Characterization of *Salmonella enterica* serovar Typhimurium aconitase A

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Aconitases (Acn) are iron–sulfur proteins that catalyse the reversible isomerization of citrate and isocitrate via the intermediate cis-aconitate in the Krebs cycle. Some Acn proteins are bi-functional and under conditions of iron starvation and oxidative stress lose their iron–sulfur clusters and become post-transcriptional regulators by binding specific mRNA targets. Many bacterial species possess two genetically distinct aconitase proteins, AcnA and AcnB. Current understanding of the regulation and functions of AcnA and AcnB in dual Acn bacteria is based on a model developed in *Escherichia coli*. Thus, AcnB is the major Krebs cycle enzyme expressed during exponential growth, whereas AcnA is a more stable, stationary phase and stress-induced enzyme, and both *E. coli* Acns are bi-functional. Here a second dual Acn bacterium, *Salmonella enterica* serovar Typhimurium (S. Typhimurium), has been analysed. Phenotypic traits of *S*. Typhimurium *acn* mutants were consistent with AcnB acting as the major Acn protein. Promoter fusion experiments indicated that *acnB* transcription was ~10-fold greater than that of *acnA* and that *acnA* expression was regulated by the cyclic-AMP receptor protein (CRP, glucose starvation), the fumarate nitrate reduction regulator (FNR, oxygen starvation), the ferric uptake regulator (Fur, iron starvation) and the superoxide response protein (SoxR, oxidative stress). In contrast to *E. coli*, *S*. Typhimurium *acnA* was not induced in the stationary phase. Furthermore, *acnA* expression was enhanced in an *acnB* mutant, presumably to partially compensate for the lack of AcnB activity. Isolated *S*. Typhimurium AcnA protein had kinetic and mRNA-binding properties similar to those described for *E. coli* AcnA. Thus, the work reported here provides a second example of the regulation and function of AcnA and AcnB proteins in a dual Acn bacterium.

**INTRODUCTION**

Aconitase (Acn, aconitase hydratase; EC 4.2.1.3) is a dehydratase–hydratase enzyme that catalyses the reversible isomerization of citrate and isocitrate via the intermediate cis-aconitate, which is an essential component of the Krebs cycle. Acn activity is dependent upon the presence of a [4Fe-4S]$^{2+}$ cluster at the active site. The Acn structural prototype consists of three structural domains (1, 2 and 3) that pack around the iron–sulfur cluster, and a fourth domain (4) that is joined to domain 3 by a long linker sequence to create the active-site cleft (Robbins & Stout, 1989). Three of the four Fe atoms of the iron–sulfur cluster are coordinated by three conserved cysteine residues located in domain 3; the fourth Fe atom (Fe$_{3}$) is the binding site for the substrate (Beinert et al., 1996). Oxidation of the Acn [4Fe-4S]$^{2+}$ cluster to form a [3Fe-4S]$^{+}$ cluster leads to loss of activity due to ejection of Fe$_{3}$, but activity is restored in the presence of Fe(II) under reducing conditions (Robbins & Stout, 1989). Two genetically distinct bacterial Acns have been identified (Gruer et al., 1997a, b). AcnA was first isolated and characterized in *Escherichia coli* by virtue of its relatively stable iron–sulfur cluster and is closely related to the cytoplasmic Acns (cAcn) of higher eukaryotes (Gruer et al., 1997a; Prodromou et al., 1991). The expression of *E. coli* *acnA* was enhanced under stress conditions (stationary phase, iron starvation, oxidative stress) (Cunningham et al., 1997; Gruer & Guest, 1994). In contrast, *E. coli* *acnB* is expressed during exponential growth, and the iron–sulfur cluster of the AcnB protein is less stable *in vitro* than that of AcnA (Cunningham et al., 1997; Jordan et al., 1999). The AcnB proteins form a separate branch in the Acn protein superfamily (Gruer et al., 1997a). In contrast to other Acns,
AcnB proteins have five (5-4-linker-1-2-3) rather than the usual four (1-2-3-linker-4) domains (Williams et al., 2002). Thus, AcnB proteins are cyclically permuted with an N-terminal region consisting of an additional HEAT-like domain (domain 5) followed by domain 4 and a short linker to domains 1-2-3 (Williams et al., 2002).

The cAcns of higher organisms are bi-functional proteins. When in possession of a [4Fe-4S] cluster, cAcns have aconitase activity and convert citrate to isocitrate; however, under conditions (iron starvation, oxidative stress) that promote conversion to the apo-form, they become mRNA-binding post-transcriptional regulators known as iron-responsive proteins (IRPs) (IRP-1) (Wang & Pantopoulos, 2011). When iron availability is low, IRP-1 exhibits high affinity binding to stem–loop structures known as iron-responsive elements (IREs) present in the untranslated regions of target mRNAs. This has the effect of enhancing or inhibiting the synthesis of proteins that control cellular iron homeostasis (Theil, 1994). It is now known that the apo-forms of bacterial AcnA and AcnB proteins also interact with IRE-like sequences and are thus, like cAcn, bi-functional proteins (Alén & Sonenshein, 1999; Banerjee et al., 2007; Pechter et al., 2013; Tang & Guest, 1999; Tang et al., 2002, 2004, 2005). Therefore, the switching of bacterial Acn proteins between catalytic and regulatory modes links environmental signals (e.g. iron availability and oxidative stress) to gene expression and central metabolism.

The motivation for the work presented here arose from the interrogation of the National Center for Biotechnology Information genome database (March 2013), which identified 432 bacterial species with acnA genes, 227 with acnB and 133 bacterial species that possess both acnA and acnB genes. Therefore, it was decided to test the E. coli paradigm describing the roles and regulation of Acns in bacteria (i.e. AcnB is the Krebs cycle enzyme used during exponential growth; AcnA is a more stable, stationary phase, stress-induced enzyme and both are bi-functional) in another enteric bacterium with both acn genes, S. Typhimurium. It was concluded that AcnB is the major S. Typhimurium aconitase and that AcnA is a secondary aconitase that is induced under conditions of glucose starvation, iron starvation and oxidative stress.

**Overproduction and purification of AcnA.** The acnA expression plasmid was constructed using ligation-independent cloning (LIC) as previously described (Li & Evans, 1997). The acnA-coding region was amplified and isolated as a 2.7 kb product by PCR using S. Typhimurium genomic DNA as the template. For ligation into pET30a Ek/LIC vector (Novagen) the forward primer (GCGGAGC-AAGATGTCGTCAACCCCTACGAG) contained a sequence (underlined) immediately upstream from the ATG start codon, and the reverse primer (GAGAGAAAACGGGTATTTGCATATTTC-ATTG) contained a sequence (underlined) downstream of the stop codon that anneal to the LIC site in the vector. The PCR product was incubated with T4 DNA polymerase and dATP and the processed DNA was then annealed with the pET30a Ek/LIC vector. The authenticity of the construct (pGS2345) was confirmed by DNA sequencing before being used to transform E. coli BL21(DE3) to create the expression strain JRG6432 (Table 1). Recombinant AcnA with a vector encoded N-terminal His-tag was overproduced by culturing the host strain in 2 l flasks containing 500 ml LB with 50 μg ml⁻¹ kanamycin inoculated 1:100 from an overnight culture. Cultures were grown aerobically at 37°C to an optical density at 600 nm (OD₆₀₀) of 0.5. At this point, IPTG (100 μg ml⁻¹) was added and the culture was incubated at 30°C with shaking for a further 4 h. The bacteria were then pelleted by centrifugation at 15 000 g for 15 min at 4°C. The cell pellets were resuspended in 18 ml binding buffer (10 mM MES, 5 mM MgCl₂, pH 6.0) and disrupted by two passages through a French pressure cell at 16 000 p.s.i. After clarification (27 000 g for 20 min at 4°C) AcnA was isolated from the soluble cell-free extract using Ni²⁺ affinity chromatography on HiTrap chelating columns (GE Healthcare). Protein purity was monitored by SDS-PAGE (Laemmli, 1970) followed by Coomassie blue staining, and protein concentration was estimated by the Bradford assay (Bradford, 1976) with BSA as standard.

**Construction of S. Typhimurium crp, fnr fur and soxR mutants.** Disruption of the crp, fnr, fur and soxR genes was achieved by linear transformation based on the method of Datsenko & Wanner (2000). Oligonucleotides containing 3’ sequences complementary to the first or last 20 bp of the chloramphenicol-resistance cassette of plasmid pACYC184 (Martinez et al., 1988) and 5’-end sequences flanking crp, fnr or fur genes were obtained. For soxR, oligonucleotides containing sequences complementary to the first or last 20 bp of the kanamycin-resistance cassette of plasmid pKD4 (Datsenko & Wanner, 2000) were constructed. Linear DNA carrying the resistance cassette and flanking regions was generated by PCR. Cultures of S. Typhimurium containing the plasmid pKD46 (Amp<sup>+</sup>), which carries the <i>λ</i> red recombinase genes under the control of a λ-arabinose inducible pBAD promoter, were grown overnight at 30°C and diluted (1:100) in LB containing ampicillin (100 μg ml⁻¹) and λ-arabinose (20 mM) and grown to OD₆₀₀ ~0.3. Electrocompetent cells were prepared and transformed with ~5 μg PCR product then incubated in 1 ml LB broth at 37°C for 1 h before plating on selective medium. The resulting colonies (Cm<sup>+</sup> or Kan<sup>+</sup>) were immediately cured of pKD46, and the presence of the mutation was confirmed by PCR and DNA sequencing. Further transfer of the crp, fur, fnr and soxR mutations into clean genetic backgrounds was achieved using bacteriophage P22-mediated transduction (Sambrook & Russell, 2001).

**Enzymology.** Aconitase activity was assayed as described by Gruer & Guest (1994). Cultures (1.5 ml) were harvested in early stationary phase, resuspended on ice in 300 μl Tris/citrature buffer (20 mM, pH 8.0), and disrupted by two ultrasonic treatments of 10 s at an amplitude of 10 μm separated by a 30 s interval. Cell debris was removed by centrifuging (10 000 g, 5 min) and the supernatants (cell-free extracts) were usually assayed immediately. Acn activity was measured spectrophotometrically by monitoring the time-dependent conversion of isocitrate to cis-aconitate at 240 nm using an absorption coefficient of 3.6 mM⁻¹ cm⁻¹ as described by Kennedy.

**METHODS**

**Bacterial strains, plasmids and growth media.** Bacterial strains and plasmids are listed in Table 1. Standard procedures were used to isolate and manipulate DNA (Sambrook & Russell, 2000). Unless indicated, all bacteria were grown in Luria–Bertani broth (LB) (Sambrook & Russell, 2000) containing antibiotics (ampicillin, 100 μg ml⁻¹; kanamycin, 50 μg ml⁻¹ and tetracycline, 30 μg ml⁻¹) as required. The media were amended with 2, 2'-bipyridyl (1.6 mM), glucose (1 %); or methyl viologen (0.2 mM) as indicated. Citrate-free minimal medium (Cole & Guest, 1980) was used with glucose (20 mM) supplemented with L-methionine (0.2 mM). Cultures were grown at 37°C under aerobic conditions in shaking (250 r.p.m.) conical flasks or under anaerobic conditions in sealed bottles.

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Table 1. Strains and plasmids used in this study

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et al. (1983) at 25 °C. One unit of activity (U) represents 1 μmol cis-aconitate formed min⁻¹.

β-Galactosidase activities were determined according to Miller (1972) using S. Typhimurium strains transformed by derivatives of the plasmid pRWSO (TetR) carrying the lacZ reporter gene fused to the acnA (pGS2220) or acnB (pGS2221) promoter. Cultures were grown to stationary phase (OD₆₀₀=0.8−1.2) in unsupplemented LB, LB plus glucose (1%), LB plus 2,2′-bipyridyl (1.6 mM), or LB plus methyl viologen (0.2 mM for 1 h before sampling). Specific activities (Miller units) were averaged from at least three independent cultures.

Western blotting. SDS-PAGE was performed as described by Laemmli (1970) before transfer of the separated protein samples to nitrocellulose. The blots were immunostained using rabbit sera raised against the E. coli Acn proteins that have been previously shown to be specific for AcnA and AcnB (Gruer et al., 1997b). Horseradish peroxidase-conjugated anti-rabbit antibodies (GE Healthcare) were used to detect AcnA and AcnB according to the method of Prodromou et al. (1991). ImageJ software was used for densitometric quantification.

In vitro transcription and gel shift assays. Template DNA was obtained by annealing primers with a T7 RNA polymerase promoter sequence upstream of 30 nt of human ferritin IRE (upstream primer TAATAGCTCCATATAG and downstream primer GTTCCG-TTCAAAACTGTTGAAGCAACCTATAGTGAGTCGATATG).

The human ferritin IRE sequence is underlined. The procedure and reagents for the in vitro transcription reaction were purchased from Ambion (MEGAscript kit). Reactions (20 μl) contained 100 ng DNA template; 20 μCi [p⁴⁰P]-UTP (800 Ci mmol⁻¹); 75 mM ATP, CTP and GTP; 7.5 mM unlabelled UTP, 10× reaction buffer and 25 units of T7 RNA polymerase mix. The reactions were incubated at 37 °C overnight. DNase (1 mg ml⁻¹) was added and mixed to remove any template DNA and then incubated again at 37 °C for 15 min. After extraction with phenol (pH −5), RNA was precipitated with 0.5 M ammonium acetate and 1 vol. isopropanol at −20 °C. The labelled RNA was dissolved in nuclease-free water. To allow proper folding, the RNA was heated to 85 °C and then slowly cooled to 4 °C.

The radiolabelled RNA fragment (6 pmol per reaction) was incubated with different concentrations of AcnA in a binding reaction mixture
(20 μl) containing 1 mg ml⁻¹ yeast RNA. The reactions were incubated at room temperature for 15 min and then applied to a running 5% non-denaturing polyacrylamide gel (1 x Tris/borate-EDTA buffer, constant current, 25 mA) at room temperature for 20–30 min. The gel was then dried and visualized by autoradiography.

RESULTS AND DISCUSSION

Growth of S. Typhimurium acnB and acnAB double mutants is attenuated in minimal medium

In quantitative growth tests in glucose minimal medium under aerobic conditions, the values for maximal growth rate (1.04 ± 0.034 h⁻¹) and yield (final OD₆₀₀ 2.47 ± 0.28) for the parental S. Typhimurium strain were similar to those of an isogenic acnA mutant (1.03 ± 0.05 h⁻¹, 2.33 ± 0.04) (Fig. 1). This suggests that, as is the case in E. coli (Gruer et al., 1997b), any effect of the acnA mutation is compensated for by the presence of the acnB gene. Under the same conditions, the maximum growth rate (0.65 ± 0.05 h⁻¹) and final yield (OD₆₀₀ 1.52 ± 0.04) were compromised for the S. Typhimurium acnB mutant (Fig. 1), but much less so compared to the E. coli acnB mutant (Gruer et al., 1997b). The S. Typhimurium acnAB double mutant, like the E. coli acnAB mutant, failed to grow on glucose minimal medium under aerobic conditions. Under anaerobic conditions, the growth rates of the S. Typhimurium parent and acnA mutant strains were similar (0.96 ± 0.04 and 0.93 ± 0.05 h⁻¹, respectively), whereas that of the acnB mutant (0.37 ± 0.05 h⁻¹) was impaired, and the acnAB double mutant did not grow, suggesting that aconitase activity (particularly AcnB activity) is required to support maximal growth rates during aerobic respiratory and anaerobic fermentative growth in S. Typhimurium.

Expression of acnA is repressed by the presence of acnB

The growth tests suggested that the activity of AcnB was able to fully compensate for the absence of AcnA activity.

This would be consistent with relatively low-level expression of acnA under the conditions tested; in E. coli, acnA encodes a more stable aconitase that is expressed under stress conditions (Cunningham et al., 1997; Gruer & Guest, 1994; Jordan et al., 1999). In contrast, the activity of AcnA could only partially compensate for the absence of acnB in the growth tests, suggesting that capacity to induce acnA expression was limited under the conditions tested. Therefore, acnA and acnB promoter regions (P_acnA and P_acnB) were fused to lacZ in the low copy number plasmid pRW50 (Lodge et al., 1992) to measure the activity of the promoters in the parent, acnA and acnB mutants. For the parent, β-galactosidase activity from P_acnA-lacZ was ~12-fold lower than that of P_acnB-lacZ (Fig. 2a and b), suggesting that transcription from the acnB promoter is stronger than that from acnA under the conditions tested. In the acnB mutant, P_acnA was approximately threefold more active (Fig. 2a), suggesting that in the absence of

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**Fig. 2.** Expression of S. Typhimurium acnA is enhanced in an acnB mutant. (a) β-Galactosidase activities (Miller units) of stationary phase aerobic cultures of S. Typhimurium (JRG6013; parent) and an acnB mutant (JRG6404) transformed with the plasmid-based P_acnA-lacZ reporter fusion (pGS2220) were measured after growth in LB broth. (b) β-Galactosidase activities of S. Typhimurium (JRG6014; parent) and an acnB mutant (JRG6403) transformed with the plasmid-based P_acnB-lacZ reporter fusion (pGS2221) were measured as in (a). For both (a) and (b) the mean values and sds (n=3) are shown. (c) Below each chart in (b) are Western blots developed with anti-AcnA or anti-AcnB for the indicated strains and a Coomassie blue-stained gel showing the relative protein loadings of the cell-free extracts used.

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**Fig. 1.** Growth of S. Typhimurium parent (TR6583) and acn mutants (JEG5216, JEG5992 and JEG5993) in defined minimal medium. Bacteria were grown as aerobic batch cultures in glucose minimal medium. Results (mean and se, n=3) are shown for: parent (square), acnA mutant (diamond), acnB mutant (triangle) and acnAB double mutant (circle).
AcnA protein present in the acnB cyclic-AMP receptor protein (CRP), and S regulation of mutations in regulators that have been implicated in the 969 bp upstream of the P acnB reflected in the Acn protein content of the bacteria. Thus, the transcriptional changes reported above were approximately threefold lower than in the parent (Fig. 2c). Western blotting of clarified cell-free extracts from the parent, acnA and acnB mutants showed that the amount of AcnA protein present in the acnB mutant was ~3.5-fold greater than in the parent (Fig. 2c). Furthermore, the amount of AcnB protein present in the acnA mutant was approximately threefold lower than in the parent (Fig. 2c). Thus, the transcriptional changes reported above were reflected in the Acn protein content of the bacteria.

Expression of S. Typhimurium acnA is regulated by CRP, FNR, Fur and SoxR

Enhanced expression of the acnA gene in the acnB mutant suggested that P acnA was regulated. Two acnA promoters have been identified in E. coli. P1 is an RpoS-regulated gearbox promoter located 407 bp upstream of the acnA start codon and is responsible for stationary phase induction of acnA expression. P2 has a typical σ70 –10 element (TATCTT) and is located 50 bp upstream of the acnA start codon (Cunningham et al., 1997). Therefore, the P acnA-lacZ reporter plasmid (pGS2220) which included 969 bp upstream of the S. Typhimurium acnA ORF was used to transform S. Typhimurium strains carrying mutations in regulators that have been implicated in the regulation of E. coli acnA. The global regulator Fis had no significant effect on acnA expression (Fig. 3a). However, P acnA was subject to activation by the glucose-responsive cyclic-AMP receptor protein (CRP), and β-galactosidase activity was ~1.8-fold greater in the parent compared to the crp mutant (Fig. 3a). Activation by CRP implied that acnA expression would be lower in the presence of glucose (glucose inactivates CRP by lowering cellular cAMP concentrations). Accordingly, P acnA-driven lacZ expression in the parent strain was 1.8-fold lower in the presence of glucose (Fig. 3a). Expression of acnA was lower in the absence of O2: 4.1-fold lower β-galactosidase activity was measured for anaerobic compared to aerobic cultures of S. Typhimurium transformed with the P acnA-lacZ reporter plasmid pGS2220 (Fig. 3a). This lowering of acnA transcription was not observed when β-galactosidase activity from P acnA-lacZ was measured in anaerobic cultures of the S. Typhimurium fur mutant, suggesting that FNR represses acnA expression (Fig. 3a). In contrast to the reported Fur-mediated activation of the E. coli acnA promoter (Cunningham et al., 1997; Gruer & Guest, 1994; Fig. 3b), acnA expression was increased in the S. Typhimurium fur mutant (Fig. 3a), implying Fur-mediated repression. This suggested that P acnA-driven lacZ expression should be greater when S. Typhimurium cultures were iron starved. Growth in the presence of the iron chelator 2, 2′-bipyridyl (1.6 mM) decreased bacterial growth (final OD600 with and without 2,2′-bipyridyl, 1.3 ± 0.3 and 0.49 ± 0.07, respectively) but increased β-galactosidase activity of S. Typhimurium pGS2220 cultures 1.8-fold compared to iron-replete cultures, with a similar enhancement in acnA expression observed for the fur mutant strain (Fig. 3a). Thus, it was concluded that the Fur–Fe(II) complex mediates repression of acnA expression. Expression of acnA under aerobic conditions was only slightly lowered (1.2-fold) in a soxR mutant (Fig. 3a). The oxidative stress-responsive regulator SoxR is activated by

\[ \beta \text{Galactosidase activity (Miller units)} \]

**Fig. 3.** Effects of CRP, Fis, FNR, Fur and SoxR on P acnA-lacZ expression. (a) β-Galactosidase activities (Miller units) of stationary phase aerobic cultures (except where indicated by ‘minus O2’) of S. Typhimurium parent strain (JRG6013) and the indicated mutants (Table 1) transformed with the plasmid-based P acnA-lacZ reporter fusion (pGS2220) were measured after growth in LB broth amended with glucose (Glc; 1%), methyl viologen (MV, 0.2 mM for 1 h), 2,2′-bipyridyl (Bip, 1.6 mM) as indicated. Values are the means and SD s(n=3). (b) Regulation of acnA in E. coli (upper panel; Cunningham et al., 1997) and S. Typhimurium (lower panel). The effects of transcriptional regulators acting directly or indirectly to regulate acnA expression are shown (↑, activation; ↓, repression).
the redox stress reagent methyl viologen. After exposure of exponential phase cultures to methyl viologen (0.2 mM) for 1 h, the OD<sub>600</sub> of the sosR mutant was similar to that of the parent (2.25 ± 0.1 and 1.99 ± 0.1, respectively). However, acnA expression was enhanced (1.7-fold) in the parent, but not in the sosR mutant, which is consistent with SoxR activation of P<sub>acnA</sub> (Fig. 3a). The activation of acnA expression by CRP and SoxR and repression by FNR are in accord with observations reported for the E. coli acnA promoter (Cunningham et al., 1997; Fig. 3b) and indicate that these are conserved regulatory features in both bacteria.

Measurement of β-galactosidase activity throughout the aerobic growth cycle of S. Typhimurium pGS2220 (P<sub>acnA-lacZ</sub>) indicated that acnA expression was relatively constant (~50–60 Miller units) and was not, in contrast to observations in E. coli, increased in stationary phase (not shown).

The pattern of regulation reported above was supported by inspection of the DNA sequence upstream of the S. Typhimurium acnA ORF. This showed that compared to E. coli, the P1 acnA gearbox promoter was relatively poorly conserved in S. Typhimurium (~10 region has 4 out of 8 matches to the consensus compared to 6 out of 8 for E. coli; ~35 region has 1 out of 6 matches to the consensus compared to 4 out of 6 for E. coli). In contrast, the oxidative stress-responsive P2 acnA promoter, including the ~10 element (both TATCCCT, 4 out 6 matches to the g<sup>36</sup> consensus) and the hybrid CRP-FNR site (both TTGGGTTGTATACAA, 8 out 10 matches to the FNR consensus and 6 out of 10 matches to the CRP consensus) were conserved (Cunningham et al., 1997). Thus, the overall pattern of acn gene expression in S. Typhimurium is similar to that described for E. coli, i.e. transcription of acnB is greater than transcription of acnA, which in turn is induced under stress conditions. However, in contrast to E. coli, S. Typhimurium acnA was not induced in stationary phase and was repressed, rather than activated, by Fur (Fig. 3b). This pattern of regulation suggested that AcnB is likely to be the major Acn in S. Typhimurium but that AcnA could play an important role under oxidative stress and iron-starvation conditions. Consistent with this interpretation, exposure of aerobic LB exponential phase cultures to the oxidative stress reagent hydrogen peroxide (500 mM for 60 min) resulted in survival of 36 ± 3% colony forming units detected compared to those measured at the point of hydrogen peroxide addition (mean ± S<sub>D</sub>, n=3) of parental, 27 ± 2% of acnB mutant but only 6.3 ± 0.5% of acnA mutant bacteria, indicating that the acnA mutant was the most sensitive to oxidative stress.

### Catalytic activity of the S. Typhimurium AcnA is more stable than that of AcnB

The catalytic activity of AcnB in cell-free extracts of E. coli is rapidly lost because its [4Fe-4S]-cluster, which is essential for activity, readily converts to an inactive [3Fe-4S] form (Jordan et al., 1999). Analysis of Acn activity in cell-free extracts of S. Typhimurium parent, acnA and acnB mutant strains after aerobic growth in LB broth showed that the specific activity of the acnB mutant (51 ± 4 mU mg<sup>-1</sup>) was much greater than that of the parent (14 ± 2 mU mg<sup>-1</sup>) or the acnA mutant (3 ± 1 mU mg<sup>-1</sup>). Thus, those strains (parent and acnA mutant) that express acnB had low Acn activity, whereas the acnB mutant, which overproduces AcnA (Fig. 2), had higher activity, which is consistent with AcnA activity being more stable than AcnB activity in cell-free extracts.

### S. Typhimurium AcnA protein is bifunctional

The S. Typhimurium AcnA protein was isolated as a His-tagged fusion by nickel affinity chromatography after overproduction from E. coli JRG6432 (Table 1). The His-AcnA protein was monomeric as judged by size-exclusion chromatography (M<sub>r</sub> ~100,000; not shown) and had a UV-visible spectrum typical of Acn proteins exhibiting a broad absorbance around 420 nm with an estimated extinction coefficient of ~8000 M<sup>-1</sup> cm<sup>-1</sup> and an A<sub>420</sub>/A<sub>280</sub> ratio of ~0.05 broadly in agreement with values reported for other Acn proteins (Emptage et al., 1983; Jordan et al., 1999).

Acn proteins catalyse the reversible isomerization of citrate and isocitrate via the intermediate cis-aconitate. Accordingly, the S. Typhimurium AcnA protein was active when citrate, cis-aconitate and isocitrate were supplied as substrates. The K<sub>m</sub> values (Table 2) for these substrates were comparable to those measured for E. coli AcnA (Jordan et al., 1999) and were close to the range of intracellular concentrations that have been reported for citrate (2–9 mM), cis-aconitate (0.016 mM) and isocitrate (0.5 mM) in E. coli (Bennett et al., 2009; El-Mansi et al., 2015; Hausladen & Fridovich, 1994). The k<sub>cat</sub>/K<sub>m</sub> values for citrate and isocitrate were similar and ~25-fold lower than the value for cis-aconitate (Table 2).

The AcnA group of Acns are closely related to the bifunctional cAcn of eukaryotes (Gruer et al., 1997a). When the iron–sulfur cluster of cAcn is absent as a result of iron starvation or oxidative stress, the apo-form of the protein (IRP-1) is able to act as a post-transcriptional regulator by specifically interacting with mRNA structures, IREs, found in the untranslated regions of target transcripts (Wang & Pantopoulos, 2011). The effect of the IRP-1–IRE interaction can be to inhibit or promote the translation of the protein encoded by the mRNA. Several bacterial apo-Acn proteins have also been shown to bind IRE-like structures (Alén & Sonenshein, 1999; Banerjee et al., 2007; Pechter et al., 2013; Tang & Guest, 1999; Tang et al., 2004). Inspection of the IRP-1–IRE X-ray structure (Walden et al., 2006) and alignment of the IRP-1 and AcnA amino acid sequences revealed that, of 21 amino acid residues that make contact with the IRE, 17 are conserved in AcnA. Of the four non-conserved residues, two substitute amino acids with strongly conserved properties (S371A, K379R; numbering for IRP-1), one is a replacement with an amino acid...
acid with weakly similar properties (N685S) and the fourth is not conserved (R688P). Therefore, in the absence of an identified S. Typhimurium AcnA mRNA target, the 3′-UTR of E. coli acnA, which is a target for E. coli AcnA (Tang & Guest, 1999), is not well conserved in S. Typhimurium LT2; binding to a synthetic RNA sequence based on the human ferritin IRE was tested. Low-affinity binding comparable to that observed with E. coli AcnA at the 3′-UTR of the acnA transcript (Tang & Guest, 1999) was evident, perhaps reflecting the relative stability of the AcnA iron–sulfur cluster resulting in only a small proportion of RNA-binding apo-AcnA in the samples (Fig. 4). Further work is needed to fully characterize the nature and significance of S. Typhimurium AcnA–RNA interactions, but nevertheless it appears that, like the Bacillus subtilis, E. coli and Mycobacterium tuberculosis AcnA proteins, S. Typhimurium AcnA binds RNA.

Conclusions

Previous analyses of the regulation, biochemistry and physiology of E. coli acn genes and proteins have provided the framework for understanding the functions of genetically distinct Acns in bacteria. AcnB has been designated the major Krebs cycle enzyme operating in exponential growth phase, whereas AcnA is a more stable enzyme that is induced by iron starvation and oxidative stress (Jordan et al., 1999). Moreover, the apo-forms of AcnA and AcnB act as post-transcriptional regulators by interacting with mRNA targets (Tang & Guest, 1999; Tang et al., 2002; Tang et al., 2005). Here, a second example of the regulation and functions of Acns in a dual acn bacterium (S. Typhimurium) that largely conforms to the E. coli paradigm is presented. Thus, catalytic activity of S. Typhimurium AcnA is more stable than that of AcnB, and acnA expression is enhanced two- to threefold under conditions that either inactivate (acnB mutant) or are likely to impair (iron starvation and oxidative stress) AcnB aconitase activity and is lowered under conditions (anaerobic growth) that are likely to protect AcnB activity. However, the regulation of S. Typhimurium acnA differs from the E. coli paradigm in exhibiting relatively constant expression throughout the growth cycle and Fur repression, rather than the stationary phase induction of acnA and Furactivation observed in E. coli. Nevertheless, taken as a whole, the data obtained from this analysis of S. Typhimurium Acns suggest that the regulation and functions of AcnA and AcnB proteins in enteric bacteria with dual Acn proteins are mostly conserved.

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