Transcriptional control of several aerobically induced cytochrome structural genes in *Rhodobacter sphaeroides*

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To decipher how the synthesis of energy-transducing enzymes responds to environmental cues, the response of three *Rhodobacter sphaeroides* aerobic cytochrome gene promoters was analysed under different conditions. Two of these promoters are upstream of structural genes (*ctaD* and *coxII*) for individual subunits of the cytochrome *aa*₃ respiratory complex. The third promoter is that for the *cycFG* operon, which encodes two c-type cytochromes of unknown function, cytochrome *c*₅₅₄ and CycG. Primer extension analysis identified a single oxygen-responsive transcription start site for each gene. Utilizing operon fusions to *Escherichia coli lacZ* as a measure of promoter activity, transcription from the *ctaD*, *coxII* and *cycFG* promoters was approximately twofold higher when cells were grown at high (30%) oxygen tensions than under low (2%) oxygen or anaerobic (photosynthetic) conditions. Analysis of promoter function using specific host mutations indicated that loss of the *R. sphaeroides* FNR homologue, FnrL, causes a small, but reproducible, increase in *cycFG* and *coxII* transcription when cells are grown at 2% oxygen. However, neither the Δ FnrL mutation nor alterations in sequences related to a consensus target site for the *E. coli* FNR protein increased function of any of these three promoters to that seen under aerobic conditions in wild-type cells. From this we conclude that FnrL is not solely responsible for reduced transcription of these three aerobic cytochrome genes under low oxygen or anaerobic conditions. When activity of these three promoters was monitored after cells were shifted from anaerobic (photosynthetic) conditions to a 30% oxygen atmosphere, it took several cell doublings for LacZ levels to increase to those found in steady-state 30% oxygen cultures. From these results, it appears that activity of these promoters is also regulated by a stable molecule whose synthesis or function responds slowly to the presence of high oxygen tensions.

Keywords: cytochromes, gene expression, transcriptional control, anaerobic regulation, oxygen regulation

INTRODUCTION

Most facultative bacteria contain multiple energy-generating pathways whose synthesis is often controlled by overlapping global regulatory networks. By coordinating expression of specific respiratory components, these networks help direct electron flow through particular energy-generating pathways under

different environmental conditions. This study sought to define metabolic and genetic elements that control expression of cytochromes known to either function or to be induced under aerobic respiratory conditions in the facultative phototroph *Rhodobacter sphaeroides*. While genes encoding components of the *R. sphaeroides* photosynthetic apparatus are known to respond to oxygen, light and tetrapyrrole availability (Lee & Kaplan, 1992; Schilke & Donohue, 1992; Eraso & Kaplan, 1994; Penfold & Pemberton, 1994; Gomelsky & Kaplan, 1995), factors governing expression of

Abbreviations: cyt *aa*₃, cytochrome *aa*₃ oxidase; LacZ, β -galactosidase.

aerobic electron carriers in this facultative phototroph have received relatively little attention.

Like most bacterial species, *R. sphaeroides* contains a branched aerobic respiratory chain (García-Horsman *et al.*, 1994a). The cytochrome *aa₃* oxidase (cyt *aa₃*) of this bacterium is a well-characterized prokaryotic homologue of the mitochondrial cytochrome oxidase and a member of the haem-copper oxidase superfamily (Cao *et al.*, 1991, 1992; Shapleigh & Gennis, 1992). From studies on many haem-copper oxidases, a paradigm has developed in which the cyt *aa₃* family members are maximally expressed in highly aerated bacteria because they have a lower affinity for oxygen than most of the alternative cytochrome oxidases (García-Horsman *et al.*, 1994a; Trumpower & Gennis, 1994). To test if such differential expression of aerobic cytochromes occurs in *R. sphaeroides*, we examined transcription of two cyt *aa₃* genes (*ctaD* and *coxII*), both in steady-state cultures grown at different oxygen tensions and when photosynthetic (anaerobic) cultures were shifted to high (30%) oxygen conditions. Since previous work suggested that the elevated levels of cytochrome *c₅₅₄* found under high (30%) oxygen growth conditions reflects increased *cycFG* transcription (Flory & Donohue, 1995), we also analysed function of this promoter. In considering how these genes might be regulated by changes in oxygen tension, the existence of sites similar to the consensus sequence for the *Escherichia coli* global anaerobic regulator FNR in the *cycFG*, *coxII* and *ctaD* control regions (Cao *et al.*, 1991; Shapleigh & Gennis, 1992; Flory & Donohue, 1995) make it possible that their expression is repressed under anaerobic conditions by the *R. sphaeroides* FNR homologue, FnrL (Zeilstra-Ryalls & Kaplan, 1995). All three of these promoters are oxygen-regulated since β -galactosidase (LacZ) levels from specific operon fusions to *E. coli lacZ* are reduced under low oxygen or anaerobic (photosynthetic) growth conditions. However, by monitoring promoter function from mutant reporter genes either in wild-type cells or in a defined FnrL null mutant it appears that this *R. sphaeroides* global anaerobic transcription factor is not solely responsible for normal oxygen control of their transcription. Indeed, the time-dependent increases in promoter activity when oxygen is added to anaerobic (photosynthetic) cultures suggest that *cycFG*, *coxII* and *ctaD* transcription is also controlled by a stable, anaerobic repressor or an activator that is synthesized during growth in the presence of high (30%) oxygen.

METHODS

Cell growth, media and genetic techniques. *R. sphaeroides* cells (Table 1) were grown on solid media or in liquid cultures of Sistrom's minimal medium A at 32 °C (Donohue *et al.*, 1986). Aerobic cultures (100 ml) were sparged either with a gas mixture known to repress photosynthetic membrane synthesis (30% O₂, 69% N₂, 1% CO₂; Chory *et al.*, 1984) or at low oxygen tensions (2% O₂, 97% N₂, 1% CO₂). Steady-state anaerobic (photosynthetic) cells were grown in filled tubes in front of a tungsten light bank (Donohue *et al.*, 1986).

For shifting cultures from anaerobic (photosynthetic) to

respiratory (30% oxygen) growth conditions, illuminated 500 ml cultures were sparged for several doublings with 95% N₂ and 5% CO₂. When indicated, the gas mixture was changed to 30% O₂, 69% N₂, 1% CO₂ and illumination terminated. Cultures were diluted into fresh medium, as necessary, to maintain exponential growth during the course of the experiment.

E. coli cells were grown in LB on a rotary shaker or on solid media at 37 °C (Sambrook *et al.*, 1989). To maintain plasmids (Table 1) in *E. coli*, ampicillin (50 µg ml⁻¹), kanamycin (25 µg ml⁻¹), spectinomycin (25 µg ml⁻¹) or tetracycline (10 µg ml⁻¹) were used. For *R. sphaeroides*, spectinomycin and kanamycin were used at 25 µg ml⁻¹ and tetracycline was added to 1 µg ml⁻¹. After diparental mating between *E. coli* S17-1 (Simon *et al.*, 1983) and *R. sphaeroides* recipients (Donohue *et al.*, 1988), cells were plated aerobically on media containing spectinomycin and kanamycin to select for exconjugants.

DNA manipulations, cloning and sequencing techniques.

Restriction enzymes, T4 DNA ligase, T4 DNA polymerase and Klenow fragment DNA polymerase were used according to suppliers' specifications and standard techniques (Sambrook *et al.*, 1989). Plasmid DNA was prepared with Qiagen kits; DNA was isolated from agarose gels using Prep A Gene kits (Bio-Rad). *Taq* DNA polymerase was used for dideoxy DNA sequencing with deazanucleotide triphosphates (Promega) and either vector (Stratagene) or *R. sphaeroides*-specific primers (Genosys Biotechnologies). Sequence analysis was aided by University of Wisconsin Genetics Computer Group software (Devereux *et al.*, 1984).

Use of pRKK200 to construct *lacZ* operon fusions. Plasmid pRKK200 (R. K. Karls & T. J. Donohue, unpublished) is a kanamycin-resistant pKT231 derivative (Bagdasarian & Timmis, 1982) that contains a promoterless *E. coli lacZ* gene downstream of a strong transcription terminator on the Ω Sp^r cassette (Prentki & Krish, 1984). This plasmid has unique *KpnI* and *StuI* sites for directional cloning of DNA fragments upstream of *lacZ*. Similar IncQ plasmids exist at 4–10 copies per *R. sphaeroides* cell (Donohue & Kaplan, 1991).

***cycFG* operon fusions.** To construct a *cycFG::lacZ* fusion, a 140 bp *MscI* restriction fragment of *cycFG* promoter DNA (Flory & Donohue, 1995) was cloned into the *StuI* site of pRKK200. DNA sequencing identified a plasmid in which the *cycFG* promoter was fused to *lacZ* (pRKKcycF).

For creating *cycFG* promoter mutations, the same DNA was cloned into the *SmaI* site of pUC119 (pTC119F) and transformed into RZ1032 (Kunkel, 1985). Single-stranded DNA (Kunkel, 1985) was hybridized to mutagenic oligonucleotides *cycFFNR* (CGATCACATTAAAGCTACATTTTCTAGT-ATGTCTGTCG) to create a mutant FNR site (*cycFG-1* allele), or *cycFREP* (CCAGTATGTCTGTCGTAGTTAG-CGGGCTAGATTTC) to destroy an inverted repeat in the *cycFG* promoter spacer (*cycFG-2* allele). After confirming individual mutations (indicated by underlining) by DNA sequencing, *KpnI* and *HincII* were used to isolate mutant *cycFG* promoter fragments for cloning into *KpnI* and *StuI*-digested pRKK200. This mutagenesis scheme added 2 bp of upstream DNA and 17 bp before the start of *lacZ* compared to the wild-type *cycFG::lacZ* fusion. The additional downstream DNA accounts for the size difference in primer extension products from a wild-type *cycFG::lacZ* fusion and those from analogous *cycFG-1* or *cycFG-2* reporter genes (see Results).

***coxII* operon fusions.** A 282 bp *coxII* DNA fragment (Cao *et al.*, 1991) was amplified by PCR using the primers *coxIIPCR1*

Table 1. Bacterial strains and plasmids

Designation	Relevant feature	Source or reference
Strains		
2.4.1	<i>R. sphaeroides</i> , wild-type	Laboratory strain
JZ1678	<i>R. sphaeroides</i> , <i>fnrL::kan</i>	Zeilstra-Ryalls & Kaplan (1995)
RZ1032	<i>E. coli</i> , <i>dut ung</i>	Kunkel (1985)
S17-1	<i>E. coli</i> , <i>mob</i> ⁺	Simon <i>et al.</i> (1983)
Plasmids		
pRKK200	Promoterless <i>lacZ</i> plasmid	R. K. Karls (this laboratory)
pRKKcycF	pRKK200 + <i>cycFGp</i>	This work
pRKKF-FNR	pRKK200 + <i>cycFGp</i> FNR site mutant (<i>cycFG-1</i>)	This work
pRKKF-REP	pRKK200 + <i>cycFGp</i> spacer mutant (<i>cycFG-2</i>)	This work
pRKKctaD	pRKK200 + <i>ctaDp</i>	This work
pRKKcoxII	pRKK200 + <i>coxIIp</i>	This work
pRKKIIFNR	pRKK200 + <i>coxIIp</i> FNR site mutant (<i>coxII-1</i>)	This work
pUC119	Phagemid vector	Clonetech
pTC119F	pUC119 + <i>cycFGp</i>	This work
pTC119FNR	pUC119 + <i>cycFGp</i> FNR site mutant (<i>cycFG-1</i>)	This work
pTC119REP	pUC119 + <i>cycFGp</i> spacer mutant (<i>cycFG-2</i>)	This work
pBSIIKS-	Phagemid vector	Stratagene
pBScoxII	pBSIIKS- + <i>coxIIp</i>	This work
pBSIIFNRII	pBSIIKS- + <i>coxIIp</i> FNR site mutant (<i>coxII-1</i>)	This work
pJS3	pT3T718 + <i>ctaD</i> coding sequence	Shapleigh & Gennis (1992)

(GCTGGTACCCTCGGAGGCATCGTAAC) and *coxIIPC-R2* (AGAGTCCGTCCGACCCGTTGTTCTGAACCG). The ~300 bp product was digested with *KpnI* and *HincII* (underlined), ligated into *KpnI* and *StuI*-digested pRKK200, and sequenced to identify a wild-type *coxII::lacZ* reporter plasmid (pRKKcoxII).

For mutagenesis, this PCR product was cloned into *KpnI* and *HincII*-digested pBSIIKS-. After finding a plasmid where no mutations were introduced into the *coxII* promoter by PCR (pBScoxII), the *Fnr* site was mutagenized (Kunkel, 1985) with the *coxFNR* primer (CATGACTACTGTCCCTAGTTCA-GACTAGATGATGACGCTTCG). A mutant *coxII* promoter fragment (*coxII-1*) was isolated by digestion with *KpnI* and *HincII* and cloned into *KpnI* and *StuI*-digested pRKK200 to generate a *coxII-1::lacZ* reporter plasmid (pRKKIIFNR).

***ctaD* operon fusions.** A 226 bp restriction fragment of the *ctaD* upstream region was isolated as a *SmaI*-*MscI* fragment from pJS3 (Shapleigh & Gennis, 1992) and ligated into the *StuI* site of pRKK200. DNA sequencing identified a plasmid with *lacZ* under *ctaD* promoter control (pRKKctaD).

RNA techniques. Cellular RNA was prepared (Zhu & Kaplan, 1985), quantified (Donohue *et al.*, 1986) and used in primer extension assays (Karls *et al.*, 1989) with avian myeloblastosis virus reverse transcriptase (Molecular Genetic Resources). Primers were: *cycF10* (AGGCCGTGGCGCCTTCGAT), *coxII-1* (AGGTCGTGGAATGTCTCATG), and *ctaD-1* (CTGCGTCGGCCATTGGCTTG). Products were separated on denaturing urea/polyacrylamide gels alongside DNA sequencing ladders.

LacZ assays. *R. sphaeroides* cells containing individual transcriptional fusions were assayed in triplicate for LacZ activity (Schilke & Donohue, 1992). Cells were harvested in the exponential phase of growth to minimize unwanted secondary effects on promoter activity. Data presented are the mean $\pm$ SD

of at least three independent cultures. The Student's *t* test was used to assess if LacZ levels in individual cultures were significantly different from each other.

Bacteriochlorophyll analysis. For these assays, 25 ml culture samples were harvested, stored at -20 °C and resuspended in 0.2 ml deionized water to determine the specific bacteriochlorophyll content. Half of the sample was used for protein determination (Markwell *et al.*, 1978); the remainder was assayed for bacteriochlorophyll (Cohen-Bazire *et al.*, 1957).

RESULTS AND DISCUSSION

Activity of the *ctaD*, *coxII* and *cycFG* promoters is increased under aerobic conditions

By primer extension assays the start site for *coxII* transcription maps 80 nt upstream of the initiator methionine, while that for *ctaD* is 54 nt upstream of the start of translation (summarized in Fig. 1). Each gene appears to be transcribed from a single promoter because the primer extension product is the same when RNA from aerobically or anaerobically grown cells is used (Fig. 2 for *coxII*, data not shown for *ctaD*). Both primer extension assays and Northern blot analysis suggested that the *ctaD* and *coxII* promoters were oxygen-regulated since there was more mRNA in 30% oxygen cells than in those grown under anaerobic (photosynthetic) conditions (see Fig. 2 for *coxII* primer extension assays, data not shown for *ctaD*).

To facilitate the study of oxygen-dependent control of *ctaD*, *coxII* and *cycFG* transcription, individual *lacZ* operon fusions were constructed. To verify that these low-copy reporter genes were valid indicators of pro-

E. coli FNR consensus recognition site

aaaTGAATatATCAAtt

Fig. 1. Promoter sequences used to construct *lacZ* operon fusions to *cycFG* (accession no. L36880; positions 245–388; Flory & Donohue, 1995), *coxII* (M57680; 1–280; Cao *et al.*, 1991) and *ctaD* (X62645; 4–229; Shapleigh & Gennis, 1992). The transcription start sites (+1, outlined), potential FNR target sequences (underlined), mutations to generate mutant promoters (above the line) and the start of translation (italics) are indicated. Sequences present in the *lacZ* operon fusions that are not found in their genomic counterparts (lower case letters) and endonuclease recognition sites destroyed during cloning (parentheses) are also shown. An inverted repeat in the *cycFG* sequence (arrows), the half-site substitution (below the line) and the position of an additional transcription start site in the *cycFG*-2 mutant promoter that resulted from this mutation (asterisk) are indicated. The consensus binding site for *E. coli* FNR and bases proposed to make site-specific protein contacts (carets) are presented at the bottom (Spiro *et al.*, 1990; Lazazzera *et al.*, 1993).

moter activity, primer extension assays showed that the *lacZ*-specific transcripts had the same 5' end as their genomic counterparts (data not shown). Cells containing any of these three reporter genes produced LacZ activity well over the background observed from a control strain containing a promoterless *lacZ* gene

Fig. 2. *coxII* mRNA levels are increased under aerobic conditions. Genomic primer extension products using 40 µg RNA from wild-type cells grown under anaerobic (photosynthetic) (lane 1) or 30% oxygen (lane 2) conditions are shown.

(pRKK200, <6 Miller units). In addition, LacZ levels in cells containing the *ctaD*, *coxII* or *cycFG* reporter genes were approximately twofold higher when cells were grown at high (30%) oxygen levels rather than under anaerobic (photosynthetic) conditions (Fig. 3). This suggests that a portion of the increased mRNA abundance is due to elevated transcription. While a contribution of additional *cis*-acting sequences to promoter function is still possible, these data indicate that the amount of each promoter DNA which is fused to *lacZ* (268 bp for *coxII*, 149 bp for *ctaD* or 89 bp for *cycFG*) is sufficient to increase transcription at 30% oxygen.

To further evaluate the effects of oxygen tension on *ctaD*, *coxII* and *cycFG* expression, LacZ levels were measured in cells grown under a 2% oxygen atmosphere, a condition sufficient to induce photosynthetic membrane assembly (Cohen-Bazire *et al.*, 1957). Activity of the *ctaD*, *coxII* and *cycFG* promoters in cells grown in a 2% oxygen environment was similar to that found in anaerobic (photosynthetic) cultures (Fig. 3). Such a reduction in *ctaD* and *coxII* promoter function at reduced oxygen tensions was the response expected given the low affinity of cyt *aa*₃ complexes for oxygen (Trumpower & Gennis, 1994). Indeed, the *E. coli* equivalent to cyt *aa*₃ (cytochrome *bo*₃ oxidase) is also transcribed less at low oxygen (Iuchi *et al.*, 1990; Minagawa *et al.*, 1990). Thus, these estimates of *coxII*

Fig. 3. LacZ specific activities of wild-type cells containing the indicated reporter genes in cells grown under 30% oxygen (■), 2% oxygen (□) or anaerobic (photosynthetic) (▨) conditions. Reported values are the mean $\pm$ SD of at least three independent assays and are corrected for activity from the vector alone under the same conditions (pRK200, <6 Miller units). Note that LacZ levels from the *ctaD* reporter fusion are reported at one-tenth of the actual values. Relevant reporter plasmids are as follows (Table 1): *cycFG*, pRKKcycF; *coxII*, pRKKcoxII; *ctaD*, pRKKctaD.

and *ctaD* promoter function provide a provisional indication that cyt *aa*₃ levels are lower at reduced oxygen tensions. However, the modest reduction in *ctaD* and *coxII* promoter activity at 2% oxygen or anaerobic conditions suggests these cells contain a considerable pool of cyt *aa*₃ complexes. If *R. sphaeroides* regulates synthesis of its high affinity oxidase(s) as in other bacteria (García-Horsman *et al.*, 1994a, b) they should be maximally expressed at low oxygen tensions (Luchi *et al.*, 1990; Fu *et al.*, 1991; Moshiri *et al.*, 1991).

Given that CtaD and CoxII are found in equimolar amounts within the cyt *aa*₃ complex, we were surprised that *ctaD* promoter activity was approximately ninefold higher than its *coxII* counterpart (Fig. 3). This difference could reflect deletion of important *cis*-acting sequences on either reporter gene. However, differences in stability of *ctaD* and *coxII* transcripts could also explain both the apparent strengths of the *coxII* and *ctaD* promoters and our difficulty in reproducibly obtaining *ctaD* primer extension products (see above). Alternatively, *ctaD* transcription could generate a stable pool of CtaD for assembly of the cyt *aa*₃ complex. A similar role as a membrane-bound nucleation site for newly synthesized enzyme complexes has been proposed for the H subunit of the *R. sphaeroides* reaction centre complex (Chory *et al.*, 1984).

Function of the aerobic cytochrome promoters increases slowly after exposure to 30% oxygen

To examine how oxygen controls *ctaD*, *coxII* and *cycFG* promoter function, the kinetics of LacZ accumulation from individual reporter genes was monitored after photosynthetic cells were exposed to 30% oxygen (Fig. 4). Photosynthetically grown *R. sphaeroides* cells contain considerable cytochrome oxidase activity (García-Horsman *et al.*, 1994a, b), so it is not surprising that cell growth continues when these cells are exposed to 30% oxygen. When oxygen was introduced into steady-state anaerobic (photosynthetic) cultures, there was a slow, progressive increase in LacZ activity from each operon fusion. Indeed, about 24 h (six cell doublings) after the introduction of 30% oxygen were required for LacZ levels from each reporter fusion to increase to that found in steady-state cells grown under these conditions. The slow increase in activity of these three promoters seems to preclude function of either a pre-existing oxygen sensor or a rapidly metabolized intermediate as negative transcriptional regulators. Rather, the slow increase in transcription could reflect time required for an activator or co-inducer of *ctaD*, *coxII* and *cycFG* promoter function to accumulate after photosynthetic cells are shifted to a 30% oxygen atmosphere. Another equally plausible alternative is that *ctaD*, *coxII* and *cycFG* promoter function is repressed by an oxygen-insensitive transcription factor that uses a component of the photosynthetic apparatus or a stable compound that is accumulated under low oxygen conditions as a co-repressor. In either of these two latter cases, *coxII*, *ctaD* and *cycFG* promoter function would only increase after the putative repressor, pre-existing photosynthetic units, or a potential co-repressor was diluted by growth at 30% oxygen.

The rapid cessation of photosynthetic membrane synthesis that occurs when *R. sphaeroides* is exposed to high oxygen tensions (Cohen-Bazire *et al.*, 1957) allowed us to test if the increased activity of these three promoters under these conditions could be correlated with dilution of a regulatory factor from photosynthetic cultures. When we assayed the specific bacteriochlorophyll content of these cells (as a marker for photosynthetic membrane content), we observed the expected time-dependent decrease in this parameter (Fig. 4) as pre-existing photosynthetic membranes were diluted by cell division after oxygen was introduced (Cohen-Bazire *et al.*, 1957). When all the data are considered together, it appears that increased function of these three promoters parallels the loss of pre-existing components from photosynthetic cells.

The *R. sphaeroides* FNR homologue, FnrL, is not sufficient to mediate oxygen regulation of *ctaD*, *coxII* and *cycFG* expression

In considering potential molecular explanations for the increased activity of these three promoters at high oxygen tensions, we noted that sequences related to a

Fig. 4. The effect of oxygen on anaerobic cultures of cells containing individual LacZ operon fusions. Photosynthetic cultures of each strain were shifted to aerobic, dark conditions at time zero (dotted line) and were later diluted to fresh medium for continued aerobic growth (arrow). Top panel: □, culture density (OD₆₀₀). Bottom panel: ○, LacZ activity in Miller units; ▲, specific bacteriochlorophyll content [μg bacteriochlorophyll (mg protein)⁻¹].

Fig. 5. Effect of mutations in *fnrL* or the putative FnrL target sites on expression from *cycFG*, *coxII* or *ctaD* reporter genes. LacZ activities of wild-type *R. sphaeroides* (WT) or an FnrL null strain (Δ FnrL; JZ1678) containing the indicated reporter plasmids grown under 30% oxygen (■), 2% oxygen (□) or anaerobic (photosynthetic) (▨) conditions. Reported values are the mean $\pm$ SD of at least three independent assays and are corrected for activity from the vector alone under the same conditions (pRK200, <6 Miller units). (a) Wild-type (WT) *cycFG*::lacZ fusion (plasmid pRKKcycF); *cycFG-1*::lacZ fusion (plasmid pRKKF-FNR). (b) Wild-type (WT) *coxII*::lacZ fusion (plasmid pRKKcoxII); *cycFG-1*::lacZ fusion (plasmid pRKKII-FNR). (c) Wild-type *ctaD*::lacZ fusion (plasmid pRKKctaD).

consensus target site for the *E. coli* global anaerobic transcription factor, FNR, were present upstream of each gene (Fig. 1). While the site in the *cycFG* promoter (centred at -42.5) has only 5/10 matches to the *E. coli* FNR consensus, those in the *coxII* (-71.5) and *ctaD* (-60.5) promoters are identical to this consensus in both half sites. *E. coli* FNR represses transcription of

several aerobic respiratory enzymes (Sharrocks *et al.*, 1991). In the case of the *E. coli ndh* promoter, FNR represses transcription of this aerobic respiratory enzyme under anaerobic conditions by binding to independent sites centred at -50.5 and -94.5 bp upstream of the start of transcription (Green & Guest, 1994). Thus, it would not be surprising to find that

Fig. 6. Mutation of the putative FNR target sites does not alter the 5' end of *coxII* and *cycFG* transcripts. Primer extension assays using a *lacZ*-specific primer with RNA from cells containing: lane 1, (from left) a wild-type *coxII::lacZ* fusion (plasmid pRKKcoxII; 15 µg RNA); lane 2, a *coxII-1::lacZ* fusion (plasmid pRKKIIFNR; 15 µg RNA); lane 3, a wild-type *cycFG::lacZ* fusion (plasmid pRKKcycF; 30 µg); or lane 4, a *cycFG-1::lacZ* fusion (plasmid pRKKF-FNR; 30 µg) are shown. As described in Methods, the 17 nt difference between *cycFG* primer extension products 1 and 2 reflects the addition of vector sequences during construction of the *cycFG-1::lacZ* fusion.

transcription of *cycFG* or genes encoding individual subunits of the *R. sphaeroides* cyt *aa₃* were negatively regulated by either the *R. sphaeroides* FNR homologue, FnrL, or a related protein that recognized these sites.

E. coli FNR is believed to make specific contacts at the first, third and fourth nucleotides of the consensus half-site, TTGAT (Lazazzera *et al.*, 1993; Spiro *et al.*, 1990). As a test of whether *R. sphaeroides* FnrL might repress transcription by binding to regions that are related to the *E. coli* FNR consensus, a single mutant derivative of the *cycFG* (*cycFG-1*) and *coxII* (*coxII-1*) promoters was generated in which all of the putative contact points were replaced with non-consensus bases that are known to abolish DNA binding by the *E. coli* protein (Fig. 1;

Ziegelhoffer & Kiley, 1995). *R. sphaeroides* FnrL has considerable amino acid sequence identity with *E. coli* FNR in the putative DNA binding domain, so it is expected that the site-specific protein-DNA contacts would be similar (Zeilstra-Ryalls & Kaplan, 1995). Within experimental error, LacZ levels from cells containing the mutant reporter genes were indistinguishable from those of cells harbouring a wild-type reporter fusion under all tested conditions (Fig. 5a-c). Thus, it appears that these FNR sites are not essential for the reduced activity of the *cycFG* and *coxII* promoters under low oxygen or anaerobic conditions. Control experiments suggest these mutations did not introduce unwanted secondary effects on promoter function since primer extension assays showed that the transcription start sites from these mutant reporter genes were the same as from their wild-type counterparts (Fig. 6).

As an independent test of the role of FnrL in *ctaD*, *coxII* and *cycFG* promoter function, LacZ levels produced from wild-type and mutant reporter plasmids were measured in the *R. sphaeroides* FnrL null mutant, JZ1678 (Fig. 5a-c). Because the FnrL null mutant is photosynthetically incompetent (Zeilstra-Ryalls & Kaplan, 1995), LacZ levels were only measured from cultures grown in the presence of 30% or 2% oxygen (a condition which approximates the effects of anaerobic growth on *ctaD*, *coxII* and *cycFG* promoter function, Fig. 3). At high (30%) oxygen tensions, LacZ levels from the *cycFG-1* (Fig. 5a) and *coxII-1* (Fig. 5b) fusions were nearly identical to their wild-type counterparts in both wild-type cells and the FnrL null mutant. Normal activity of the *coxII-1* and *cycFG-1* mutant promoters in the FnrL null mutant at 30% oxygen is a clear indication that this global regulator is not functioning under these conditions. Similarly, LacZ levels produced from the *coxII-1* promoter at 2% oxygen in the ΔFnrL mutant were indistinguishable from those measured in cells containing a wild-type *coxII* reporter gene (Fig. 5b). This provides an indication that this target site is not solely responsible for reducing function of the *coxII* promoter under these conditions. In contrast, there was a slight, but reproducible, increase in activity of the *cycFG* (Fig. 5a) and *coxII* (Fig. 5b) promoters (25–30%) at 2% oxygen in the FnrL null mutant compared to wild-type cells (Fig. 5). While this reproducible increase in LacZ levels suggests that FnrL has some role in reducing transcription from the *cycFG* and *coxII* promoters at low oxygen tension, the magnitude of the effect is below the twofold increase expected if this protein were the sole negative regulator of these genes under these conditions.

When the above results are considered together, it appears likely that FnrL is not solely responsible for reducing *ctaD*, *coxII* and *cycFG* promoter activity under the conditions we have tested [2% oxygen and anaerobic (photosynthetic) growth]. However, it is still possible that FnrL or a related protein contributes to reduced transcription of these *R. sphaeroides* aerobic cytochrome genes under these or other conditions we did not test. For example, action of *E. coli* FNR can often require

Fig. 7. Effect of the *cycFG-2* mutation on promoter activity. LacZ activities of wild-type *R. sphaeroides* 2.4.1 or an FnrL null strain (Δ FnrL; JZ1678) containing either a wild-type (WT) *cycFG::lacZ* fusion (plasmid pRKKcycF) or the *cycFG-2::lacZ* fusion (plasmid pRKKF-REP) grown under 30% oxygen (■), 2% oxygen (□) or anaerobic (photosynthetic) (▨) conditions are shown. Reported values are the mean $\pm$ SD of at least three independent assays and are corrected for activity from the vector alone under the same conditions (pRKK200, <6 Miller units). Note that under 2% oxygen conditions, the SD in LacZ levels from wild-type cells carrying the wild-type *cycFG* plasmid and Δ FnrL cells carrying the promoter mutant plasmid were each <5 Miller units.

additional proteins (Spiro & Guest, 1991), so the failure of the target site or host mutations to fully increase *cycFG*, *coxII* or *ctaD* transcription at low oxygen tensions could reflect the action of additional transcription factors. *E. coli* FNR interacts at two sites within the *ndh* promoter (Green & Guest, 1994), so it is possible that additional non-consensus FNR sites in these promoters might prevent us from observing a strong effect of the mutations we made. *R. sphaeroides* is also one of a growing number of eubacteria which have been found to contain more than one protein in the FNR family (Cuypers & Zumft, 1993; Tosques *et al.*, 1996). Thus, the potential for related proteins, such as NnrR from *R. sphaeroides* strain 2.4.3 (Tosques *et al.*, 1996), to alter *ctaD*, *coxII* or *cycFG* promoter function in the presence or absence of FnrL can not be ruled out at this time (if this protein exists in strain 2.4.1).

An inverted repeat overlapping the *cycFG* promoter is not essential for oxygen regulation

We also tested whether a previously noted inverted repeat (centred at -24.5) regulated *cycFG* promoter function (Flory & Donohue, 1995). When a mutation that destroyed the downstream half-site (without changing the upstream half-site that overlaps a potential -35 element; Fig. 1) was generated (*cycFG-2*), LacZ levels from this mutant reporter gene increased approximately twofold in cells grown at 30% oxygen (Fig. 7). The

Fig. 8. The *cycFG-2* mutation creates a new promoter. Primer extension assays of 20 μ g RNA from photosynthetically grown wild-type cells containing: lane 1, a wild-type *cycFG::lacZ* fusion (plasmid pRKKcycF); or lane 2, a *cycFG-2::lacZ* fusion (plasmid pRKKF-REP) are shown. Primer extension product 1 is the wild-type 5' end; product 2 depicts the wild-type transcript displaced 17 nt by addition of vector sequence during construction of the *cycFG-2* allele (Methods); product 3 denotes the new 5' end created by the *cycFG-2* allele.

cycFG-2 allele appears to have created a new promoter since these cells contain a second primer extension product that is 14 nt longer than the wild-type transcript (Fig. 8). Even though the *cycFG-2::lacZ* fusion probably contains multiple promoters, transcription under 2% oxygen and anaerobic conditions was reduced to an extent comparable to its wild-type counterpart. Thus, we can conclude that the downstream half-site is not essential for decreased *cycFG* promoter function at low oxygen tensions. Finally, changes in oxygen tension must affect both potential promoters since the abundance of both primer extension products is lowered under photosynthetic conditions (data not shown).

Is oxygen regulation of the *coxII*, *ctaD* and *cycFG* promoters independent of systems which control photosynthesis genes?

In summary, our data show that transcription of genes for cyt *aa*₃ subunits as well as the *cycFG* operon is reduced at low oxygen tension. The slow, gradual

increase in *ctaD*, *coxII* and *cycFG* transcription after oxygen is added to photosynthetic cultures contrasts dramatically with the rapid induction of photosynthetic gene expression seen when respiratory cultures of purple bacteria like *R. sphaeroides* are shifted to low oxygen or anaerobic conditions that trigger intracytoplasmic membrane formation (Cohen-Bazire *et al.*, 1957; Chory *et al.*, 1984). Thus, it will be interesting to define the regulators of these aerobic cytochrome genes, explore if these transcription factors interact with proteins or products accumulated under photosynthetic conditions and determine if the similar patterns of *ctaD*, *coxII* and *cycFG* promoter function reflects their existence in a regulon of genes with similar metabolic functions.

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