Aerobic and anaerobic regulation of the *ubiCA* operon, encoding enzymes for the first two committed steps of ubiquinone biosynthesis in *Escherichia coli*

Britta Søballe, Robert K. Poole*

Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK

Received 22 July 1997

Abstract The *ubiCA* operon of *Escherichia coli* encodes enzymes for the first two steps of ubiquinone biosynthesis. A monolysogen (*ubiC-lacZ* operon fusion) was constructed to study *ubiCA* regulation. Expression was higher during aerobic growth than anaerobically, and increased with rate of oxygen supply. Although ubiquinone is implicated in antioxidant roles, *ubiC* expression was not elevated in response to hydrogen peroxide or the redox cycling agent, paraquat. Glucose repressed expression and mutation of *cya* (encoding adenylate cyclase) increased expression. Anaerobically utilised electron acceptors (nitrite, nitrate, fumarate) did not affect expression. *ubiC* expression appears to be negatively regulated by Fnr and IHF.

Key words: Ubiquinone biosynthesis; *Ubi* genes; Gene expression (*E. coli*); IHF; Fnr

1. Introduction

Quinones are lipophilic molecules that function in electron and proton transport in the respiratory chains of bacteria and mitochondria. However, the realisation that quinones also occur in non-respiring membranes, generate oxygen radicals in mitochondria (i.e. have pro-oxidant properties), and may act (in the quinol form) as antioxidants, has revolutionised ideas about the biological functions of quinones. Clinical use of ubiquinone in the treatment of heart disease [1] has further stimulated interest in quinones, their metabolism and synthesis.

Escherichia coli synthesises three types of quinones [2,3]: ubiquinone (Q_8) and two naphthoquinones, i.e. menaquinone (MQ_8) and demethylmenaquinone (DMQ_8). All have an octaprenyl side-chain rendering them soluble in the cytoplasmic membrane. The quinone pool allows coupling between appropriate pair(s) of dehydrogenases and terminal oxidoreductases to allow *E. coli* to grow by aerobic or anaerobic respiration. The biosynthetic pathway was determined [4] from identification of intermediates accumulated by Q_8-deficient mutant strains (*ubi*) obtained after chemical mutagenesis. Previously, we cloned and sequenced three *ubi* genes: the *ubiCA* operon (91.5 min [5]), encoding enzymes for the first two steps in the pathway and *ubiG* (50.3 min [6]). The functions of the different quinones in aerobic and anaerobic respiration have been studied through analyses of *ubi* mutants and strains defective in MQ_8 and DMQ_8 (*men*) [2].

*Corresponding author. Fax: (44) (114) 272 8697.
E-mail: r.poole@sheffield.ac.uk

Little is known of *ubi* gene regulation in any bacterium. This information is important for understanding integration of energetic mechanisms and for attempts to engineer ubiquinone production commercially [7]. Clearly, regulation of quinone synthesis does occur: aerobically grown *E. coli* cells contain more Q_8 than MQ_8 and DMQ_8, whereas in anaerobic cells this profile is reversed [3,8,9]. Recently, Shestopalov et al. [9] have presented evidence that these changes are unaffected by chloramphenicol, implicating post-translational regulation of Q_8, MQ_8 and DMQ_8 levels. Here we present complementary data demonstrating that an operon required for Q_8 biosynthesis is regulated at the level of transcription.

2. Materials and methods

2.1. Strains, plasmids, phage and growth conditions

E. coli K-12 strains, plasmids, and λ specialised transducing bacteriophage used in this study are described in Table 1. Methods for genetic crosses, restriction endonuclease digestion and ligation of DNA, plasmid DNA isolation and transformation of bacteria were described by Poole et al. [12]. Cells were grown in LB broth, initial pH 7.0, or in 3-(N-morpholino)propanesulfonic acid (MOPS)-buffered minimal medium (initial pH 7.8) with 40 mM glucose or other carbon source as specified. Kanamycin, chloramphenicol, and ampicillin were used at final concentrations of 50, 25 and 150 μg/ml, respectively.

2.2. Culture conditions

These have been described before [12]. Briefly, starter cultures were grown with shaking overnight in MOPS defined medium containing 5 mM glucose; such carbon-depleted cultures were inoculated (1%) into the same medium but supplemented with the carbon sources specified (40 mM). Sodium fumarate, $NaNO_3$ or $NaNO_2$ were added as autoclaved solutions to MOPS-buffered media at final concentrations of 40 mM, 40 mM and 5 mM, respectively, as indicated. Aerobic cultures (10 ml) were shaken (200 rpm) in 250 ml conical flasks with matched glass tubes of Klett dimensions as a side arm. For anaerobic growth, cells were grown in screw-capped glass tubes, filled to the brim with MOPS medium supplemented with 0.3% casamino acids (Difco) and containing two glass balls (approximately 1 mm diameter) to aid resuspension of cells that had settled during static culture. Turbidity was measured with a Klett colorimeter (Manostat Corporation) fitted with a red filter. After growth to the mid-exponential phase (about 60 Klett units or equivalent OD_{600}), chloramphenicol (100 μg/ml) or spectinomycin (for chloramphenicol-resistant strains, 300 μg/ml) was added to prevent adaptation to anaerobiosis or other change, and incubation continued for 5 min before harvest.

The oxygen transfer coefficient, K_La was determined for shake flasks containing different volumes of solution using the sodium sulphite method [13]. In a typical experiment, the volume of culture medium (LB) was varied from 5 ml to 200 ml in 250 ml conical flasks to alter K_La, a measure of the efficiency of oxygen absorption.

2.3. β-Galactosidase assay

Assays were carried out at room temperature as described before [12]. Cell pellets were suspended in 2.5 to 4 ml of buffer and stored on ice. β-Galactosidase activity was measured in $CHCl_3$- and sodium

PII S0014-5793(97)01041-7

Table 1
Strains and plasmids used

Strain or plasmid	Relevant genotype/properties	Reference or source
E. coli strains		
RK4353	*araD139 Δ(argF-lac)U169 gyrA219 non-9 rpsL150*	[10]
RKP4153	Same as VJS676 but Φ(*ubiC-lacZ*)*1*	This work
RKP4164	Same as RKP4153 but *cya::kan*	This work
RKP4169	Same as RKP4153 but *narL215::*Tn*10*	This work
RKP4173	Same as RKP4153 but *fnr271::*Tn*10*	This work
RKP4213	Same as RKP4153 but *himA::cat*	This work
RKP4239	Same as RKP4153 but *fnr271::*Tn*10 himA::cat*	This work
VJS676	*Δ(argF-lac)U169*	V.J. Stewart
Plasmids		
pBRP23	Ap^R *ubiCA*$^+$ in pBluescript SK$^+$	[5]
pRS415	Ap^R, *bla-Tl4-lacZ'* operon fusion vector	[11]
Phage		
λBS1	Φ(*ubiC-lacZ*)*1* (gene fusion)	This work
λRS45	*bla'-lacZ*$_{SC}$ *att*$^+$ *int*$^+$ *imm*^{21}	[11]

dodecyl sulphate-permeabilised cells by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. Activities are expressed in terms of the OD_{600} of cell suspensions using the formula of Miller [12]. Each culture was assayed in triplicate; results were confirmed in at least two independent experiments.

2.4. Construction of a ubiC operon fusion

Fusions of *ubiC* to *lacZ* were constructed on plasmids and then transferred to λ phage by recombination in vivo, using the method of Simons et al. [11]. A 0.76 kbp DNA fragment was excised from pBRP23 [5] using *Eco*RI and *Sma*I and ligated into the site created by similar digestion of pRS415. The required recombinant plasmid was isolated by transformation of strain RK4353 (*Δlac*). The fusion was recombined onto λRS45 to make λBS1. Several single-copy fusions to the chromosome of VJS676 (*Δlac*) were isolated and verified using β-galactosidase assays and Ter tests as described before [12]. One such fusion strain (RKP4153) was used into which the following mutant alleles were transduced (Table 1): *narL* (RK5278 [10]), *fnr* (VJS1741, courtesy of V. Stewart), *cya* (MK1010, see [12]), and *himA* (GS019 [14]).

3. Results

3.1. Effects of medium composition and mutant alleles on Φ(ubiC-lacZ) expression

The activities of the enzymes encoded by the *ubiCA* operon (chorismate pyruvate-lyase and 4-hydroxybenzoate octaprenyltransferase) are not easy to assay and so a Φ(*ubiC-lacZ*) operon fusion was used to monitor expression during growth under various conditions. Strain RKP4153 carries a single-copy operon fusion comprising 62 bp of the coding region of *ubiC* and the promoter region up to 379 bp upstream of the translational start site. In defined medium containing glucose as carbon and energy source, expression of the fusion was 2.4-fold higher aerobically than anaerobically (Table 2). Replacement of glucose with maltose, glycerol, lactate or lactose as carbon sources all gave levels of expression that were 1.3–1.8-fold higher than glucose (not shown). Changes in Φ(*ubiC-lacZ*) expression during batch growth and the repressive effect of glucose on Φ(*ubiC-lacZ*) expression are shown in Fig. 1. Addition of glucose to a culture pre-grown in minimal medium containing succinate as sole carbon source stimulated growth but prevented the gradual rise in fusion activity characteristic of the later stages of exponential growth. Under all growth conditions tested (Table 2), deletion of *cya* (encoding adenylate cyclase) increased *ubiC* expression 1.5–1.7-fold.

The addition of nitrite to glucose-containing medium gave no significant increase in anaerobic expression, nor did replacement of the glucose with glycerol and supplementation with nitrate. With fumarate as electron acceptor, anaerobic expression was slightly enhanced (Table 2). The transcriptional regulator Fnr controls expression of many genes required for anaerobic metabolism in response to oxygen availability, but the expression of MQ_8 and DMQ_8 appears to be *fnr*-independent [15]. Therefore, we investigated the role of Fnr in *ubiCA* expression. An *fnr* mutation caused a slight decrease in *ubiC-lacZ* expression aerobically, but anaerobically the mutation enhanced expression up to 2.7-fold in glucose-containing media. When nitrate or fumarate were provided as anaerobic electron acceptors, glucose was provided as carbon source (since *fnr* mutants grew extremely poorly with glycerol

Table 2
Effects of electron acceptors and regulatory mutations on Φ(*ubiCA-lacZ*)*1* expression[a]

Strain	Genotype[b]	β-Galactosidase specific activity[c]						
		Glucose					Glycerol	
		$+O_2$	No O_2	No O_2+NO_2^-	No O_2+NO_3^-	No O_2+fumarate	No O_2+NO_3^-	No O_2+fumarate
RKP4153	Wild type	170	70	91	ND	ND	95	120
RKP4164	*cya*	290	120	150	ND	ND	150	180
RKP4173	*fnr*	120	190	160	190	180	ND	ND
RKP4213	*himA*	330	140	80	ND	ND	150	150
???	*himA fnr*	1150	380	320	290	690	ND	ND
RKP4169	*narL*	180	100	82	ND	ND	74	93

[a]Strains were cultured in MOPS defined medium in the presence of the indicated carbon sources and electron acceptors.
[b]All strains carry Φ(*ubiC-lacZ*)*1*
[c]Miller units; ND, not determined.

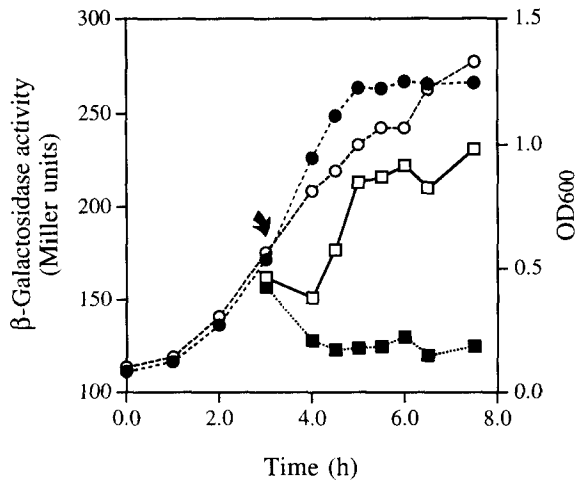

Fig. 1. Effects of glucose on growth and Φ(*ubiC-lacZ*) expression. At the time indicated by the arrow, glucose was added to 40 mM, and the effects on growth (○, ●) and Φ(*ubiC-lacZ*) expression (□, ■) determined. Open symbols: no glucose; filled symbols: added glucose.

and these acceptors); levels of expression were consistently higher than in *fnr*$^+$ strains (Table 2). The effects of a mutation in *himA* (encoding Integration Host Factor) were broadly similar to those of *fnr*, but when mutant alleles of *fnr* and *himA* were introduced together, Φ(*ubiC-lacZ*) expression activity was increased by 7-fold aerobically and 5-fold anaerobically, when glucose was used as carbon source. Similar increases, 4–6-fold, were seen during anaerobic growth with fumarate or nitrate. A mutation in *narL* (encoding a nitrate-responsive regulator that functions with its cognate sensor NarX [2]) reduced Φ(*ubiC-lacZ*) expression during anaerobic growth on glycerol plus nitrate by only about 20% (Table 2).

The following mutations were without significant effect, aerobically or anaerobically, enhancing Φ(*ubiC-lacZ*) expression only up to 1.4-fold: *arcA*, *fruR*, *fur*, *narP*, *oxyR*, *rob*, *rpoS*, *soxR* and *soxS* (not shown).

3.2. Effects of oxygen supply and oxidative stress

Because quinones have been implicated in responses to oxidative stress, we examined Φ(*ubiC-lacZ*) expression at various oxygen supply rates in bath cultures. At the highest rate of oxygen supply tested (achieved by shaking 5 ml of culture in a 250 ml Erlenmeyer flask), the K_La value was 146 mmol O_2/l/h and Φ(*ubiC'lacZ*) expression was 162 Miller units (data not shown). Expression declined as the oxygen transfer rate was lowered; at a K_La value of 36 mmol O_2/l/h, Φ(*ubiC-lacZ*) expression was 131 Miller units, decreasing to 93 Miller units in shaken flasks that contained 80% of their volume of medium. Neither paraquat (up to 0.1 mM) or hydrogen peroxide (up to 1 mM) caused any significant change in Φ(*ubiC-lacZ*) expression (data not shown).

4. Discussion

There have been few attempts to study transcription of the quinone biosynthesis genes. Suzuki et al. [16] used plasmid-borne fusions, which may be problematic, and reported that Φ(*ubiA-lacZ*) expression was catabolite-repressed by glucose. Gibert et al. [17] used a *Bam*HI site on a cloned *Pst*I-*Pst*I fragment to make a Φ(*ubiC-lacZ*) fusion. Interpretation of these results is complicated by uncertainty about the direction of transcription of *ubiG*, but it was reported that expression of the fusion was subject to catabolite repression. The recent finding that aeration-dependent changes in the quinone profile of *E. coli* are independent of the presence of chloramphenicol suggests that post-translational regulation is also important in regulation of quinone synthesis and composition. Indeed, 2-octaprenylphenol accumulated anaerobically is converted by 'aerobic' mono-oxygenases to Q_8 without enzyme synthesis [18]. These findings are complemented by the present data which show that most transcriptional regulators involved in the expression of other respiratory chain components are without significant effect on regulation of Φ(*ubiC-lacZ*) expression. Since the nature of the electron donor can affect quinone usage [2], various carbon sources were tested: although glucose was consistently observed to give lower levels of Φ(*ubiC-lacZ*) expression than other carbon sources, no Crp box [19] was observed in the *ubiCA* promoter region. The possibility that the faster growth rate supported by glucose contributes to the lower Φ(*ubiC-lacZ*) expression observed can only be rigorously tested in chemostat cultures at fixed growth (= dilution) rates.

Mutations in *fnr* and *himA*, either separately or, more notably, together, caused enhanced Φ(*ubiC-lacZ*) expression. Other genes known to be regulated by Fnr and IHF include *focA-pfl*, *nuoA-N*, *narGHJI*, *narK*, *hemA*, and *sodA* [20,21]. In the case of *nuoA-N*, both regulators have negative effects [22], as observed here for *ubiC*. For *sodA*, the effect of FNR may be indirect, via ArcA [23]. Analysis of the promoter region of *ubiCA* (Fig. 2) reveals putative, overlapping binding sites for both Fnr and IHF. The sequence TTGC(T) (boxed) resembles the TTGA(T) half-site motif, within which the G is common to the core motifs for both Fnr and Crp interactions. The first T is that proposed to provide discrimination between Fnr and Crp interactions. The half-site ((A)TCAA) constituting the anticipated palindrome, however, is present instead as (A)TCTC (boxed). However, membership of *ubiCA* in the Fnr modulon can be demonstrated unequivocally only by Fnr binding site-specifically to the target site and repressing transcription. Modest enhancement of Φ(*ubiC-lacZ*) expression was observed aerobically (Table 2). Although Fnr is

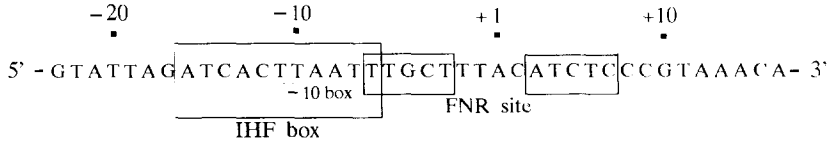

Fig. 2. Localisation of putative sites (boxed) for Fnr and IHF in the *ubiCA* promoter region. The underlined sequence is the putative −10 region.

sometimes regarded solely as an anaerobic regulator, the aerobic activities of Fnr are now recognised (reviewed by Guest et al. [21]).

Overlapping the putative Fnr-binding site is a sequence (Fig. 2; boxed) that bears resemblance to the consensus IHF box (WATCAANNNNTTR; W = A or T, R = A or G, N = any base [24]). For several operons in *E. coli*, IHF has been shown to inhibit transcription. In the case of *ubiC*, the putative IHF-binding site overlaps with the −10 region of the promoter; as well as a possible Fnr site. Thus IHF could inhibit transcription directly or indirectly through modulation of a second regulator protein.

Here we have focused on the genes encoding the first steps in the biosynthetic pathway. The dosage of *ispB*, which encodes the polyprenyl pyrophosphate synthetase, may also influence ubiquinone$_8$ pool size [7]. In future, data on the regulation of *ubiCA*, *ispB* and other *ubi* genes will allow assessment to be made of the relative roles of transcriptional and post-translational regulation of quinone pools and facilitate further trials of ubiquinone overproduction [7].

Acknowledgements: This work was supported by a BBSRC Studentship to B.S. and a BBSRC Research Grant to R.K.P. B.S. acknowledges additional financial support from the Danish Research Academy. We thank Dr G. Wu for valuable advice and Dr G. Cook for K_La determinations.

References

[1] Ernster, L. and Dallner, G. (1995) Biochim. Biophys. Acta 1271, 195–204.
[2] Gennis, R.B. and Stewart, V. (1996) in: *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology (Neidhardt, F.C., Curtiss, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M. and Umbarger, H.E., Eds.), pp. 217–261, American Society for Microbiology, Washington, DC.
[3] Meganathan, R. (1996) in: *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology (Neidhardt, F.C., Curtiss, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M. and Umbarger, H.E., Eds.), pp. 642–656, American Society for Microbiology, Washington, DC.
[4] Gibson, F. (1973) Biochem. Soc. Trans. 1, 317–326.
[5] Wu, G., Williams, H.D., Gibson, F. and Poole, R.K. (1993) J. Gen. Microbiol. 139, 1795–1805.
[6] Wu, G., Williams, H.D., Zamanian, M., Gibson, F. and Poole, R.K. (1992) J. Gen. Microbiol. 138, 2101–2112.
[7] Zhu, X., Yuasa, M., Okada, K., Suzuki, K., Nakagawa, T., Kawamukai, M. and Matsuda, H. (1995) J. Ferment. Bioeng. 79, 493–495.
[8] Ingledew, W.J. and Poole, R.K. (1984) Microbiol. Rev. 48, 222–271.
[9] Shestopalov, A.I., Bogachev, A.V., Murtazina, R.A., Viryasov, M.B. and Skulachev, V.P. (1997) FEBS Lett. 404, 272–274.
[10] Stewart, V. (1982) J. Bacteriol. 151, 1320–1325.
[11] Simons, R.W., Houman, F. and Kleckner, N. (1987) Gene 53, 85–96.
[12] Poole, R.K., Anjum, M.F., Membrillo-Hernández, J., Kim, S.O., Hughes, M.N. and Stewart, V. (1996) J. Bacteriol. 178, 5487–5492.
[13] D'mello, R., Purchase, D., Poole, R.K. and Hill, S. (1997) Microbiology 143, 231–237.
[14] Altuvia, S., Almiron, M., Huisman, G., Kolter, R. and Storz, G. (1994) Mol. Microbiol. 13, 265–272.
[15] Unden, G. (1988) Arch. Microbiol. 150, 499–503.
[16] Suzuki, K., Ueda, M., Yuasa, M., Nakagawa, T., Kawamukai, M. and Matsuda, H. (1994) Biosci. Biotech. Biochem. 58, 1814–1819.
[17] Gibert, I., Llagostera, M. and Barbé, J. (1988) J. Bacteriol. 170, 1346–1349.
[18] Knoell, H.-E. (1981) FEMS Microbiol. Lett. 10, 59–62.
[19] Berg, O.G. and von Hippel, P.H. (1988) J. Mol. Biol. 200, 709–723.
[20] Unden, G., Becker, S., Bongaerts, J., Holighaus, G., Schirawski, J. and Six, S. (1995) Arch. Microbiol. 164, 81–90.
[21] Guest, J.R., Green, J., Irvine, S. and Spiro, S. (1996) in: Regulation of Gene Expression in *Escherichia coli* (Lin, E.C.C. and Lynch, A.S., Eds.), pp. 317–342, R.G. Landes, Georgetown, TX.
[22] Bongaerts, J., Zoske, S., Weidner, U. and Unden, G. (1995) Mol. Microbiol. 116, 521–534.
[23] Compan, I. and Touati, D. (1994) Mol. Microbiol. 11, 955–964.
[24] Goosen, N. and van de Putte, P. (1995) Mol. Microbiol. 16, 1–7.