ANTIOXIDANTS & REDOX SIGNALING
Volume 16, Number 8, 2012

DOI: 10.1089/ars.2011.4051

COMPREHENSIVE INVITED REVIEW

Bacterial Adaptation of Respiration from Oxic to Microoxic and Anoxic Conditions: Redox Control

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Abstract

Under a shortage of oxygen, bacterial growth can be faced mainly by two ATP-generating mechanisms: (i) by synthesis of specific high-affinity terminal oxidases that allow bacteria to use traces of oxygen or (ii) by utilizing other substrates as final electron acceptors such as nitrate, which can be reduced to dinitrogen gas through denitrification or to ammonium. This bacterial respiratory shift from oxic to microoxic and anoxic conditions requires a regulatory strategy which ensures that cells can sense and respond to changes in oxygen tension and to the availability of other electron acceptors. Bacteria can sense oxygen by direct interaction of this molecule with a membrane protein receptor (*e.g.*, FixL) or by interaction with a cytoplasmic transcriptional factor (*e.g.*, Fnr). A third type of oxygen perception is based on sensing changes in redox state of molecules within the cell. Redox-responsive regulatory systems (*e.g.*, ArcBA, RegBA/PrrBA, RoxSR, RegSR, ActSR, ResDE, and Rex) integrate the response to multiple signals (*e.g.*, ubiquinone, menaquinone, redox active cysteine, electron transport to terminal oxidases, and NAD/NADH) and activate or repress target genes to coordinate the adaptation of bacterial respiration from oxic to anoxic conditions. Here, we provide a compilation of the current knowledge about proteins and regulatory networks involved in the redox control of the respiratory adaptation of different bacterial species to microxic and anoxic environments. *Antioxid. Redox Signal.* 16, 819–852.

Reviewing Editors: *Timothy Donahue, Lars Hederstedt, Woojun Park, and James Shapleigh*

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I. Introduction

Respiration is a fundamental process to all living cells in which electrons produced from oxidation of low-redox-potential electron donors such as NADH are transferred sequentially through a series of membrane-bound or membrane-associated protein carriers, the electron transport chain (ETC), which terminates in the reduction of the high-redox-potential electron acceptor, oxygen (Fig. 1a). The free energy released during this electron transfer process is used to drive the translocation of protons across the membrane to generate an electrochemical gradient that can be used for a variety of purposes, such as ATP synthesis and active transport (Fig. 1a). In contrast to the respiratory systems found in the mitochondria of many higher eukaryotic organisms, prokaryotic cells can induce branched-respiratory chains terminating in multiple oxidases with different affinities for the oxygen or use alternative electron acceptors, which contributes to their ability to colonize many microoxic and anoxic environments [reviewed in (61, 183, 186, 197)] (Fig. 1b).

The oxidation of organic molecules in cell respiration releases electrons that are transferred to membrane mobile quinones which constitute the link between the electron donating enzymes and the electron accepting enzymes (Fig. 1b). Quinones are small, freely diffusible, lipophilic, membrane-entrapped organic molecules that can carry two electrons and two protons when fully reduced, that is, in the quinol state [for a review, see (221)]. Different kinds of quinones have different electrochemical potentials, and many bacteria can synthesize more than one type of quinone. *Escherichia coli* synthesize three types of quinones, a benzoquinone (UQ-8), and two naphthoquinones, menaquinone (MK) and demethylmenaquinone. Ubiquinone (UQ) predominates under aerobic conditions, and MK predominates under anaerobic conditions when the cellular state is more reduced (221). From the lipophilic hydroquinones, electrons can be carried to two different types of terminal oxidases: cytochrome *c* oxidases or quinol oxidases, where dioxygen is reduced to water (Fig. 1b). When oxygen is not present in the medium, electrons can be transmitted to alternative reductases that reduce substrates such as nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), dimethyl-sulphoxide (DMSO), trimethylamine N-oxide, sulfate, sulfite, and fumarate as final electron acceptors. Among them, nitrate is one of the essential environmental components in the biosphere. It serves as a nutrient for plants and microorganisms, and is used as an electron acceptor by many bacteria, archaea, and also by several eukaryotes (109, 125, 266). During anaerobic respiration, nitrate can be reduced to dinitrogen gas (N_2) or to ammonium. The anaerobic reduction of nitrate to N_2 gas is called denitrification, and it constitutes one of the more important processes in the N-cycle (141). This reductive process occurs in four stages, reduction of NO_3^- to NO_2^- and *via* the gaseous intermediates NO and N_2O to N_2. The enzymes involved in denitrification are nitrate-, nitrite-, nitric oxide-, and nitrous oxide reductase, encoded by *nar/nap*, *nir*, *nor*, and *nos* genes, respectively [reviewed in (125, 247, 248)]. In agriculture and wastewater treatment, denitrification by microorganisms is an important issue due to the economical, environmental, and public health implications of this process (10, 105).

The bacterial respiratory flexibility requires a regulatory strategy which ensures that prokaryotic cells sustain life under different environments in response to changing oxygen tension. To date, three main modes to sense O_2 have been described in bacteria: two types by direct interaction of O_2 with a membrane protein receptor (as in the heme-based sensor kinase FixL in rhizobia [reviewed in (83, 101, 205)] or by interaction with a regulatory protein such as the Fe-S-based fumarate and nitrate reductase regulatory protein (Fnr) in *E. coli* [reviewed in (65, 68, 84, 53, 101, 261)]. In addition, a third type of O_2 perception is based on monitoring environmental oxygen concentration by sensing changes in the redox state of molecules or pools of molecules within the cell. These changes are detected by various protein sensors that convert the redox signals into regulatory output at the transcriptional or post-transcriptional level. Redox signals are many and diverse, and can involve redox-active cofactors such as heme, flavins, pyridine nucleotides, iron-sulfur clusters, and redox-sensitive amino-acids chains such as cysteine thiols among others [reviewed in (3, 14, 102)]. This article focuses on a comparative study about the regulatory mechanisms used by different bacterial groups to undergo respiratory shifts from oxic to microoxic and anoxic environments.

II. Aerobic Respiration Under Microoxic Conditions

The branched ETC present in many bacteria often contains several terminal oxidases (Fig. 1b), which can be grouped into two main types based on the substrates used as electron donors [reviewed in (61, 177, 183)]. Reduced *c*-type cytochromes donate electrons to cytochrome oxidases, whereas hydroquinones deliver electrons to quinol oxidases (Fig. 1b). Both types of oxidases belong to the extensively studied family of heme-copper oxidases (HCOs). The common denominator in HCOs is a membrane-integral subunit I that carries as cofactors a low-spin heme and a high-spin heme-copper binuclear center (Cu_B site) where reduction of O_2 to H_2O takes place (Fig. 2). Among this family, quinol oxidases possess a cofactor-free subunit II, whereas cytochrome *c* oxidases have cofactors bound to subunit II. In most cases, this is a binuclear Cu–Cu center (Cu_A site) that is liganded by six highly conserved amino acids (87, 176). The HCO superfamily is very versatile due to the wide range of electron donors they can use, their subunit composition, and the types of heme groups that they

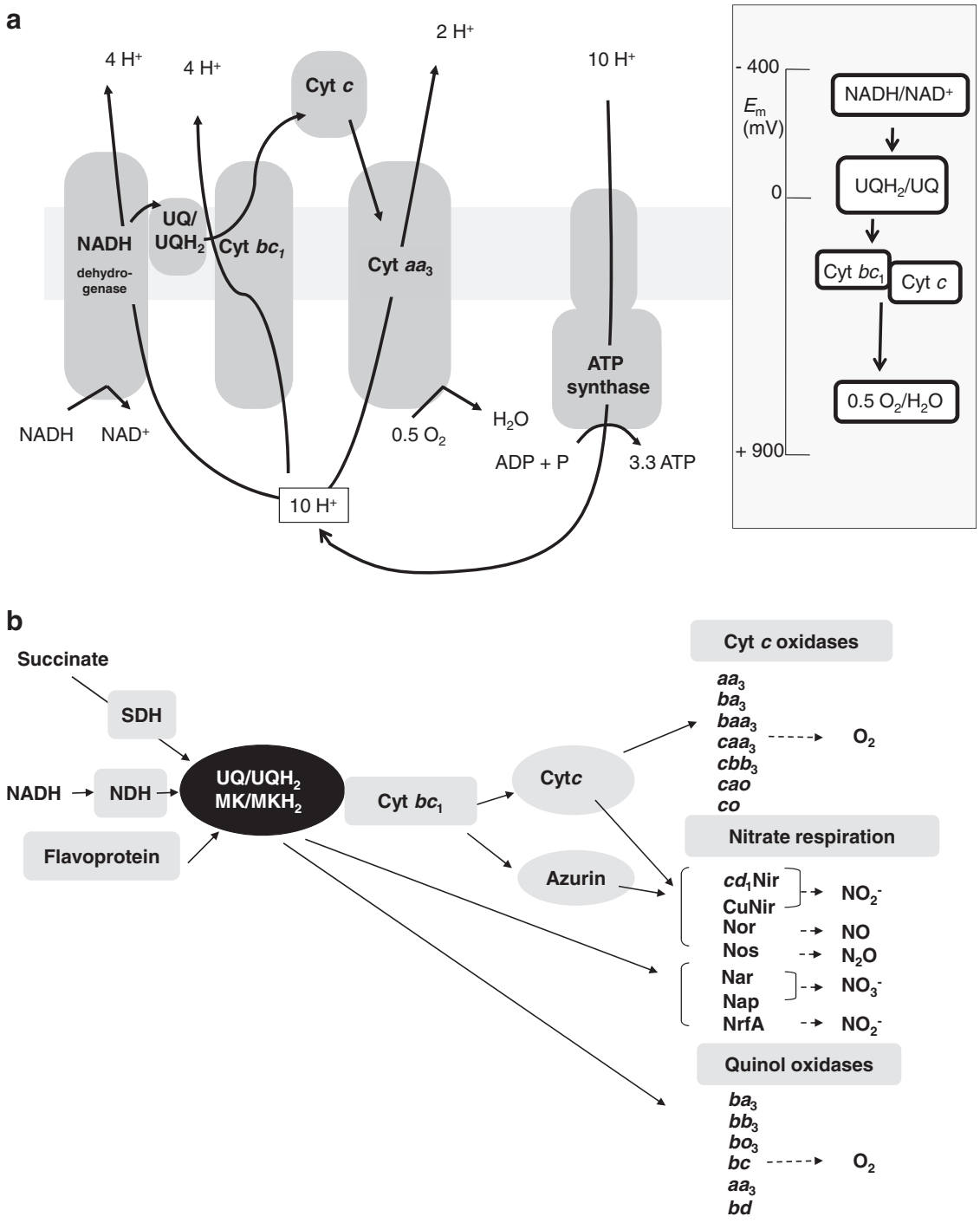

FIG. 1. Respiratory chain(s) in mammalian mitochondrion and bacteria. (a) A summary of the topology and bioenergetics of a basic aerobic respiratory electron transport system of a mammalian mitochondrion is shown. This figure is adapted from ref. (197). **(b)** Schematic representation of aerobic and anaerobic nitrate respiration pathways in bacteria. MK, menaquinone; UQ, ubiquinone; Cyt, cytochrome; SDH, succinate dehydrogenase; NDH, NADH dehydrogenase; cd_1Nir, cd_1-type nitrite reductase; CuNir, Cu-type nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase; Nar, membrane-bound nitrate reductase; Nap, periplasmic nitrate reductase; NrfA; cytochrome *c* nitrite reductase; UQH_2, ubihydroquinone; MKH_2, menahydroquinone.

contain. Studies of evolutionary relationships led to classifying the HCO enzymes in three different types (176, 177) (Fig. 2): (i) type A oxidases (oxidases aa_3), which are structurally and functionally close to mitochondrial oxidases, (ii) type B oxidases, grouped as cytochrome oxidases bo_3, and (iii) type C oxidases, which include the cytochrome *c* oxidases cbb_3. To gain new insights into the oxygen respiration process, C-family HCOs from pathogenic bacteria have been recently structurally characterized, and it has been shown that C-HCOs differs from the two other HCO families, A and B (40).

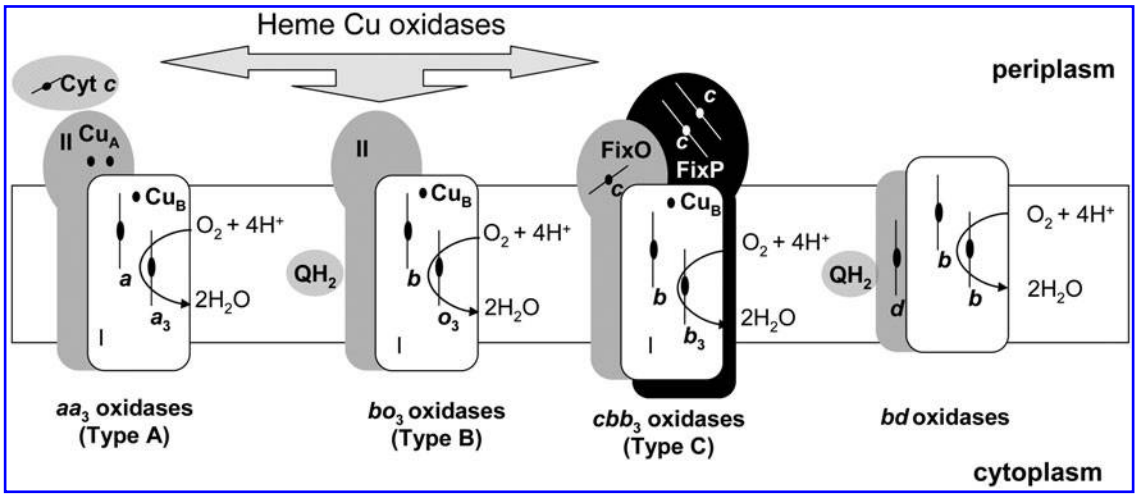

FIG. 2. Summary of the main types of heme–copper oxygen reductases belonging to the A, B, and C families and the *bd*-type quinol oxidase.

The X-ray structure of the C-family cbb_3 oxidase from *Pseudomonas stutzeri* shows an electron supply system different from families A and B.

Under O_2 limitation (microoxic conditions), many bacteria induce high-affinity oxidases to respire traces of molecular oxygen. The cbb_3-type cytochrome oxidase is known to have a high affinity for oxygen. Thus far, the enzyme from *Bradyrhizobium japonicum* is the only cytochrome cbb_3 oxidase in which substrate affinity has been measured showing a Km for dioxygen in the order of 7 n*M* (185). As a consequence, it allows respiration under microoxic conditions, such as those encountered by rhizobia in the legume nodule ($[O_2]$ estimated to be 30 n*M*) (61). This oxidase has a subunit II (CcoO or FixO) that is a monoheme *c*-type cytochrome instead of the Cu_A-containing protein (67, 176). Subunit III in cbb_3-type oxidases, which is a diheme cytochrome *c* (CcoP or FixP), is thought to relay the electrons from the cytochrome bc_1 complex *via* CcoO to the redox centers of subunit I (126, 265). Subunit III of all other HCOs is cofactor-free, just like the nonconserved small subunit IV (176). Homology modelling and recent studies on the thermodynamic properties of the redox centers in cbb_3 oxidase from *Rhodobacter sphaeroides* using a combination of optical and electron paramagnetic resonance redox titrations have allowed the characterization of the cbb_3 active site (191). Furthermore, it has been recently shown that the redox-coupled proton movement in the proximal cavity of cbb_3-enzymes contributes to the low redox potential of heme b_3, and suggests its potential implications for the high oxygen affinity of these enzymes (219).

In addition to C-family cbb_3 oxidases, another family of high-affinity oxidases comprises cytochrome *bd*-type oxidases (29). Among them, the *bd* oxidase of *E. coli* is known to have a high affinity for oxygen showing the $Kd(O_2)$ value of 0.28 μM (19). The *bd*-type oxidases are quinol oxidases that show no sequence homology to any subunit of heme-cooper family members. They do not contain any copper atoms in the catalytic center, but probably replace the Cu_B atom with a second heme (Fig. 2). In contrast to the heme-copper enzymes that are found at most branches of the tree of life, *bd*-type oxidases are only found in bacteria and archaea (183). The *bd*-type cytochromes do not pump protons and, therefore, have a lower total energetic efficiency compared with the heme-copper type oxidases (29).

A. *Oxygen control of* cbb_3 *oxidase expression*

As just mentioned, cbb_3 oxidase is quite distinct from other bacterial HCOs in terms of its strategy for receiving electrons, the heme prosthetic group present in the active site, and its affinity for oxygen. Cytochrome cbb_3 oxidases have been purified from several organisms, including *Paracoccus denitrificans*, *Rho. sphaeroides*, *Rhodobacter capsulatus*, and *Br. japonicum* [reviewed in (179)]. The biogenesis of this multisubunit enzyme, encoded by the *ccoNOQP* operon, depends on the *ccoGHIS* gene products, which are proposed to be specifically required for cofactor insertion and maturation of cbb_3-type cytochrome *c* oxidases (185). In the facultative photosynthetic model organism *Rho. capsulatus,* CcoN, CcoO, and CcoQ assemble first into an inactive 210 kDa sub-complex, which is stabilized *via* its interaction with CcoH and CcoS. Binding of CcoP, and probably subsequent dissociation of CcoH and CcoS, generates the active 230 kDa complex (126). Recent results have proposed that CcoH behaves more similar to a subunit of the cbb_3 oxidase rather than a transient assembly factor *per se* (173). The insertion of the heme cofactors into the *c*-type cytochromes CcoP and CcoO precedes sub-complex formation, while the cofactor insertion into CcoN could occur either before or after the 210 kDa sub-complex formation during the assembly of the cbb_3-type oxidase (126). CcoQ is required for optimal cbb_3-type oxidase activity, because it stabilizes the interaction of CcoP with the CcoNO core complex, leading subsequently to the formation of the active 230-kDa cbb_3-type oxidase complex (178).

Genes encoding the cbb_3 complex were initially isolated from rhizobia and named *fixNOQP* due to its requirement for symbiotic nitrogen fixation (185). Since then, orthologous genes called *ccoNOQP* were identified in other Proteobacteria including photosynthetic and pathogenic bacteria, suggesting that this oxidase is not only required for diazotrophs to sustain N_2 fixation, but also for the successful colonization of anoxic tissues by human pathogens [reviewed in (50, 61, 179)]. Regulation of *ccoNOQP* expression has been investigated in

many bacteria with oxygen being the key effector that controls its expression. To date, four different strategies that activate the *ccoNOQP* operon have been described (Fig. 3): (a) in *Rho. sphaeroides*, *Pa. denitrificans*, *Pseudomonas aeruginosa*, *Rhizobium leguminosarum* bv. *viciae* (UPM791), and *Rho. capsulatus*, Fnr acts as oxygen sensor and transcriptional factor; (b) in rhizobial species, such as *Sinorhizobium meliloti*, *Br. japonicum*, and *Azorhizobium caulinodans*, FixLJ/FixK is the system implicated in the activation of *ccoNOQP* under oxygen deprivation; (c) in *Rhi. leguminosarum* (VF39) and *Rhizobium etli* CFN42, a mixed regulation strategy operates that involves both Fnr and FixK proteins; (d) and finally, in the human pathogenic bacteria *Helicobacter pylori* and *Campylobacter jejuni* (ε-Proteobacteria), which lack *fnr* and *fixLJ-fixK* genes, it has been proposed that cbb_3 may be constitutively expressed allowing the growth under microoxic conditions in its hosts. In addition to the positive control of Fnr on *fixNOQP* expression, it has been shown in *Rhi. etli* that two novel Fnr-type transcriptional regulators (StoRd and StoRf, symbiotic terminal oxidase regulators) have a negative control on microoxic expression of *fixNOQP* genes (100).

1. Involvement of Fnr. The O_2-sensitive protein Fnr belongs to the cyclic AMP (cAMP) receptor protein (Crp) superfamily of transcription factors that trigger physiological changes in response to a variety of metabolic and environmental cues [reviewed in (53, 65, 68, 84, 101, 103, 124, 261)]. All members of this family are predicted to be structurally related to Crp. They consist of four functionally distinct domains: (i) a sensor domain, (ii) a series of β-strands (β-roll structure) that form a loop-like structure making contact with the RNA polymerase holoenzyme (RNAP), (iii) a long α-helix acting as a dimerization interface, and (iv) a C-terminal helix-turn-helix motif (H-T-H) involved in DNA binding (Fig. 4a). The best-characterized Fnr protein is that of *E. coli*. In *E. coli*, Fnr acts as a direct oxygen sensor and is the primary transcriptional regulator of the switch between aerobic and anaerobic growth. Thus, under anoxic conditions, Fnr is in its active state and is able to bind specific palindromic sequences of DNA (Fnr box). Once bound to DNA, Fnr activates target gene expression by recruiting RNAP or represses transcription by preventing the formation of productive RNAP:DNA complexes (53). Active Fnr regulates genes involved in the adaptation of *E. coli* for anaerobic growth and controls one of the best-studied gene regulatory networks in the cell. Very recently, the Fnr regulatory network has been reviewed by Tolla and Savageau (238). Generally, Fnr activates genes encoding products involved in anaerobic metabolism, such as the *nar* operon (nitrate reductase), the *dms* operon (DMSO reductase), and the *frd* operon (fumarate reductase), and represses genes encoding products involved in aerobic metabolism, such as the *sdh* operon (succinate dehydrogenase) and *ndh* (NADH dehydrogenase II). Fnr is activated under anoxic conditions by the acquisition of one $[4Fe\text{-}4S]^{2+}$ cluster per monomer (designated 4Fe-Fnr). Each $[4Fe\text{-}4S]^{2+}$ cluster is ligated by four cysteine residues (Cys^{20}, Cys^{23}, Cys^{29}, and Cys^{122}), and the presence of the cluster promotes Fnr dimerization, increasing its capacity to bind specifically to DNA (52, 54, 132) (Fig. 4a). The $[4Fe\text{-}4S]^{2+}$ cluster is susceptible to be attacked by O_2, resulting in rapid and reversible conversion to a $[2Fe\text{-}2S]^{2+}$ and finally to apoFnr on exposure to high oxygen concentrations. In addition to its reaction with oxygen, the Fnr $[4Fe\text{-}4S]^{2+}$ cluster is also sensitive to NO. On exposure to NO, $[4Fe\text{-}4S]^{2+}$ becomes nitrosylated, forming a combination of monomeric and dimeric dinitrosyl-iron-cysteine complexes, again abolishing its ability to bind DNA (53, 57). It has been reported that Fnr represses genes involved in NO detoxification (*e.g.*, *hmp*, which encodes the flavohemoglobin) (182). When cultures of *E. coli* are exposed to NO, Fnr repression of *hmp* is relieved, suggesting that the reaction between Fnr and NO is physiologically significant. This suggestion is supported by transcript profiling experiments which reveal that the abundances of many Fnr-activated genes are lower, and many Fnr-repressed genes are higher, when *E. coli* is exposed to NO (119, 187).

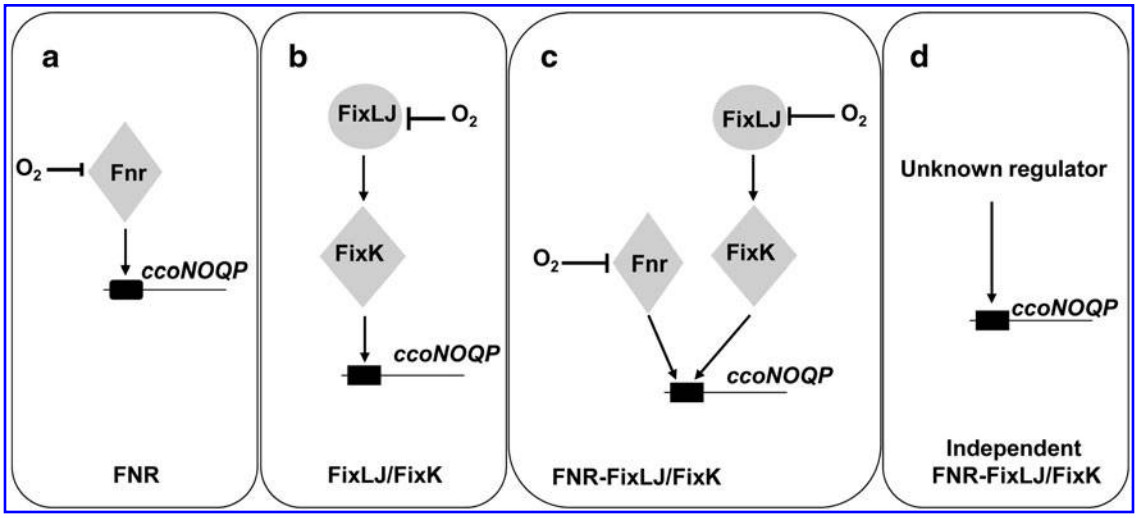

FIG. 3. Regulation strategies of *ccoNOQP* gene transcription. Four regulation strategies have been described in several bacterial groups (see text for details) that involve **(a)** Fnr, **(b)** FixLJ-FixK, **(c)** Fnr and FixLJ-FixK, or **(d)** an unknown regulator. Gene transcriptional activation is indicated by *arrows*. Protein inactivation by O_2 is indicated by *perpendicular lines*. This figure is adapted from ref. (50).

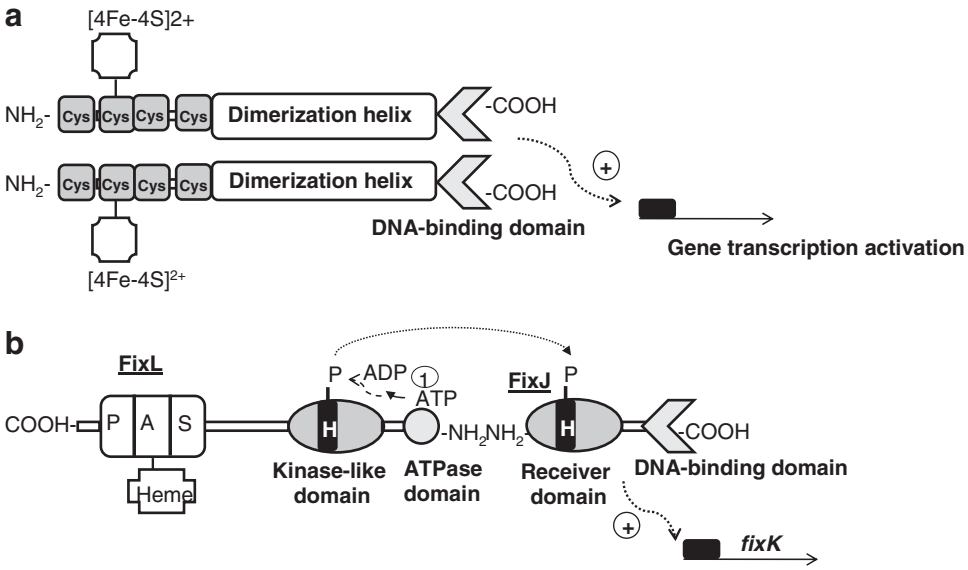

FIG. 4. Functional domains and model of Fnr and FixLJ mediated oxygen activation. (a) Fnr senses oxygen *via* an iron-sulfur center, which is coordinated with four cysteine residues. Under anoxic conditions, Fnr is in its active state and it promotes DNA-binding by formation of a homodimer leading to activation of gene transcription. **(b)** FixL is an oxygen receptor in which oxygen binds directly to a heme group that is coordinated to a histidine residue within a PAS domain. Detection of low oxygen tension changes the conformation of the input PAS domain, rendering increased autophosphorylation activity of the transmitter domain in His residue and repressing the phosphatase activity of FixL. FixL activates FixJ transcriptional activity by transferring the phosphoryl group to the N-terminal domain of FixJ (+) gene transcription activation.

2. Involvement of FixLJ/K. In some rhizobial species, the *fixNOQP* operon is under the control of one Fnr-type transcriptional regulator named FixK, which is also able to recognize Fnr boxes but lacks $[4Fe–4S]^{2+}$ clusters. FixK is thereby unable to sense oxygen directly. In this case, it is the two-component regulatory system FixLJ that ensures oxygen sensing under low-oxygen conditions and activates the transcription of the *fixK* gene. This regulatory system FixLJ/FixK has been studied in rhizobial species where it controls the expression of genes for microoxic respiration, as well as various other functions, in response to low oxygen (28, 56, 83, 145, 160). Interestingly, it has been shown that *Rhodopseudomonas palustris* FixK is not only required for optimal microaerobic growth, but is also required for phototrophic and autotrophic growth, and for growth on aromatic compounds (194). These authors also propose that FixK helps the control of the transition of *R. palustris* to anaerobic growth by activating the expression of the oxygen-sensing Fnr homolog, AadR.

The biochemistry of the FixLJ two-component system has been characterized in *Si. meliloti* and *Br. japonicum* (96, 211, 223). FixL is a histidine kinase that binds heme at an amino-terminal PAS domain (Fig. 4b). PAS domains are present in many proteins involved in the sensing of oxygen, redox, or light. They were first found in eukaryotes, and were named after homology to the *Drosophila* period protein (PER), the aryl hydrocarbon receptor nuclear translocator protein (ARNT), and the *Drosophila* single-minded protein (SIM). The binding of oxygen to heme inactivates FixL kinase activity. FixL, and the response regulator and transcription factor FixJ form a complex in which FixJ allosterically stimulates autophosphorylation of FixL. At very low oxygen concentrations, FixL transphosphorylates FixJ, which, in turn, activates expression of the transcription factor gene *fixK* (Fig. 4b). In *Br. japonicum*, the nitrogen-fixing root-nodule endosymbiont of soybean (*Glycine max*), a FixK-like protein called $FixK_2$, acts as the key distributor of the low-oxygen signal perceived at the level of the FixLJ two-component regulatory system. $FixK_2$ is one of the 16 (Crp/Fnr)-type transcriptional regulators whose genes are present in the *Br. japonicum* genome [reviewed in (146)]. $FixK_2$ recognizes a palindromic sequence motif (TTG-N_8-CAA, termed the $FixK_2$ box) that is located around position–41 upstream of the transcription start site in the regulated promoters. Microoxically induced targets of $FixK_2$ include the operons *fixNOQP* and *fixGHIS*, both essential for microoxic respiration, several heme biosynthesis genes (*hemA*, *hemB*, $hemN_1$, and $hemN_2$), denitrification genes, and some hydrogen oxidation genes (*hup* genes) (16, 160). Recently, the *Br. japonicum* $FixK_2$ regulon was unraveled by using a transcriptomics approach (145). DNA binding site predictions, together with a $FixK_2$-dependent *in vitro* transcription assay, demonstrated that the *fixNOQP* operon is a direct target for $FixK_2$. The latter studies, carried out with purified $FixK_2$ protein, showed that $FixK_2$ is sufficient to activate transcription *in vitro* without any identified effector (148). This is puzzling in view of the fact that all Crp/Fnr-like proteins can be positively or negatively modulated in their activity through bound cofactors or intrinsic, reactive amino acids (101). However, recent findings have reported that post-translational control occurs at $FixK_2$, whereby a critical cysteine at position 183 in the polypeptide chain is a target for oxidation. This provides a second, important means of affecting $FixK_2$ activity, in addition to the regulation of its expression by FixLJ (147).

III. Redox Control of the Adaptation of Aerobic Respiration from Oxic to Microoxic Conditions

A. E. coli *ArcBA*

E. coli has three terminal quinol oxidases, of which one is a heme-copper bo_3 oxidase and two are *bd*-type oxidases [reviewed in (183, 186)]. The quinol bo_3 oxidase contains a heme-Cu cofactor (Cu_B) and is essential when oxygen is present at high concentrations. The protein complex is encoded by the *cyoABCDE* operon (186). Under conditions of low aeration, the high-affinity *bd* oxidase is the preferred terminal reductase in *E. coli* (29). The structural components are encoded by the genes *cydAB*, which form a heterodimer. CydC and D are encoded in the same operon as CydA and B and thought to form a heme transporter necessary for cytochrome *bd* oxidase biogenesis (29). There is a third terminal oxidase present in *E. coli* encoded by the genes *cyxAB*. It is a *bd* type oxidase but its function is unclear. It appears to be expressed under microoxic conditions (29).

Cytochrome *bd* oxidase prevails under oxygen-limiting conditions. Consistent with these properties, transcription of the *cydAB* operon is activated when oxygen becomes limiting. Under fully anoxic conditions, *cydAB* expression is subsequently repressed to an intermediate level relative to microoxic and oxygen-rich conditions (241). It is now solidly established that this oxygen control is achieved through the combined action of the oxygen sensing transcription factor Fnr and the aerobic respiration control (Arc)B/ArcA two-component system (217). Under anoxic conditions, Fnr acts as a repressor of the *cydAB* operon (Fig. 5). ArcBA is a two-component regulatory system involved in the transcriptional regulatory network that allows facultative aerobic bacteria to sense and respond to various respiratory conditions. While the Fnr protein is directly implicated in sensing oxygen, the ArcBA system activity is proposed to be modulated by the redox state of the quinones in the membrane (137, 138). The ArcBA system comprises the ArcB protein, a tripartite membrane-bound sensor kinase, and the cognate response regulator ArcA (Fig. 6a). ArcB belongs to the subclass of hybrid sensor kinases that also includes BarA, BvgS, EvgS, LemA (GacS), RteA, and TorS (2, 138). Under limiting oxygen conditions, ArcB autophosphorylates residue His^{292} and, through a phosphorelay system involving Asp^{576} and His^{717}, transphosphorylates ArcA (Fig. 6a). It has been previously reported that histidine kinases act as homodimers and that they autophosphorylate by an intermolecular reaction (229). That is, the gamma-phosphoryl group of ATP, which is bound to one monomer in the homodimer, is transferred to the other monomer. On the contrary, it has been recently proposed that ArcB autophosphorylates through an intramolecular and not through an intermolecular reaction as previously proposed (175).

On a shift to aerobic conditions, ArcB can form intermolecular disulphide bonds *via* Cys^{180} and Cys^{241} located in the PAS domain (Fig. 6a). The kinase activity of ArcB is highly dependent on this covalent linkage (137). The regulatory mechanisms that underlie the function of the ArcBA two-component system have been the subject of numerous studies. ArcB does not sense oxygen directly but is thought to sense the redox state of the UQ-ubiquinol pool in the aerobic respiratory chain, because UQ inhibits ArcB autokinase activity (137). However, this regulatory network becomes complex, as recent observations could not demonstrate a correlation between ArcB activation and oxygen availability (18). Bekker and colleagues (18) proposed that the *in vivo* activity of ArcB in *E. coli* is also modulated by the redox state of the MK pool and that the UQ/ubiquinol ratio is not the only determinant of ArcB activity (Fig. 6b). This combined regulation by the redox state of the UQ pool and MK pool provides a mechanism to explain the observed complex regulation of ArcB activation at variable rates of oxygen supply (Fig. 6b). Menaquinols would be the dominant activators under anoxic conditions, where the size of the UQ pool is approximately five times less than the size of the MK pool (18). Recent work has used controlled chemostat cultures subject to gradually decrease oxygen concentration to study the effects of oxygen availability on the transcriptome, redox state of the UQ-ubiquinol, outer membrane protein profiles, and terminal oxidase proteins of *E. coli* K-12 (208). A probabilistic model was used to predict the activity of ArcBA across the aerobiosis range. The model implied that the activity of the regulator ArcA correlated with aerobiosis, but not with the redox state of the UQ pool. Rolfe and colleagues (208) have proposed that, at least under the conditions used in this work, the rate of fermentation product synthesis (*q*acetate) exerts a greater influence on ArcA activity than the redox state of the quinone

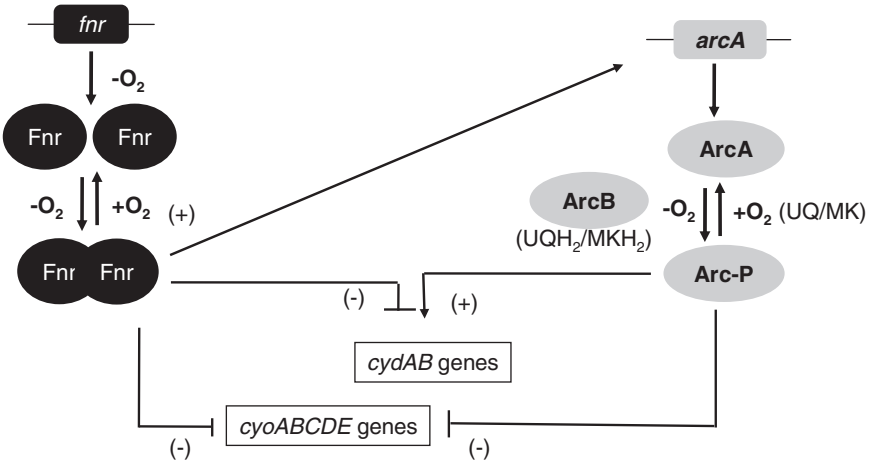

FIG. 5. Regulation of *bd* and bo_3 terminal oxidase expression in *Escherichia coli*. Fnr and ArcB provide negative or positive transcriptional control in response to oxygen availability. The genes *cydAB* and *cyoABCDE* encode *bd* and bo_3 oxidase polypeptides, respectively. Positive regulation (+) is denoted by *arrows*, and negative regulation (−) is indicated by *perpendicular lines*.

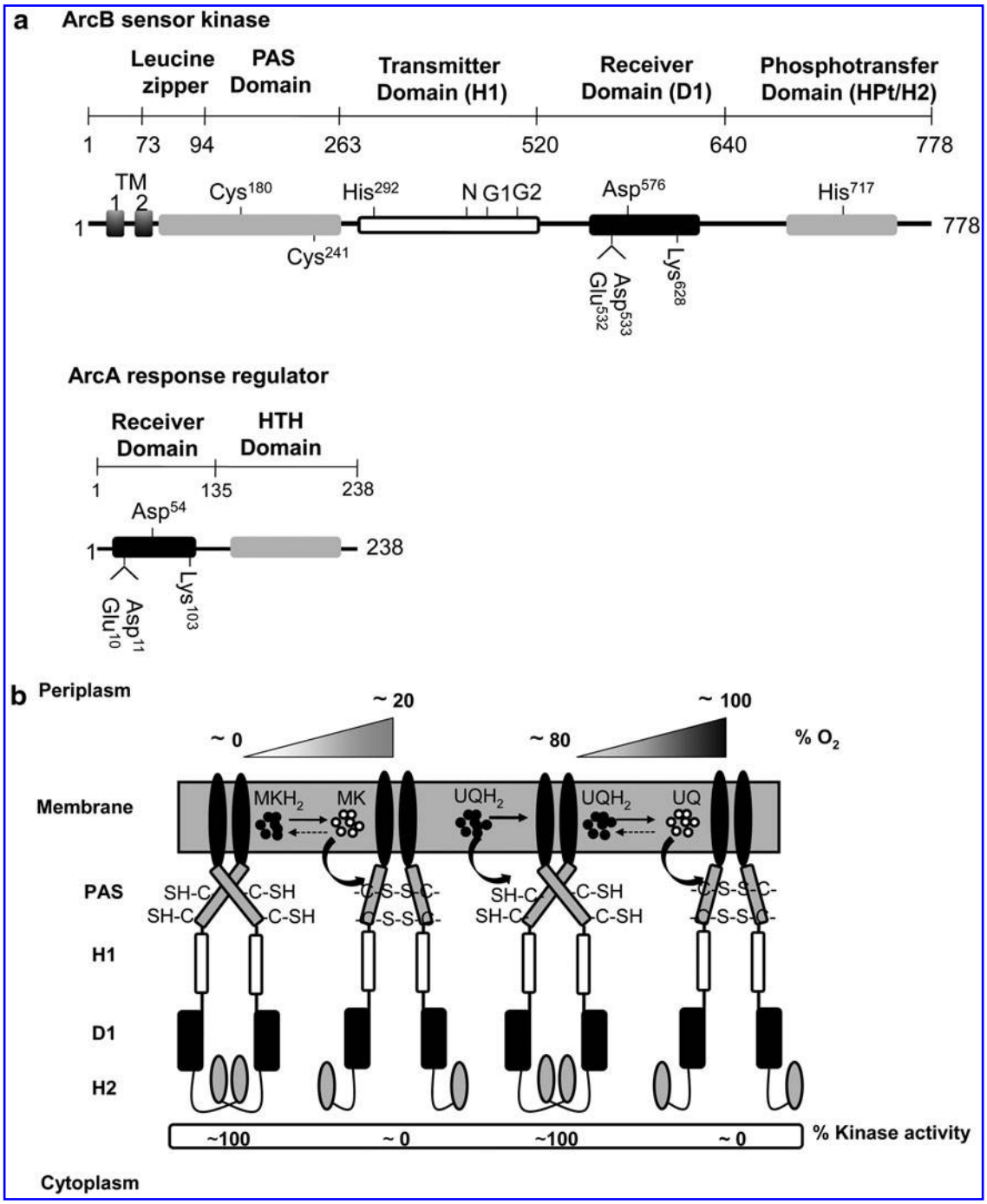

FIG. 6. Functional domains and model of ArcBA-mediated redox control. (a) ArcB is attached to the membrane by two transmembrane regions (TM). A linker region containing a putative leucine-zipper and a PAS domain connects TM2 with the catalytic domains. The two cysteine-residues (Cys^{180} and Cys^{241}) in the linker region are able to undergo oxidation and to form intermolecular disulfide bonds between both ArcB monomers. The transmitter domain (H1) contains the conserved His^{292} together with the G1 and G2 nucleotide-binding motifs. The receiver domain (D1) harbors the conserved Asp^{576}, and the histidine phosphotransfer domain (Hpt/H2) contains the conserved His^{717}. The ArcA component is represented with its N-terminal receiver domain carrying the conserved Asp^{54} and its C-terminal H-T-H) domain. This figure is adapted from ref. (138). **(b)** MKH_2, which predominates under anaerobic conditions, would be oxidized in the presence of low levels of oxygen (~20% aerobiosis), leading to increased levels of MKs and inactivation of ArcB kinase activity. UQH_2 predominate under microoxic conditions (~80% aerobiosis), giving rise to activation of ArcB kinase activity. In fully aerobic conditions (100%), the UQH_2 pool is subjected to oxidation, which results in increasing levels of UQs and oxidation of the key cysteines, leading to inactivation of the ArcB kinase activity. This figure is adapted from ref. (18).

pools. It is perhaps not surprising that, as a regulator of genes that have fundamental roles in different processes of the bacterial metabolism (aerobic respiration, anaerobic respiration, and fermentation), ArcB should integrate the response to multiple signals (*e.g.*, UQ, MK, acetate, and other fermentation products) in response to different growth conditions.

Phosphorylated ArcA activates genes or operons needed to use traces of oxygen in the medium, for example, those for terminal oxidase *bd*, and represses expression of the aerobic cytochrome bo_3 terminal oxidase (136). Regulation of *bd* and cytochrome bo_3 terminal oxidases in response to oxygen concentration variation is also controlled by Fnr as just described (Fig. 5b). Fnr inhibits *cyd* and *cyo* gene activation from 0% to 10% oxygen tension. However, ArcA represses *cyo* expression and activates *cyd* transcription when O_2 concentration is between 10% and 20% (241) (Fig. 5). These authors proposed that when the oxygen tension drops, ArcA is phosphorylated by ArcB and activates *cydAB* transcription reaching a maximum at $<2\%$ O_2 tension. When oxygen is decreased further, Fnr becomes active and represses *cydAB* transcription. Fnr repression of *cydAB* requires the presence of a functional ArcA protein (51, 99). According to this observation, Fnr has been proposed to serve as an antiactivator by counteracting ArcA-mediated activation, rather than directly repressing transcription. This indirect regulation of *cydAB* repression by Fnr through ArcA is supported by gene expression profiling analyses where nearly two-thirds of the genes for which expression is affected by Fnr are also affected by ArcA (209, 210). In addition to controlling genes encoding respiratory oxidases, ArcA∼P represses the expression of genes encoding dehydrogenases of the tricarboxylic acid cycle and enzymes of the glyoxylate shunt. Recent studies have demonstrated that ArcAB exerts an important influence on carbon and redox balances in *E. coli* growing under carbon-limited conditions with a restricted oxygen supply (163).

B. Rhodobacter *species RegBA/PrrBA*

Photosynthetic bacteria such as *Rho. Capsulatus* and *Rho. sphaeroides* conserve energy under oxic or microoxic conditions by driving electrons through branched electron transfer chains to terminal oxidases with different affinity for oxygen (Fig. 7). One ubiquinol oxidase is found in *Rho. capsulatus* (*cydAB*), while two are found in *Rho. sphaeroides* (*qoxBA* and *qxtAB*). The cytochrome *c* oxidases depend on the cytochrome bc_1 complex (*petABC* in *Rho. capsulatus and fbcFBC* in *Rho. sphaeroides*), and cytochromes c_2 (*cycA*) or c_y (*cycY*). While *Rho. capsulatus* contains only one cbb_3-type oxidase (*ccoNOQP*), an aa_3- (*ctaDC*) and a cbb_3-type oxidase (*ccoNOQP*) are found in *Rho. sphaeroides* [reviewed in (72, 120, 166, 233, 258)]. Gene expression experiments using the *lacZ* as reporter gene revealed that the cbb_3 oxidase from *Rho. capsulatus* and aa_3 oxidase from *Rho. sphaeroides* are considered low-oxygen-affinity oxidases which are induced under oxic conditions (233). However, the *Rho. capsulatus* ubiquinol *bd* oxidase and *Rho. sphaeroides* cbb_3 oxidase are classified as high-affinity terminal oxidases and are highly induced under oxygen-limiting conditions (233).

RegBA/PrrBA are members of the family of the two-component regulatory systems present in a large number of Proteobacteria. These proteins are named RegBA in *Rho. capsulatus*, *Rhodovulum sulfidophilum*, and *Roseobacter denitrificans* [reviewed in (72, 258)], PrrBA in *Rho. sphaeroides* (106, 167), ActSR in *Si. meliloti* (73) and *Agrobacterium tumefaciens* (8), RegSR in *Br. japonicum* (15), and RoxSR in *Ps. aeruginosa* (46). In *Rhodobacter* species, the RegBA/PrrBA regulon encodes proteins involved in numerous energy-generating and energy-utilizing processes such as photosynthesis, carbon fixation, nitrogen fixation, hydrogen utilization, aerobic and anaerobic respiration, and aerotaxis [reviewed in (72, 233, 258)]. Recent analyses of the transcriptome and the proteome of *Rho. sphaeroides prrA* mutant revealed that, in addition to the numerous PrrA gene targets already known, genes encoding proteins whose functions are involved in intermediary metabolism, repair of DNA and protein damage, cell motility and secretion, and translation constituted new targets for PrrA regulation (77).

The RegBA/PrrBA two-component systems comprise the membrane-associated RegB/PrrB histidine protein kinase, sensing changes in redox state and its cognate PrrA/RegA

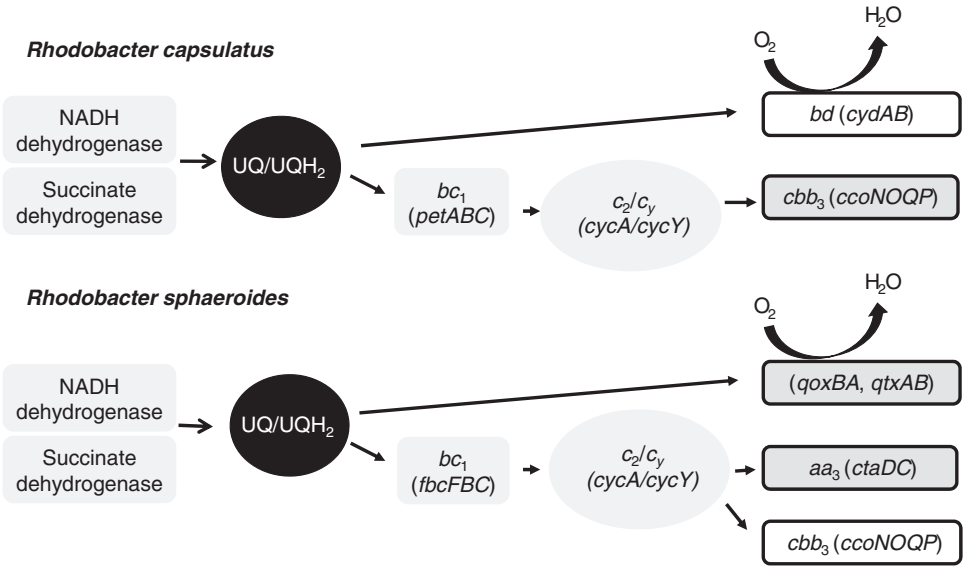

FIG. 7. Aerobic respiratory chains in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. The quinone-reducing (*left*) and quinol-oxidizing (*right*) branches with terminal oxidases induced under oxic conditions (*gray boxes*), and microoxic conditions (*white boxes*), are shown. *Black arrows* indicate the influx of reducing equivalents.

response regulador. Under conditions where the redox state of the cell is altered due to generation of an excess of reducing potential, produced by either an increase in the input of reductants into the system (*e.g.*, presence of reduced carbon source) or a shortage of the terminal respiratory electron acceptor (*e.g.*, oxygen deprivation), the kinase activity of RegB/PrrB is stimulated relative to its phosphatase activity (72). This increases phosphorylation of the partner response regulators RegA/PrrA, which are transcription factors that bind DNA and activate or repress gene expression. The membrane-bound sensor kinase proteins RegB/PrrB contain an H-box site of autophosphorylation (His^{225}), a highly conserved quinone binding site (the heptapeptide consensus sequence GGXXNPF, which is totally conserved among all known RegB homologues), and a conserved redox-active cysteine (Cys^{265}, located in a "redox box"). The mechanism by which RegB controls kinase activity in response to redox changes has been an active area of investigation. A previous study demonstrated that RegB Cys^{265} is partially responsible for redox control of kinase activity. Under oxidizing growth conditions, Cys^{265} can form an intermolecular disulfide bond to convert active RegB dimers into inactive tetramers (235) (Fig. 8a). The highly conserved sequence, GGXXNPF, located in a short periplasmic loop of the RegB transmembrane domain has also been implicated in redox sensing by interacting with the UQ pool [Swem *et al.* (234)] (Fig. 8a). Recently, kinase activity assays together with isothermal titration calorimetry (ITC) measurements indicated that RegB with a substitution in the cytosolic cysteine by serine in position 265 (RegB $C^{265}S$) binds both oxidized and reduced UQ with almost equal affinity. However, only the oxidized UQ inhibits RegB kinase activity (259). The observation that the RegB $C^{265}S$ mutant is still redox responsive suggests that UQ binding is a signal input able of functioning independently from Cys^{265}. However, the contribution of each redox sensing inputs is unknown.

In *Rho. sphaeroides*, the PrrB histidine kinase is a bifunctional enzyme that possesses both kinase and phosphatase activities (168). Several reports proposed that the cbb_3 oxidase transduced an inhibitory signal to the PrrBA under oxic conditions to prevent gene expression (165, 166). The dual function of the cbb_3 oxidase as both terminal oxidase and O_2/redox sensor and modulator of PrrB kinase/phosphatase activity represents a new model of redox sensing. In this model (Fig. 8b), the UQ binding site within the PrrB

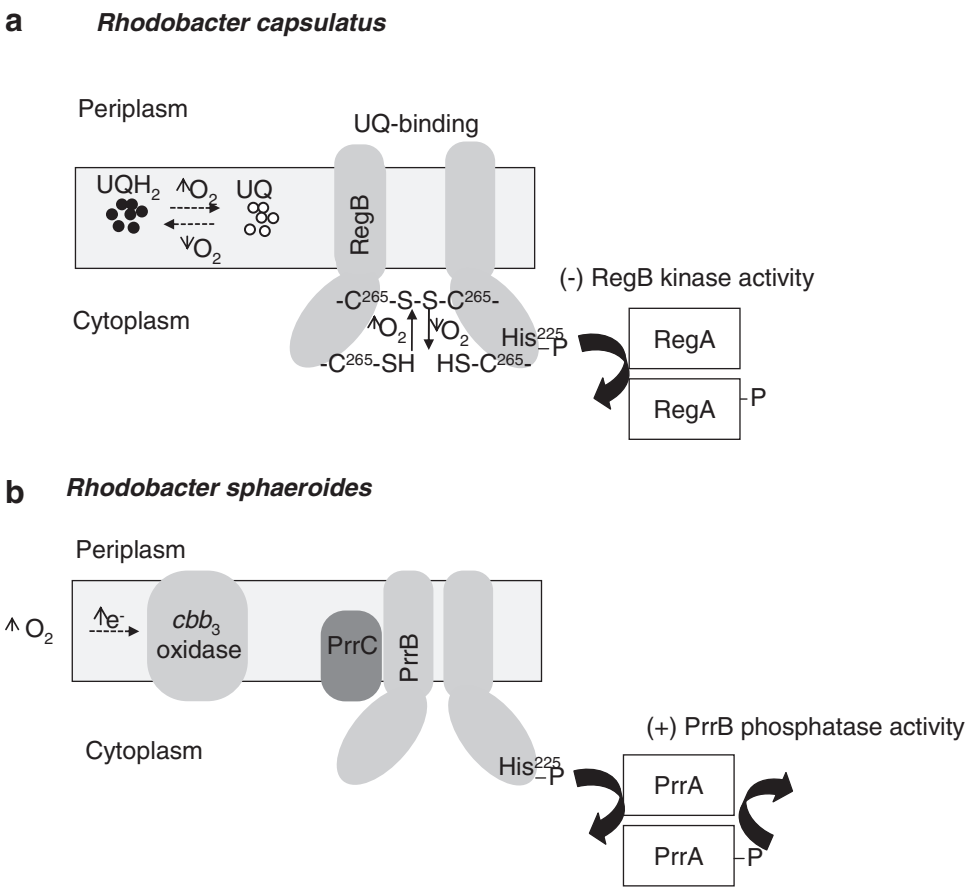

FIG. 8. Proposed models for redox-mediated sensing by RegBA/PrrBA. (a) In *Rho. capsulatus*, the conserved cysteine C^{265} of RegB functions as a redox switch that controls RegB kinase activity through a metal-dependent formation of a disulfide bond in response to redox changes (235). Binding of UQ to the RegB transmembrane domain has also been proposed to inhibit RegB kinase activity, whereas UQH_2 does not affect RegB kinase activity (234). **(b)** In *Rho. sphaeroides*, the cbb_3 oxidase generates an "inhibitory" signal that can directly stimulates the phosphatase activity of PrrB. Under low oxygen conditions when the electron flow is reduced, the inhibitory signal from cbb_3 is weakened, and PrrB would retain its kinase activity (165). PrrC has been proposed as a signal mediator between the cbb_3 oxidase and the sensor kinases PrrB (7).

transmembrane domain is not required for monitoring the PrrB kinase activity. Instead, a control based in direct interaction between components of the terminal oxidase cbb_3 and PrrB is strengthened (122).

The photosynthetic regulatory response protein (PrrC) is a Sco homolog present in *Rho. sphaeroides* (75). Sco is thought to be involved in donating copper to the Cu_A center and, thus, it has a central role in cytochrome oxidase synthesis (12). *Rho. sphaeroides* PrrC, which reduces Cu^{2+} to Cu^{+}, and possesses disulfide reductase activity, is required for the correct functioning of the sensor kinase/phosphatase PrrB (7). Similarly, the *Rho. capsulatus* SenC protein, homologous to PrrC, which is required for synthesis of a functional cytochrome *c* oxidase (232), might act as a signal mediator between the Q-pool and the sensor kinase RegB. However, at present, there is no direct evidence that SenC or cbb_3 oxidase directly modulates the activity of the RegBA regulatory system.

RegA/PrrA contain conserved domains that are typical in two component response regulators such as a phosphate accepting aspartate, an "acid box" containing two highly conserved aspartate residues and a H-T-H DNA-binding motif (72). The phosphorylated form of RegA/PrrA has increased DNA binding capacity (129, 189). Under oxidizing conditions, RegB/PrrB shifts the relative equilibrium from the kinase to the phosphatase mode resulting in a dephosphorylated inactive RegA/PrrA form. Despite this evidence, it has been reported that inactivation of the *regA* gene affects expression of many different genes under oxidizing (aerobic) conditions, suggesting that both phosphorylated and unphosphorylated RegA/PrrA may be active transcriptional regulators (72, 233). In this context, it has been shown that both phosphorylated and unphosphorylated forms of RegA/PrrA are capable of binding DNA *in vitro* and activating transcription (189). Based on recent findings, there are two types of PrrA binding sites within the promoter of *Rho. sphaeroides hemA*, which codes for one of two isoenzymes catalyzing 5-aminolevulinate synthesis: (i) one site for which unphosphorylated PrrA has greater affinity, and (ii) another for which phosphorylated PrrA has greater affinity (190). Several consensus DNA binding sequences have been previously reported for PrrA (128, 139) and its homologs RegA in *Rho. capsulatus* (233, 253) and RegR in *Br. japonicum* (73, 134). While there is a measure of agreement in terms of the assignment of two imperfect quasi-symmetrical inverted repeats, or half-sites separated by a spacer region possessing variable length, the DNA sequence *per se* shows a high degree of degeneracy. Very recently, it has been demonstrated that PrrA can bind *in vitro* to DNA sequences with different lengths in the spacer regions between the half sites (76).

In *Rho. capsulatus*, RegA has been reported to activate the expression of both the *bd* and cytochrome cbb_3 oxidases (Fig. 9a). An interesting paradox is that RegA activates expression of cytochrome cbb_3 oxidase under oxic conditions, but represses expression of this oxidase under anoxic conditions (233). In many respects, this is similar to what occurs in *E. coli* where ArcA acts as an oxic activator, as well as an anoxic repressor of cytochrome bo_3 oxidase (see section III.A; Fig. 5b).

Terminal oxidase expression in *Rho. capsulatus* involves a complex set of regulators beyond that of RegBA. In response to oxygen availability, these regulators provide negative or positive transcriptional control to coordinate enzyme synthesis for optimal growth. A regulatory scheme for control of terminal oxidases in response to oxygen availability is shown in Figure 9a. Specifically, in *Rho. capsulatus* cytochrome cbb_3, oxidase expression is regulated by RegA and FnrL, as well as moderately regulated by HvrA, an activator of various photosynthetic components under low-light conditions [(Swem *et al.* (231)]. Ubiquinol *bd* oxidase expression was found to be regulated by RegA and HvrA as well as by AerR and CrtJ, which are aerobic repressors of photosystem gene expression (231). In *Rho. sphaeroides*, microarray analyses and quantitative reverse transcriptase–polymerase chain reaction showed that both PrrA and the repressor of genes involved in photosynthesis, PspR, affect the transcription of the *ccoNOQP* operon (Fig. 9b). The absence of PrrA prevented the expression of the *cco* genes, and a *prrA-pspR* double mutant leads to derepresion of *ccoNOQP* operon (34). The fact that PpsR binds directly the *cco* promoter indicates that PrrA/PspR could act as an antirepressor/repressor system in which PspR acts as the direct repressor of the *cco* operon and PrrA is the PspR antirepressor regulator (34). Thus, RegBA/PrrBA appears to be just one component of a more complex regulatory network that controls many cellular processes. In this context, recent comparative genomics analyses and characterization of *Rho. sphaeroides* FnrL regulon have revealed that *ccoNOQP* is also positively controlled by this protein (68).

C. Pseudomonas *species RoxSR*

Pseudomonads are opportunistic pathogens that inhabit a wide variety of environments, including soil, water, and animal, human,- and plant roots. They are endowed with versatile respiratory chains that can be adapted to changing oxygen concentrations. Although they preferentially obtain energy from aerobic respiration, some of them, such as *Ps. aeruginosa*, can also grow anaerobically using nitrate as a final electron acceptor, or can even ferment arginine and pyruvate (254). The genome sequence and some biochemical studies have revealed that *Pseudomonads* have at least five terminal oxidases for aerobic respiration [reviewed in (183)] (Fig. 10a). In *Ps. aeruginosa*, UQs can be oxidized by either a bo_3-type oxidase, a cyanide-insensitive oxidase (named CIO), or a bc_1 complex that transfers electrons to a cytochrome *c*, which, in turn, can be oxidized by an aa_3 oxidase or by two related cbb_3 oxidases, named cbb_3-1 and cbb_3-2 (46, 47, 49). The *Pseudomonas putida* genome contains genes coding for all these terminal oxidases (161). Each oxidase is expected to have a specific affinity for oxygen, efficiency of proton translocation, and redox potential. *Ps. aeruginosa* has three predicted high-affinity oxidases; CIO, cbb_3-1, and cbb_3-2, which are believed to be important in the adaptation to oxygen-limiting conditions during growth under microoxic conditions. In this context, Alvarez-Ortega and Harwood (1) reported that any one of CIO, cbb_3-1 and cbb_3-2, could support growth under 2% oxygen concentrations and that either of cbb_3-1 and cbb_3-2 was indispensable for the growth under 0.4% oxygen.

In *Ps. aeruginosa*, the Anr global transcriptional regulator plays a pivotal role in adaptation to microoxic or anoxic conditions. Anr is a homolog of *E. coli* Fnr and regulates expression of the enzymes required for nitrate respiration (4, 213), and of at least some of the aerobic terminal oxidases, such as the CIO oxidase and cytochrome cbb_3-2 oxidase (47, 49). Recently, it has been shown that under low-oxygen conditions, Anr is involved in the repression of the CIO genes and

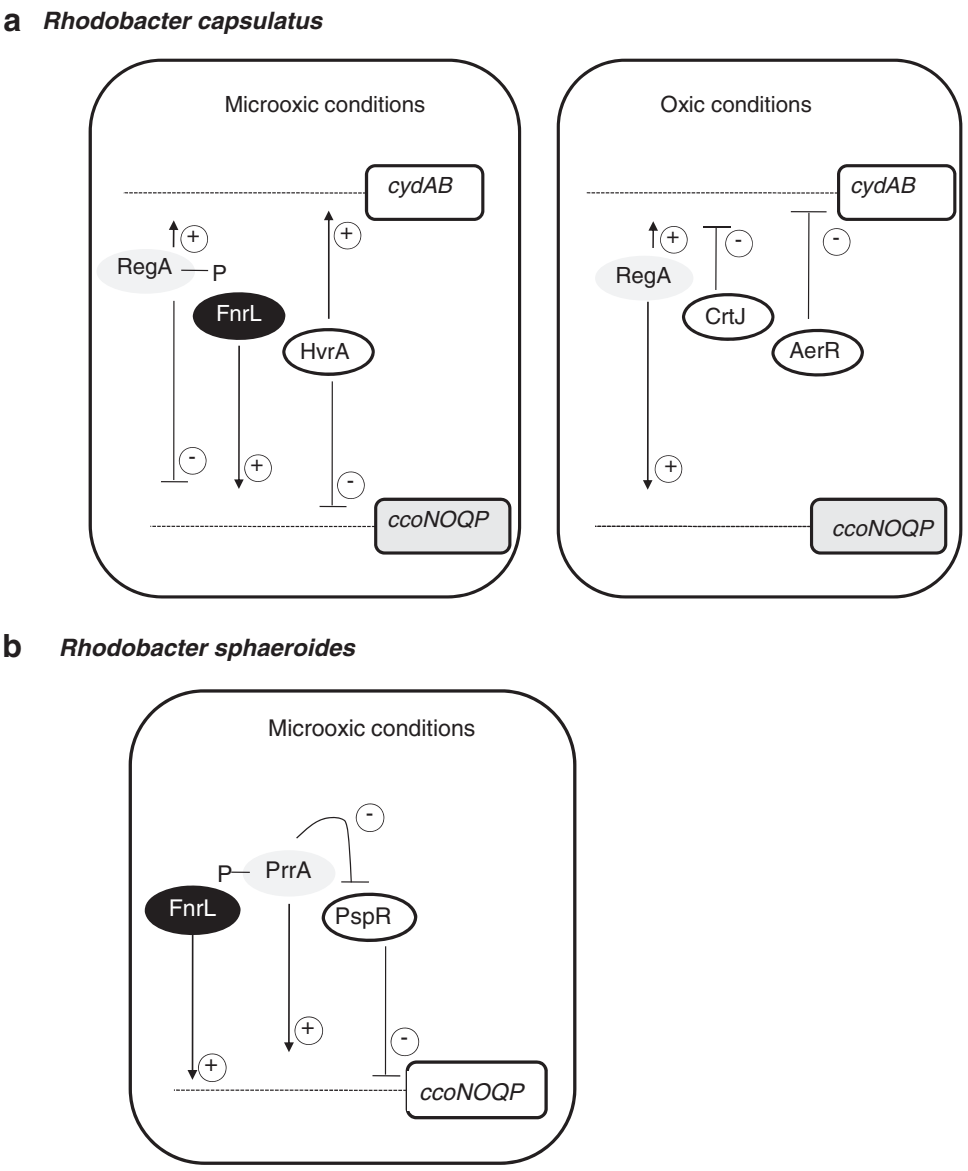

FIG. 9. Regulation of *bd* and cbb_3 terminal oxidases expression in *Rho. capsulatus* (a) and *Rho. sphaeroides* (b). In *Rho. capsulatus*, RegA regulates gene transcription in response to both oxic and anoxic conditions, FnrL, HvrA, in response to anaerobiosis, and AerR and CrtJ in response to aerobiosis. In *Rho. sphaeroides*, FnrL, PrrA, and PspR regulate gene transcription in response to anoxic conditions. Genes *cydAB* and *ccoNOQP* encode *bd* and cytochrome cbb_3 oxidase polypeptides, respectively. Positive regulation (+) is denoted by *arrows*, and negative regulation (−) is indicated by *perpendicular lines*.

induction of the cbb_3-2 genes, while the other three terminal oxidases are not significantly regulated by Anr (Fig. 10b) (121). Similarly, in *Ps. Putida,* it has been shown that, under oxygen limitation, inactivation of *anr* led to an increased expression of the bo_3-type and the CIO terminal oxidases, and to a much lower expression of cbb_3-1 (equivalent to *Ps. aeruginosa* cbb_3-2), suggesting that Anr is a transcriptional activator of cbb_3-1 and a repressor of CIO genes (244).

In addition to Anr, another redox-responsive regulatory system has been proposed to be important for the regulation of terminal oxidases in *Pseudomonas* species, the RoxSR system. RoxSR belongs to the family of RegBA and PrrBA regulatory systems. It has been postulated in *Ps. aeruginosa* that the activation signal perceived by RoxS might depend on electron transport signals emerging from the oxidase cbb_3-1 (47). Microarray analyses performed in *Ps. putida* (152) showed that a *cyo* mutation leads to a significant change in the transcriptome profile. They showed that the absence of bo_3-type oxidase was compensated by upregulation of CIO and cbb_3-1 (corresponding to cbb_3-2 of *Ps. aeruginosa*). A regulatory system monitoring the electron flow through the bo_3-type oxidase, which is similar to PrrBA activity modulation by the cbb_3 oxidase, might be operative in *Ps. putida*.

Microarray studies have reported that RoxRS is involved in the regulation of the terminal oxidases genes in *Ps. putida* (78) and *Ps. aeruginosa* (121). In *Ps. aeruginosa*, RoxSR functions as a positive regulator of the putative high-affinity oxidases CIO, cbb_3-1 and cbb_3-2, and at the stationary phase where the dissolved oxygen concentration is low due to the high cell density, as a negative regulator of the putative low-affinity oxidase aa_3 (121) (Fig. 10b). The RoxSR regulon of *Ps. putida* includes genes for respiratory function and maintenance of the redox balance, genes involved in sugar and amino acid metabolism, and the sulfur starvation response (78). These authors also showed that RoxSR participates in cell density signal transduction in *Ps. putida*. In *Ps. aeruginosa*, however, only a few genes related to sugar and amino-acid metabolism were identified as the members of the RoxSR regulon (121). The number of the putative RoxSR-regulated genes in *Ps. aeruginosa* seemed to be low compared with that in *Ps. putida*.

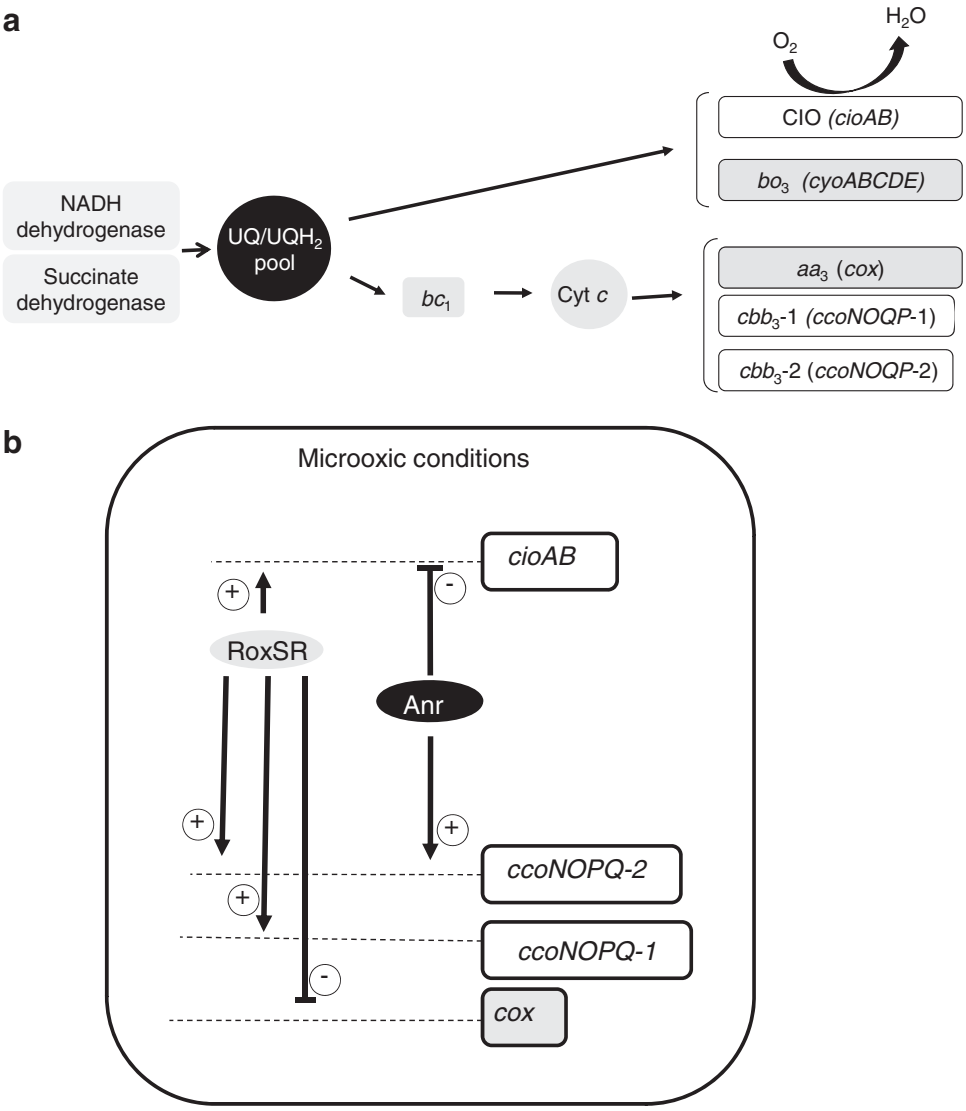

FIG. 10. Aerobic respiratory chains (a) and regulation of CIO, cbb_3, and aa_3 terminal oxidase expression (b) in *Pseudomonas aeruginosa*. In **(a),** the quinone-reducing (*left*) and quinol-oxidizing (*right*) branches with terminal oxidases induced under oxic conditions (*gray boxes*), and microoxic conditions (*white boxes*), are shown. In **(b),** Anr and RoxSR regulators provide negative or positive transcriptional control, in response to oxygen availability. Genes *cox*, *cioAB*, and *ccoNOQP* encode aa_3, CIO, and cbb_3 oxidase polypeptides, respectively. Positive regulation (+) is denoted by *arrows*, and negative regulation (−) is indicated by *perpendicular lines*. CIO, cyanide insensitive oxidase.

These differences are presumably due to the different growth conditions used for the microarray analysis in the two *Pseudomonas* species.

D. Br. japonicum *RegSR*

The genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (*Ensifer*), collectively referred to as rhizobia, are members, along with other genera, of the bacterial order *Rhizobiales* of the α-Proteobacteria. Rhizobia are soil bacteria with the unique ability to establish a N_2-fixing symbiosis on legume roots and on the stems of some aquatic legumes. During this interaction, bacteroids, as rhizobia are called in the symbiotic state, are contained in intracellular compartments called symbiosomes within a specialized organ, the nodule, where they fix N_2 [reviewed in (116, 169)]. In the nodule, maintenance of nitrogenase activity is subject to a delicate equilibrium. A high rate of O_2 respiration is necessary to supply the energy demands of the N_2-reduction process; but, on the other hand, O_2 also irreversibly inactivates the nitrogenase complex. In order to keep the steady-state concentration of free-O_2 low, the cortex of nodules acts as a diffusion barrier, which greatly limits permeability to O_2 [reviewed in (150)]. Oxygen is delivered to the symbiosomes by the plant O_2-carrier leghemoglobin (Lb), which transports O_2 at a low, but stable, concentration, allowing for the simultaneous operation of nitrogenase activity and bacteroid respiration [(66) and references therein]. N_2-fixing bacteroids deal with the low levels of free O_2 by inducing a high-affinity cytochrome cbb_3-type oxidase [reviewed in (61)].

Similar to many other anaerobic facultative bacteria, *Br. japonicum* adapts to different environmental O_2 concentrations by inducing multiple terminal oxidases with different affinities for O_2. *Br. japonicum* has eight terminal oxidases, of which two are *bd*-type oxidases, and six are heme-copper oxidases, the latter being further divided into two quinol oxidases and four cytochrome oxidases [reviewed in (38)] (Fig. 11). Of particular interest is the *fixNOQP*-encoded cbb_3-type oxidase, because it supports *Br. japonicum* aerobic respiration under free-living microoxic conditions and enables endosymbiotic *Br. japonicum* cells (bacteroids) to support the

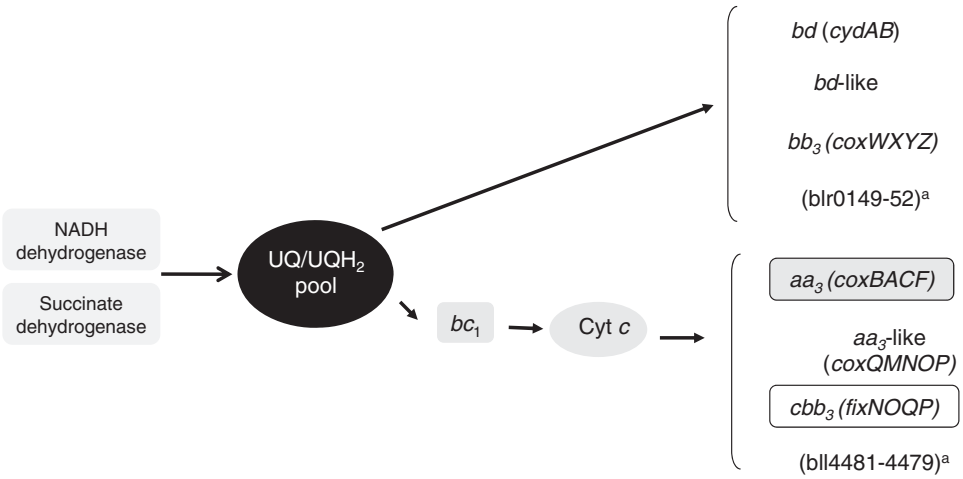

FIG. 11. Aerobic respiratory chains in *Bradyrhizobium japonicum*. The quinone-reducing (*left*) and quinol-oxidizing (*right*) branches with terminal oxidases are shown. The *coxBACF*-encoded cytochrome aa_3 is the predominant heme-copper oxidase for aerobic growth (*gray box*), and the *fixNOQP*-encoded cbb_3-type oxidase (*white box*) supports respiration under free-living microoxic and under symbiotic conditions. [a]Gene number according to RhizoBase (http://genome.kazusa.or.jp/rhizobase).

nitrogen fixation process. Cytochrome cbb_3 oxidase has been purified from *Br. japonicum* (185), where the experimentally determined K_m for O_2 is in the order of 7 n*M*, a value that is consistent with its function in the bacteroid.

The contribution of *Br. japonicum* CoxG and ScoI to the biogenesis of the cbb_3 oxidase has been recently investigated (38). These proteins are the respective paralogs of the mitochondrial chaperones Cox11 and Sco1 involved in the formation of Cu_B and Cu_A centers of cytochrome aa_3 (12). Analyses of *Br. japonicum coxG* and *scoI* mutants revealed that disparate pathways are used for aa_3- and cbb_3-type oxidases biogenesis [(Bühler *et al.* (38)].

Oxygen concentration is the primary effector of *fixNOQP* expression in *Br. japonicum*. Under O_2 limitation, the FixJ protein of the FixLJ system is phosphorylated by the oxygen-inhibitable, heme-based sensor kinase FixL (see section II.A Fig. 4b). The only known target of FixJ in *Br. japonicum* is $fixK_2$, whose product, $FixK_2$, has been shown to activate genes involved in anoxic or microoxic energy metabolism, including the *fixNOQP* operon (161). By using a transcriptomics approach (145), and $FixK_2$-dependent *in vitro* transcription assay (148), the involvement of $FixK_2$ as a direct transcriptional regulator of cbb_3 has been demonstrated.

RegSR are members of the family of two-component regulatory redox-responsive proteins. In *Br. japonicum*, RegSR induces expression of the *fixR-nifA* operon, which encodes the NifA regulatory protein (15) (see Fig. 15). Targets of NifA include *nif* and *fix* genes, which are directly involved in nitrogen fixation, and also genes that are indirectly related to nitrogen fixation or have a unknown function in this process (83, 108, 162). Although *fixNOQP* genes are involved in nitrogen fixation, these genes were not identified as targets of NifA in microarray-based experiments (108). Transcription of *fixR-nifA* is dependent on NifA and on RegSR, respectively (Fig. 15). RegR induces expression of the *fixR-nifA* operon in both oxic and anoxic conditions, suggesting that RegSR responds to the overall redox state of the cell rather than to oxygen directly (15). *Br. japonicum* RegS possesses a highly conserved quinone binding site and a conserved redox-active cysteine, which suggests that the redox state of the membrane-localized quinone pool or the redox-active cysteine might be involved in redox sensing in *Br. japonicum.* However, the precise nature of the signal that is transduced by the *Br. japonicum* RegSR is unknown.

Null mutations in the *regR* gene led to a dramatically decreased nitrogen fixation activity (15). Surprisingly, the phenotypic properties of *regS* mutants were largely indistinguishable from the wild type. Although it has been demonstrated, *in vitro*, that RegS is both a functional autokinase and a kinase (74), the *in vivo* role of RegS in RegR-mediated transcriptional activation is still unclear. In addition to *fixR-nifA*, a large number of novel members of the RegR regulon have been identified by transcriptomic analyses (134). Among them, a putative operon that encodes a predicted multidrug efflux system required for an efficient symbiosis specifically with soybean has been recently characterized (133).

In addition to oxygen respiration, *Br. japonicum* is able to obtain energy and grow from nitrate reduction to N_2 through denitrification when cultured under oxygen-limiting conditions with nitrate as the terminal electron acceptor. In *Br. japonicum*, denitrification reactions depend on *napEDABC*, *nirK*, *norCBQD*, and *nosRZDYFLX* genes encoding the nitrate-, nitrite-, nitric oxide-, and nitrous oxide reductases, respectively (see section V.C). Inactivation of *Br. japonicum cycA* or *napC*, which encode cytochromes *c* involved in the electron transfer through denitrification pathway, decreases the expression of *Br. japonicum* cbb_3 oxidase during nitrate-dependent anaerobic growth (35, 37). These results suggest that a change in the electron flow through the denitrification pathway may affect the quinone redox state, leading to alterations in cbb_3 expression. To further explore the possibility of redox-dependent regulation of *Br. japonicum* cbb_3 oxidase, the effect of reduced and oxidized carbon substrates on β-galactosidase activity from a *fixP′–′lacZ* fusion was investigated. Levels of *fixP′–′lacZ* expression were largely dependent on the oxidized or reduced nature of the carbon source in the medium (37). In order to study the involvement of *Br. japonicum* RegR in the redox-dependent regulation of *fixNOQP* genes, Bueno and colleagues (37) analyzed the expression of the *fixP′–′lacZ* fusion in a *Br. japonicum regR* mutant. When cells were grown under denitrifying conditions with an oxidized carbon source, there was a significant decrease in β-galactosidase activity in the *regR* mutant compared with the expression levels detected in the wild-type strain. These observations suggest that *Br. japonicum fixNOQP* operon might be under the control of RegR. However, a whole-genome transcription-profiling analysis of the *Br. japonicum regR* mutant grown under microoxic

conditions demonstrated that transcript levels of *fixNOQP* operon were not RegR-dependent (134). In recent studies from our group (Maria J. Torres *et al.*, unpublished results), *fixNOQP* genes were not identified as RegR targets after transcriptomic analyses of the *regR* mutant grown under denitrifying conditions with succinate (an oxidized carbon source).

E. Bacilus subtilis *ResDE and Rex*

The endospore-forming Gram-positive bacterium *Bacilus subtilis* utilizes a branched ETC under aerobic conditions (250). To date, three terminal oxidases have been identified in *Ba. subtilis* (Fig. 12a). Both cytochromes aa_3 and caa_3 have been identified as heme-copper oxidases. The third oxidase has been shown to be a member of the cytochrome *bd* family encoded by the *cydABCD* operon (255). In addition, the presence of a fourth terminal oxidase of *bd* type, YthAB, can be predicted from the genome sequence (6).

Cytochrome *bd* is produced under conditions of low-oxygen tension and in cells grown in the presence of glucose. A perfect 16-bp palindromic sequence, upstream of the translation start site for *cydA*, was proposed as a potential operator binding site for a regulatory protein (255). Gel-shift and DNase I footprinting analyses identified YdiH as a negative regulator of *cyd* genes (212). YdiH is a redox sensor protein whose activity is regulated by the levels of NAD^+ and NADH in the cell (104). YdiH has been renamed Rex, as it is an ortholog of Rex in *Streptomyces coelicolor* (31) where this redox-sensitive transcription regulator was first described. In *St. coelicolor*, the Rex-DNA target region (Rex operator, ROP) is an 8-bp inverted repeat located upstream of genes encoding respiratory proteins, such as heme biosynthetic enzymes, NADH dehydrogenase, Rex itself, and cytochrome *bd* oxidase. In *Staphylococcus aureus,* it has been recently demonstrated, by combining various protein–DNA interaction studies with transcriptional analyses, that Rex acts on a multitude of anaerobically induced genes (172).

Brekasis and Paget (31) showed by electrophoretic mobility shift assay (EMSA) experiments that *St. coelicolor* Rex DNA-binding properties are influenced by the $NADH/NAD^+$ ratio. Under aerobic conditions, raised NAD^+/NADH ratio results in an increased binding of NAD^+ to Rex, leading to its activation (Fig. 12b). Activated Rex binds to ROP, and inhibits the expression of several respiratory genes. However, under oxygen-limiting conditions, NADH replaces NAD^+ bound to Rex, triggering a conformational change that dissociates Rex from the *cyd*-ROP sequence. Derepression of the *cyd* operon

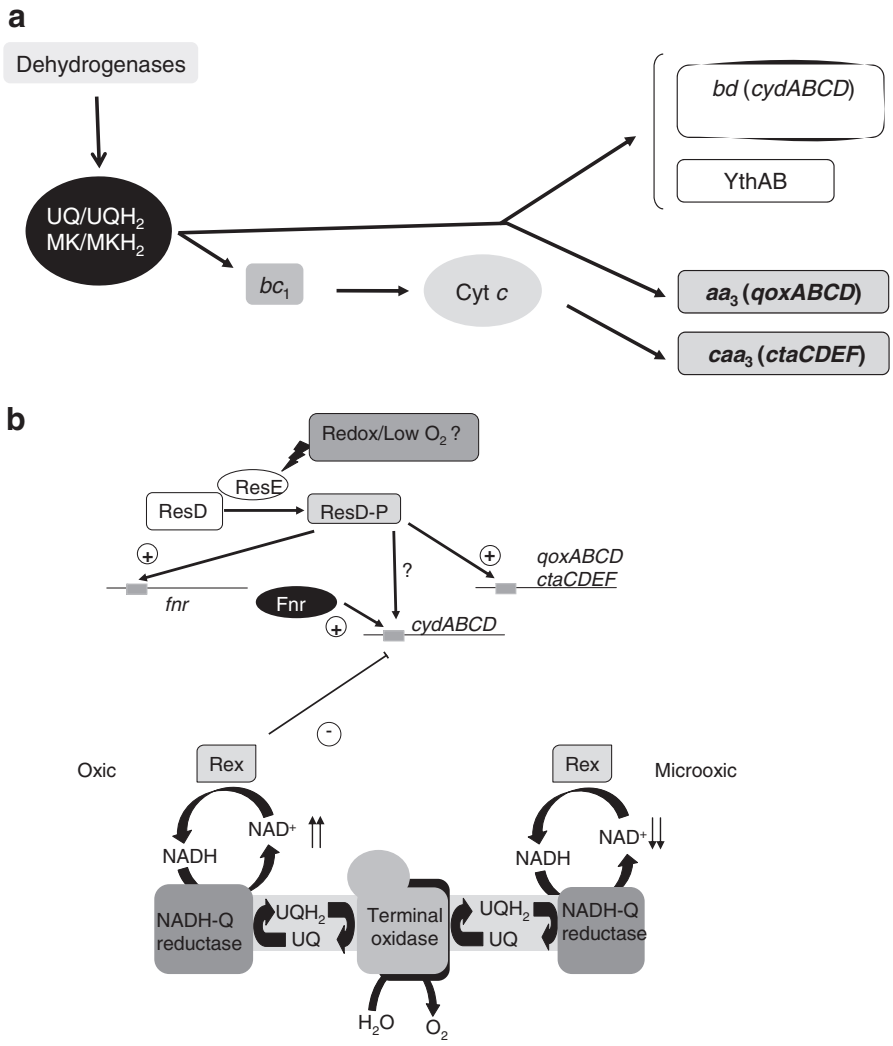

FIG. 12. Aerobic respiratory chains (a) and regulation of *bd*, aa_3, and caa_3 terminal oxidase expression (b) in *Bacilus subtilis*. In **(a),** the quinone-reducing and quinol-oxidizing branches with terminal oxidases induced under oxic conditions (*gray boxes*), and microoxic conditions (*white boxes*), are shown. In **(b),** ResDE and Fnr provide positive transcriptional control in response to oxygen availability. ResD, but not ResE, is required for transcription of *cyd* operon (this complex regulation is indicated with a question mark in the figure). Redox-dependent repression of *cydABCD* (*bd* oxidase) by Rex is controlled by NAD^+/NADH ratio in the cells. Operons *qox* and *cta* encode the aerobic terminal oxidases aa_3 and caa_3. Positive regulation (+) is denoted by *arrows*, and negative regulation (−) is indicated by *perpendicular lines*.

enables the cell to respire micromolar concentration of oxygen (31) (Fig. 12b). DNA binding studies of *Ba. subtilis* Rex with NADH or NAD^+ showed that NAD^+ boosted the binding activity of Rex, but that NADH seemed to have a negligible effect or a partial negative effect on DNA-binding activity (104). By contrast, in *St. coelicolor*, NADH completely inhibits DNA-binding activity. These data suggest that DNA-binding determinants of *Ba. subtilis* Rex are distinct from those of *St. coelicolor* Rex (31). Gyan and colleagues (104) proposed that Rex and NADH dehydrogenase together form a regulatory loop which functions to prevent a large fluctuation in the NADH/NAD+ ratio in *Ba. subtilis.*

In order to understand the conformational modifications involved in the redox sensing by Rex, Sickmier and colleagues (220) performed X-ray studies of the Rex homolog in the thermophile *Thermus aquaticus* (T-Rex). Its structure comprises two main domains, an N-terminal domain that adopts a winged H-T-H fold and which most likely is the region that interacts with DNA and a C-terminal NADH binding Rossmann fold domain (220). These studies revealed that T-Rex possesses the same functional characteristics as the *St. coelicolor* homolog Rex, which lead to speculatation that the mechanism of repression of gene activation in response to the $NADH/NAD^+$ redox pair is conserved among Rex family members. Small-angle X-ray scattering has been recently applied to obtain solution structures for *Ba. subtilis* apo B-Rex, B-Rex:NADH, and B-Rex:NAD^+ showing that B-Rex presents rigid conformations for the complexes with NADH and NAD^+ (251). Results from these studies indicated that the domain movements of B-Rex from the DNA bound form to the NADH (DNA-free) form could be different from the reported mechanism for T-Rex which is based on crystal structures (220).

In addition to Rex, transcription of the *Ba. subtilis* cytochrome *bd* oxidase during the transition between aerobic to anaerobic lifestyle is subjected to the action of other regulatory proteins such as Fnr, CcpA (carbon catabolite regulator protein), and the two-component system ResDE (188).

The ResD/ResE two-component signal transduction system has an important role in the regulation of both aerobic and anaerobic respiration [reviewed in (92)]. ResE is a membrane-bound sensor histidine kinase that, by responding to a redox signal(s), which have been postulated to be the redox state of intracellular MK, autophosphorylates and subsequently donates a phosphate to its cognate response regulator, ResD. It has been demonstrated that ResD activates transcription by interaction with the C-terminal domain of the alpha subunit of RNAP (91). The ResD/ResE system activates transcription of genes involved in the respiratory pathway that transfers electrons to oxygen (under oxic conditions) or to nitrate (under anoxic conditions) as well as genes encoding transcriptional factors such as Fnr (91, 92) (Fig. 12b). Since ResDE is essential for both *ctaA* and *ctaB* expression, which are required for heme A biosynthesis, *resDE* mutant strains lack cytochromes aa_3 and caa_3. ResD also activates the genes *ctaCDEF*, encoding the structural proteins for cytochrome caa_3 (264). By using a *cydA:lacZ* transcriptional fusion in *resD* and *resE* mutant backgrounds, it was shown that ResD, but not ResE, is required for the transcription of the *cydA* promoter, suggesting that another sensor might be involved in *cydA* activation by ResD. The direct interaction of ResD with the *cydA* promoter was found around −58 and −108 nucleotides upstream from the transcription start site by using a DNase I footprinting assay (188).

IV. Nitrate Respiration: Denitrification and Ammonification

When faced with a shortage of oxygen, many bacterial species are able to use nitrate as an electron acceptor of the respiratory chain. This switch from oxygen to nitrate respiration leads to a reduction in the ATP yield rates, but allows bacteria to survive and multiply (221). Denitrification has been defined as the dissimilatory reduction of nitrate or nitrite to a gaseous N-oxide concomitant with free energy transduction (266). The process requires four separate enzymatically catalyzed reactions:

$$2NO_3^- + 4e^- + 4H^+ \rightarrow 2NO_2^- + 2H_2O \text{ (reaction 1)}$$
$$2NO_2^- + 2e^- + 4H^+ \rightarrow 2NO + 2H_2O \text{ (reaction 2)}$$
$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \text{ (reaction 3)}$$
$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \text{ (reaction 4)}$$

For many years, it was believed that denitrification is performed exclusively by eubacteria. However, there are indications that some fungi (*e.g.*, the pathogenic species *Fusarium oxysporum*) and archaea are also able to denitrify (236, 266). It has also been shown that nitrifiers also have genes involved in denitrification (44). Some bacteria such as *E. coli* or *Ba. subtilis* are able to perform nitrate respiration and release gaseous nitrogen oxides, but they do not denitrify with dinitrogen as a product. Instead, they reduce nitrate to ammonium, so-called nitrate-ammonification (45). In many species of nitrate ammonifying bacteria, there are two biochemically distinct nitrate reductases, one membrane-bound with the active site located in the cytoplasm and the other in the periplasm. These are coupled to two nitrite reductases (Nir) that provide independent pathways for nitrate reduction to ammonium (nitrate-ammonification) in the two cellular compartments. In the cytoplasm, nitrate is reduced to nitrite by a membrane-bound respiratory nitrate reductase system (NarGHI):

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \text{ (reaction 5)}$$

The nitrite produced can then be further reduced to ammonium by a siroheme-containing Nir (NirB):

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O \text{ (reaction 6)}$$

In the periplasm, the process involves two different enzymes, a periplasmic nitrate reductase (NapA) that reduces nitrate to nitrite and a periplasmic cytochrome *c* Nir that further reduces nitrite to ammonium. This process of nitrate ammonification produces nitric oxide and nitrous oxide as side-products (95, 149, 222, 225).

Products of denitrification and nitrate ammonification have manifold, mainly adverse, effects on the atmosphere, soils, and waters and thus, have both an agronomic and environmental impact (10, 105). When nitrate is converted to gaseous nitrogen by denitrifying bacteria in agricultural soils, nitrogen is lost as an essential nutrient for the growth of plants. In contrast to ammonium, which is tightly bound in soil, nitrate is easily washed out and flows to the groundwater where it (and its reduction product nitrite) adversely affects water

quality. In addition, nitrogenous oxides released from soils and waters are in part responsible for the depletion of the ozone layer above the Antarctic, and in part for the initiation of acid rain and global warming (192). Among them, N_2O, commonly known as laughing gas, has received special attention in the last years, because it is a powerful greenhouse gas that can persist for up to 150 years while it is slowly broken down in the stratosphere. Although N_2O only accounts for around 0.03% of total greenhouse gas emissions, it has a 300-fold greater potential for global warming effects, based on its radiative capacity compared with that of carbon dioxide (10, 13, 192, 195).

A. Respiratory nitrate reductases

The first reaction of denitrification and ammonification (reactions 1 and 5), the conversion of nitrate to nitrite, is catalyzed by a membrane-bound nitrate reductase (Nar), or a Nap [reviewed in (98, 153, 154, 184, 198, 199, 201, 247)].

The Nar enzyme employs a redox loop to couple quinol oxidation with proton translocation and energy conservation, which permits cell growth under oxygen-limiting conditions (23, 221). Nap is also linked to quinol oxidation, but does not transduce the free energy in the QH_2-NO_3^- couple into proton motive force (PMF) to synthesize ATP (221). NO_3^-reduction *via* Nap can only be coupled to free-energy transduction if the primary quinone reductase, for example NADH dehydrogenase or formate dehydrogenase, generates an H^+-electrochemical gradient (221). In contrast to Nar, which has a respiratory function, Nap systems have a range of physiological functions that include the disposal of reducing equivalents during aerobic growth on reduced carbon substrates and anaerobic nitrate respiration as a part of bacterial ammonification or denitrification pathways (184).

1. Membrane-bound respiratory nitrate reductase. The *E. coli* and *Paracoccus* Nar enzymes have been the focus of the most biochemical and genetic studies (reviewed in references just mentioned). Nar is a structurally defined 3-subunit enzyme composed of NarGHI (Fig. 13a) (24, 118). NarG is the catalytic subunit of about 140 kDa that contains a *bis*-molybdopterin guanine dinucleotide (*bis*-MGD) cofactor and a [4Fe-4S] cluster. NarH, of about 60 kDa, contains four additional iron-sulfur centers: one [3Fe-4S] and three [4Fe-4S]. NarG and NarH are located in the cytoplasm and associate with NarI. NarI is an integral membrane protein of about 25 kDa with five transmembrane helices and the N-terminus facing the periplasm (Fig. 13a). Nar proteins are encoded by genes of a *narGHJI* operon. The organization of this operon is conserved in most species that express Nar. The *narGHI* genes encode the structural subunits, and *narJ* encodes a dedicated chaperone required for the proper maturation and membrane insertion of Nar (27). *E. coli* has a functional duplicate of the *narGHJI* operon named *narZYWV*. The subunits of the two enzymes encoded by both operons are interexchangeable, but physiologically, NarZYWV has a function during stress response rather than anaerobic respiration *per se* (26, 224). A number of *nar* gene clusters in denitrifying bacteria also have a gene encoding a nitrogen ox anion transporter NarK that can transport nitrate into the cell and nitrite out of the cell (97, 257), a homolog of which is also found in many nitrate-ammonifying bacteria (45). A variation of the Nar system occurs in some archaea and bacteria where the NarGH subunits are on the outside, rather than the inside of the cytoplasmic membrane. These systems have thus far been less well studied biochemically, but a nitrate transporter is not needed for these systems (142).

2. Periplasmic nitrate reductase. Nap is widespread in all classes of denitrifying and non-denitrifying proteobacteria [reviewed in (98, 153, 154, 184, 198, 199, 201, 247)]. The best-studied Nap enzymes were isolated from *Paracoccus pantotrophus E. coli*, *Rho. sphaeroides*, and *Desulfovibrio desfuromonas* (5, 64, 115). Nap is commonly a 2-subunit enzyme composed of the NapAB complex located in the periplasm and associates with a transmembrane NapC component (Fig. 13a). The catalytic subunit NapA contains the *bis*-MGD cofactor at its active site and an FeS center. NapB is diheme cytochrome c_{552}, and NapC is a *c*-type tetra-heme membrane-anchored protein that is involved in the electron transfer from the quinol pool to NapAB (43, 207). Mono-subunit forms of NapA also occur, which may interact with small FeS proteins as well as cytochromes (114, 115, 140).

In contrast to the *nar* operon, the *nap* operons present considerable heterogeneity in gene composition as well as in ordering. Eight different genes have been identified as components for operons that encode Naps in different organisms (199). Except for *Shewanella oneidensis, Wollinella succinogenes, Desulfobacterum hafniensi*, and *C. jejuni*, which lack *napB* or *napC*, all operons studied thus far have the *napABC* genes in common. The remaining *napDEFKL* genes encode for different proteins that are not directly involved in the nitrate reduction. NapD is a cytoplasmic protein that acts as a chaperone. NapF is a cytoplasmic iron–sulfur containing protein with four loosely bound [4Fe–4S] clusters, and is thought to participate in the assembling of the iron–sulfur cluster of NapA (164, 170). The *napEKL* genes encode for proteins with so far unknown functions. In *E. coli*, the *nap* operon includes *napGH* genes encoding a periplasmic and an integral membrane protein with [4Fe–4S] clusters. NapH and NapG interact, making an electron transfer supercomplex that can channel electrons from both menaquinol and ubiquinol to NapA (32, 33).

B. Other enzymes in bacterial denitrification

The enzymes involved in denitrification are nitrate-, nitrite-, nitric oxide-, and nitrous oxide reductase, encoded by *nar*/*nap*, *nir*, *nor*, and *nos* genes, respectively. A scheme of the denitrification reactions is shown in Figure 13a. Comprehensive reviews covering the physiology, biochemistry, and molecular genetics of denitrification have been published elsewhere (11, 16, 110, 125, 199, 247, 248, 266).

1. Nitrite reductases. In denitrifying bacteria, two types of respiratory Nir have been described: the NirS cd_1 nitrite reductase, a homodimeric enzyme with hemes *c* and d_1, and the NirK, a copper-containing Nir [reviewed in (202, 203, 246, 247)]. Both are located in the periplasmic space, and receive electrons from cytochrome *c* and/or a blue copper protein, pseudoazurin *via* the cytochrome bc_1 complex (Fig. 13a). They catalyze the one-electron reduction of nitrite to nitric oxide. Neither of the enzymes is electrogenic.

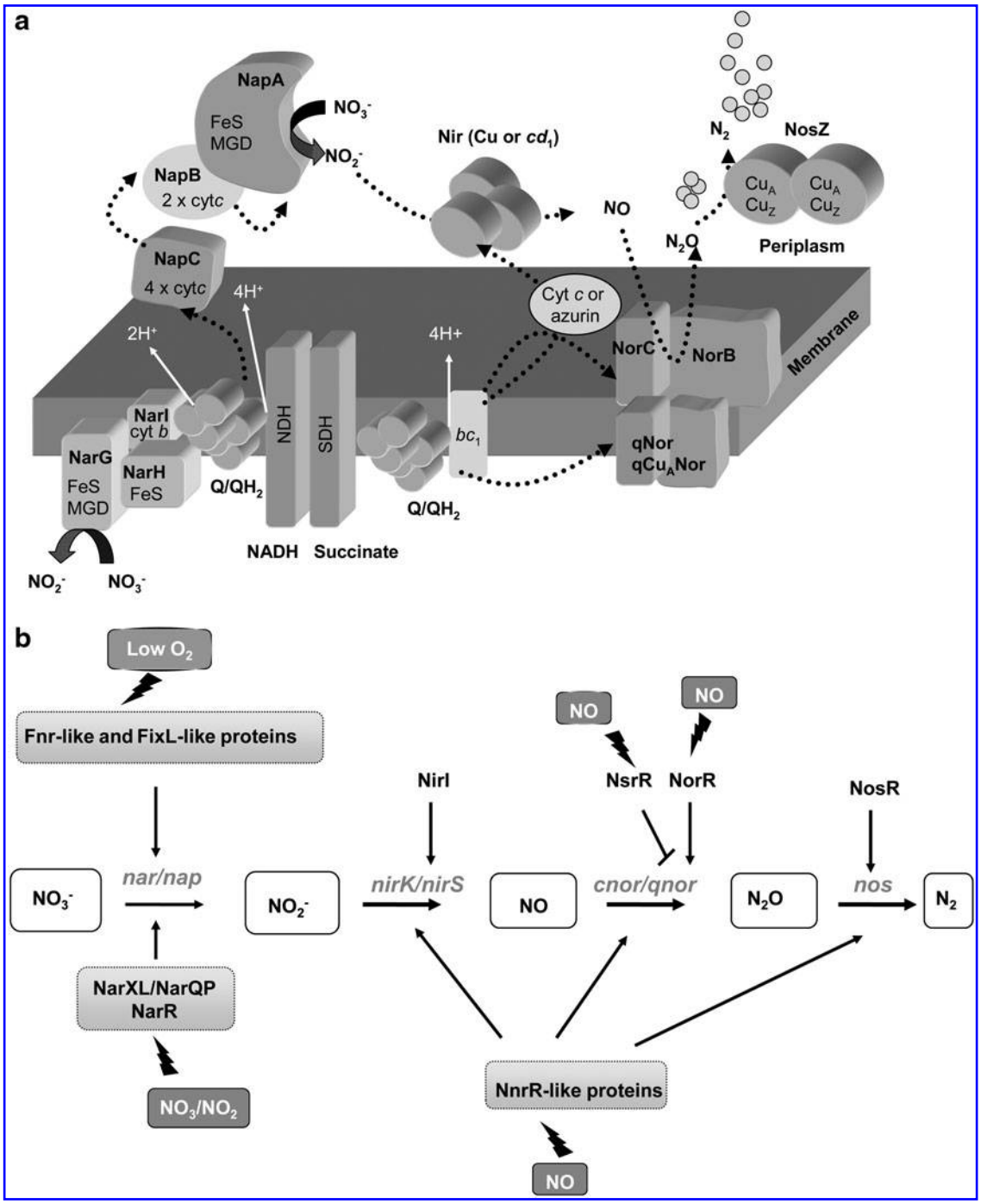

FIG. 13. Denitrification pathway (a) and regulation (b) in bacteria. In **(a),** the topological organization of denitrification enzymes is shown. The membrane-bound (NarGHI), and periplasmic, (NapABC) nitrate reductases as well as the nitrite reductases (Cu-type or cd_1-type), nitric oxide reductases (cNor, qNor, and qCu_ANor), and nitrous oxide reductase (NosZ) are shown. **(b)** Regulatory network of denitrification genes in response to O_2 concentration, nitrate/nitrite, and nitric oxide (NO). Positive regulation is denoted by *arrows*, and negative regulation is indicated by *perpendicular lines*. Further details are given in the text.

The cd_1-type nitrite reductase (cd_1Nir) is a homodimer, and each monomer carries a small heme *c* binding domain and a larger heme d_1-binding domain. Electrons are transferred from the electron donor *via* the heme *c* of NirS to heme d_1, where nitrite binds and is reduced to nitric oxide (202). The *nirS* gene encoding the cd_1-Nir polypeptide is part of a *nir* gene cluster. The best-characterized clusters are those from the denitrifying species *Ps. aeruginosa* (*nirSMCFDLGHJEN*), *Pa. denitrificans* (*nirXISECFDLGHJN*), and *Ps. stutzeri* (*nirSTBMCFDLGH* and *nirJEN*, the two clusters being separated by a part of the *nor* gene cluster encoding nitric oxide reductase [Nor]).

Cu-type nitrite reductases (CuNir) are homotrimeric complexes harboring three type I copper centers, and three type II copper centers, which form the active site. Nitrite binds to the copper ion in the type II center, replacing an exogenous ligand (water or chloride), and by electron transfer from the type I copper site, nitrite is reduced to nitric oxide. The *nirK* gene encodes the CuNir (203).

2. Nitric oxide reductases. To date, three types of Nor have been characterized, and they are classified according to the nature of their electron donor as *c*-type nitric oxide-reductase (cNor), qNor, and qCu_ANor [reviewed in (59, 247, 248, 267)]. As a unique case, the Nor of *Ros. denitrificans* is similar to cNor, but differs in that it contains copper (143). The best-studied Nor is the cNor that uses membrane or soluble *c*-type cytochromes or small soluble blue copper proteins (azurin, pseudoazurin) as physiological electron donors. The qNor uses quinol or menaquinol as electron donors and it is found not only in denitrifying archaea and soil bacteria, but also in pathogenic microorganisms that do not denitrify (60). The qCu_ANor has thus far been found only in the Gram-positive bacterium *Bacillus azotoformans* (230). This enzyme is bifunctional using both menahydroquinone (MKH_2) and a specific *c*-type cytochrome c_{551} as electron donor. It was suggested that the MKH_2-linked activity of qCu_ANor serves detoxification and the c_{551} pathway has a bioenergetic function.

The cNor is an integral membrane enzyme harboring two subunits, NorC with a heme *c* group and NorB containing hemes *b* and a non-heme iron (Fig. 13a). Electron transfer from donor molecules to cNor is mediated by the cytochrome bc_1 complex and a soluble cytochrome *c* or pseudoazurin (174). Electrons are transferred to the heme *c* in subunit II and then *via* the heme *b* to the active site. There, two molecules of nitric oxide are reduced. The molecular mechanism of the NO reduction by Nor has been extensively studied through chemical, biochemical, and physicochemical techniques (80, 81, 252). The crystal structure of Nor from *Ps. aeruginosa* has been recently solved (112). Although the overall structure of Nor is closely related to the heme copper oxidases, neither the D- nor K-proton pathway, which connect the HCO active center to the intracellular space, was observed. This confirmed early studies with NorCB in intact cells of *Rho. capsulatus* which showed that it was not a proton pump or electrogenic (196). Site-specific mutagenesis has suggested that protons required for the Nor reaction are probably provided from the periplasmic side of the membrane *via* a channel that involves conserved glutamate residues (41, 85, 86, 237).

cNOR is encoded by the *norCBQD* operon. The *norC* and *norB* genes encode subunit II and subunit I, respectively. The *norQ* and *norD* genes encode proteins essential for activation of cNor. Some more specialized denitrifiers have additional *norEF* genes, the products of which are involved in maturation and/or stability of Nor activity (107).

3. Nitrous oxide reductase. The final step in denitrification consists of the two-electron reduction of nitrous oxide to dinitrogen gas. This reaction is performed by a copper containing homodimeric soluble protein located in the periplasmic space, the nitrous oxide reductase (Nos) [reviewed in (246–248, 268)] (Fig. 13a). The enzyme has been purified from a large number of denitrifying strains, including *Pa. denitrificans Pa. Pantotrophus*, and *Ps. stutzeri*. Nos is a homo-dimer of a 65 kDa copper-containing subunit. Each monomer is made up of two domains: the "Cu_A domain" and the "'Cu_Z domain." The recently reported structure of purple N_2OR from *Ps. stutzeri* has revealed that N_2O bound side-on at Cu_Z, in close proximity to Cu_A (181). Electron input into Cu_A is usually *via c*-type cytochromes or cupredoxins (20, 22). The *nos* gene clusters often comprise the *nosRZDFYLX* genes. The *nosZ* gene encodes the monomers of Nos. The *nosDFYL* genes encode proteins that are apparently required for copper assemblage into Nos, although their specific role still remains unknown. The NosRX proteins have roles in transcription regulation, activation, and Cu assemblage of Nos (268).

C. Oxygen and nitrogen oxides control of nitrate respiration

The key molecules that act as signals for the regulation of nitrate respiration through denitrification or ammonification pathways are oxygen, nitrate, nitrite, and NO. Sensing of each of these molecules involves several types of sensor proteins (see Fig. 13b).

1. Oxygen sensors. There are two types of oxygen sensors involved in regulation of nitrate respiration, Fnr and FixL (reviewed in section II.A; see Fig. 4). Fnr controls the expression of genes involved in anaerobic respiration when O_2 is absent. For example, the *nar* and *nap* operons in *E. coli* and *Ba. subtilis* (193, 228, 238, 241) are activated by Fnr under anaerobic conditions (Fig. 13b). *Pa. denitrificans* FnrP controls expression of the *nar* gene cluster and the *cco*-gene cluster encoding the cbb_3-type oxidase (30, 249). Oxygen tension is sensed in *Ps. aeruginosa* by the Anr regulator, which carries a $[4Fe\text{-}4S]^{2+}$ cluster (262).

2. Nitrate and nitrite sensors. In denitrifying species and species that anaerobically reduce nitrate to amonium, there are three types of nitrate/nitrite sensing systems: NarXL, NarQP, and NarR. NarXL and NarQP are members of two-component regulatory systems (Fig. 13b). The NarX and NarQ proteins are the signal sensors, and NarL and NarP proteins are their cognate response regulators, respectively (227). In *E. coli* NarL and NarP bind DNA to control induction of the *nar* and *nap* operons and repression of genes encoding alternate anaerobic respiratory enzymes (58, 227, 228). The effects of nitrate and nitrite on the *E. coli* transcriptome during anaerobic growth have been investigated, resulting in a new list of operons that are regulated by Fnr, NarL, and NarP (48). To date, *narXL* and *narQP* genes are confined to species classified in the γ and β subdivisions of the proteobacteria such as *Escherichia, Salmonella, Klebsiella,Yersinia, Burkholderia*, *Ralstonia*, *Neisseria*, and *Pseudomonas* species among others. In *Ps. aeruginosa,* the regulators Anr, Dnr, and NarL in concert with integration host factor (IHF) activate transcription of the *narK1K2GHJI* operon encoding nitrate reductase and two transporters in response to oxygen limitation, nitrate, and N-oxides (213). Recently, it has been shown that during anaerobic growth of *Ps. aeruginosa* PAO1, NarL directly represses expression of Nap, while inducing maximal expression of membrane nitrate reductase (245).

NarR is a member of the Fnr family of transcription activators, but it lacks a [4Fe-4S] cluster. NarR of *Pa. pantotrophus*

and *Pa. denitrificans* is specifically required for transcription of the *narKGHJI* genes in the presence of nitrite (249, 256) (Fig. 13b). Genes encoding NarR are found in the α-proteobacteria *Brucella suis*, *Brucella melitensis*, *Pa. Denitrificans*, and *Pa. pantotrophus*. There are no indications that they have counterparts of *narXL*. It, therefore, seems that NarR substitutes the NarXL system in the α-proteobacteria. A recent proteomic analysis of *Pa. denitrificans fnrP*, *nnr*, and *narR* mutant strains grown oxically, microoxically, and microoxically in the presence of nitrate has revealed new proteins involved in the FnrP, Nnr, and NarR regulons (30). Data from this work are in full agreement with the current view on the FnrP protein being a sensor for oxygen and on the Nnr and NarR proteins being sensors for intermediates of nitrate respiration. This study has also revealed that FnrP, Nnr, and NarR control transcription of target genes having both activating and repressing functions (30).

3. NO sensors. NO is a toxic, free radical gas with diverse biological roles in eukaryotes and bacteria. Several bacterial transcriptional regulators sense this molecule and regulate the expression of genes involved in both denitrification and NO detoxification [reviewed in (206, 226, 243, 267)]. Bacterial NO regulatory proteins can be roughly divided into (i) proteins whose primary role is not to sense NO, but which contain metal centers or cysteines that are nitrosylated by NO and (ii) those that appear to be solely dedicated to sensing NO (*i.e.*, they regulate genes involved in NO detoxification). An example of the first class is Fnr, whose primary role is to sense oxygen but that has also been shown to sense and respond to NO in *E. coli* (see section II.A).

The second class of NO sensors includes nitrite and nitric oxide reductase regulator (NnrR), NorR, and NsrR. NnrR also belongs to the Fnr family of transcription activators, but, similar to NarR, it lacks the cysteines to incorporate a [4Fe-4S] cluster. NnrR orthologs, sometimes named as Nnr, DnrR, or FnrD, are found in nearly all species containing the reductases Nir and Nor. In the presence of NO, these regulators orchestrate the expression of the *nir* and *nor* gene clusters. In many species, *nnrR* gene and its target genes are situated in close proximity on the chromosome, suggesting a local specificity. The promoters of these operons contain NnrR binding sites that resemble the consensus Fnr-box to a large extent. Nnr homologs, including NnrR in *Rho. sphaeroides* (131) and Dnr in *Ps. aeruginosa*, most likely sense NO *via* a ligated heme (93, 94). In denitrifying bacteria, knockout mutagenesis has shown that the Dnr and NnrR regulators are needed for the transcriptional control of the *nir*, *nor*, and *nos* operons.

NorR is another NO-responsive protein that was first identified in *Ralstonia eutropha* (180). This bacterium has two copies of the *norR* gene, both of which are located upstream of their *norAB* gene clusters where *norB* encodes a single-subunit Nor of the qNor type. NorR is a member of the NtrC family of response regulators. The absence of possible phosphorylation sites as well as the presence of a conserved GAF domain indicative for signal perception suggest that the protein belongs to a sub-family of response regulators which sense their signal themselves rather than *via* a cognate signal sensor (55). In response to anaerobiosis and the presence of NO, the NorR proteins specifically activate transcription of the σ^{54}-dependent *norAB* promoters and repress their own synthesis *via* negative autoregulation (39). NorR has also been found in *E. Coli*, which senses NO directly through a mononuclear non-heme iron center and responds by switching on expression of the flavorubredoxin NorVW to detoxify NO (242).

NsrR is an iron–sulfur-containing protein that senses NO directly *via* a [2Fe–2S] cluster. Nitrosylation of this cluster leads to a loss of DNA binding activity and, hence, derepression of NsrR target genes. In *E. coli*, this transcription repressor was shown to sense reactive nitrogen species and to switch on a regulon of at least 60 genes, including genes involved in nitrate respiration (82, 226, 243). In denitrifying bacteria, NsrR appears to have a specific role in coordinating production of the nitrite and NO reductase enzymes to prevent the build up of NO (243). Intriguingly, the same role is performed by Nnr homologs in denitrifying bacteria that do not contain NsrR. Two components of the denitrification pathway are transcriptionally regulated by NsrR in *Neisseria meningitidis*: the membrane-bound nitrite reductase AniA and the respiratory NO reductase NorB (111). In the closely related *Neisseria gonorrhoeae*, NsrR represses both *norB* and *aniA* expression in the absence of NO and, most notably, also exerts an autoregulatory effect on its own expression (113, 171).

4. Other regulators in denitrifying bacteria. In addition to the regulatory proteins that can monitor oxygen, nitrate, nitrite, and NO, regulation of the on-set and fine-tuning of nitrate respiration in some bacteria involves copper responsive regulators, redox sensing mechanisms and the NosR and NirI proteins (Van Spanning, 2011). The NosR is a membrane-bound protein containing six transmembrane helices, a large periplasmic domain, and cysteine-rich cytoplasmic domains that resemble the binding sites of [4Fe-4S] clusters. The periplasmic domain of NosR has a structural similarity with the FMN-binding domain of the NqrC subunit of the Na^+-translocating NADH:quinone oxidoreductase and may play a role in activating or repairing oxygen damaged Nos (260). Although NosR proteins do not have properties of transcription factors, they are required for transcription activation of the *nos* genes in denitrifying bacteria. However, the regulatory function of NosR is not well understood (268). In *Pa. Denitrificans*, a homolog of *nosR*, *nirI*, representing thus far a singular case among the denitrifying bacteria, has been studied for its role in the transcription of *nirS*, encoding the cytochrome cd_1Nir.

V. Redox Control of Nitrate Respiration

Nitrate reduction by the membrane-bound Nar generates an H^+-motive force (PMF) across the cytoplasmic membrane of the cell, allowing ATP synthesis (197, 221). During denitrification, the periplasmic Nir and Nos cannot themselves be involved in H^+-movement across the membrane. Although Nor is an integral membrane protein, several lines of evidence show that H^+ required for reduction of NO are taken from the periplasm and thus, no net charge movement occurs (see section IV.B). However, e-transfer from ubiquinol to NO_2^-, NO or N_2O is coupled to H^+-translocation across the membrane, because this involves the cytochrome bc_1 complex (197). Quinol oxidation by the periplasmic Nap is not directly coupled to the generation of a PMF and is independent of the cytochrome bc_1 complex. In many bacteria, Nap has a role in redox balancing under aerobic conditions (20, 42); indeed it seems to be biochemically tuned for this function (88). The need to dissipate reductants is likely to be most acute during

the metabolism of a reduced carbon substrate under conditions that are both oxygen and energy sufficient. Accordingly, expression of the Nap is significantly higher in *Paracoccus* species grown aerobically with butyrate or caproate to that when grown with malate or succinate (69, 71, 196, 215, 216). Taken together, these results suggest that Nap constitutes an example of redox-dependent regulation of the respiratory network in *Paracoccus* species. Two crucial regulatory sequences that control expression of the *nap* operon in response to the redox status of the cell were identified in *Pa. pantotrophus*, but the redox responsive transcriptional regulator has yet to be described (70). The use of Nap in redox homoeostasis has been also shown in photosynthetic bacteria where it regulates the redox poise of the cyclic photosynthetic electron transport system (117), particularly during photoheterotrophic growth on reduced carbon substrates (89, 200).

Many bacteria can also recruit Nap for anaerobic nitrate respiration as a part of bacterial ammonification (*Enterobacteriaceae*) or to promote denitrification (*Rhizobiaceae*). In fact, some rhizobia species (*e.g.*, *Pseudomonas* sp. G179 [actually *Rhizobium galegae*], *Br. japonicum*) can express *nap* genes under anaerobic conditions, and disruption of the *nap* genes is lethal for growth under denitrifying conditions (17, 62). In addition, in *Rho. sphaeroides* f. sp. *denitrificans*, which can express both Nar and Nap, inactivation of Nap is lethal for anaerobic denitrification (135). Thus, in these organisms, the physiological role of Nap is in anaerobic denitrification. When considered as single enzymes, the energy coupling of Nar and Nap appear markedly different: $q+/2e^- = 6$ (Nar) and 4 (Nap) with NADH as electron donor and 2 (Nar) and 0 (Nap) with succinate as electron donor. However, when considered in the context of the entire denitrification pathway, the $q+/2e^-$ ratio is 24 (Nar) or 22 (Nap) with NADH and 8 (Nar) or 6 (Nap) with succinate. Thus, the energetic loss of using Nap rather than Nar is only 8% when NADH is the electron donor to the respiratory system (197).

To date, the knowledge about the redox-dependent control of anaerobic nitrate respiration as well as the identification of the regulators and the signal transduction mechanism involved in this response is limited. In this section, we will focus on recent advances in understanding nitrate respiration redox-dependent control in *Rho. sphaeroides, Ag. tumefaciens, Br. japonicum, and Ba. subtilis.*

A. Rho. sphaeroides

Rho. sphaeroides strains are photosynthetic bacteria able to induce the denitrification pathway by expressing the enzymes Nap, Nir, and cNor, which are encoded by the *nap*, *nirK*, and *nor* genes, respectively, when oxygen concentration is limited and nitrate or nitrite is present in the medium. In *Rho. sphaeroides* 2.4.3., Nap is the enzyme responsible for denitrification as judged by the fact that strains with mutations in the *nap* gene cluster lost nitrate reductase activity as well as the ability to grow with nitrate under anaerobic-dark conditions (135). In the strain IL106 of *Rho. sphaeroides*, *nap* genes showed the same level of expression under both oxic and denitrifying conditions with nitrate. Expression increases in the presence of the reduced carbon compound butyrate, indicating that this protein probably plays a role in redox homeostasis [reviewed in (218)]. A slightly different *nap* expression pattern was observed in *Rho. sphaeroides* strain 2.4.1, where expression of the *nap* operon increases significantly when cells are shifted from anoxic photosynthetic conditions to oxic conditions and do not require nitrate [reviewed in (218)].

Although the expression of *nap* has been shown to be variable, the expression of the genes encoding Nir and Nor only occurs under microoxic conditions. In *Rho. sphaeroides* 2.4.3, *nir* and *nor* expression requires low oxygen and also requires the presence of a nitrogen oxide. It has been shown that NnrR is necessary for the expression of *nirK* and *nor* during denitrification (240). Studies on regulation by NnrR demonstrated that the presence of NO is required for transcriptional activation of *nirK* and *nor*, yet the mechanism whereby NnrR senses NO is not known. Analysis of the *Rho. sphaeroides* 2.4.3 genome and expression studies demonstrated that genes designated *paz* and *norEF* are also members of the NnrR regulon involved in denitrification (107).

Genes for anaerobic growth in many photosynthetic bacteria are also regulated by the two-component PrrBA system. In *Rho. sphaeroides* strain 2.4.1 where *nap* genes are maximally expressed under oxic conditions, the phosphorylated form of the response regulator PrrA is responsible for repressing *nap* expression under low oxygen [reviewed in (218)], see Fig. 14a). By contrast, in *Rho. sphaeroides* 2.4.3, inactivation of *prrA* eliminated the ability to grow both photosynthetically and anaerobically in the dark on nitrite-amended medium (130). The PrrA-deficient strain exhibited a severe decrease in both Nir activity and expression of a *nirK-lacZ* fusion (130) when environmental oxygen tension was limited. This regulation is not mediated by NnrR, as *nnrR* is fully expressed in a PrrA mutant background (Fig. 14a). By using truncated versions of the *nirK* promoter fused to the *lacZ* maker gene, Laratta and colleagues (130) revealed that the DNA region expanded upstream to the putative NnrR binding site is required for full activation of *nirK*. These results suggested that this DNA region could act as a binding site of PrrA or additional regulatory proteins in order to control *nirK* expression. In fact, the presence upstream of the *nirK* start site of 4 imperfect repeats proposed as binding sites for RegR (a PrrA homolog in *Br. japonicum*) suggests again that PrrA could bind directly to such sites. However, binding assays using purified PrrA would be required to confirm this. Unlike the *nirK* promoter region, the *nor* promoter has no sites with similarity to the imperfect repeats proposed as binding sites for PrrA homologs, suggesting that PrrA may not directly regulate *norB* expression (130). The model proposed by Laratta and colleagues (130) to understand the mechanism of *nirK* control by PrrBA is that under low oxygen tensions, the kinase activity of PrrB increases relative to its phosphatase activity, resulting in an increase in the concentration of PrrA-P. Under microoxic conditions and in the presence of NO, NnrR together with PrrA-P activates transcription of *nirK* (Fig. 14a). As mentioned in section III.B (Fig. 8c), inactivation of genes encoding the cbb_3 oxidase in *Rho. sphaeroides* has been shown to lead to high levels of PrrA-P under both oxic and microoxic conditions (190). Unexpectedly, loss of the cbb_3 oxidase resulted in a significant decrease in *nirK* expression and Nir activity (130). Recent results have demonstrated that the cbb_3 mutant retains Nir activity at very low oxygen concentrations (107). The simplest explanation for this result is that, under transition conditions, loss of the cbb_3 oxidase results in high residual oxygen levels in the culture, which prevents expression of *nirK* and *nor*.

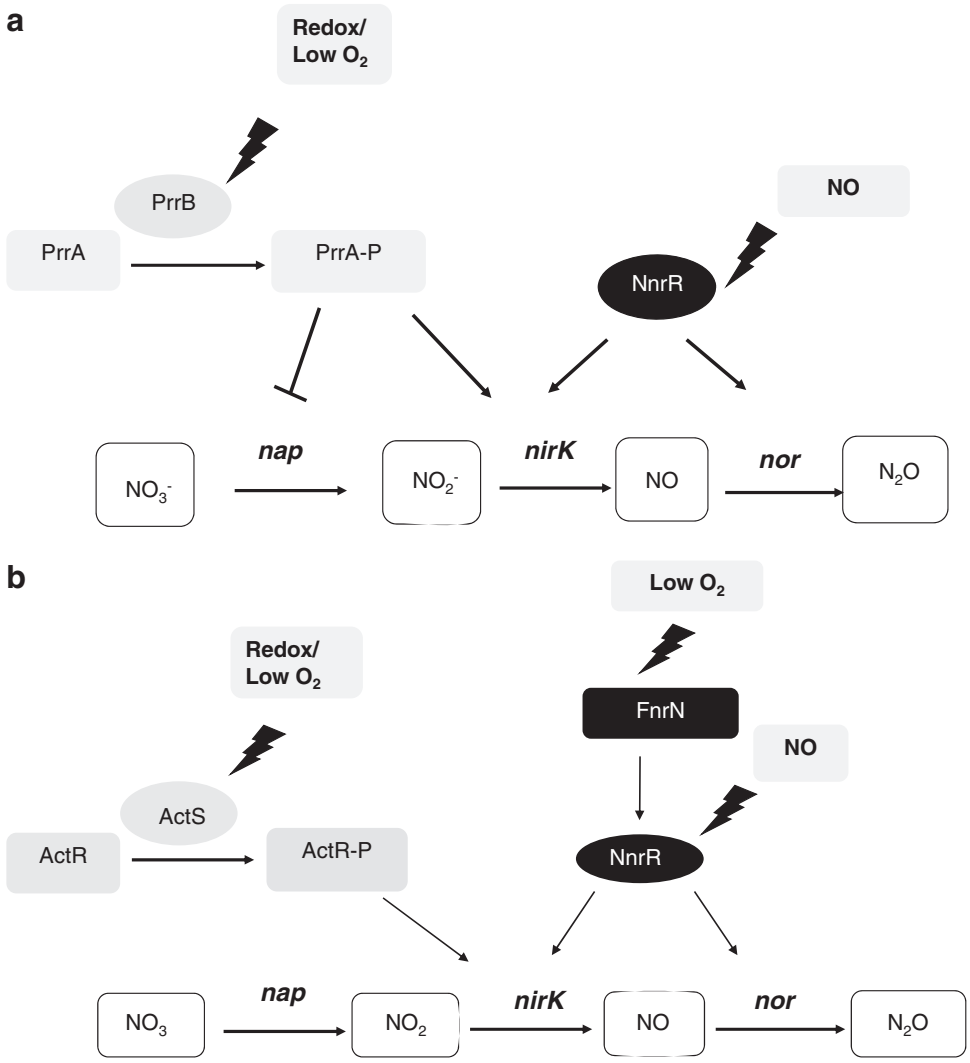

FIG. 14. Schematic representation of denitrification control in *Rho. sphaeroides* (a) and *Agrobacterium tumefaciens* (b). (a) In *Rho. Sphaeroides*, PrrBA provides control of *nirK* expression in response to external redox variations, and NnrR provides control of expression of *nirK* and *nor* genes in response to NO. This figure is adapted from ref. (218). **(b)** In *Ag. tumefaciens*, ActSR provides control of *nirK* expression presumably in response to external redox variations, and FnrN and NnrR provide control of *nirK* and *nor* expression in response to low oxygen tension and presence of NO, respectively. Positive regulation is denoted by *arrows*, and negative regulation is indicated by *perpendicular lines*.

B. Ag. tumefaciens

Ag. tumefaciens is a plant pathogen that can grow under free-living conditions in the absence of oxygen *via* denitrification. The ability to respire nitrogen oxides could be significant for the survival and growth of free-living and plant-associated *Ag. tumefaciens* cells. The ability to respire nitrate has been shown to be advantageous to bacteria in the rhizosphere, as nitrate provides an alternative oxidant when oxygen concentrations are low (25). In addition, the ability to reduce nitrogen oxides may be useful for countering some of the plant defenses against plant pathogens (63, 79). *Ag. tumefaciens* is considered a partial denitrifier, as it lacks the genes encoding Nos (9). Consequently, nitrous oxide is the final product of denitrification.

Ag. tumefaciens has a *nap* cluster that is maximally expressed under denitrifying conditions, but is not influenced by nitrate (218). The regulator(s) responsible for expression of *nap* in *Ag. tumefaciens* are uncertain, as there are no conserved sequence motifs in the promoter region that might indicate the involvement of a particular family of regulators. Similar to *Rho. sphaeroides*, activation of *nirK* and *norCBQD* expression requires low oxygen concentration and the presence of NO (8, 9, 21). By using the *lacZ* marker gene fused to the *nirK* and *nor* promoter and by *in vitro* binding assay experiments, it has been demonstrated that activation of *nirK* and *nor* requires NnrR. This protein is directly activated by FnrN when oxygen tension in the environment becomes depleted (Fig. 14b). However, activation of *nirK* and *nor* is raised when NO, as a reduction product of nitrate or nitrite, is present in the medium, due to the maximal NO-dependent activation of NnrR. In *Ag. tumefaciens*, electron transfer through the denitrification pathway is facilitated by soluble small cytochromes such as the copper-containing pseudoazurin, encoded by the *paz* gene, whose expression also depends on FnrN and NnrR. In addition to FnrN and NnrR, the ActRS system is involved in denitrification (Fig. 14b). As observed in *Rho. sphaeroides*, insertional inactivation of the response regulator ActR significantly reduced *nirK* expression and Nir activity but not *nnrR* expression. In *Ag. tumefaciens*, a putative ActR binding site was identified in the *nirK* promoter region using mutational analysis and an *in vitro* binding assay (8). These studies also show that purified ActR bound to the *nirK* promoter but not to the *nor* or *nnrR* promoter. Additional experiments revealed that expression of *paz* was highly reduced in the *actR* or *fnrN* mutants and that ActR binds to the *paz* promoter (8). Due to the similarity to the PrrBA/RegBA systems, the ActSR system is believed to mediate gene activation on sensing redox

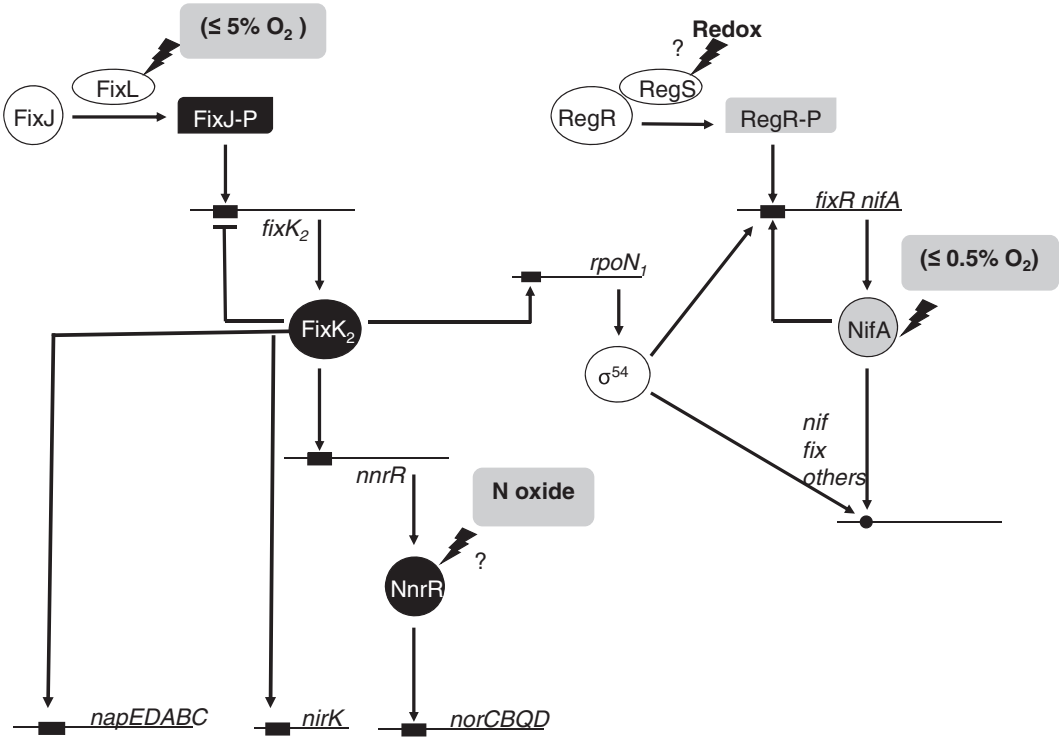

FIG. 15. Schematic representation of the regulatory network of denitrification in *Br. japonicum*. FixLJ regulates *fixK*$_2$ gene transcription in response to a shortage of oxygen, and FixK$_2$ provides control of transcription on *nap* and *nirK* denitrification genes as well as the *nnrR* gene, whose product, in turn, activates transcription of *nor* genes. RegSR activates expression of *fixRnifA* operon presumably by sensing changes in the redox state of the cell. The NifA regulatory protein activates gene expression at very low oxygen concentrations. Control of denitrification genes by NifA has been demonstrated by our group (36). Furthermore, recent results have shown that NorC expression is significantly reduced in a *Br. japonicum regR* mutant (239). Sigma^{54} links both regulatory cascades. Positive regulation is denoted by *arrows*, and negative regulation is indicated by *perpendicular lines*.

changes originated from the ETC through *cbb*$_3$-oxidase (PrrB) or through the redox state of the UQ pool (RegB). However, there is no experimental evidence as yet to confirm these hypotheses.

With all of this experimental evidence, we can see that activation of denitrification in *Ag. tumefaciens* depends on regulatory proteins involved in sensing different environmental signals. While the Fe-S-containing FnrN protein is an oxygen sensor, NnrR activates denitrification genes in response to NO. In this complex regulatory process, the Prr/Reg orthologs protein ActSR would adjust the denitrification machinery in response to redox status as an indirect measure of external oxygen tension (Fig. 14b).

C. Br. japonicum

Similar to *Ag. tumefaciens*, the nitrogen-fixing root nodule symbiont of soybean *Br. japonicum* has the ability to sustain cell growth by using nitrate as respiratory substrate when oxygen concentrations are very low. In contrast to *Ag. tumefaciens*, *Br. japonicum* contains all the denitrification enzymatic machinery necessary to reduce sequentially nitrate to molecular nitrogen.

In *Br. japonicum*, denitrification is dependent on the *napEDABC*, *nirK*, *norCBQD*, and *nosRZDYFLX* genes that encode an Nap, a Cu-containing Nir (NirK), a cNor, and an Nos, respectively (16). In addition, accessory cytochromes are necessary to support electron transport during denitrification. For example, the cytochrome c_{550}, encoded by *cyc*A, is essential for the electron delivery to the NirK reductase (35). Very recently, it has also been found that the cytochrome c_6, encoded by the *cycS* gene, has a role in denitrification, although its precise biochemical function still remains unknown (145). Neither azurin- nor pseudoazurin-like copper proteins has been annotated in the genome sequence of *Br. japonicum* (www.kazusa.jp/rhizobase/).

In *Br. japonicum*, maximal expression of denitrification genes requires both the absence of oxygen and the presence of nitrate or a derived N-oxide. Microoxic induction of transcriptional fusions from the *nap*, *nir*, *nor*, and *nos* promoter regions to the *lacZ* reporter gene depends on the FixLJ/FixK$_2$ regulatory cascade (Fig. 15) (16, 204). Sequences homologous to the consensus DNA binding sites of the Fnr and FixK proteins are present in the promoter region of each denitrification gene. Regulatory studies indicated that, in addition to low oxygen, nitrate, or an N-oxide derived from it, presumably either NO_2^- or NO or both, are required for maximal induction of *Br. japonicum* denitrification genes. This N oxide-mediated induction of *nap*, *nir*, and *nor* genes depended on NnrR (144, 204). Thus, NnrR expands the FixLJ/FixK$_2$ regulatory cascade by an additional control level that integrates the N oxide signal required for maximal induction of the denitrification genes (Fig. 15). Induction of the *norCBQD* promoter is completely abolished in the absence of a functional

nnrR gene. By contrast, microoxic induction of the *nap* or *nirK* promoters is retained in a *nnrR* mutant background, implying that the *nap* or *nirK* and the *norCBQD* promoters exhibit slight differences with regard to their dependence on $FixK_2$. In this context, recent results from our group have demonstrated that purified $FixK_2$ activates transcription from *nap*- or *nirK*-dependent promoters but not from *nor*-dependent promoter (E. Bueno, unpublished work). By contrast, ITC allowed us to demonstrate that NnrR bound to a specific DNA fragment from the promoter region of the *nor* genes, but not to those from the *nap* and *nirK* genes and that this interaction requires anoxic conditions but not the presence of an N oxide (unpublished results from our laboratory). Supporting these observations, a genome-wide transcription profiling of *Br. japonicum fixJ* and $fixK_2$ mutant strains grown in free-living microoxic conditions have shown that *nap*, *nirK*, and *nnrR* but not *nor* are targets of $fixK_2$ regulatory gene (145).

In addition to FixLJ/$FixK_2$-NnrR, in *Br. Japonicum*, a second oxygen responsive regulatory cascade has been described, the RegSR/NifA cascade (Fig. 15). NifA activates gene expression in concert with the RNAP containing the alternative sigma factor (σ^{54}), which, in *Br. japonicum*, is encoded by the two highly similar and functionally equivalent genes ($rpoN_1$ and $rpoN_2$) (127). Since $rpoN_1$ is under the control of $FixK_2$, this gene represents the link between the two regulatory cascades.

While the FixLJ/$FixK_2$-NnrR cascade activates gene transcription at a moderately low oxygen concentration ($\leq 5\%$), activation of the RegSR/NifA system requires oxygen concentration $\leq 0.5\%$ (214). Recent results from our group showed that NifA is required for maximal expression of *nap*, *nirK*, and *nor* genes, suggesting a role for the RegSR/NifA regulatory cascade in the control of the denitrification process in *Br. japonicum* (36). In that study, it was shown that disruption of *nifA* caused a growth defect in *Br. japonicum* cells when grown under denitrifying conditions, as well as decreased activity of Nap and Nir enzymes. Furthermore, expression of *napE–lacZ*, *nirK–lacZ*, or *norC–lacZ* transcriptional fusions, as well as levels of *nirK* transcripts, were significantly reduced in the *nifA* mutant after incubation under nitrate-respiring conditions. These results suggest that maximal induction of denitrification genes is only triggered once the cellular oxygen falls to a sufficiently low level, that is, within the sensory range of the NifA-dependent regulatory cascade. Similarly, in *Ag. tumefaciens*, a study of expression and activity of Nir and Nor as a function of oxygen has shown that *nirK* and *nor* are expressed once oxygen reaches $< 0.5\ \mu M$ or approximately 0.2% of air-saturating oxygen (21). These oxygen concentrations are low enough to allow formation of ActR-P necessary for *nirK* activation in *Ag. tumefaciens*.

Concerning the involvement of *Br. japonicum* RegSR in denitrification, it has been shown that free-living growth of *regR* mutants under anoxic conditions with nitrate as the terminal electron acceptor was severely impaired [Bauer *et al.* (15)]. Furthermore, expression of NorC is significantly lower in membranes from the *regR* mutant (239). As just mentioned, it has been shown that disruption of *Rho. sphaeroides prrA* or *prrB* causes a significant decrease in both *nirK* expression and Nir activity. Similarly, in *Ag. tumefaciens*, it has been shown that purified ActR binds to the *nirK* promoter, but not to the *nor* or *nnrR* promoters. In *Br. japonicum*, the involvement of RegR in *nirK* expression is at the moment unknown, and further investigations are needed to demonstrate the involvement of RegR in *norCBQD* gene expression and to establish whether these genes are direct, or indirect, targets of RegR.

D. Ba. subtilis

Once the oxygen present in the medium has been depleted by the *bd* oxidase (see section III.E; Fig. 12a), *Ba. subtilis* has the ability to exploit the use of an alternative final electron acceptor such as nitrate (158). Two distinct operons specifying two nitrate reductases have been characterized: (i) *narGHJI* encoding a respiratory nitrate reductase, which is required for nitrate respiration and is dependent on Fnr, and (ii) *nasBC* encoding a assimilatory nitrate reductase involved in the assimilation of nitrogen from nitrate under oxic conditions (159) and references therein) (Fig. 16). *Ba. subtilis* does not contain *nirK*, *nor*, or *nos* denitrification genes. Under anoxic conditions with nitrate or nitrite, this bacterium reduces nitrite to ammonium by the Nir NasDE encoded by *nasD* and *nasE* (Fig. 16) (*i.e.*, reactions 5 and 6). The NasDE Nir was previously identified as an assimilatory enzyme [(157) and references therein]. Under aerobic conditions of nitrogen limitation, this enzyme catalyzes the reduction of nitrite to ammonia for the anabolic incorporation of nitrogen into biomolecules. It also functions catabolically in anaerobic respiration, which involves the use of nitrite as a terminal electron acceptor (157). Nitrite reduction catalyzed by *Ba. subtilis* Nir does not result in a proton gradient and coupled ATP generation. Instead, nitrite enhances anaerobic growth by serving as an electron sink to recycle the cellular pyridine nucleotide pool (157).

The *nasDE* genes, together with *nasBC* (encoding assimilatory nitrate reductase) and *nasF* (required for Nir siroheme cofactor formation), constitute the *nas* operon (157). Transcription of *nasDEF* is driven not only by the *nasBCDEF* operon promoter, but also from an internal promoter residing between the *nasC* and *nasD* genes. Transcription from both promoters is activated by nitrogen limitation during aerobic growth by the nitrogen regulator, TnrA. However, under conditions of oxygen limitation, *nasDEF* expression and Nir activity were significantly induced (157, 158).

The expression of genes involved in nitrate respiration in *Ba. subtilis* is also regulated by the ResDE two-component signal transduction system (Fig. 16). To successfully switch from aerobic growth to nitrate respiration, the ResDE two-component signal transduction system should be activated, which allows induction of genes that function in anaerobic metabolism including *fnr*, *nasDEF*, and *hmp* (encoding flavohemoglobin) [(90) and references therein]. Footprinting analysis revealed that ResD tandemly binds to the -41 to -83 region of *hmp* and the -46 to -92 region of *nasD* (90). *In vitro* run-off transcription experiments showed that ResD is necessary and sufficient to activate transcription of the ResDE regulon. Although phosphorylation of ResD by ResE kinase greatly stimulated transcription, unphosphorylated ResD, and ResD with a phosphorylation site (Asp^{57}) mutation, it was able to activate transcription at a low level (90). These results suggest that ResD itself, in addition to its activation through phosphorylation-mediated conformation change, senses oxygen limitation *via* an unknown mechanism leading to anoxic gene activation. Although ResDE was initially believed to sense anoxic conditions, subsequent studies have revealed that NO is also required (151, 155). It has been

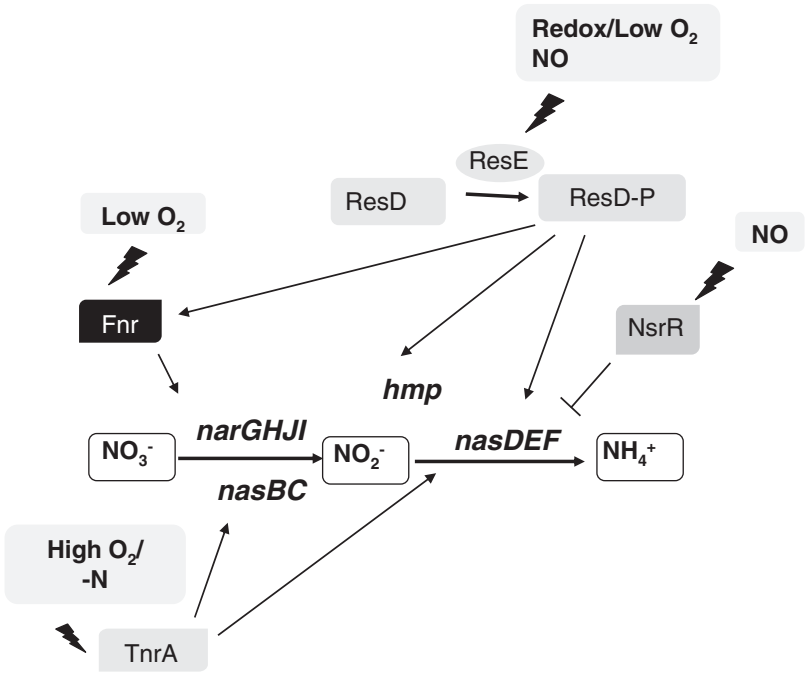

FIG. 16. Schematic representation of denitrification control in *Ba. subtilis*. ResDE regulates gene transcription in response to both redox/oxygen changes and presence of NO, Fnr provides control in response to low oxygen concentration, NsrR in response to NO, and TnrA in response to high oxygen concentration and limited nitrogen availability. *narGHJI* encodes a respiratory nitrate reductase. *nasBC* encodes an assimilatory nitrate reductase; *nasDEF*, a nitrite reductase; and *hmp*, a flavohemoglobin. Positive regulation is denoted by *arrows*, and negative regulation is indicated by *perpendicular lines*.

proposed that *Ba. subtilis* senses NO and responds through activation of ResDE-dependent transcription (156). In this context, it has been recently discovered that the NO-sensitive transcription factor NsrR is involved in ResDE-dependent gene regulation (156). In *Ba. subtilis*, NsrR represses transcription of the Nir (*nasDEF*) genes that are under positive control of ResDE (Fig. 16). Derepression is achieved by reaction of NO with NsrR. Anaerobically purified *Ba. subtilis* NsrR (BsNsrR) bears a [4Fe–4S] cluster that, on exposure to NO, forms dinitrosyl iron complexes (263). [4Fe–4S]-NsrR binds around the -35 element of the *nasD* promoter with much higher affinity than apo-NsrR, and this binding is sensitive to NO. RNAP and phosphorylated ResD make a ternary complex at the *nasD* promoter, and NsrR dissociates the preformed ternary complex (123).

VI. Concluding Remarks

Genetic, biochemical, and transcriptomic analyses have revealed that in many bacterial species, the adaptation of respiratory metabolism to changing environments with different oxygen conditions is controlled by Fnr-type transcriptional regulators that directly sense O_2 or by the Fnr paralog, FixK; which senses O_2 through the FixLJ two-component system. In addition to external oxygen concentration, other signals such as redox changes can regulate the expression of genes involved in respiration. Two-component regulatory systems (*e.g.*, ArcBA, RegBA/PrrBA, RoxSR, RegSR, ResDE, and ActSR) from different bacterial species integrate the response to multiple redox signals. The redox state of the membrane-localized UQ/ubiquinol pool as well as the redox state of the conserved cysteine[265] were considered as major indicators of redox variations detected by these regulatory systems. Recently, it has been proposed that both signals are able of functioning independently (259). In addition, a third model has been proposed in *Rho. sphaeroides and Pseudomonas*, where redox changes are sensed by the terminal oxidase cbb_3, which modulates PrrB and RoxS kinase/phosphatase activity (47, 122, 152). Those redox-dependent responses are complex, as recent reports have proposed that the activity of the sensor kinase ArcA from *E. coli* can also be modulated by the redox state of the MK pool (18) or the presence of fermentation products (208). Particulary interesting is the case for the Gram-positive bacteria such as *Ba. subtilis*, where, in addition to the Fnr-type and the ResDE two-component system, the respiratory shift from oxic to microoxic conditions is also controlled by the repressor Rex, whose activity is influenced by the balance of the $NADH/NAD^+$ pool in the cell (31, 92).

The respiratory shift in response to changes in oxygen concentration becomes even more sophisticated in bacterial species that use nitrate as respiratory substrate through denitrification or ammonification pathways. This shift is controlled not only by oxygen limitation (FixL and Fnr-type), but also by the presence of nitrate/nitrite (NarXL, NarQP, and NarR) or/and NO (NnrR, NorR, and NsrR). Furthermore, recent findings have shown that redox-responsive two-component regulatory systems (*e.g.*, PrrBA, ActSR, RegSR, and ResDE) are also involved in the regulation of genes needed for anaerobic nitrate respiration. Intriguingly, *Ba. subtilis* ResDE requires anaerobiosis and the presence of NO to activate transcription of nitrate respiration genes (151, 155, 156). In this context, it has been recently discovered that the NO-sensitive transcription factor NsrR plays a role in ResDE-dependent gene regulation (123, 156). However, in Gram negative bacteria, the involvement of such NO-sensitive repressor in regulation of denitrification genes by others redox-responsive regulators (PrrBA, ActSR, RegSR, *etc*) is at the moment unknown.

In summary, it is clear that bacteria adopt a range of strategies to monitor the redox cellular state to coordinate the respiratory shift from oxic to anoxic environments. This control is based on a complex regulatory network where different regulators responding to diverse redox signals interact and control expression of target genes. The study and comparison

of regulatory pathways in different bacterial groups is very useful to better understand how bacteria integrate different signals and how the regulatory systems respond to these signals. In spite of their diversity, regulatory networks from different bacteria share common proteins as well as their complexity. With the advent of the post-genomic era, and the move toward more predictive biology, a better understanding of how these complex regulatory circuits interact to integrate transcriptional responses to multiple environmental cues will be achieved.

Acknowledgments

The authors would like to thank all of their colleagues who have contributed to the research arising from their laboratories that is discussed in this article and also Junta de Andalucía (grant RMN-4746 partially funded by FEDER), CYTED (grant 107PICO312), and Ministerio de Ciencia e Innovación (grants AGL2010-18607 and 200940I116 partially funded by FEDER) for financial support. Support from the Junta de Andalucía (BIO-275) is also acknowledged. D.J.R. thanks the Royal Society and Wolfson Foundation for a Merit Award Fellowship and the Biotechnology and Biological Research Council for financial support for N-cycle research.

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Date of first submission to ARS Central, May 09, 2011; date of final revised submission, November 16, 2011; date of acceptance, November 18, 2011.

Abbreviations Used

Arc = aerobic respiration control
cd_1Nir = cd_1type nitrite reductase
CIO = cyanide insensitive oxidase
Crp = cAMP receptor protein
CuNir = Cu-type nitrite reductase
Cys = cysteine
Cyt = cytochrome
DMSO = dimethyl sulfoxide
ETC = electron transport chain
Fnr = fumarate and nitrate reductase regulatory protein
HCO = heme-copper oxidase
His = histidine
H-T-H = helix-turn-helix motif
ITC = isothermal titration calorimetry
MK = menaquinone
MKH_2 = menahydroquinone
NDH = NADH dehydrogenase
Nap = periplasmic nitrate reductase
Nar = membrane-bound nitrate reductase
Nir = nitrite reductase
NnrR = nitrite and nitric oxide reductase regulator
Nor = nitric oxide reductase
Nos = nitrous oxide reductase
NrfA = cytochrome *c* nitrite reductase
PMF = proton motive force
RNAP = RNA polymerase
SDH = succinate dehydrogenase
TM = transmembrane
UQ = ubiquinone
UQH_2 = ubihydroquinone

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1. R. Martin Roop II, Clayton C. Caswell. 2012. Redox-responsive regulation of denitrification genes in Brucella. *Molecular Microbiology* **85**:1, 5-7. [CrossRef]