A role for DNA supercoiling in the regulation of the cytochrome \textit{bd} oxidase of \textit{Escherichia coli}

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

**Keywords:** \textit{arcA}, \textit{fnr}, oxygen, respiration, DNA gyrase

INTRODUCTION

The branched aerobic respiratory chain of \textit{Escherichia coli} has two well-characterized terminal oxidases, cytochrome \textit{bo′} (or \textit{bo}) and cytochrome \textit{bd} \cite{Poole, et al., 1987; Gennis & Stewart, 1996}. A third terminal oxidase encoded by the \textit{cbdAB} operon is called cytochrome \textit{bd} II, based on its high degree of homology to cytochrome \textit{bd}, although its function is poorly understood \cite{Dassa et al., 1994; Atlung & Brøndsted, 1994; Sturr et al., 1996; Gennis & Stewart, 1996}. Cytochrome \textit{bo′} and cytochrome \textit{bd} differ in their structural and functional properties and these differences have been invoked to explain why cytochrome \textit{bo′} is favoured at high and cytochrome \textit{bd} at low oxygen concentrations \cite{Gennis & Stewart, 1996}. Cytochrome \textit{bo′} belongs to the haem–copper oxidase superfamily \cite{Garcia-Horsman et al., 1994}. It has a moderate affinity for \textit{O}_2 \left(K_m \sim 0.2 \mu\text{M}\right) and it is an energetically efficient oxidase, as in addition to functioning in a redox loop it also acts as an electrogenic proton-pump \cite{Gennis & Stewart, 1996}. Cytochrome \textit{bd} is encoded by the \textit{cydAB} operon \cite{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 (\textit{CydA}, 58 kDa) and subunit II (\textit{CydB}, 43 kDa; Miller & Gennis, 1983). Subunit I contains the haem \textit{b}_{595} and it is probably the site of quinol oxidation \cite{Lorenz et al., 1987}. The oxidase has two further haems, haem \textit{b}_{595} and haem \textit{d}, which both bind exogenous ligands. The catalytic site, where oxygen is reduced to water, is thought to be a...
haem d–haem $b_{58}$ binuclear centre analogous to the haem–Cu$_B$ binuclear centre of the haem–copper oxidases (Poole et al., 1983; Rothery & Ingledeu, 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 O$_2$ ($K_m = 3–5$ mM, D’Mello et al., 1996). It is energetically less efficient than cytochrome bo$_{3}$ 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/ArcB ratio. However, numerous reports on the regulation of cydAB in a ΔarcA Δfnr 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 O$_2$ 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 (4·0 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 λGC101 (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 λGC101 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 immunity including zimM21, zimM434 and λvir. Approximately 10 independent lysogens were then grown and assayed for β-galactosidase expression to screen single from multiple lysogens. In each λGC101 λysogen 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, toposoisomers were separated in 1% agarose gels containing 25 μg chloroquine ml$^{-1}$ (Ni Bhriain et al., 1989). The mobility of the different toposoisomers varies depending on the chloroquine concentration and at 25 μg ml$^{-1}$ the more relaxed toposoisomers 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 μg 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 ΔarcA Δfnr 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
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 ΔarcA Δfnr 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 ΔarcA Δfnr 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 µg ml⁻¹ had no effect and up to 150 µg 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 µg 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 µg ml⁻¹ delayed the normal induction of cydA–lacZ expression (Fig. 4). At 150 µg novobiocin ml⁻¹, expression remained at a low basal level.
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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 µg ml$^{-1}$.

Fig. 3. Effect of DNA gyrase inhibitors on cydA expression. E. coli HW456 [Φ(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 µg nalidixic acid ml$^{-1}$ the induction of cydAB expression was reduced compared to that in HW456, while with 2.5 µg 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 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 µg novobiocin ml$^{-1}$ had no effect on the amount of spectrally detectable cytochrome d in membranes, while
whether these DNA gyrase inhibitors modified on either the ArcAB regulon or Fnr, we determined HW457 ∆ at 150 µg novobiocin ml⁻¹ was delayed, while induction was abolished with β

to determine whether novobiocin and nalidixic acid expression is independent of the ArcAB regulon and 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 ∆arcA ∆fnr background. The data in Fig. 5 show that both novobiocin and nalidixic acid reduced cydA–lacZ expression in the ∆arcA ∆fnr double mutant HW457. With 50 µg novobiocin ml⁻¹, the reduced level of induction observed in the ∆arcA ∆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 ∆arcA ∆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 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 ∆arcA ∆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
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 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 ΔarcA Δfnr 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 (Siegel & Kolter, 1993, Siegel 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


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