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 aa₃ respiratory complex. The third promoter is that for the cycFG operon, which encodes two c-type cytochromes of unknown function, cytochrome c₅₅₉ 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 ΔFnrl 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 aa₃, cytochrome aa₃ oxidase; LacZ, β-galactosidase.
Like most bacterial species, \textit{R. sphaeroides} contains a branched aerobic respiratory chain (García-Horsman \textit{et al.}, 1994a). The cytochrome \textit{aa}, oxidase (\textit{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 \textit{et al.}, 1991). From studies on many haem-copper oxidases, a paradigm has developed in which the \textit{cyt aa}, 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 \textit{et al.}, 1994a; Trumpower & Gennis, 1994). To test if such differential expression of aerobic cytochromes occurs in \textit{R. sphaeroides}, we examined transcription of two \textit{cyt aa}, genes (\textit{ctaD} and \textit{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 \textit{c554} found under high (30\%) oxygen growth conditions reflects increased \textit{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 \textit{Escherichia coli} global anaerobic regulator FNR in the \textit{cycFG}, \textit{coxII} and \textit{ctaD} control regions (Cao \textit{et al.}, 1991; Shapleigh & Gennis, 1992; Flory & Donohue, 1995) make it possible that their expression is repressed under anaerobic conditions by the \textit{R. sphaeroides} FNR homologue, FnrL (Zeilstra-Rylls & Kaplan, 1995). All three of these promoters are oxygen-regulated since \textit{\beta}-galactosidase (LacZ) levels from specific operon fusions to \textit{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 \textit{R. sphaeroides} global anaerobic transcription factor is not solely responsible for normal oxygen control of their transcription. Indeed, the time-dependent increase in promoter activity when oxygen is added to anaerobic (photosynthetic) cultures suggest that \textit{cycFG}, \textit{coxII} and \textit{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.

\section*{METHODS}

\textbf{Cell growth, media and genetic techniques.} \textit{R. sphaeroides} cells (Table 1) were grown on solid media or in liquid cultures of Sistrom's minimal medium A at 32 \textdegree C (Donohue \textit{et al.}, 1986). Aerobic cultures (100 ml) were sparged either with a gas mixture known to repress photosynthetic membrane synthesis (30\% \textit{O}2, 69\% \textit{N}2, 1\% \textit{CO}2; Chory \textit{et al.}, 1984) or at low oxygen tensions (2\% \textit{O}2, 97\% \textit{N}2, 1\% \textit{CO}2). Steady-state anaerobic (photosynthetic) cells were grown in filled tubes in front of a tungsten light bank (Donohue \textit{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\% \textit{N}2 and 5\% \textit{CO}2. When indicated, the gas mixture was changed to 30\% \textit{O}2, 69\% \textit{N}2, 1\% \textit{CO}2 and illumination terminated. Cultures were diluted into fresh medium, as necessary, to maintain exponential growth during the course of the experiment.

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

\textbf{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 \textit{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 \textit{R. sphaeroides}-specific primers (Genosys Biotechnologies). Sequence analysis was aided by the Wisconsin Genetics Computer Group software (Devereux \textit{et al.}, 1984).

\textbf{Use of pRKK200 to construct lacZ operon fusions.} Plasmid pRKK200 (R. K. Karls & T. J. Donohue, unpublished) is a kanamycin-resistant \textit{kT23} derivative (Bagdasarian & Timmis, 1982) that contains a promoterless \textit{E. coli} lacZ gene downstream of a strong transcription terminator on the \textit{lacZ} operon (Frentki & Krish, 1984). This plasmid has unique \textit{KpnI} and \textit{Stul} sites for directional cloning of DNA fragments upstream of \textit{lacZ}. Similar IncQ plasmids exist at 4–10 copies per \textit{R. sphaeroides} cell (Donohue & Kaplan, 1991).

\textbf{cycFG operon fusions.} To construct a \textit{cycFG}::\textit{lacZ} fusion, a 140 bp Max restriction fragment of \textit{cycFG} promoter DNA (Flory & Donohue, 1995) was cloned into the \textit{Stul} site of pRKK200. DNA sequencing identified a plasmid in which the \textit{cycFG} promoter was fused to \textit{lacZ} (pRKKcycF).

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

\textbf{coxII operon fusions.} A 282 bp \textit{coxII} DNA fragment (Cao \textit{et al.}, 1991) was amplified by PCR using the primers coxII-PCR1
(GCTGGTACCTCAGGACAGCATCCTACG) and coxIPC-R2 (AGAGGACGACCGCTTCTCCTGAACCC). The ~300 bp product was digested with KpnI and HimII (underlined), ligated into KpnI and Stul-digested pRKK200, and sequenced to identify a wild-type coxII::lacZ reporter plasmid (pRKKcoxII).

For mutagenesis, this PCR product was cloned into KpnI and HimII-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 avian myeloblastosis primer extension assays (Karls et al., 1986) and used (Fig. 2 for coxII primer extension product) because the primer extension product is the same when data not shown for coxII). 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 thectaD, 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-

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**Table 1. Bacterial strains and plasmids**

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motar 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 (pRRK200, <6 Miller units). In addition, LacZ levels in cells containing thectaD, 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 is equivalent to cyt aa3 complexes for oxygen (Trumpower & Gennis, 1994). Indeed, the E. coli equivalent to cyt aa3 (cytochrome bo oxidase) is also transcribed less at low oxygen (Iuchi et al., 1990; Minagawa et al., 1990). Thus, these estimates of coxII

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.

E. coli FNR consensus recognition site

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 FNR and bases proposed to make site-specific protein contacts (caret) are presented at the bottom (Spiro et al., 1990; Lazazzera et al., 1993).
and ctaD promoter function provide a provisional indication that cyt aa3 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 aa3 complexes. If R. sphaeroides regulates synthesis of its high affinity oxidase(s) as in other bacteria (Garcia-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 aa3 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 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 aa3 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 (Garcia-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, FnrIL, 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

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**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±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 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. 

Aerobic cytochrome expression
**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 (ΔFnrL; JZ1678) containing the indicated reporter plasmids grown under 30% oxygen (■), 2% oxygen (□) or anaerobic (photosynthetic) (○) conditions. Reported values are the mean ± SD of at least three independent assays and are corrected for activity from the vector alone under the same conditions (pRKK200, <6 Miller units). (a) Wild-type (WT) cycFG::lacZ fusion (plasmid pRKKcycF); cycFG-1::lacZ fusion (plasmid pKKF-nfr). (b) Wild-type (WT) coxII::lacZ fusion (plasmid pRKKcoxII), cycFG-1::lacZ fusion (plasmid pKKF-nfr). (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...
transcription of cycFG or genes encoding individual subunits of the R. sphaeroides cyt aa, 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-I) and coxI1 (coxI1-1) 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 coxI1 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, coxI1 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, coxI1 and cycFG promoter function, Fig. 3). At high (30%) oxygen tensions, LacZ levels from the cycFG-I (Fig. 5a) and coxI1-I (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 coxI1-I and cycFG-I 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 coxI1-I promoter at 2% oxygen in the ΔFnRL mutant were indistinguishable from those measured in cells containing a wild-type coxI1 reporter gene (Fig. 5b). This provides an indication that this target site is not solely responsible for reducing function of the coxI1 promoter under these conditions. In contrast, there was a slight, but reproducible, increase in activity of the cycFG (Fig. 5a) and coxI1 (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 coxI1 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, coxI1 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 (ΔFnrl; 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 (O), 2% oxygen (0) or anaerobic (photosynthetic) (69) conditions are shown. Reported values are the mean ± 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 ΔFnrl cells carrying the promoter mutant plasmid were each <5 Miller units.

Fig. 8. The cycFG-2 mutation creates a new promoter. Primer extension assays of 20 μg 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.

additional proteins (Spiro & Guest, 1991), so the failure of the target site or host mutations to fully increase cycFG, coxlI 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, coxlI 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 −245) 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 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 coxlI, ctaD and cycFG promoters independent of systems which control photosynthesis genes?

In summary, our data show that transcription of genes for cyt aa₃ 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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REFERENCES


