A role for DNA supercoiling in the regulation of the cytochrome *bd* oxidase of *Escherichia coli*

Keren J. Bebbington and Huw D. Williams

Author for correspondence: Huw D. Williams. Tel: +44 20 7594 5383. Fax: +44 20 7584 2056.
e-mail: h.d.williams@ic.ac.uk

Department of Biology,
Imperial College of
Science, Technology and
Medicine, Imperial College
Road, London SW7 2AZ,
UK

The *cydAB* operon of *Escherichia coli* encodes cytochrome *bd*, a terminal oxidase in the aerobic respiratory chain. The high oxygen affinity of this oxidase explains its increased synthesis under low-oxygen conditions. Expression of the *cydAB* operon is controlled by the ArcA/ArcB two-component system and the oxygen-sensing transcriptional regulator Fnr. However, *cydAB* expression is still induced upon entry into stationary phase or following a shift to anaerobic conditions in a mutant deleted for *arcA* and *fnr* [Cotter, P. A. & Gunsalus, R. P. (1992), *FEMS Microbiol Lett* 91, 31–36]. Indeed, such a mutant contains 60% of the wild-type levels of spectrally detectable cytochrome *bd*. A possible mechanism to account for this regulation is that changes in negative supercoiling, which occur during a shift to low-oxygen or anaerobic conditions, may contribute to the regulation of the *cydAB* operon. This paper reports several lines of evidence in support of this idea. Firstly, the expression of *cydAB*, and the final level of spectrally detectable cytochrome *bd*, is sensitive to inhibitors of DNA gyrase, the enzyme responsible for introducing negative supercoils into DNA. Both nalidixic acid and novobiocin reduce *cydA-lacZ* expression in a concentration-dependent manner. Secondly, in a *gyrA* mutant, defective in DNA gyrase activity, expression of *cydAB* is reduced to a basal level that is no longer sensitive to the oxygen status. Both gyrase inhibitors and the *gyrA* mutation reduce *cydAB* expression in a strain deleted for *arcA* and *fnr*, indicating that their effects are not mediated indirectly through ArcA or Fnr, but rather that they are likely to be direct effects on *cydAB* expression. In conclusion, the authors have shown that changes in DNA supercoiling play a role in the induction of *cydAB* expression and may provide a general way of increasing cytochrome *bd* levels in the cell in response to environmental stress.

Keywords: *arcA*, *fnr*, oxygen, respiration, DNA gyrase

INTRODUCTION

The branched aerobic respiratory chain of *Escherichia coli* has two well-characterized terminal oxidases, cytochrome *bo'* (or *bo₃*) and cytochrome *bd* (Poole *et al.*, 1994; Poole & Ingledew, 1987; Gennis & Stewart, 1996). A third terminal oxidase encoded by the *cbdAB* operon is called cytochrome *bd* II, based on its high degree of homology to cytochrome *bd*, although its function is poorly understood (Dassa *et al.*, 1991; Atlung & Brøndsted, 1994; Sturr *et al.*, 1996; Gennis & Stewart, 1996). Cytochrome *bo'* and cytochrome *bd* differ in their structural and functional properties and these differences have been invoked to explain why cytochrome *bo'* is favoured at high and cytochrome *bd* at low oxygen concentrations (Gennis & Stewart, 1996).

Cytochrome *bo'* belongs to the haem–copper oxidase superfamily (García-Horsman *et al.*, 1994). It has a moderate affinity for O₂ ($K_m \sim 0.2 \mu\text{M}$) and it is an energetically efficient oxidase, as in addition to functioning in a redox loop it also acts as an electrogenic proton-pump (Gennis & Stewart, 1996). Cytochrome *bd* is encoded by the *cydAB* operon (Green *et al.*, 1988) and it has no homology to members of the haem–copper oxidase superfamily. The enzyme is a heterodimer of two integral membrane polypeptides, subunit I (CydA, 58 kDa) and subunit II (CydB, 43 kDa; Miller & Gennis, 1983). Subunit I contains the haem *b*₅₅₈ and it is probably the site of quinol oxidation (Lorence *et al.*, 1987). The oxidase has two further haems, haem *b*₅₉₅ and haem *d*, which both bind exogenous ligands. The catalytic site, where oxygen is reduced to water, is thought to be a

haem *d*-haem b_{595} binuclear centre analogous to the haem-Cu_B binuclear centre of the haem-copper oxidases (Poole *et al.*, 1983; Rothery & Ingledew, 1989; D'Mello *et al.*, 1996; Hill *et al.*, 1993), although some data contradict this idea (Junemann, 1997). In *E. coli* the expression of the cytochrome *bd* complex increases at low oxygen tension (Rice & Hempfling, 1978; Cotter *et al.*, 1990; Iuchi *et al.*, 1990) and the oxidase has a very high affinity for O₂ ($K_m = 3\text{--}5$ nM, D'Mello *et al.*, 1996). It is energetically less efficient than cytochrome *bo'* as it does not function as a proton pump and it is relatively insensitive to inhibition by the classical cytochrome oxidase inhibitor KCN (Rice & Hempfling, 1978; Pudek & Bragg, 1974).

There is clear evidence for the role of the ArcA/ArcB two-component regulatory system and the oxygen-sensing transcription factor Fnr in the control of the *cydAB* operon (Lynch & Lin, 1996; Cotter *et al.*, 1990, 1997; Iuchi *et al.*, 1990, Cotter & Gunsalus, 1992). Two *cydAB* promoters have been identified by primer extension and both ArcA and Fnr regulate the transcription from the P1 promoter in response to anaerobiosis, with ArcA activating and Fnr repressing transcription under low-oxygen conditions (Cotter *et al.*, 1997). However, more recent evidence makes it likely that Fnr exerts its effect indirectly by both increasing the ArcA levels in anaerobic cells and increasing the ArcA-P/ArcA ratio. However, numerous reports on the regulation of *cydAB* in a $\Delta arcA \Delta fnr$ background indicate that the *cydAB* operon is still upregulated upon oxygen limitation, to a similar two- to fivefold degree as found in the wild-type strain, even though the absolute levels of transcription are lower in these mutants (Cotter *et al.*, 1990, 1997; Iuchi *et al.*, 1990; Cotter & Gunsalus, 1992). This suggests that in addition to ArcA/ArcB and Fnr there are undiscovered factors that both sense the availability of oxygen and upregulate *cydAB* expression when oxygen is limited. Extensive studies on the regulation of oxygen-controlled genes in *E. coli* have not identified another transcriptional regulator involved in this process. However, one possibility is that a change in DNA structure or topology at the *cydAB* promoter could enhance the productive interaction of the RNA polymerase. Since the onset of anaerobiosis coincides with an increase in negative DNA supercoiling (Dorman *et al.*, 1988), and cytochrome *bd* expression is known to be induced maximally under low O₂ conditions, we hypothesized that changes in DNA supercoiling may regulate cytochrome *bd*. The aim of this work was to test this hypothesis.

METHODS

Bacterial strains, media and growth conditions. The *E. coli* K-12 strains used in this work are listed in Table 1. Bacteria were routinely grown in LB medium, while TB was used for phage propagation (Silhavy *et al.*, 1984). Minimal Medium A (MMA) (Miller, 1972) contained either DL-lactate (0.2%, w/v) or glucose (0.6%, w/v) and was supplemented with the following amino acids where appropriate: arginine (0.9 mM), histidine (4.4 mM), isoleucine (4 mM), and phenylalanine, tryptophan and tyrosine (all 0.2 mM). Aerobic cultures were grown in

Erlenmeyer flasks containing one-fifth the flask volume of culture medium. Starter cultures were prepared by inoculating a single colony from a plate into 5 ml of the appropriate growth medium in 25 ml sterile Universal tubes and incubating overnight in an orbital shaker. These starter cultures were then diluted 50-fold to inoculate growth experiments. All cultures were grown at 37 °C and shaken at 200 r.p.m. Anaerobic growth was achieved by filling a 25 ml Universal tube with medium and incubating in an anaerobic jar (GasPak, Becton Dickinson). Growth was followed by measuring the increase in OD_{600 nm} in a Shimadzu MPS-2000 spectrophotometer.

High-titre λ lysates were prepared by induction of a strain carrying λ GC101 (Georgiou *et al.*, 1988) by growing it to early exponential phase and then adding mitomycin C to 2 mg ml⁻¹. The selection and identification of lysogens was carried out as described in Silhavy *et al.* (1984). Briefly, a high-titre lysate of λ GC101 was applied to a lawn of *E. coli* MC4100 and after overnight growth cells were purified from turbid plaques and their immunity checked by testing their ability to grow through phage streaks of different immunities including λ imm21, λ imm434 and λ vir. Approximately 10 independent lysogens were then grown and assayed for β -galactosidase expression to screen single from multiple lysogens. In each λ GC101 lysogen the *cydAB* genes were intact, as the *lacZ* fusions were stably integrated into the λ attachment site on the chromosome. P1vir transduction was carried out as described by Silhavy *et al.* (1984).

Spectrophotometry. Reduced-minus-oxidized difference spectra of membranes, resuspended in 50 mM potassium phosphate buffer, were obtained as described previously (Cunningham & Williams, 1995), using a Shimadzu MPS-2000 spectrophotometer, reducing and oxidizing samples with sodium dithionite and ammonium persulphate, respectively. The concentration of cytochrome *d* was determined using a millimolar absorption coefficient of 18.5 and a wavelength pair of 630–650 nm. Protein was quantified by the method of Markwell *et al.* (1978).

Analysis of *in vivo* plasmid supercoiling. To determine the level of *in vivo* plasmid supercoiling, topoisomers were separated in 1% agarose gels containing 25 μ g chloroquine ml⁻¹ (Ni Bhriain *et al.*, 1989). The mobility of the different topoisomers varies depending on the chloroquine concentration and at 25 μ g ml⁻¹ the more relaxed topoisomers migrate fastest. Electrophoresis was carried out in a cold-room at constant voltage of 3 V cm⁻¹ for 20 h with recirculation of the running buffer. The buffer contained 90 mM Tris (pH 8.3), 90 mM borate, 10 mM EDTA and chloroquine at the same concentration as in the gel. Chloroquine was washed from the gel by soaking in distilled water for at least 4 h before staining with ethidium bromide (5 μ g ml⁻¹).

β -Galactosidase assays. β -Galactosidase activity was assayed in cells grown in MMA with either glucose or lactate as carbon source as described previously (Georgiou *et al.*, 1988). β -Galactosidase values represent the mean of three experiments with a variation of less than 10%.

RESULTS

Cytochrome *bd* regulation in a $\Delta arcA \Delta fnr$ mutant

There have been a number of reports of the effects of mutation of *arcA* and *fnr* on *cydAB* expression (Cotter *et al.*, 1990, 1997; Iuchi *et al.*, 1990, Cotter & Gunsalus, 1992). We looked at this again here, but additionally

Table 1. *E. coli* K-12 strains

Strain	Relevant characteristic(s)	Source or reference
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>flbB5301 relA1 rpsL150 deoC1</i> , <i>ptsF25 rbsR</i>	Silhavy <i>et al.</i> (1984)
JRG1728	<i>lacX74 galU galK rpsL</i> Δ (<i>ara-leu</i>)	J. R. Guest, University of Sheffield, UK
PC35	Δ (<i>tyrR-fnr-rac-try</i>) <i>zdd-230::Tn9</i> , Cm ^R	
	MC4100 Δ <i>arcA</i> , Km ^R	Km ^R results from the kanamycin-resistance gene from pUC-4K (Pharmacia), inserted into <i>arcA</i> (Cotter & Gunsalus, 1992)
CSCG 6601	F ⁻ <i>zeg-298::Tn10 gyrA261</i> λ^- , Tc ^R Nal ^R	B. Bachmann, <i>E. coli</i> Genetic Stock Center
HW434	HW456 <i>zeg-298::Tn10 gyrA261</i> Φ (<i>cydA'-lacZ</i>), Tc ^R Nal ^R Ap ^R	P1.CSCG 6601 $\times$ HW456, selecting for Tc ^R and screening for Nal ^R
HW443	MC4100 Δ (<i>tyrR-fnr-rac-try</i>) <i>zdd-230::Tn9</i> , Cm ^R	P1.JRG1728 $\times$ MC4100, selecting for Cm ^R
HW445	MC4100 Δ <i>arcA</i> Δ (<i>tyrR-fnr-rac-try</i>) <i>zdd-230::Tn9</i> , Cm ^R Km ^R	P1.PC35 $\times$ HW443, selecting for Km ^R
HW447	HW445 <i>zeg-298::Tn10 gyrA261</i> , Cm ^R Km ^R Tc ^R Nal ^R	P1.HW434 $\times$ HW445, selecting for Tc ^R and screening for Nal ^R
HW456	MC4100 Φ (<i>cydA'-lacZ</i>), Ap ^R	λ GC101 $\times$ MC4100, selecting for Ap ^R
HW457	HW445 Φ (<i>cydA'-lacZ</i>), Ap ^R Cm ^R Km ^R	λ GC101 $\times$ HW445, selecting for Ap ^R
HW461	HW447 Φ (<i>cydA'-lacZ</i>), Cm ^R Km ^R Tc ^R Nal ^R Ap ^R	λ GC101 $\times$ HW447 selecting for Ap ^R

Fig. 1. Effect of deletion of *arcA* and *fnr* on *cydA* expression. *E. coli* HW456 [Φ (*cydA-lacZ*)] (■) or *E. coli* HW457 Δ *arcA* Δ *fnr* [Φ (*cydA-lacZ*)] (□). β -Galactosidase activity was assayed as a function of growth in lactate-containing MMA.

followed *cydA-lacZ* expression throughout the growth curve and looked at final cytochrome *bd* levels in membranes using difference spectrophotometry. During aerobic growth in lactate minimal medium, *cydA-lacZ* expression increased during exponential growth to a maximum as the culture approached stationary phase (Fig. 1). A similar pattern of expression was seen in HW457 Δ *arcA* Δ *fnr* except that the final expression levels were about half those found in HW456. In both strains expression was induced about fourfold during growth.

We determined whether the level of spectrally detectable cytochrome *bd* in HW456 and HW457 reflected the pattern of *cydA-lacZ* expression observed. Membranes from both strains had the characteristic signals of cytochrome *bd* in reduced-minus-oxidized spectra (data not shown): a weak maximum at 595 nm due to cytochrome *b595*, a prominent peak at 628 nm due to

reduced cytochrome *d* and a trough at 650 nm resulting from the oxygenated form of cytochrome *d* (the dominant species in the 'oxidized' reference cuvette (Miller & Gennis, 1983; Poole *et al.*, 1983). Quantification of cytochrome *d* levels showed that there was a 35% reduction of the levels of cytochrome *d* in HW457 Δ *arcA* Δ *fnr* compared to HW456 (Fig. 2), in contrast to a 60% reduction in β -galactosidase activity between these strains at the same point in the growth curve. This indicates that while mutation of the regulatory genes *arcA* and *fnr* leads to a reduction in cytochrome *bd* levels, a significant fraction of the wild-type activity remains.

DNA gyrase inhibitors affect *cydAB* expression

The level of DNA supercoiling *in vivo* can be perturbed by using specific inhibitors of DNA gyrase. To determine whether DNA supercoiling has a role in the regulation of *cydAB* expression, the effect of the DNA gyrase inhibitors novobiocin and nalidixic acid on *cydA-lacZ* expression were investigated. Concentrations of novobiocin up to 50 μ g ml⁻¹ had no effect and up to 150 μ g ml⁻¹ only a marginal effect on the growth rate of HW456 (data not shown). However, increasing concentrations of novobiocin led to a progressive reduction in *cydA-lacZ* expression in samples taken from late-exponential-phase cultures (Fig. 3a). Concentrations of nalidixic acid up to 2.5 μ g ml⁻¹ did not alter the growth rate and also reduced the expression of *cydA-lacZ* in late-exponential-phase cultures in a concentration-dependent manner (Fig. 3b). Both novobiocin and nalidixic acid had a substantial effect on the pattern of *cydA-lacZ* expression throughout the growth cycle (Fig. 4). Novobiocin at 50 μ g ml⁻¹ delayed the normal induction of *cydA-lacZ* expression (Fig. 4). At 150 μ g novobiocin ml⁻¹, expression remained at a low basal level

Fig. 2. Spectral levels of cytochrome *d*, determined in membrane preparations from cells grown in lactate-containing MMA to an OD_{600} of 0.8. Reduced-minus-oxidized difference spectra were recorded and the levels of cytochrome *d* quantified from $\Delta A_{628-650}$ and an absorption coefficient of 18.5 mM cm^{-1} as described in Methods. NA is nalidixic acid and NB is novobiocin and the indicated quantities of both are in units of $\mu\text{g ml}^{-1}$.

Fig. 3. Effect of DNA gyrase inhibitors on *cydA* expression. *E. coli* HW456 [$\Phi(\text{cydA-lacZ})$] was grown in lactate-containing MMA to an OD_{600} of 0.8 in the presence of the indicated concentrations of novobiocin (a) or nalidixic acid (b) and β -galactosidase activity assayed.

throughout the experiment. In the presence of $1.5 \mu\text{g}$ nalidixic acid ml^{-1} the induction of *cydAB* expression was reduced compared to that in HW456, while with $2.5 \mu\text{g}$ nalidixic acid ml^{-1} induction from the basal level was completely abolished. Under the conditions used for the *cydAB* expression experiments, we confirmed that the concentrations of novobiocin and nalidixic acid used affected the *in vivo* supercoiling levels of the plasmid pBR322. pBR322 DNA isolated from novobiocin and nalidixic acid treated cells, when examined on chloroquine-agarose gels, showed topoisomers that migrated more rapidly than plasmid from untreated

Fig. 4. Effect of DNA gyrase inhibitors on *cydA* expression throughout the growth cycle. (a) HW456 (■); HW456 + $50 \mu\text{g}$ novobiocin ml^{-1} (□); HW456 + $150 \mu\text{g}$ novobiocin ml^{-1} (○). (b) HW456 (■); HW456 + $1.5 \mu\text{g ml}^{-1}$ nalidixic acid (□); HW456 + $2.5 \mu\text{g ml}^{-1}$ nalidixic acid (○). β -Galactosidase activity was assayed as a function of growth in lactate-containing MMA.

cells, consistent with previous published results (Dorman *et al.*, 1988) (data not shown). This indicated the plasmid DNA to be more relaxed, consistent with less negatively supercoiled DNA, as would be expected with inhibition of DNA gyrase. These data are consistent with a correlation between the levels of *in vivo* supercoiling and *cydAB* expression. The levels of cytochrome *d* in late-exponential-phase cultures treated with novobiocin or nalidixic acid are shown in Fig. 2: $50 \mu\text{g}$ novobiocin ml^{-1} had no effect on the amount of spectrally detectable cytochrome *d* in membranes, while

Fig. 5. Effect of DNA gyrase inhibitors on *cydA* expression in a $\Delta arcA \Delta fnr$ background. (a) HW457 $\Delta arcA \Delta fnr$ (■); HW457 $\Delta arcA \Delta fnr$ + 50 μg novobiocin ml^{-1} (□); HW457 $\Delta arcA \Delta fnr$ + 150 μg novobiocin ml^{-1} (○). (b) HW457 $\Delta arcA \Delta fnr$ (■); HW457 $\Delta arcA \Delta fnr$ + 1.5 μg nalidixic acid ml^{-1} (□); HW457 $\Delta arcA \Delta fnr$ + 2.5 μg nalidixic acid ml^{-1} (○). β -Galactosidase activity was assayed as a function of growth in lactate-containing MMA.

150 μg ml^{-1} reduced levels to 60% of those in the untreated cells. Increasing nalidixic acid concentrations led to a progressive decrease in the levels of cytochrome *d*, to 20% of the untreated levels in membranes from cultures treated with 2 μg nalidixic acid ml^{-1} .

The effect of DNA gyrase inhibitors on *cydAB* expression is independent of the ArcAB regulon and Fnr

To determine whether novobiocin and nalidixic acid decrease *cydAB* expression via direct or indirect effects on either the ArcAB regulon or Fnr, we determined whether these DNA gyrase inhibitors modified *cydAB* expression in a $\Delta arcA \Delta fnr$ background. The data in Fig. 5 show that both novobiocin and nalidixic acid reduced *cydA-lacZ* expression in the $\Delta arcA \Delta fnr$ double mutant HW457. With 50 μg novobiocin ml^{-1} , the reduced level of induction observed in the $\Delta arcA \Delta fnr$ background was delayed, while induction was abolished with 150 μg ml^{-1} . Similarly, nalidixic acid treatment led to a concentration-dependent reduction in *cydA-lacZ* expression in a $\Delta arcA \Delta fnr$ background, with β -galactosidase levels remaining at a basal level of around 150 units throughout growth. Novobiocin at 150 μg ml^{-1} , and all concentrations of nalidixic acid

Fig. 6. Effect of *gyrA* mutation on *cydA* expression. ■, HW456; □, HW434 *gyrA261*; ○, HW461 $\Delta arcA \Delta fnr$ *gyrA261*. β -Galactosidase activity was assayed as a function of growth in lactate-containing MMA.

tested, significantly reduced cytochrome *d* levels in membranes (Fig. 2). In conclusion, these data suggest that any effect on *cydAB* expression through modifying DNA supercoiling is not a result of indirect effects on the expression of or binding of ArcA or Fnr to the *cydAB* promoters.

A *gyrA* mutation affects cytochrome *bd* expression

Since gyrase inhibitors are capable of reducing cytochrome *bd* expression, we further examined the effect of a *gyrA* mutation on *cydA-lacZ* expression. HW434 *gyrA261* showed a dramatic reduction in *cydA-lacZ* expression throughout growth compared to its isogenic parent, HW456 (Fig. 6). No induction of expression was observed above the basal level present at the start of exponential growth. Transduction of the *gyrA261* mutation into a $\Delta arcA \Delta fnr$ background, to construct HW461, led to a similarly severe repression of *cydA-lacZ* expression. Spectral analysis supported the gene fusion data, in that cytochrome *d* spectral signals were markedly reduced in *gyrA261* backgrounds (Fig. 2). Analysis of the distribution of topoisomers of plasmid DNA isolated from HW456 and HW434 backgrounds confirmed that the plasmid DNA was indeed more relaxed in the *gyrA261* background (data not shown), which is indicative of a reduction in negative supercoiling leading to a reduction in cytochrome *bd* expression.

Effect of mutation of *gyrA* on the anaerobic expression of cytochrome *bd*

Finally, we examined the effect of a *gyrA* mutation on the anaerobic expression of cytochrome *bd*. Negative DNA supercoiling is increased under anaerobic compared to aerobic conditions (Dorman *et al.*, 1988). Therefore, if our model is correct, and this increase in negative supercoiling is in part responsible for the induction of *cydAB* under anaerobic conditions, then we would predict that anaerobic induction of *cydAB* would be abolished in a *gyrA* background. For this experiment

Fig. 7. Effect of *gyrA* mutation on *cydA* expression during (a) aerobic and (b) anaerobic growth in MMA medium with glucose as the carbon source. ■, HW456; □, HW434 *gyrA261*; ○, HW461 $\Delta arcA \Delta fnr gyrA261$. β -Galactosidase activity was assayed as a function of growth.

we grew cultures with glucose as the fermentable carbon source, and so we first confirmed that *gyrA261* abolishes induction of *cydAB* during aerobic growth on glucose. This indeed was the case in both *gyrA261* and *gyrA261* $\Delta arcA \Delta fnr$ backgrounds (Fig. 7a). In addition, Fig. 7(b) clearly shows that anaerobic induction of *cydA-lacZ* is abolished in *gyrA261* backgrounds.

DISCUSSION

It is well established that the *cydAB* operon is under the control of the Arc regulon and Fnr (Lynch & Lin, 1996). However, it is clear that there is a further level of regulation that has not been elucidated. Evidence for this comes from the finding that strains deleted for *arcA* and *fnr* still show significant induction of expression under anaerobic conditions (Lynch & Lin, 1996; Cotter *et al.*, 1997; this study, Fig. 7), and during entry into stationary phase, when oxygen levels will be depleted (Fig. 1). However, no further transcriptional regulators have been found to have a role in cytochrome *bd* regulation. It is well established that many prokaryotic genes have their transcription modified by the levels of negative DNA supercoiling in the cell (Schrum & Hassan, 1992; Drlica, 1992; Dimri & Das, 1988; Ni Bhriain *et al.*, 1989; Dorman, 1995; Dorman *et al.*, 1988; Graeme-Cook *et al.*, 1989; Grau *et al.*, 1994; Kareem & Foster, 1993; O'Byrne *et al.*, 1992; Sun & Fuchs, 1994). Furthermore, there is an increase in the

levels of DNA supercoiling following a shift from high- to low-oxygen conditions (Dorman *et al.*, 1988). Therefore, we hypothesized that the increase in DNA supercoiling that occurs upon a shift to low-oxygen conditions might have a role in inducing *cydAB* expression. In support of this we report several lines of evidence. Firstly, expression of *cydAB* is sensitive to two inhibitors of DNA gyrase, the enzyme responsible for introducing negative supercoiling into DNA, that differ in their mode of action. Secondly, expression is altered in mutants with lesions in the *gyrA* gene, which encodes DNA gyrase. DNA supercoiling is modulated by the actions of topoisomerase I, which causes DNA relaxation by making single-strand breaks in the DNA, and DNA gyrase, which is responsible for both relaxation and increasing negative supercoiling by an ATP-dependent mechanism (Drlica & Coughlin, 1989). The activity of DNA gyrase is inhibited by nalidixic acid, which affects the A subunit by trapping the gyrase-DNA complex (Drlica, 1992), and by novobiocin, which blocks ATP hydrolysis (Fisher *et al.*, 1992). We report here that both novobiocin and nalidixic acid decreased expression of a *cydA-lacZ* fusion in a concentration-dependent manner and at concentrations that reduced the negative supercoiling of a reporter plasmid.

Besides gyrase inhibitors, the level of DNA supercoiling can be perturbed by mutation of the *gyrA* gene. In an isogenic *cydA-lacZ* fusion strain carrying a *gyrA* mutation there was a marked reduction of *cydA-lacZ* expression throughout the growth cycle. The levels of spectrally detectable cytochrome *bd* broadly followed *cydA-lacZ* expression levels, indicating that changes in supercoiling alter the effective cytochrome *bd* oxidase levels in the cell. We considered the possibility that changes in supercoiling might be affecting *cydAB* expression by altering the ability of the *cydAB* promoter to respond to regulatory proteins ArcA and Fnr, or by altering the expression of *arcA*, *arcB* or *fnr*. To investigate this possibility the effects of DNA gyrase inhibitors and a *gyrA* mutant on *cydA-lacZ* expression were investigated in a $\Delta arcA \Delta fnr$ background. However, it is clear that both gyrase inhibitors and *gyrA* mutation reduce *cydAB* expression in a strain background deleted of *arcA* and *fnr* (Figs 5 and 6). These data do not prove but are consistent with a role for DNA supercoiling in the regulation of *cydAB* operon expression. As our experiments involve artificial perturbation of supercoiling levels they cannot provide definitive evidence that DNA supercoiling regulates cytochrome *bd*, while DNA supercoiling measured using reporter plasmids may not give a perfectly accurate picture of the levels of chromosomal supercoiling. However, the fact that we obtained similar results with two different gyrase inhibitors and with a *gyrA* mutant, together with the previous demonstrations that changes in oxygen availability perturb cellular supercoiling levels, gives us confidence in concluding that DNA supercoiling has a role in regulating the levels of cytochrome *bd* in *E. coli*.

Entry into stationary phase through carbon starvation

alters the level of DNA supercoiling (Dorman *et al.*, 1988; Balke & Gralla, 1987). It is known that cytochrome *bd* is required for effective exit from stationary phase (Siegele & Kolter, 1993; Siegele *et al.*, 1996; Goldman *et al.*, 1996). Therefore, perhaps control of *cydAB* expression by changes in DNA supercoiling provides a general way of upregulating *cydAB* levels in response to environmental stresses that might lead to cessation of growth. *E. coli* has a branched aerobic respiratory chain; the relatively low oxygen affinity but energetically efficient oxidase, cytochrome *bo'*, is preferred under high-oxygen conditions. It would be interesting to examine if it is also regulated by DNA supercoiling perturbations and if so whether they affect it in the opposite direction to *cydAB* in order to achieve co-ordinate regulation of these oxidases.

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REFERENCES

- Atlung, T. & Brøndsted, L. (1994). Role of the transcriptional activator AppY in regulation of the *cyx appA* operon of *Escherichia coli* by anaerobiosis, phosphate starvation, and growth phase. *J Bacteriol* **176**, 5414–5422.
- Balke, V. L. & Gralla, J. D. (1987). Changes in the linking number of supercoiled DNA accompany growth transitions in *Escherichia coli*. *J Bacteriol* **169**, 4499–4506.
- Cotter, P. A. & Gunsalus, R. P. (1992). Contribution of the *fnr* and *arcA* gene products in coordinate regulation of cytochrome *o* and *d* oxidases (*cyoABCDE* and *cydAB* genes) in *Escherichia coli*. *FEMS Microbiol Lett* **91**, 31–36.
- Cotter, P. A., Chepuri, V., Gennis, R. B. & Gunsalus, R. P. (1990). Cytochrome *o* (*cyoABCDE*) and *d* (*cydAB*) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH and the *fnr* gene product. *J Bacteriol* **172**, 6333–6338.
- Cotter, P. A., Melville, S. B., Albrecht, J. A. & Gunsalus, R. P. (1997). Aerobic regulation of cytochrome *d* oxidase (*cydAB*) operon expression in *Escherichia coli*: roles of Fnr and ArcA in repression and activation. *Mol Microbiol* **25**, 605–615.
- Cunningham, L. & Williams, H. D. (1995). Isolation and characterisation of mutants defective in the cyanide insensitive respiratory pathway of *Pseudomonas aeruginosa*. *J Bacteriol* **177**, 432–438.
- Dassa, E., Fsihi, H., March, C., Dion, M., Kieffer-Bontemps, M. & Boquet, P. L. (1991). A new oxygen-regulated operon in *Escherichia coli* comprises the genes for a putative third cytochrome oxidase and for pH 2.5 acid phosphatase (*appA*). *Mol Gen Genet* **229**, 341–352.
- Dimri, G. P. & Das, H. K. (1988). Transcriptional regulation of nitrogen fixation genes by DNA supercoiling. *Mol Gen Genet* **212**, 360–363.
- D'Mello, R., Hill, S. & Poole, R. K. (1996). The cytochrome *bd* quinol oxidase in *Escherichia coli* has an extremely high apparent affinity for oxygen and two oxygen-binding haems: implications for regulation of activity *in vivo* by substrate (oxygen) inhibition. *Microbiology* **142**, 755–763.
- Dorman, C. J. (1995). DNA topology and the global control of bacterial gene expression: implications for the regulation of virulence gene expression in pathogenic bacteria. *Microbiology* **141**, 1271–1280.
- Dorman, C. J., Barr, G. C., Ni Bhriain, N. & Higgins, C. F. (1988). DNA supercoiling and the anaerobic and growth phase regulation of *tonB* gene expression. *J Bacteriol* **179**, 2816–2826.
- Drlica, K. (1992). Control of bacterial DNA supercoiling. *Mol Microbiol* **6**, 425–433.
- Drlica, K. & Coughlin, S. (1989). Inhibitors of DNA gyrase. *Pharmacol Ther* **44**, 107–121.
- Fisher, L. M., Austin, C. A., Hopewell, R., Margerrison, E. E. C., Oram, M., Patel, S., Plummer, K., Sng, J.-H. & Sreedharan, S. (1992). DNA supercoiling and relaxation by ATP-dependent DNA topoisomerases. *Philos Trans R Soc Lond Ser B Biol Sci* **336**, 83–91.
- García-Horsman, J. A., Barquera, B., Rumbley, J., Ma, J. & Gennis, R. B. (1994). The superfamily of haem-copper respiratory oxidases. *J Bacteriol* **176**, 5587–5600.
- Gennis, R. B. & Stewart, V. (1996). Respiration. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, 2nd edn, pp. 217–261. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Georgiou, C. D., Dueweke, T. J. & Gennis, R. B. (1988). Regulation of expression of cytochrome *d* of *Escherichia coli* is transcriptional. *J Bacteriol* **170**, 961–966.
- Goldman, B. S., Gabbert, K. K. & Kranz, R. G. (1996). The temperature-sensitive growth and survival phenotypes of *Escherichia coli* *cydDC* and *cydAB* strains are due to deficiencies in cytochrome *bd* and are corrected by exogenous catalase and reducing agents. *J Bacteriol* **178**, 6348–6351.
- Graeme-Cook, K. A., May, G., Bremer, E. & Higgins, C. F. (1989). Osmotic regulation of porin expression: a role for DNA supercoiling. *Mol Microbiol* **3**, 1287–1294.
- Grau, R., Gardiol, D., Gilkin, G. C. & di Mendoza, D. (1994). DNA supercoiling and thermal regulation of unsaturated fatty acid synthesis in *Bacillus subtilis*. *Mol Microbiol* **11**, 933–941.
- Green, N. G., Fang, H., Lin, R.-J., Newton, G., Mather, M., Georgiou, C. & Gennis, R. B. (1988). The nucleotide sequence of the *cyd* locus encoding the two subunits of the cytochrome *d* terminal oxidase complex of *Escherichia coli*. *J Biol Chem* **263**, 13138–13143.
- Hill, J. J., Alben, J. O. & Gennis, R. B. (1993). Spectroscopic evidence for a haem-haem binuclear center in the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*. *Proc Natl Acad Sci USA* **90**, 5863–5867.
- Iuchi, S., Chepuri, V., Fu, H.-A., Gennis, R. B. & Lin, E. C. C. (1990). Requirement for terminal cytochromes in generation of the aerobic signal for the *arc* regulatory system in *Escherichia coli*: study using deletions and *lac* fusions of *cyo* and *cyd*. *J Bacteriol* **172**, 6020–6025.
- Junemann, S. (1997). Cytochrome *bd* terminal oxidase. *Biochim Biophys Acta* **132**, 107–127.
- Karem, K. & Foster, J. W. (1993). The influence of DNA topology on the environmental regulation of a pH regulated locus in *Salmonella typhimurium*. *Mol Microbiol* **10**, 75–86.
- Lorence, R. M., Carter, K., Green, G. N. & Gennis, R. B. (1987). Cytochrome *b-558* monitors the steady-state redox state of the ubiquinone pool in the aerobic respiratory chain of *Escherichia coli*. *J Biol Chem* **262**, 10532–10536.
- Lynch, A. S. & Lin, E. C. C. (1996). Responses to molecular oxygen. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, 2nd edn, pp. 1526–1538. Edited by F. C.

Neidhardt and others. Washington, DC: American Society for Microbiology.

Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**, 206–210.

Miller, J. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Miller, M. J. & Gennis, R. B. (1983). The purification and characterisation of the cytochrome *d* terminal oxidase complex of the *Escherichia coli* aerobic respiratory chain. *J Biol Chem* **258**, 9159–9165.

Ni Bhriain, N., Dorman, C. J. & Higgins, C. F. (1989). An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. *Mol Microbiol* **3**, 933–942.

O'Byrne, C. P., Ni Bhriain, N. & Dorman, C. J. (1992). The DNA sensitive supercoiling sensitive expression of the *Salmonella typhimurium* *his* operon requires the *his* attenuator and is modulated by anaerobiosis and by osmolarity. *Mol Microbiol* **6**, 2467–2476.

Poole, R. K. & Ingledew, W. J. (1987). Pathways of electrons to oxygen. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 170–200. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.

Poole, R. K., Kumar, C., Salmon, I. & Chance, B. (1983). The 650 nm chromophore in *Escherichia coli* is an oxy-compound or oxygenated compound, not the oxidized form of cytochrome oxidase *d* – an hypothesis. *J Gen Microbiol* **129**, 1335–1344.

Poole, R. K., Salmon, I. & Chance, B. (1994). The high-spin cytochrome *o'* component of the cytochrome *bo*-type quinol oxidase in membranes from *Escherichia coli*: formation of the primary oxygenated species at low temperatures is characterized by a slow 'on' rate and low dissociation constant. *Microbiology* **140**, 1027–1034.

Pudek, M. R. & Bragg, P. D. (1974). Inhibition by cyanide of the respiratory chain oxidases of *Escherichia coli*. *Arch Biochem Biophys* **164**, 682–693.

Rice, C. W. & Hempfling, W. P. (1978). Oxygen-limited continuous culture and respiratory energy conservation in *Escherichia coli*. *J Bacteriol* **134**, 115–124.

Rothery, R. & Ingledew, W. J. (1989). The cytochromes of anaerobically grown *Escherichia coli*. An electron paramagnetic study of the cytochrome *bd* complex in situ. *Biochem J* **262**, 437–443.

Schrum, L. W. & Hassan, H. M. (1992). Transcriptional regulation of Mn-superoxide dismutase gene (*sodA*) of *Escherichia coli* is stimulated by DNA gyrase inhibitors. *Arch Biochem Biophys* **209**, 185–192.

Siegele, D. A. & Kolter, R. (1993). Isolation and characterization of an *Escherichia coli* mutant defective in resuming growth after starvation. *Genes Dev* **7**, 2629–2640.

Siegele, D. A., Imlay, K. R. & Imlay, J. A. (1996). The stationary-phase-exit defect of *cydC* (*surB*) mutants is due to the lack of a functional terminal cytochrome oxidase. *J Bacteriol* **178**, 6091–6096.

Silhavy, T. J., Berman, M. L. & Enquist, L. W. (1984). *Experiments with Gene Fusions*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sturr, M. G., Krulwich, T. A. & Hicks, D. B. (1996). Purification of a cytochrome *bd* terminal oxidase encoded by the *Escherichia coli* *app* locus from a $\Delta cyo \Delta cyd$ strain complemented by genes from *Bacillus firmus* OF4. *J Bacteriol* **176**, 1742–1749.

Sun, L. & Fuchs, J. A. (1994). Regulation of the *Escherichia coli* *nrd* operon: role of DNA supercoiling. *J Bacteriol* **176**, 4617–4626.

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