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NOTES

Coordination of Ubiquinol Oxidase and Cytochrome cbb_3 Oxidase Expression by Multiple Regulators in *Rhodobacter capsulatus*

Danielle L. Swem and Carl E. Bauer*

Department of Biology, Indiana University, Bloomington, Indiana 47405

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Rhodobacter capsulatus **utilizes two terminal oxidases for aerobic respiration, cytochrome cbb_3 and ubiquinol oxidase. To determine the transcription factors involved in terminal oxidase expression, *ccoN-lacZ* and *cydA-lacZ* protein fusions were assayed in a variety of regulatory mutants. The results of this and previous studies indicate that cytochrome cbb_3 expression is controlled by *regB-regA*, *fnrL*, and *hvrA* and that ubiquinol oxidase expression is controlled by *regB-regA*, *fnrL*, *hvrA*, *crtJ*, and *aerR*.**

The facultative photosynthetic bacterium *Rhodobacter capsulatus* demonstrates substantial metabolic plasticity through its ability to grow in response to a variety of different environmental conditions (25). For example, while growing anaerobically in light, *R. capsulatus* utilizes a photosystem to generate energy. However, when growing in the presence of oxygen, the cells repress synthesis of the photosystem and instead obtain energy by aerobic respiration using two cytochrome oxidases, cytochrome cbb_3 and ubiquinol oxidase, as terminal electron acceptors (1, 11, 12, 26) (Fig. 1). Both oxidases reduce oxygen to water in an effort to remove excess reducing power concomitant with proton translocation. The generated proton potential is utilized for multiple cellular processes, including ATP synthesis via ATPase (13–15) (Fig. 1).

Like many organisms, *R. capsulatus* has developed a means of ensuring that all available oxygen is used as an electron acceptor before switching to lower-yielding terminal electron acceptors, such as nitrate or dimethyl sulfoxide. The cytochrome cbb_3 and ubiquinol oxidases are thought to have different affinities for oxygen. Cytochrome cbb_3 is thought to have a low affinity for oxygen, as evidenced by high expression under oxygen-saturating conditions and low expression under oxygen-limiting conditions, while ubiquinol oxidase is thought to have a higher affinity for oxygen due to its low expression under oxygen-saturating conditions and high expression under oxygen-limiting conditions. In order to regulate expression of these terminal electron acceptors in response to oxygen tension, it is necessary that they be differentially regulated by transcription factors that respond to different amounts of oxygen. For example, it was recently demonstrated that the RegB-RegA two-component global signal transduction system from *R. capsulatus* directly controls aerobic/anaerobic expression of numerous cellular processes, including that of the two terminal oxidases (21). In this study, we reveal that the regulation of terminal oxidase expression in *R. capsulatus* involves a complex set of regulators beyond that of RegB-RegA. Specifically, we demonstrate that cytochrome cbb_3 oxidase expression is regulated by RegB-RegA and FnrL, as well as moderately regulated by HvrA, an activator of various photosynthetic components under low-light conditions. Ubiquinol oxidase expression was found to be regulated by RegB-RegA, FnrL, and HvrA as well as by AerR and CrtJ, which are aerobic repressors of photosystem gene expression.

Ubiquinol oxidase expression. Expression of ubiquinol oxidase was assayed using a translational *lacZ* fusion to the first gene in the *cydAB* operon that contains 920 bp of DNA upstream from *cydA* (21). This plasmid was mobilized as described previously (24) into *regA* (19), *crtJ* (20), *hvrA* (3), *aerR* (20), and *fnrL* (20) single-mutant strains, as well as *regA-crtJ* (20) and *regA-fnrL* (20) double-mutant strains. Each of the constructed strains was tested for β-galactosidase activity under aerobic, semiaerobic, and anaerobic (photosynthetic) growth conditions as reported by Buggy and Bauer (2).

The expression pattern observed for ubiquinol oxidase in wild-type *R. capsulatus* was similar to that reported by Swem et al. (21) (Fig. 2). Specifically, expression was lowest under aerobic conditions, intermediate under anaerobic conditions (1.8-fold higher), and highest (3.2-fold higher) under semiaerobic growth conditions. The effect of a disruption of *regA* was also similar to that reported by Swem et al. (21), in which expression was significantly lower than for the wild type (by 81 to 87%) under all tested growth conditions.

We also assayed the effect of disrupting *hvrA* on expression of ubiquinol oxidase. HvrA is a member of the HNS family of histone-like DNA-binding proteins and is cotranscribed with RegA (3). Gel shift experiments indicate that HvrA may cooperatively interact with phosphorylated RegA (10). In the *hvrA* mutant strain, there is a 64% reduction in anaerobic ubiquinol oxidase expression compared to the 81% reduction observed for the *regA* mutant. Interestingly, HvrA has no effect

* Corresponding author. Mailing address: Department of Biology, Jordan Hall, Bloomington, IN 47405. Phone: (812) 855-6595. Fax: (812) 856-4178. E-mail: cbauer@bio.indiana.edu.

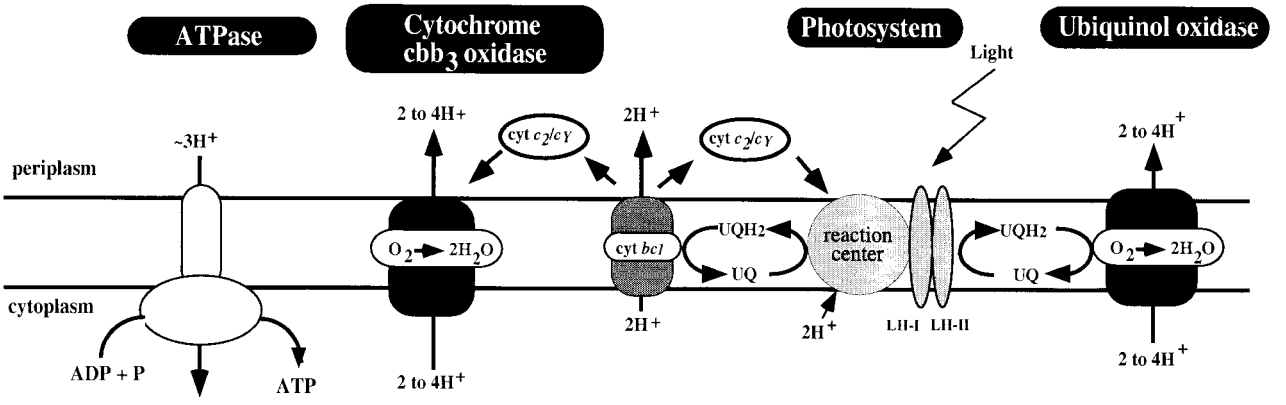

FIG. 1. A model of the electron transport system and ATP synthase in the *R. capsulatus* membrane. Energy production in the form of ubihydroquinone (UQH_2) occurs in the photosystem reaction center. Ubiquinol oxidase utilizes UQH_2 to reduce oxygen to water, while cytochrome cbb_3 oxidase obtains reducing power from cytochrome c_2 or c_y via the cytochrome bc_1 complex. Electron transfer is coupled to proton translocation at the sites indicated. Protons can reenter the cell via ATP synthase to make ATP. Electrons also shuttle back to the reaction center via cytochrome c_2 or c_y.

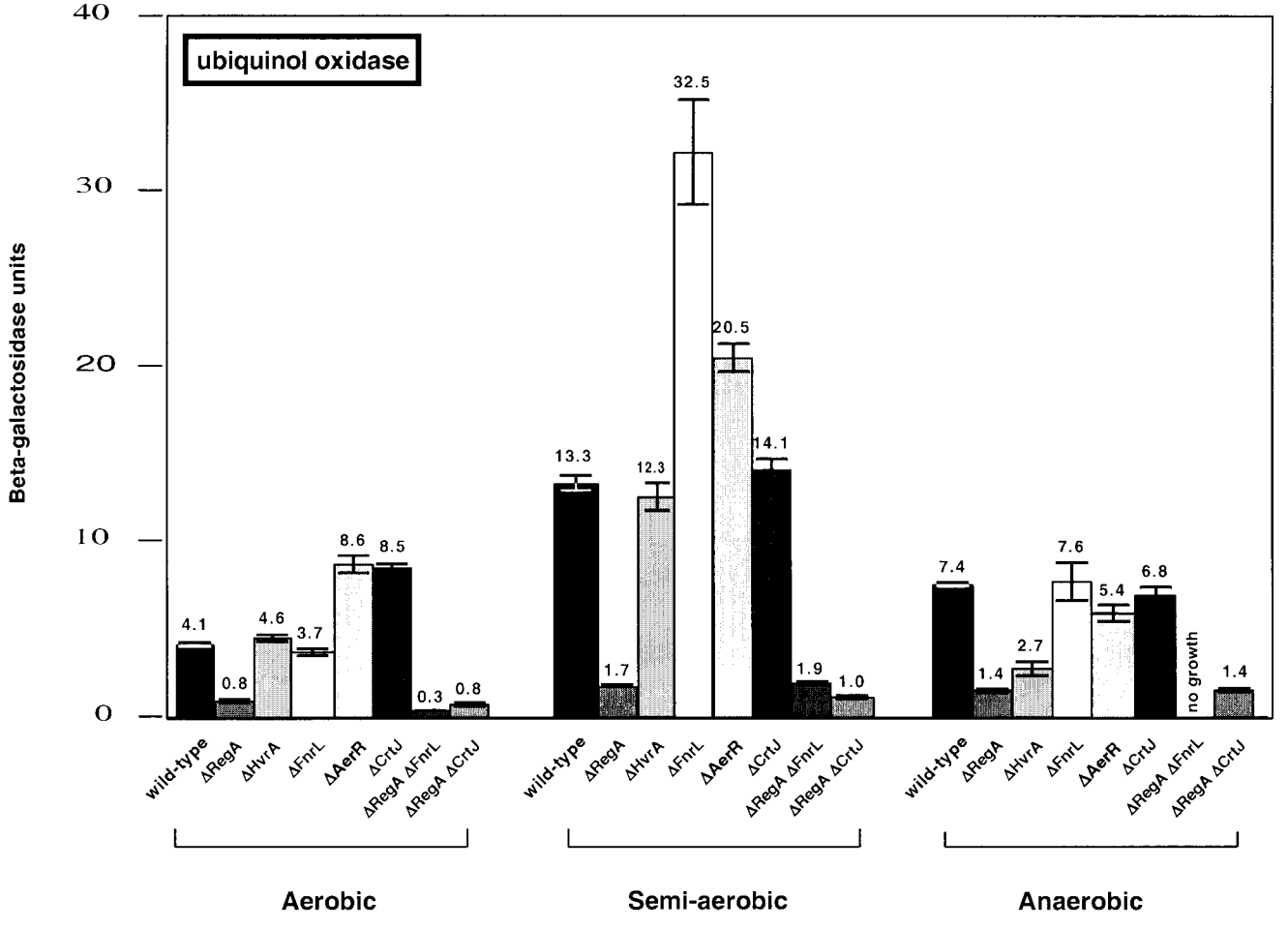

FIG. 2. β-Galactosidase analysis of aerobic, semiaerobic, and anaerobic photosynthetic ubiquinol oxidase gene expression patterns in the wild-type parent strain SB1003 and various regulatory mutants, as indicated below each bar. The values represent averages of at least three independent assays (standard deviations indicated by the error bars). Units of activity refer to the number of micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

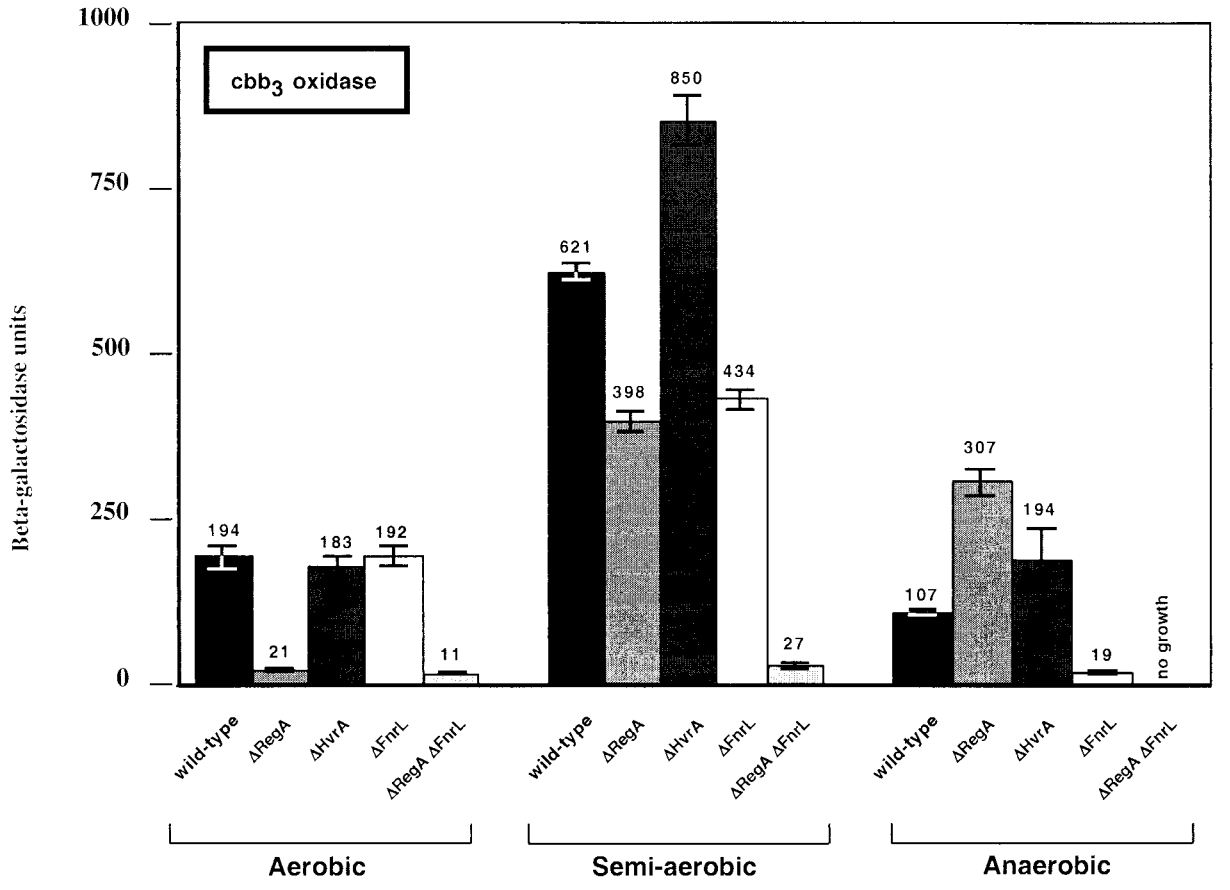

FIG. 3. β-Galactosidase analysis of aerobic, semiaerobic, and anaerobic photosynthetic cytochrome cbb_3 oxidase gene expression patterns in the wild-type parent strain SB1003 and various regulatory mutants as indicated below each bar. The values represent averages of at least three independent assays. Units of activity refer to the number of micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

on expression aerobically or semiaerobically, though RegA does.

In *Escherichia coli*, Fnr is one of several regulators of terminal oxidase gene expression (4). Since *R. capsulatus* contains a homolog of Fnr (27), we constructed a mutation in the chromosomal copy of *fnrL* and tested the mutant strain for its effect on terminal oxidase gene expression. The bar graph in Fig. 2 shows that there is no effect of disrupting *fnrL* on ubiquinol oxidase expression when the cells are grown strictly aerobically or anaerobically. In contrast, there is a reproducible 2.5- to 3-fold increase in ubiquinol oxidase expression from that in the wild type under semiaerobic growth conditions. This pattern has also been observed for cytochrome *o* oxidase expression in *E. coli*, where *fnr* mutations only show a significant effect under semiaerobic growth conditions (22).

In addition to the above tested "global regulators" that are found in many photosynthetic and nonphotosynthetic species, we also tested whether two aerobic repressors of the photosystem, CrtJ and AerR, are also involved in controlling ubiquinol oxidase gene expression. Analysis of ubiquinol oxidase expression indicates that *aerR* and *crtJ* mutants exhibit a twofold increase in expression aerobically and no effect anaerobically. This is very similar to the effect on *bch*, *crt*, and *puc* expression that is also observed upon disruption of these regulators (5, 8).

We also addressed the issue of dominance by constructing *regA-fnrL* and *regA-crtJ* double mutants. The ubiquinol oxidase expression pattern exhibited by the *regA-crtJ* double mutant was the same as that observed with the *regA* mutant under all three growth conditions. The *regA* mutant phenotype also prevailed in the *regA-fnrL* mutant when grown aerobically and semiaerobically. However, under anaerobic (photosynthetic) conditions, the *regA-fnrL* mutant showed a rather unexpected phenotype of no growth.

Cytochrome cbb_3 oxidase expression. To assay the expression of cytochrome cbb_3 oxidase, a translational *lacZ* fusion to the first gene in the *ccoNOPQ* operon was constructed (pDSccoN2) that contained 466 bp of DNA upstream of *ccoN* (20). This plasmid was mobilized into the same set of regulatory mutants and assayed in the same manner as described above for ubiquinol oxidase.

The expression pattern that we observed for cbb_3 oxidase in wild-type *R. capsulatus* was also similar to that reported by Swem et al. (21) (Fig. 3). Specifically, expression was highest under semiaerobic conditions, intermediate under aerobic conditions, and lowest under anaerobic growth conditions.

The effect of disrupting *regA* was also similar to that reported by Swem et al. (21) in that expression was significantly reduced under aerobic conditions, partially reduced under

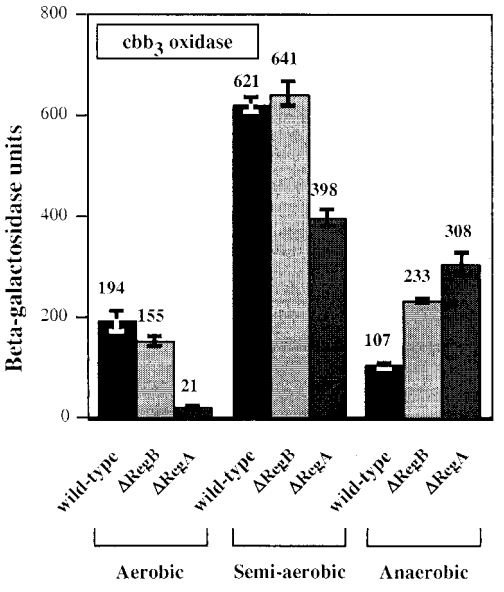

FIG. 4. β-Galactosidase analysis of aerobic, semiaerobic, and anaerobic photosynthetic cytochrome cbb_3 oxidase gene expression gene expression patterns in the wild-type parent strain SB1003, as well as in *regA*- and *regB*-disrupted strains. The values represent averages of at least three independent assays. Units of activity refer to the number of micromoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

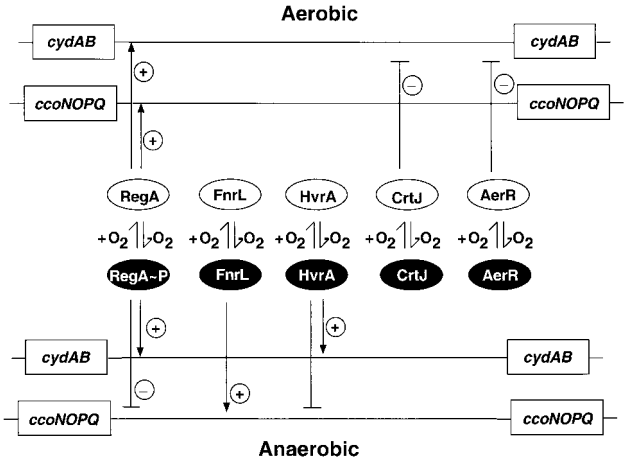

FIG. 5. Regulatory scheme for control of the terminal oxidase operons in *R. capsulatus*. In response to oxygen availability, the regulators provide negative or positive transcriptional control to coordinate enzyme synthesis for optimal growth. The regulators provide control as follows: (i) RegB-RegA provides regulation in response to both aerobiosis and anaerobiosis, (ii) FnrL provides regulation in response to anaerobiosis, (iii) HvrA provides regulation in response to anaerobiosis, (iv) CrtJ provides regulation in response to aerobiosis, and (v) AerR provides regulation in response to aerobiosis. The operons code for the following genes: *cydAB*, ubiquinol oxidase; and *ccoNOPQ*, cytochrome cbb_3 oxidase. Positive control (transcriptional activation) (+) and negative control (transcriptional repression) (−) of the genes are indicated.

semiaerobic conditions, and increased twofold under anaerobic conditions. This suggests that perhaps dephosphorylated RegA functions as an activator and that phosphorylated RegA functions as a repressor of cbb_3 oxidase expression. To test this possibility, we also assayed cbb_3 oxidase expression in a regB mutant strain that would just contain dephosphorylated RegA (Fig. 4). In this strain, we observed that the *regB* mutant has no phenotype under aerobic and semiaerobic growth conditions, which are conditions where RegB should not exhibit significant phosphorylation activity (6). This is contrasted by a rather significant reduction in expression observed upon disruption of *regA* under these conditions. Under anaerobic conditions, the *regB* mutant displays an expression level that is intermediate for wild-type cells and for a *regA* mutant.

The effect of an *hvrA* disruption on cbb_3 oxidase expression is different from that observed with ubiquinol oxidase. In this case, there is no effect on aerobic gene expression, but there is a reproducible increase in semiaerobic expression. This indicates that HvrA may have a repressing effect on cbb_3 oxidase expression.

One of the significant effects on cbb_3 oxidase expression occurs upon disruption of *fnrL*. In this case there is no effect on aerobically grown cells, a slight (30%) reduction under semiaerobically grown cells, and an 82% reduction in anaerobically grown cells. The effect of FnrL is even more obvious in semiaerobically grown cells when the *fnrL* mutation is present in conjunction with a *regA* mutation. In this case the effect is a 96% reduction in expression.

The *crtJ* and *aerR* regulatory mutants were also assayed for effects on *ccoN*::*lacZ* expression. Neither of these mutants exhibited a significant difference in expression from that found in the wild type under any growth conditions (data not shown).

The involvement of multiple regulators. There are two primary conclusions that can be derived from the results of this study. One conclusion is that *R. capsulatus* uses multiple regulators to ensure preferential and complete use of O_2 as an electron acceptor by varying the level of cytochrome cbb_3 oxidase and ubiquinol oxidase expression in response to changes in oxygen tension (Fig. 5). The second conclusion is that many transcription factors involved in controlling photosystem gene expression are involved in differentially controlling respiratory gene expression.

The terminal oxidases utilized by *E. coli* follow an expression pattern similar to that for the terminal oxidases of *R. capsulatus.* The *E. coli* cytochrome *o* and cytochrome *d* oxidases have different affinities for oxygen, with cytochrome *o* oxidase having a low affinity for oxygen (K_m = 1.4 to 2.9 μM) and cytochrome *d* having a high affinity for oxygen (K_m = 0.23 to 0.38 μM) (18). Therefore, the cell utilizes cytochrome *o* oxidase for aerobic respiration when oxygen is plentiful. As oxygen becomes depleted, the cell utilizes cytochrome *d* for respiration because its higher affinity for oxygen allows it to function even when oxygen is scarce. In accordance with this, it has been demonstrated that the cell regulates an inverse expression of these oxidases in response to changes in oxygen tension (22).

The actual affinities of the *R. capsulatus* cytochrome cbb_3 and ubiquinol oxidases for oxygen have not yet been established. However, as is the case for *E. coli* (22), both of the *R. capsulatus* oxidases are expressed maximally under semiaerobic conditions. Also like *E. coli*, the aerobic and anaerobic expression patterns of the two *R. capsulatus* oxidases are reciprocal (Fig. 2 and 3). This pattern of expression suggests that cytochrome cbb_3 is the low-affinity oxidase and that ubiquinol

is the high-affinity oxidase. In order to maintain the reciprocal pattern of expression of the terminal oxidases, the cell must regulate transcription of each oxidase according to oxygen availability.

It has been demonstrated that the RegB-RegA regulatory system plays a key role in the transcriptional regulation of the terminal oxidases in *R. capsulatus.* In addition to RegA, *R. capsulatus* utilizes a homolog of *E. coli* Fnr to differentially control terminal oxidase gene expression. In *E. coli* Fnr functions as a repressor of cytochrome *o* oxidase semiaerobically but has no effect anaerobically. For cytochrome *d* oxidase, Fnr switches roles by functioning as an activator semiaerobically and as a repressor anaerobically (4). Similarly, the *fnrL* mutant in *R. capsulatus* also has no effect anaerobically on ubiquinol oxidase expression but acts as a repressor semiaerobically (Fig. 2). Conversely, FnrL acts as an activator of cbb_3 oxidase expression both semiaerobically and anaerobically (Fig. 3). The phenotype of the *regA-fnrL* double mutant in *R. capsulatus* is intriguing though presently unexplainable. In addition to the substantially lowered terminal oxidase expression observed in these mutants, there is also the lack of growth under photosynthetic (anaerobic) conditions. The exact explanation for the nongrowth phenotype observed has yet to be uncovered, but it may be noted that an *arcA-fnrL* double mutant in *E. coli* also demonstrates insignificant expression of cytochrome *d* oxidase regardless of the availability of oxygen (9).

In addition to the regulators with functional similarities to *E. coli* oxidase regulators, there are other "photosystem specific" oxygen-responsive regulators utilized by *R. capsulatus*. Specifically, aerobic repression of ubiquinol oxidase appears to be promoted by the photopigment repressors CrtJ and AerR. These two repressors are known to be responsible for aerobic repression of bacteriochlorophyll, carotenoid, light harvesting, and reaction center gene expression (5, 8, 17). Our observation that CrtJ and AerR also aerobically repress expression of ubiquinol oxidase (Fig. 2) further supports the recent observation that *R. capsulatus* coordinates many different aerobic and anaerobic processes, such as photosynthesis (16), respiration (21), nitrogen fixation (7), carbon fixation (23), and hydrogen utilization (7). This appears to be mediated in part through the use of global response regulators such as RegB-RegA, as well as by CrtJ and AerR, which have overlapping photosynthesis and nonphotosynthesis target genes.

The utilization of multiple regulators for this process may be explained through their various sensitivities to oxygen that can be observed in this study (Fig. 5). As oxygen becomes depleted, some regulators may lose activity while others gain activity. For this reason it would be necessary for multiple regulators to be utilized so that the cell can optimally coordinate the synthesis of respiratory, photosynthetic, and biosynthetic processes according to the available levels of oxygen. The remaining challenge will be to determine mechanistic details of how the two terminal oxidase promoters are controlled by the aforementioned transcription factors and whether many of the observed effects observed are the direct result of interactions of these regulators with these respiratory promoters.

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