *lacnA-lacZ* and *lfum-lacZ* monolysogens were only made with the correspond-

ing parental (GC4468) and *soxR* mutant (DJ901) hosts (Table 1).

Media and growth tests. The complex media were L broth for routine subculture and peptone broth for specific growth tests (Guest, 1981), supplemented as required with ampicillin (100 $\mu\text{g ml}^{-1}$), chloramphenicol (25 $\mu\text{g ml}^{-1}$), kanamycin (15 $\mu\text{g ml}^{-1}$) and glucose (50 mM). The citrate-free minimal medium (Cole & Guest, 1980) was used with glucose (20 mM), sodium acetate (40 mM), sodium succinate (40 mM), sodium DL-lactate (40 mM) or sodium pyruvate (40 mM) as carbon sources, and supplements of thiamin hydrochloride (5 $\mu\text{g ml}^{-1}$) and L-glutamate (2 mM) or L-proline (0.22 mM), as required. Cultures (10 ml) were grown at 37 °C, either aerobically in shaking 250 ml conical flasks or anaerobically in stationary tubes filled to the neck. Inocula were prepared from stationary-phase cultures grown in the same medium (with appropriate antibiotics), washed in saline, and added to give a starting OD₆₅₀ of 0.1.

Enzymology. For assaying aconitase, cultures (1.5 ml) were harvested in early stationary phase, resuspended at 0 °C in 300 μl Tris/citrate buffer (20 mM, pH 8.0), and disrupted by two ultrasonic treatments of 10 s at 10 MHz separated by a 30 s interval. Cell debris was removed by centrifuging (10000 g,

Table 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference*
W3110	Prototroph	Laboratory collection
JRG2789	<i>acnA::kan</i> ^R derivative of W3110	This work
DH5 α	$\Delta(\text{argF-lac})\text{U169}$ ($\phi 80\Delta\text{lacZM15}$) <i>recA</i>	Sambrook <i>et al.</i> (1989)
MC1000	ΔlacX74 $\Delta(\text{araABC-len})$	Silhavy <i>et al.</i> (1984)
JRG1999	MC1000 ΔcrpT8	S. T. Cole ¹
BW3218	$\Delta(\text{argF-lac})\text{U169}$ $\Delta(\text{serB-arcA-thr})$	R. Buxton ²
RK4353	$\Delta(\text{argF-lac})\text{U169}$	V. Stewart ³
RK5279	RK4353 <i>fnr-250</i>	V. Stewart
MC4100	$\Delta(\text{argF-lac})\text{U169}$ <i>rpsL</i>	Silhavy <i>et al.</i> (1984)
H1941	MC4100 Δfur	K. Hantke ⁴
GC4468	$\Delta(\text{argF-lac})\text{U169}$ <i>rpsL sup</i>	Greenberg <i>et al.</i> (1990)
DJ901	GC4468 ΔsoxR	Greenberg <i>et al.</i> (1990)
pMAK705	Rep ^{ts} , Cm ^R	Hamilton <i>et al.</i> (1989)
pGS526	pMAK705 <i>acnA::kan</i> ^R , Cm ^R	This work
pNM482	Gene fusion vector, Ap ^R	Minton (1984)
pGS563	pNM482 <i>acnA-lacZ</i> , Ap ^R	This work
pGS279	<i>crp</i> ⁺ , Ap ^R Cm ^R	Spiro <i>et al.</i> (1989)
pCH21	<i>fnr</i> ⁺ , Ap ^R Cm ^R	C. Higgins ⁵
pRB38	<i>arcA</i> ⁺ , Cm ^R	R. Buxton
pMH15	<i>fur</i> ⁺ , Cm ^R	K. Hantke

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5 min) and the supernatants (cell-free extracts) were usually assayed immediately but could be stored for 2 d at -20°C . Aconitase was assayed spectrophotometrically at room temperature by following the formation of *cis*-aconitate from isocitrate at 240 nm, using an absorption coefficient of 3.6 mM^{-1} , as described by Kennedy *et al.* (1983). One unit of activity (U) represents $1\text{ }\mu\text{mol cis-aconitate formed min}^{-1}$. In some experiments isocitrate (20 mM) was replaced by citrate (20 mM) or *cis*-aconitate (0.2 mM). Protein concentrations were determined by the Bio-Rad micro-assay procedure. In the reactivation studies, $100\text{ }\mu\text{l}$ samples of cell-free extract were incubated at 0°C with 10 mM dithiothreitol, 1.0 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and 20 mM Tris/HCl, pH 8.0, and then assayed at 10 min intervals.

β -Galactosidase activities were determined according to Miller (1972) using monolysogens containing *lacZ* fusion phages (see above) grown to early stationary phase ($\text{OD}_{650} = 1.2$ for $\lambda\text{acnA-lacZ}$) or late exponential phase ($\text{OD}_{650} = 0.8$ for $\lambda\text{fum-lacZ}$), in unsupplemented L broth or L broth containing glucose (0.4 or 1%). In experiments involving Fur, available iron was depleted by adding bipyridyl (32 mM) at $\text{OD}_{650} = 0.4$, and in the SoxRS studies, oxidative stress was imposed by adding methyl viologen (0.2 mg ml^{-1}) 1 h before sampling. Specific activities (Miller units) were averaged from at least three independent cultures, the variation between samples being $< 10\%$.

Western blotting. PAGE was performed as described by Laemmli (1970) using denaturing conditions (0.1% SDS in all buffers and 15%, w/v, acrylamide) and biotinylated size markers (M_r): phosphorylase *b* (97400); catalase (58100); alcohol dehydrogenase (39800); carbonic anhydrase (29000). Samples in SDS-polyacrylamide gels were transferred to nitrocellulose with a Bio-Rad Transblot Electrophoretic Transfer Cell according to the manufacturer's instructions. The blots were immuno-stained using aconitase A antiserum as the primary antiserum, biotinylated anti-rabbit antibodies and streptavidin-biotinylated horseradish peroxidase complex according to Prodromou *et al.* (1991).

DNA manipulation and Southern blotting. DNA was prepared and manipulated by standard procedures (Sambrook *et al.*, 1989). For Southern blotting, endonuclease-digested bacterial DNA was fractionated by electrophoresis (0.8% agarose, Tris/acetate/EDTA gel) and transferred to nitrocellulose membrane (Sambrook *et al.*, 1989). The blots were hybridized for 18 h at 68°C with $5 \times \text{SSC}$ and washed ($2 \times 15\text{ min}$) with $0.1 \times \text{SSC}$ plus 0.1% SDS (w/v) at 65°C [$1 \times \text{SSC}$ contains NaCl (0.15 M) and sodium citrate (0.015 M, pH 7.0)]. Digoxigenin-labelled probes containing the 1.3 kb *kan*^R cassette and the 1.6 kb *SalI*–*Bam*HI fragment of the *acnA* coding region were derived from M13 templates by primer-extension labelling using universal primer and a non-radioactive DNA-labelling kit (Boehringer-Mannheim). The corresponding restriction fragments were isolated after electrophoresis in a low-melting-point agarose gel and used in hybridization and detection according to the manufacturer's instructions.

Materials. Isocitric acid, *cis*-aconitic acid and biotinylated molecular mass markers for SDS-PAGE were obtained from Sigma. Alkaline phosphatase, T4 DNA ligase and the Non-Radioactive Digoxigenin Labelling Kit were from Boehringer-Mannheim. Biotinylated anti-rabbit antibody and the streptavidin-biotinylated horseradish peroxidase complex were from Amersham. Restriction enzymes were purchased from Northumbria Biologicals, Klenow DNA polymerase and the kanamycin resistance GenBlock were from Pharmacia, and Universal primer was from Cambridge Bioscience.

RESULTS

Construction of an *acnA* mutant by gene replacement

An *acnA* mutant was constructed by replacing the chromosomal *acnA* gene of *E. coli* W3110 by an *in vitro*-disrupted gene designated *acnA::kan*^R, according to the method of Hamilton *et al.* (1989). The 1.04 kb *SalI*–*NcoI* segment of pGS447, encoding residues 122 to 460 (all of domain 2 and parts of domains 1 and 3; Prodromou *et al.*, 1992), was first replaced by the corresponding 1.3 kb *kan*^R cassette of pMTL24-Kan, to produce pGS525 (Fig. 1). The entire *acnA::kan*^R region was then ligated between the *KpnI* and *SphI* sites in the polylinker of the thermosensitive replicon, pMAK705, to generate pGS526. Some 25 plasmid cointegrates arising by homologous recombination between pGS526 and the chromosome were obtained by direct selection for *Cm*^R and *Km*^R transformants of W3110 at 42°C . This represented 0.5% of comparable transformants obtained at 30°C . The cointegrates were streaked to single colonies and cultured to stationary phase at 30°C (5 colonies per 100 ml of L broth containing both antibiotics) to allow resolution by a second recombination event. The desired resolution products, in which the chromosomal *acnA* gene had been replaced by the *acnA::kan*^R construction,

Fig. 1. Restriction maps of the *acnA*, *fumAC* and *fumB* regions showing segments cloned in specific plasmids, the strategy for *acnA* gene disruption by the *kan*^R cassette, and the construction of the corresponding *acnA-lacZ* and *fum-lacZ* fusion phages. The polarity of the *acnA* gene (a) is clockwise in the *E. coli* chromosome, whereas the *fum* genes (b) are polarized anticlockwise. Relevant restriction sites are: *B*, *Bam*HI; *Bc*, *Bcl*I; *Bg*, *Bgl*II; *E*, *Eco*RI; *H*, *Hind*III; *K*, *Kpn*I; *N*, *Nco*I; *P*, *Pst*I; *S*, *Sal*I; *Sp*, *Sph*I; *X*, *Xho*I; the subscript (_v) refers to flanking vector sites used in subcloning.

Fig. 2. Western blot of potential *acnA* mutants. Samples of total protein from stationary phase L-broth cultures of W3110 (1) and seven potential mutants (2 to 8) were fractionated by SDS-PAGE (15% gel), blotted and immuno-stained using anti-AcnA serum. Each sample was equivalent to 10 μ l of a bacterial suspension with $OD_{650} = 0.2$. The M_r values of standard proteins (9) are indicated and the positions of AcnA and two unknown immunoreactive bands, X (M_r 60000) and Y (M_r 42000), are arrowed.

Fig. 3. Aerobic growth of *acnA* mutant and parental strains. Cultures of JRG2789 (*acnA*; broken lines and open symbols) and W3110 (*acnA*⁺; unbroken lines and filled symbols) were grown at 37 °C with shaking, in peptone medium (Δ , $\blacktriangle$) or minimal media with acetate ($\square$, $\blacksquare$) or succinate ($\circ$, $\bullet$) as substrates. Saline-washed suspensions of bacteria grown overnight in the same media (with kanamycin, 15 μ g ml⁻¹, for the mutant) were used as inocula for the 10 ml cultures.

were obtained by selecting Km^R colonies at 42 °C and then screening for Km^R Cm^S products at 30 °C. From a total of 125 Km^R colonies (25 per resolution culture), 22 had the desired phenotype and seven of these potential *acnA::kan^R* mutants were chosen for further analysis.

Western blotting showed that all seven potential *acnA* mutants lacked the immunoreactive polypeptide corresponding to AcnA (M_r 97500) in the parental strain (Fig. 2). Two additional immunoreactive polypeptides (M_r 60000 and 42000) were detected in all samples, but it is not known whether they are related to aconitase or if they correspond to contaminants in the sample of aconitase used to raise the antiserum. Other positive bands present in all samples were due to non-specific binding by the secondary antibody.

Chromosomal DNA from a representative mutant (JRG2789) was also compared to that of the parent

(W3110) by Southern blotting with two hybridization probes under high-stringency conditions. The 1.6 kb *Bam*HI–*Sal*I internal fragment of the *acnA* gene was used as an *acnA* probe and the *kan^R* cassette served as the *kan* probe. The fragments hybridizing to the *acnA* probe were exactly as predicted for *Bam*HI, *Pst*I and *Bam*HI + *Hind*III digests of both W3110 DNA (3.2, 9.2, and 3.2 kb, respectively) and JRG2789 DNA (3.5, 1.6, and 1.3 kb), assuming that the desired replacement had been effected (Fig. 1). Likewise, the *kan* probe hybridized to fragments of 3.5, 1.3, and 1.3 + 2.1 kb in respective digests of mutant DNA, but failed to bind to any fragments in digests of parental DNA. These observations confirmed that the chromosomal *acnA*⁺ gene of W3110 had been successfully replaced by the disrupted *acnA::kan^R* gene from pGS526 in the representative *acnA* mutant, JRG2789.

Nutritional characterization of the *acnA* mutant

Citrate synthase (*gltA*) and isocitrate dehydrogenase (*icd*) mutants have an absolute requirement for glutamate (or proline) during growth in glucose minimal medium and they cannot use acetate as sole carbon and energy source with or without supplementary glutamate (Lakshmi & Helling, 1976). It was therefore assumed that an aconitase mutant would possess the same nutritional phenotype. However, aerobic and anaerobic growth tests in solid and liquid media showed that the *acnA* mutants grew as well as the parental strain in unsupplemented glucose minimal medium. Added glutamate or proline produced a slight enhancement but this was the same for both the mutant and wild-type strains. The *acnA* mutation also had no significant effect on growth in lactate and pyruvate minimal media or in L broth (with or without glucose, 1% w/v). However, the *acnA* mutation reproducibly increased the lag phase during growth in peptone medium, decreased the lag phase in succinate minimal medium, and led to a slightly higher growth yield in acetate minimal medium (Fig. 3).

Enzymological characterization of the *acnA* mutant

Enzymological tests showed that the *acnA* mutants retained aconitase activity. The activities obtained, when assayed with citrate, *cis*-aconitate, and isocitrate as

Table 2. Aconitase activities of mutant and parental strains

Freshly prepared ultrasonic extracts of strains grown to early stationary phase in L broth were assayed without reactivation. One unit of aconitase activity corresponds to the formation or transformation of 1 μmol *cis*-aconitate min^{-1} . The results are means of triplicate assays with at least three independent extracts, $\pm$ SE.

Substrate	Aconitase specific activity [U (mg protein) $^{-1}$]	
	W3110 (<i>acnA</i> $^{+}$)	JRG2789 (<i>acnA::kan</i> R)
Isocitrate	0.18 $\pm$ 0.02	0.04 $\pm$ 0.02
<i>cis</i> -Aconitate	0.17 $\pm$ 0.02	0.03 $\pm$ 0.01
Citrate	0.10 $\pm$ 0.01	0.02 $\pm$ 0.01

substrates, ranged from approximately 10 to 30% of the corresponding wild-type activities at stationary phase in L-broth cultures (Table 2). The aconitase specific activity in wild-type cultures increases during the exponential phase to reach a maximum at stationary phase (Prodromou *et al.*, 1991). In contrast, the aconitase activity of the *acnA* mutant reached a somewhat earlier maximum and then decreased during the stationary phase. The residual activity detected in the mutant may thus represent a

higher proportion of the total activity of the wild-type during exponential phase.

The residual aconitase was unstable and resembled other aconitases in being reactivated by ferrous ions under reducing conditions. Thus the specific activity of a typical mutant extract declined steadily from 0.06 to 0.02 U (mg protein) $^{-1}$ during incubation for a period of 40 min at 20 °C, but then increased to 0.09 U (mg protein) $^{-1}$ when incubated at 0 °C for 60 min in the presence of dithiothreitol (DTT, 1 mM) and Fe $^{2+}$ (10 mM).

These observations show that in addition to AcnA there is at least one other analogous but genetically distinct aconitase in *E. coli*. This residual enzyme activity, designated AcnB, is assumed to be the product of a putative *acnB* gene, although the existence of more than one additional *acn* gene has not been excluded.

Regulation of aconitase synthesis

The residual aconitase activity (AcnB) of the *acnA* mutant was compared to the total activity (AcnA plus AcnB) of the parental strain, after growth in different media and with different carbon sources (Table 3). The AcnB activity was relatively constant in cultures grown in rich media, whereas total aconitase activity was significantly repressed by glucose and anaerobiosis. This indicates that AcnA synthesis is subject to catabolite and anaerobic repression and that AcnB synthesis is differently regulated. The aconitase activities of both mutant and parental cultures observed after growth in minimal media were similar with

Table 3. Effects of growth medium on aconitase synthesis

Aconitase specific activities, U (mg protein) $^{-1}$, were assayed in ultrasonic extracts of duplicate early stationary phase cultures, grown aerobically unless stated otherwise. β -Galactosidase expressed from the single-copy λ *acnA-lacZ* prophage was also assayed in early stationary phase and the specific activities (Miller units) were averaged from triplicate cultures. The variation between replicate samples was less than 15% for both enzymes.

Medium	Aconitase		β -Galactosidase
	W3110 (<i>acnA</i> $^{+}$ <i>acnB</i> $^{+}$) AcnA + AcnB	JRG2789 (<i>acnA::kan</i> R <i>acnB</i> $^{+}$) AcnB	MC1000 (λ G244) (<i>acnA</i> $^{+}$ <i>B</i> $^{+}$ <i>lacnA-lacZ</i>) AcnA- β -Gal fusion
L broth	0.20	0.04	30–50
L broth + glucose	0.10	0.03	13
L broth (anaerobic)	0.08	0.03	2
L broth + glucose (anaerobic)	0.05	0.03	3
Peptone broth	0.18	0.03	23
Glucose minimal	0.02	0.01	1.5
Glucose minimal (anaerobic)	0.02	0.01	1
Lactate minimal	0.05	0.06	7
Pyruvate minimal	0.02	0.02	7
Acetate minimal	0.03	0.03	9
Succinate minimal	0.05	0.06	10

Fig. 4. Effects of glucose and anaerobiosis on the expression of a single-copy *acnA-lacZ* fusion during the growth cycle. Growth ($\square$; OD_{650}) and β -galactosidase specific activities ($\blacksquare$; Miller units; averaged from at least three independent cultures) were monitored for cultures grown at 37 °C in: a, aerobic L broth; b, aerobic L broth plus glucose (0.4%); and c, anaerobic L broth plus glucose (0.4%).

Fig. 5. Effects of CRP, ArcA and FNR on *acnA-lacZ* expression. β -Galactosidase specific activities of stationary-phase cultures of strains containing a *lacnA-lacZ* fusion prophage (λ G244) were assayed after aerobic and anaerobic growth in L broth (containing 1% glucose, where indicated). Monolysogenic derivatives of strains containing wild-type ($\blacksquare$), mutant ($\square$), and multiple copies ($\square$) of three different regulatory genes were compared: (a) *crp*; (b) *arcA*; and (c), *fnr*. See Methods for details of individual strains.

all substrates. This could mean that AcnA synthesis is repressed during growth in relatively poor media, leaving AcnB as the sole source of aconitase activity. It can also be concluded that AcnB is operational in both the glyoxylate and citric acid cycles because the mutant can use acetate as a sole carbon and energy source and acetate-grown cultures contain aconitase.

In order to study the regulation of AcnA synthesis in the presence of AcnB, a *lacnA-lacZ* fusion phage (λ 244), containing the *acnA* promoter region and part of the *acnA* structural gene linked 'in-phase' to a β -galactosidase reporter gene, was constructed and established as a single-copy prophage in MC1000 (*acnA*⁺*B*⁺ Δ *lac*). Studies with L-broth cultures of MC1000(λ G244) showed that the β -galactosidase activity increases during the growth cycle to a maximum in stationary phase (Fig. 4a), is repressed by glucose (Fig. 4b) and further repressed by anaerobiosis (Fig. 4c). Assuming that β -galactosidase activity truly reflects AcnA synthesis, it would appear that *acnA* is a

typical citric acid cycle gene, subject to catabolite and anaerobic repression mediated by the corresponding regulators, CRP and ArcA. The effects of growth substrate on *acnA-lacZ* expression in minimal media are likewise consistent with the repression of AcnA synthesis under anaerobic conditions and on poorer substrates (Table 3). Further studies on the effects of global regulators on *acnA* gene expression were made with a series of λ G244 monolysogens, *acnA*⁺*B*⁺ Δ *lac* (*lacnA-lacZ*), each lacking a regulator or containing multiple copies of the corresponding gene.

Regulation of *acnA* gene expression by CRP, ArcA and FNR

The β -galactosidase activity expressed from a single-copy *lacnA-lacZ* fusion prophage in the *crp*⁺ parental strain was reduced 3.5-fold in the *crp* deletion strain but restored by the presence of a multicopy *crp*⁺ plasmid (Fig. 5a). The activities of the *crp*⁺ strains were also much reduced by the

Fig. 6. Effects of iron-depletion and Fur status on the expression of single-copy *acnA*-, *fumA*-, *fumB*- and *fumC*-*lacZ* fusions. The β -galactosidase specific activities were assayed after aerobic growth in L broth or L broth to which the ferrous-ion chelator bipyridyl (32 mM) was added at OD₆₅₀ = 0.4 (see Methods). Derivatives of the parental strain (*fur*⁺ Δ *lac*), the *fur* mutant (Δ *fur*) and the mutant transformed with a multicopy *fur*⁺ plasmid (*pfur*⁺) contained single copies of the following fusion prophages: □, λ acnA-*lacZ*; ▨, λ fumA-*lacZ*; ▩, λ fumB-*lacZ*; and ■, λ fumC-*lacZ*.

addition of glucose (1%). Thus it was concluded that the cAMP-CRP complex is required to activate transcription of the *acnA* gene. This is consistent with the presence of potential CRP binding sites in the *acnA* promoter region (Prodromou *et al.*, 1992). Analogous studies on the role of ArcA showed that deletion of the *arcA* gene or the presence of an *arcA*⁺ multicopy plasmid had no significant effect on *acnA*-*lacZ* expression under aerobic conditions (Fig. 5b). In contrast, expression of the fusion was repressed 13-fold under anaerobic conditions in the parental and multicopy *arcA*⁺ strains, but only 2.6-fold for the *arcA* deletion strain. This indicates that ArcA probably functions as an anaerobic repressor of *acnA* transcription. However, the fact that anaerobic repression was not totally abolished in the *arcA*-deletion strain suggests that some other mechanism of anaerobic repression may be operating. Because this could involve FNR, the effects of *fnr* mutation and multiple copies of the *fnr*⁺ gene on *acnA*-*lacZ* expression were investigated (Fig. 5c). The results indicated that FNR is not directly responsible for the residual anaerobic repression, although it could still act in conjunction with ArcA, or function as an anaerobic repressor solely in the absence of ArcA. Such effects, and also the partial repression observed with multicopy *fnr*⁺ under aerobic conditions (Fig. 5c), could be mediated via potential FNR sites in the *acnA* promoter region (Prodromou *et al.*, 1992).

Regulation of the *acnA* and *fum* gene expression by Fur

A role for the ferric uptake regulator (Fur) in *acnA* gene expression was investigated partly because there are two potential Fur binding sites in the *acnA* promoter region (Prodromou *et al.*, 1992) and because AcnA contains an iron-sulphur centre which might render its synthesis responsive to iron availability. The β -galactosidase activities observed with cultures of λ acnA-*lacZ* monolysogens grown in L broth with and without the iron chelator bipyridyl (Fig. 6) showed that iron and an active *fur* gene product are essential for good expression of the *acnA* gene, and that the deleterious effects of a *fur* deletion

were reversed by the presence of a multicopy *fur*⁺ plasmid. It would therefore appear that Fur serves as a transcriptional activator of *acnA* gene expression, although the existence of an indirect mechanism of Fur-mediated activation was not excluded.

These studies were extended to include the three fumarase genes of *E. coli* because the *fumA* and *fumB* genes encode analogous oxygen-labile enzymes (FumA and FumB) resembling aconitase in possessing [4Fe-4S] clusters, whereas the *fumC* gene encodes a stable tetrameric enzyme (FumC) resembling the mitochondrial fumarases (Miles & Guest, 1984; Woods *et al.*, 1986; Bell *et al.*, 1989; Flint *et al.*, 1992). Previous studies with a set of λ fum-*lacZ* fusion phages indicated that FumA is an aerobic citric acid cycle enzyme, the corresponding *fumA* gene being subject to catabolite repression (CRP-mediated activation) and ArcA-mediated anaerobic repression, whereas FumB is expressed anaerobically from the catabolite- and ArcA-insensitive but FNR-activated *fumB* gene (Woods & Guest, 1987; Guest & Russell, 1992). Expression of the *fumC* gene is not significantly affected by catabolite or anaerobic repression. However, it has recently been assigned to the *soxRS* regulon because FumC activity is strongly induced by oxidative stress but not in a strain lacking the SoxRS response (Liochev & Fridovich, 1992). Using the same set of λ fum-*lacZ* fusion phages, the effects of iron-depletion, *fur* deletion and *fur*⁺ gene amplification (Fig. 6) showed that as in the case of *acnA*, expression of the *fumA* gene requires iron and is activated by Fur. In contrast, *fumC* expression was not significantly affected by iron depletion or Fur status (Fig. 6). There was some indication that *fumB* expression might be iron-dependent and Fur-independent, but these tests were not done under anaerobic conditions where *fumB* is more highly expressed.

Regulation of the *acnA* and *fum* gene expression by SoxRS

The *soxRS* oxidative stress regulon responds to superoxide-generating agents such as methyl viologen, by a two-stage process in which the SoxR protein is thought

Fig. 7. Effects of SoxR and oxidative stress on the expression of single-copy *acnA*-, *fumA*-, *fumB*- and *fumC-lacZ* fusions. The β -galactosidase specific activities were assayed after aerobic growth in L broth or L broth to which methyl viologen (0.2 mg ml⁻¹) was added during the last hour of growth (see Methods). Monoclonogenic derivatives of a parental strain (*soxR*⁺ Δ *lac*) and a deletion mutant (Δ *soxR*) contained single copies of the following fusion prophages: □, *acnA-lacZ*; ▨, *fumA-lacZ*; ▩, *fumB-lacZ*; and ■, *fumC-lacZ*.

to serve as a redox-sensing activator of the adjacent *soxS* gene, followed in turn by the activation of regulon genes by the SoxS protein (Amabile-Cuevas & Demple, 1991; Hildago & Demple, 1994). Studies with *lacnA-lacZ* monolysogens of *soxR*⁺ and Δ *soxR* strains indicated that the *acnA* gene belongs to the *soxRS* regulon, because β -galactosidase activities increased about three-fold with methyl viologen and this increase was entirely dependent on the presence of the wild-type *soxR* gene (Fig. 7). Direct assays for the effects of methyl viologen on the aconitase specific activity in extracts of L-broth cultures of W3110 (*acnA*⁺*acnB*⁺) and JRG2789 (*acnA*⁻*acnB*⁺) indicated that the total activity in the parental strain increased slightly [0.18 to 0.22 U (mg protein)⁻¹] whereas that of the mutant decreased consistently [0.03 to 0.01 U (mg protein)⁻¹] under oxidative stress. If it is assumed that both enzymes are susceptible to oxidative disruption of their iron-sulphur centres, the apparent stability of the aconitase activity in W3110 (mainly AcnA) could be due to a compensatory increase in enzyme synthesis mediated by the SoxS-activation of the *acnA* promoter under oxidative stress conditions, as observed with the corresponding *acnA-lacZ* fusion. Similar experiments with monolysogens containing *λfum-lacZ* fusion prophages confirmed that *fumC*, the gene encoding the oxygen-stable fumarase, is a member of the *soxRS* regulon. In the parental strain, expression from the *fumC-lacZ* fusion increased 13-fold with methyl viologen, but the superoxide generator had no effect in the *soxR*-deletion strain, indicating that *fumC* is normally positively activated by the SoxRS system (Fig. 7). In contrast, the *fumA* and *fumB* genes encoding the oxygen-labile iron-sulphur containing fumarases appeared not to belong to the *soxRS* regulon because no *soxR*-dependent induction by methyl viologen was observed (although some potential effects may be masked by the aerobic repression of *fumB*).

DISCUSSION

The immunological and DNA hybridization properties of JRG2789 (*acnA*::*kan*^R) confirmed that the strategy for inactivating the chromosomal *acnA*⁺ gene had been

successfully applied, despite the presence of residual aconitase activity and the lack of the anticipated glutamate auxotrophy. It is extremely unlikely that the product of the mutated *acnA* gene retains aconitase activity because 38% of the structural gene (including codons for 10 out of 20 active-site residues) is replaced by the *kan*^R cassette. It would therefore appear that *E. coli* contains at least two aconitases (AcnA and AcnB), and this may explain why mutants lacking aconitase have never been detected amongst glutamate auxotrophs or acetate-non-utilizing mutants. In view of their instability and reactivation by ferrous ions under reducing conditions, both enzymes seem to be analogous iron-sulphur proteins. However, unlike the iron-sulphur-containing fumarases, FumA and FumB, they do not appear to perform specific roles in aerobic and anaerobic metabolism. Nevertheless, some underlying functional specialization, based on the differential regulation of the *acnA* and *acnB* genes, was indicated by the relative aconitase activities of mutant and parental strains grown under different conditions. The AcnB activity was relatively constant, whereas the deduced AcnA activity was repressed by glucose, anaerobiosis, and after growth in minimal media. The overall response to glucose and anaerobiosis is consistent with the earlier findings with wild-type strains (Gray *et al.*, 1966; Iuchi & Lin, 1988). The low activities with lactate, pyruvate, acetate and succinate were unexpected because *E. coli* should be more dependent on the citric acid cycle during growth with these substrates. Under most conditions the mutant and wild-type grew equally well, indicating that the two enzymes are functionally overlapping. However, with acetate and succinate as sole carbon sources the *acnA* mutant grew better than the wild-type. The reason for this is obscure, but it could indicate that adapting to the use of AcnB is delayed in wild-type bacteria, and that AcnB is used preferentially in the glyoxylate cycle. The possibility that the residual aconitase activity (AcnB) in the *acnA* mutant is due to some other hydratase-dehydratase having a broad substrate specificity that includes citrate, *cis*-aconitate and isocitrate, has not been excluded.

In *E. coli* there is now evidence that at least four of the eight steps in the citric acid cycle are catalysed by

genetically-distinct isoenzymes, including the aconitase reaction. The existence of analogous membrane-bound flavoprotein complexes (succinate dehydrogenase and fumarate reductase) catalysing the aerobic and anaerobic interconversion of fumarate and succinate is well known (Guest & Russell, 1992). Likewise for the interconversion of fumarate and malate, there are closely-related aerobic and anaerobic fumarases (FumA and FumB) in addition to an unrelated mitochondrial type of fumarase (FumC), which is expressed during aerobic and anaerobic growth but specifically induced by oxidative stress (Woods *et al.*, 1988; Woods & Guest, 1987; Liochev & Fridovich, 1992). More recently, a second form of citrate synthase has been isolated from a revertant of a *gltA* mutant lacking the hexameric citrate synthase typically found in Gram-negative bacteria (Patton *et al.*, 1993). The second enzyme closely resembles the citrate synthases of Gram-positive bacteria and eukaryotes, and is presumably expressed from a reactivated cryptic gene. In some cases it is clear how enzyme duplication has provided opportunities for functional specialization and the acquisition of independent mechanisms regulating their expression. Such a process offers considerable advantages over the constitutive expression of a single all-purpose enzyme. In contrast, the lipoamide dehydrogenase component of the pyruvate and 2-oxoglutarate dehydrogenase complexes and the glycine cleavage system of *E. coli* is the product of a single gene (*lpd*). Despite the need for coexpression with three independently-regulated complexes, it would appear that there has not been sufficient need for functional specialization to promote gene or enzyme duplication (Guest *et al.*, 1989) even though a second lipoamide dehydrogenase of uncertain function has been detected (Richarme, 1989). Isoenzymes likewise occur in the glycolytic pathway of *E. coli*, where different classes of fructose-1,6-bisphosphate aldolase may function preferentially in glycolysis or gluconeogenesis, and two related glyceraldehyde-3-phosphate dehydrogenase genes have been found, one of which may be cryptic (Perham, 1990). The presence of two classes of fumarase and two citrate synthases has been demonstrated in *Bacillus* (Reaney *et al.*, 1993; Jin & Sonenshein, 1994). Mammalian tissues contain distinct cytoplasmic and mitochondrial aconitases, and the cytoplasmic enzyme, which more closely resembles AcnA than the mitochondrial enzyme (53% versus 29% identity), also functions as an iron-responsive translational regulator or iron regulatory factor (IRF) after disruption of its iron-sulphur centre (Klausner & Rouault, 1993). No parallel regulatory function has yet been observed for either of the *E. coli* aconitases, nor is there any evidence for an unrelated stable class of aconitase analogous to FumC.

Further information about the function of AcnA was obtained using a single-copy *acnA-lacZ* fusion to study the roles of global regulators in *acnA* expression. These confirmed that the *acnA* gene resembles other citric acid cycle genes in being positively regulated by the cAMP-CRP complex and negatively regulated by ArcA. However, the retention of some anaerobic repression of

acnA-lacZ fusion activity after deleting *arcA* (Fig. 5) contrasts with the 2-3-fold anaerobic derepression of total aconitase activity observed previously with an *arcA* mutant (Iuchi & Lin, 1988). This discrepancy may reflect changing contributions of AcnB to the total aconitase activity measured in the latter work. The residual anaerobic repression suggests that other regulators may either repress *acnA* under anaerobic conditions (especially in the absence of ArcA) or activate *acnA* expression under aerobic conditions both in the presence and absence of ArcA. FNR appeared not to function as an independent anaerobic repressor, but it could serve as an anaerobic repressor in the absence of ArcA. In this context it may be relevant that several genes, including *sodA* (manganese superoxide dismutase), *sdh* (succinate dehydrogenase), *cyo* (cytochrome *o*), *cyd* (cytochrome *d*) and *pfl* (pyruvate formate lyase), are regulated by both FNR and ArcA, and this dual action has recently been ascribed to the activation of *arcA* transcription by FNR for *sodA*, *sdh*, and *cyo*, but not for *cyd* or *pfl* (Compan & Touati, 1994).

Fusion studies further indicated that *acnA* and *fumA* are subject to a Fur- and iron-dependent activation, and that *acnA* and *fumC* are activated by the SoxRS system in response to oxidative stress (superoxide generation). The involvement of Fur is potentially significant because it could provide a link between citric acid cycle activity and iron availability. It is also interesting because Fur normally acts as an iron-responsive transcriptional repressor (Bagg & Nielsens, 1987), although it has been reported to activate the iron-containing superoxide dismutase gene, *sodB* (Niederhoffer *et al.*, 1990). The mode of Fur-mediated activation is unknown, but there are two potential Fur sites upstream of two putative *acnA* promoter sequences in the 1004 bp *cysB-acnA* intergenic region (Prodromou *et al.*, 1992). In the case of *fumA*, the transcriptional start point has been defined and here the -10 and +1 regions are overlapped by a putative Fur site (coordinates 278-296; Miles & Guest, 1984). This seems an unlikely site for transcriptional activation. However, further searches have revealed a putative converging promoter in the antisense strand of the *fumA* coding region (+1 coordinate 369, 10 bp downstream of the initiation codon). This is likewise overlapped by potential Fur sites in the *fumA* coding region (coordinates 363-381, 402-420, 405-423 and 408-426). If transcription from the 'antisense' promoter inhibits *fumA* expression, differential effects of Fur on the two converging promoters could provide a mechanism for the Fur- and iron-dependent activation of the *fumA* gene. A somewhat similar mechanism has been suggested for the Fur-mediated activation of the bacterioferritin gene (Andrews *et al.*, 1989). Comparable searches of the *fumC* sequence revealed a putative Fur site in the *fumC* promoter region (coordinates 2108-2126; Woods *et al.*, 1986) but none were located in the *fumB* sequence.

The SoxRS two-stage regulatory system positively regulates genes such as the superoxide dismutase A (*sodA*) and glucose-6-phosphate dehydrogenase (*g6p*) genes, which have roles in defending *E. coli* against superoxide-generating agents. The results obtained with the *acn-lacZ*

and *fum-lacZ* fusions now confirm that *acnA* and *fumC* are members of the *soxRS* regulon. Previous studies have shown that aconitase is particularly sensitive to oxidative attack, leading to the suggestion that its inactivation performs a protective role in shutting down metabolic electron flow so as to halt the further production of superoxide radicals (Gardner & Fridovich, 1991, 1992). Further studies with fumarase indicated that the SoxRS system also operates to replace unstable enzymes such as FumA and FumB, by a stable isoenzyme like FumC, which is better able to withstand oxidative attack (Liochev & Fridovich, 1992). The observed *soxR*-dependent induction of *acnA-lacZ* expression by superoxide probably represents a compensatory response invoked to maintain or restore cellular aconitase activity under conditions of severe enzyme inactivation by oxidative attack. The response to oxidative stress could thus involve two phases, an immediate response in which AcnA, AcnB, FumA and FumB are rapidly inactivated in order to limit intracellular damage, followed by a delayed response in which the SoxRS-induced production of AcnA and FumC replaces the inactivated enzymes so as to maintain metabolism or to promote its restoration once the stress has diminished.

A SoxS binding-site consensus has yet to be defined, but attention has been drawn to an 18 bp motif (ACCC---GAAAA--A-G) present in the *sodA* and *zwf* promoters (Compan & Touati, 1993). It may thus be significant that an almost identical motif occurs upstream of the *acnA* coding region (coordinates 1186–1203; Prodromou *et al.*, 1992). No such motif was detected in the *fumC* promoter region, but there are regions of partial dyad symmetry which might provide more appropriate binding sites for a member of the AraC family of regulators, such as SoxS.

This work presents the first evidence for the existence of at least two aconitases in a single bacterial species. Current studies are aimed at characterizing the residual enzyme activity (AcnB) in the *acnA* mutant and the corresponding gene (*acnB*) in order to understand the metabolic basis for the presence of aconitase isoenzymes in *E. coli*. It will be particularly interesting to determine whether AcnB more closely resembles the mitochondrial aconitase than AcnA and whether either has an iron-responsive regulatory function like the mammalian iron regulatory factor.

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