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MINIREVIEW

Mechanisms for Generating Low Potential Electrons across the Metabolic Diversity of Nitrogen-Fixing Bacteria

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ABSTRACT The availability of fixed nitrogen is a limiting factor in the net primary production of all ecosystems. Diazotrophs overcome this limit through the conversion of atmospheric dinitrogen to ammonia. Diazotrophs are phylogenetically diverse bacteria and archaea that exhibit a wide range of lifestyles and metabolisms, including obligate anaerobes and aerobes that generate energy through heterotrophic or autotrophic metabolisms. Despite the diversity of metabolisms, all diazotrophs use the same enzyme, nitrogenase, to reduce N_2. Nitrogenase is an O_2-sensitive enzyme that requires a high amount of energy in the form of ATP and low potential electrons carried by ferredoxin (Fd) or flavodoxin (Fld). This review summarizes how the diverse metabolisms of diazotrophs utilize different enzymes to generate low potential reducing equivalents for nitrogenase catalysis. These enzymes include substrate-level Fd oxidoreductases, hydrogenases, photosystem I or other light-driven reaction centers, electron bifurcating Fix complexes, proton motive force-driven Rnf complexes, and Fd:NAD(P)H oxidoreductases. Each of these enzymes is critical for generating low potential electrons while simultaneously integrating the native metabolism to balance nitrogenase's overall energy needs. Understanding the diversity of electron transport systems to nitrogenase in various diazotrophs will be essential to guide future engineering strategies aimed at expanding the contributions of biological nitrogen fixation in agriculture.

KEYWORDS diazotrophs, electron transport, nitrogen fixation, physiology

Nitrogen in the form of ammonia, nitrate, or urea is required for primary production in terrestrial and marine ecosystems (1). Most terrestrial nitrogen is stored in the atmosphere as dinitrogen (N_2) gas and is unavailable to the majority of organisms. However, some bacteria and archaea, called diazotrophs, can convert N_2 into bioavailable ammonia (NH_3) through biological nitrogen fixation (BNF). Diazotrophs account for 60% of the fixed N_2 input into the biogeochemical nitrogen cycle (2). Diazotrophs fix N_2 via the activity of nitrogenase, which catalyzes the cleavage of the N_2 triple bond and its reduction to ammonia. BNF can be extraordinarily challenging for diazotrophs, as nitrogenase requires energy from ATP hydrolysis and low potential electrons and is sensitive to oxygen inactivation (3). Despite the aforementioned limitations of nitrogenase, diazotrophs are found in most ecological niches having a primary role in a diversity of microbial communities. Diazotrophs reside in oligotrophic marine ecosystems, the extreme environments of hot springs, the guts of termites, and in symbiotic relationships with crops (4–8). Diazotrophs can occupy these diverse niches through various physiological adaptations that shape native metabolisms to protect nitrogenase from oxygen while supplying enough ATP and reducing equivalents. This review details how a diversity of diazotrophs use electron transport enzymes in concert with physiological adaptations to support the energetic needs of nitrogenase-dependent reduction of N_2.

The enzymatic reduction of N_2 has one of the highest energy barriers in biology, requiring large amounts of reducing chemical energy and intricate metal cofactors to

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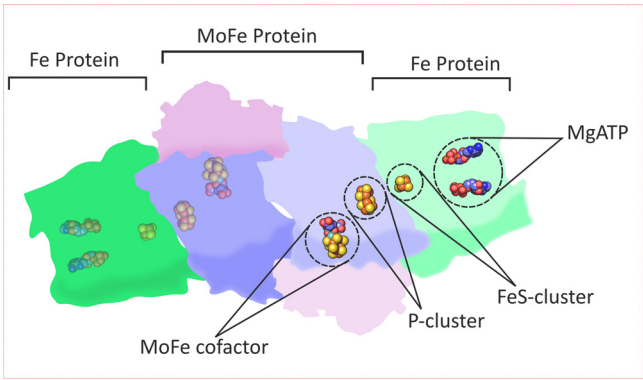

FIG 1 The nitrogenase enzyme is a heterodimer consisting of 3 subunits per dimer, the Fe protein (NifH), and the 2 subunits of the MoFe protein (NifDK). The Fe-protein contains 1 FeS cluster that accepts electrons from Fd or Fld and reduces the P-cluster in the MoFe protein in an ATP-dependent electron transfer. The P-cluster delivers electrons to the MoFe cofactor allowing electrons to be loaded for the reduction of N_2. For complete N_2 reduction, the Fe protein must deliver 8 electrons to the MoFe protein, requiring 2 ATP per electron transfer. Each metal cofactor is very sensitive to oxygen damage.

perform the reaction (9). Nitrogenase is the only enzyme that can catalyze the reduction of N_2 to NH_3 and exists in 3 related forms with different metal dependencies: Mo-dependent, V-dependent, and heterometal-independent forms. These 3 forms of nitrogenase have been demonstrated to share the same overall mechanism but have slightly different reaction stoichiometries (10). Mo-dependent nitrogenase is the most commonly occurring, extensively studied, and most efficient operating with the optimal reaction stoichiometry shown in (Equation 1) (9, 11).

$$N_2 + 8\,e^- + 16\,MgATP + 8\,H^+ \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16\,Pi \quad (1)$$

Mo-dependent nitrogenase consists of 2 separable component proteins termed the Fe protein and the MoFe protein to reflect the composition of their respective metal-containing cofactors (9, 12). The Fe protein is a homodimer containing a single [4Fe-4S] cluster and 2 binding sites for ATP (13). The MoFe protein is an $\alpha_2\beta_2$ tetramer containing two [8Fe-7S] clusters (P-clusters) and two [7Fe-Mo-9S-C-citrate] FeMo-cofactors (FeMo-cos) that are located at the sites of substrate binding and reduction (Fig. 1) (14, 15). The metal cofactors are labile to oxygen, and organisms have evolved various strategies to protect nitrogenase from oxygen inactivation (16, 17).

The Fe protein is reduced by ferredoxin (Fd) or flavodoxin (Fld) *in vivo* (18–20). Fd and Fld are small electron transfer proteins containing either FeS cluster(s) or a flavin mononucleotide (FMN), respectively (21–23). Diazotrophs typically express several Fds and Flds, and both can serve as electron donors to the Fe protein in an individual organism (24–27). During catalysis, the Fe protein switches between an ADP-bound and ATP-bound conformational state, modulating the reduction potential of the [4Fe-4S] cluster (28, 29). Fd/Fld most likely reduces the ADP-bound Fe protein preferentially, requiring Fd/Fld to have a reduction potential of at least -415 to -430 mV (Fig. 2) (30, 31) (Table 1).

Maintaining a low reduction potential is essential for efficient electron transport to nitrogenase. When discussing electron transfer mechanisms, we use standard reduction potentials ($E^{0\prime}$), which is the potential at pH 7, where the concentrations of the oxidized and reduced forms are equal. In reality, biological processes occur far from equilibrium, allowing reactions to become thermodynamically favorable, meaning a redox couple with $E^{0\prime}$ of -300 mV might be maintained at -350 mV within the cell (32). Diazotrophs utilize the disequilibrium of redox couples to sustain the production of ATP and reduce Fd for nitrogenase.

Diazotroph metabolism differs mainly in the intercellular oxidation-reduction potential and nature of primary electron carriers. For example, low potential Fd is one of the central electron carriers in anaerobic bacteria, making Fd readily available for nitrogen fixation. In

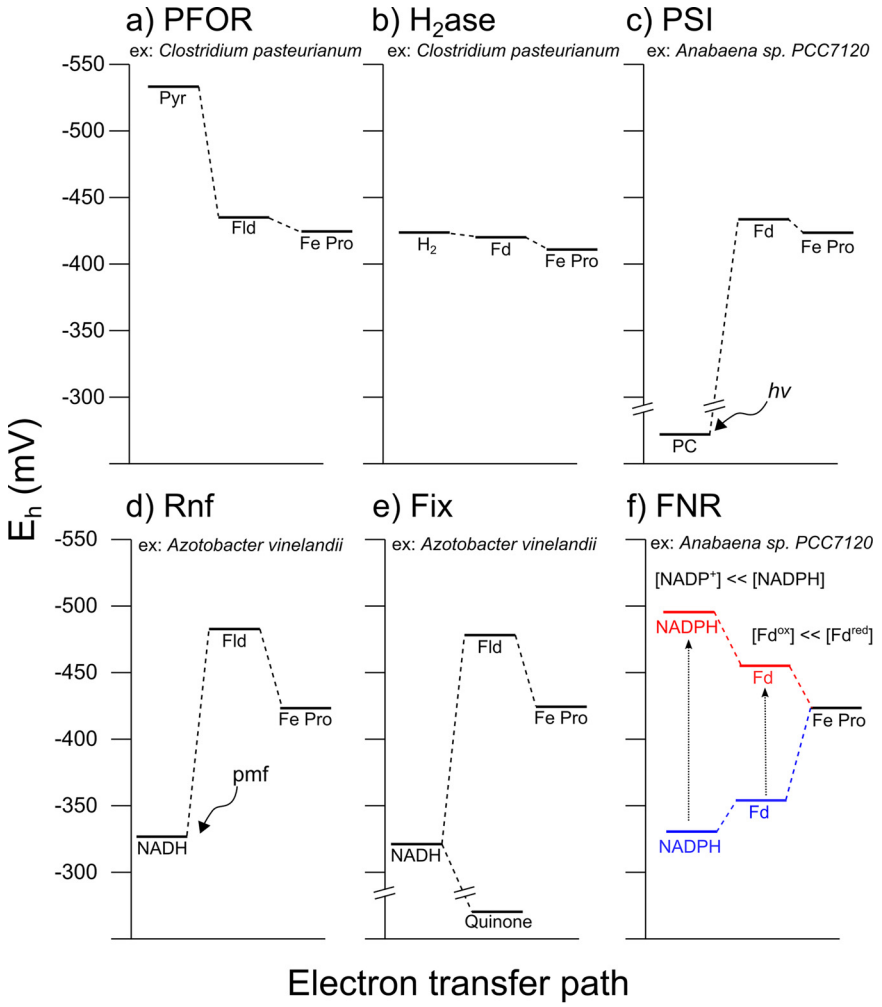

FIG 2 Electrochemical potential landscapes of electron transport mechanism to Fd/Fld and the Fe protein of nitrogenase (Fe Pro) (values in Table 1). (a) Pyruvate ferredoxin oxidoreductase (PFOR) catalyzes the reduction of Fd from pyruvate to form acetyl-CoA and CO_2. (b) Bi-directional hydrogenase (H_2ase) in *C. pasteurianum* catalyzes the reduction of Fd directly from H_2. Fd and the Fe protein of nitrogenase are at a higher potential in *C. pasteurianum* than other diazotrophs, facilitating the electron transfer. (c) Light-coupled Fd reduction in photosystem I or other reaction centers catalyzes the reduction of Fd from plastocyanin or other cytochromes. (d) Rnf catalyzes the reduction of Fd through the coupling of the proton motive force (pmf). (e) Fix couples the reduction of quinone with the reduction of Fd using electron bifurcation. (f) Ferredoxin:NADPH oxidoreductase (FNR) catalyzes Fd reduction by using the redox ratios of the NADPH and Fd. Heterocyst Fd only has a reduction potential of −351mV, which is not low enough for Fe protein reduction. An increase in the concentration of NADPH and reduced Fd will decrease the E_h (blue to red color), allowing the FNR reaction to be thermodynamically feasible.

contrast, aerobic diazotrophs carry most electrons on the NAD(P)$^+$/NAD(P)H reduction potentials at ~−320 mV and need to input energy or maintain favorable redox ratios to reduce Fd/Fld at ~−400 to −500 mV. There are 6 known or proposed mechanisms that generate low potential electrons in the form of Fd or Fld in order to donate to nitrogenase: (i) substrate-level Fd oxidoreductases, (ii) hydrogenases, (iii) photosystem I or other light-driven reaction centers, (iv) electron bifurcating Fix complexes, (v) proton motive force (*pmf*) driven Rnf complexes, and (vi) Fd:NAD(P)H oxidoreductases (Fig. 2).

OBLIGATE OR FACULTATIVE ANAEROBIC METABOLISM AND SUBSTRATE-LEVEL FD REDUCTION

During anaerobic metabolism, low potential electrons are generated through substrate-level Fd reduction. For example, obligate anaerobes *Clostridium pasteurianum* and *Desulfovibrio africanus*, and facultative anaerobes like *Klebsiella pneumoniae* and

TABLE 1 Reduction potential values and references from Fig. 2

Species	Type	Potential (mV)	Figure 2	Reference
Azotobacter vinelandii	Nitrogenase ATP bound	−430	d and e	31
Azotobacter vinelandii	Nitrogenase ADP bound	−470	d and e	31
Clostridium pasteurianum	Nitrogenase ATP bound	−415	a and b	31
Clostridium pasteurianum	Nitrogenase ADP bound	−415	a and b	31
Azotobacter vinelandii	Flavodoxin	−483	d and e	21
Azotobacter vinelandii	Ferredoxin	−619	d and e	207
Anabaena 7120	Ferredoxin heterocyst	−351	f	208
Anabaena 7120	Ferredoxin vegetative	−384	f	208
Synechocystis PCC 6803	Cytochrome c_6	+320	c	176
General	Quinone	4	e	32
General	NAD^+/ NADH (1:1)	−320	e, d, and f	32
General	NAD^+/ NADH (10:1)	−290	e, d, and f	32
General	$NADP^+$/ NADPH (1:1)	−320	e, d, and f	32
General	$NADP^+$/ NADPH (1:10)	−350	e, d, and f	32
General	$NADP^+$/ NADPH (1:100)	−380	e, d, and f	32
General	H_2	−420	b	32

Bacillus polymyxa produce Fd in CoA-dependent reactions (33–40). The half-reaction of pyruvate oxidation to acetyl-CoA and CO_2 by pyruvate-ferredoxin oxidoreductases (PFORs) has a reduction potential of −540 mV, allowing low potential Fds to act as the electron acceptor (Fig. 2a) - (Equation 2) (41–43). PFORs contain FeS clusters sensitive to oxygen, restricting PFORs from being expressed and functioning only in anaerobic conditions (44).

$$pyruvate + CoASH + 2\,Fd_{ox} \leftrightarrow acetylCoA + CO_2 + 2\,Fd_{red} + 2\,H^+ \quad (2)$$

PFORs are phylogenetically diverse, found throughout all kingdoms, and represent key steps in the Wood-Ljungdahl and reductive TCA pathways (45–47). Many PFORs are found within the genomes of diazotrophs, but only a few have been shown to support nitrogenase activity. In *K. pneumoniae*, a facultative anaerobe that only fixes nitrogen under anaerobic or microaerobic conditions, the first characterized electron transport mechanism to nitrogenase was determined to be Fd produced by PFOR (38, 48). The gene for PFOR was designated *nifJ*, and an associated flavodoxin gene was termed *nifF*. Genes homologous to *nifJ* have been found in multiple diazotrophs, but not all are associated with nitrogen fixation. In the cyanobacterium *Anabaena* 7120, its PFOR is not required for diazotrophic growth under standard conditions but is essential under Fe-limitation (49, 50). In the purple nonsulfur bacterium (PNSB) *Rhodospirillum rubrum*, there is a significant amount of PFOR activity, and purified PFOR supports pyruvate-dependent *in vitro* nitrogenase activity with purified *R. rubrum* Fd (51, 52). PFOR in *Rhodobacter capsulatus* is upregulated under nitrogen-fixing conditions, but decreased levels are found in acetate and dark aerobically grown cells (45). However, PFOR's overall contribution to nitrogen fixation in the PNSB may be low since other mechanisms, discussed later in the review, have been found to provide the primary source of low potential electrons (53–56). Overall, PFORs tend to be essential to general anaerobic metabolism and can support nitrogen fixation in obligate or facultative anaerobes.

In other anaerobic metabolisms, hydrogenases have been implicated in substrate-level Fd reduction for nitrogen fixation. Two classes of hydrogenase, [FeFe]- and [NiFe]-, catalyze the reversible oxidation of H_2 (57–59). Under standard conditions, the H^+/H_2 reduction potential is −414 mV; therefore, reducing Fd (∼ −400 to −500 mV) from H_2 is a favorable or a slightly endergonic reaction (60). Nitrogen-fixing *C. pasteurianum* produces 3 non-bifurcating [FeFe]-hydrogenases that exhibit different catalytic properties. *C. pasteurianum* hydrogenase II (CpII) is upregulated 7.5-fold under nitrogen-fixing conditions and is proposed to recapture electrons that are produced as H_2 during nitrogenase catalysis (59, 61). In *C. pasteurianum*, the standard reduction potential of Fd is −412 mV, and the ADP-bound Fe protein standard reduction potential has been

measured at −415 mV (62, 63). Thus, the reduction potentials of H_2, Fd, and Fe protein are within the thermodynamically favorable range allowing for the forward reaction and the reduction of the Fe protein from H_2 (Fig. 2b).

While hydrogenase may contribute to Fd reduction, many other reactions in anaerobic metabolisms result in Fd reduction (64). For example, during nitrogen-fixing conditions in *C. pasteurianum*, 3 distinct PFOR genes are all expressed. Under these conditions, Fld is also expressed 14-fold higher, suggesting the ability of PFORs in *C. pasteurianum* to reduce both Fd and Fld (65). Other reactions also produce reduced Fd through formate oxidation and the reduction of crotonyl-CoA to butyryl-CoA, allowing multiple pathways to deliver electrons to nitrogenase (66, 67).

Anaerobic metabolisms, either through fermentation or anaerobic respiration, yield far less ATP compared to aerobic metabolism. For example, the facultative anaerobe *K. pneumoniae* fixes nitrogen under anaerobic conditions by fermenting glucose (68). Glucose catabolism and pyruvate degradation through PFOR to acetyl-P via phosphotransacetylase, then to the final fermentation product acetate, through acetate kinase, creates 2 reduced Fds and 1 ATP (69, 70). As nitrogenase requires a 2:1 ATP to reduced Fd ratio, anaerobic nitrogen fixation is limited by ATP synthesis (71). Reductant excess is balanced by shifting to ethanol or H_2 as a final fermentation product which consumes the extra reductant but lowers the overall biomass yields (68). So, while anaerobic organisms and their reducing environment are ideal for protecting nitrogenase from oxygen and supplying electrons, overall energy expenditure is limited for optimizing growth.

ANAEROBIC LIGHT-DRIVEN FD REDUCTION

In phototrophic metabolism, reduced Fd can be generated from inputs of light energy. Bacterial FeS-type I reaction centers (RCI) found in anaerobic green sulfur bacteria and heliobacteria, as well as type I photosystems (PSI) found in cyanobacteria, reduce Fd (72) (Fig. 2c). Direct electron transfer has been proposed from Fd produced by RCI/PSI to nitrogenase. *Heliobacterium modesticaldum* is an active diazotroph with a minimal photosynthetic RC, containing just 2 subunits PshA and PshB (73). The terminal electron acceptor in PshB contains 2 Fd-like [4Fe-4S] clusters that donate electrons to Fd and is upregulated under diazotrophic conditions (74, 75). *H. modesticaldum* does have PFOR and Ferredoxin:NAD(P)H oxidoreductase (FNR) activity, but reduced diazotrophic growth rates observed under dark chemotrophic conditions suggest that RCI's have a role in providing reduced Fd for nitrogenase catalysis (76). Reduction of Fd through FeS-type RC also occurs in nitrogen-fixing green sulfur bacteria *Chlorobium tepidum*, where Fd is primarily used in the reductive TCA cycle but could also be used for nitrogen fixation (77, 78). The Fd produced by PSI in cyanobacteria can also transport electrons to nitrogenase, but other electron transport mechanisms in cyanobacteria might be favored (see section on heterocyst metabolism).

NADH OXIDATION IN AEROBIC METABOLISM

Aerobic metabolism avoids using Fd as the primary electron carrier for most redox reactions due to its sensitivity to oxygen and therefore utilizes nucleotide electron carriers $NAD(P)^+$/NAD(P)H as the primary electron source. Since nitrogenase can only accept electrons for Fd or Fld, various mechanisms are required to maintain reduced Fd/Fld production from the $NAD(P)^+$/NAD(P)H pool. Primarily, aerobic diazotrophs couple the endergonic reduction of Fd from NAD(P)H with an exergonic reaction, such as electron bifurcation or the *pmf*. Alternatively, some aerobic diazotrophs can maintain a low reduction potential by decreasing the $NAD(P)^+$/NAD(P)H ratio to overcome the thermodynamic barrier of Fd reduction in FNR.

There are multiple exergonic reactions to couple with the endergonic reduction of NAD(P)H and Fd in aerobic metabolism. One of the most studied is the flavin-based electron bifurcating enzyme Fix. The Fix enzyme was initially discovered in a genetic locus on the *Rhizobium meliloti* 2011 pSym megaplasmid that was essential for nitrogen fixation (79–

81). Some genes in this region have homology to known *K. pneumoniae nif* genes, but other genes also required for nitrogen fixation have an unknown function. These genes were given the name *fix* (82). Some of the *fix* genes, *fixABCX*, have been implicated in nitrogenase biosynthesis in *Azorhizobium caulinodans* ORS571, but others have been suggested to have a role in electron transport as FixX has amino acid sequence homology with Fd (83, 84). The FixABCX complex was finally determined to be involved in electron transfer to nitrogenase in *R. rubrum*, in which the Δ*fixABCX* strains showed an ~ 80% decrease of *in vivo* nitrogenase activity while maintaining full activity *in vitro* (54, 55).

FixABCX is a membrane-bound oxidoreductase enzyme that generates low potential electrons Fd or Fld (Fig. 2e). FixAB shares similarities to the family of electron transfer flavoproteins (ETF) found in all life, including the ETF ubiquinone oxidoreductase, which transfers electrons to the quinone pool in the membrane (85). FixABCX employs flavin-based electron bifurcation (FBEB) to overcome the energy barrier of the endergonic reduction of Fd/Fld via NADH oxidation (86, 87). FBEB couples a thermodynamically favorable exergonic reaction to drive a thermodynamically unfavorable endergonic reaction (88). Using FBEB, FixABCX couples the exergonic reduction of quinone (10 mV) and the endergonic reduction of Fd (−460 mV) to the oxidation of 2 NADH (−320 mV) in an overall thermodynamically feasible reaction (Fig. 2e) (Equation 3) (89). Recently the structure of FixABCX homolog termed EtfABCX from *Thermotoga maritima* was determined by cryo-electron microscopy providing insights into the energy landscape of this class of flavin-based electron bifurcating enzymes (90).

$$2\,NADH + CoQ + 2\,Fd/Fld^{ox} \rightarrow 2\,NAD^{+} + CoQH_2 + 2\,Fd/Fld^{Red} \quad (3)$$

Another mechanism to drive the endergonic reduction of Fd by NADH is harnessing the proton motive force. Seven genes in *R. capsulatus* were discovered to be required for nitrogen fixation called *Rhodobacter* nitrogen fixation or *rnf* (91, 92). The *R. capsulatus* genes *rnf*ABCDGEH are similar to the respiratory Na^+-dependent NADH:ubiquinone oxidoreductase (Na^+-NQR) (93, 94). While Rnf has been implicated in Fd reduction for nitrogen fixation in *R. capsulatus* (91, 92), *Pseudomonas stutzeri* (95), and *A. vinelandii* (89, 96, 97), the biochemical characterization of Rnf has been in the directionality of Fd oxidation and H^+/Na^+ pumping. In *Acetobacterium woodii*, purification and characterization of Rnf has shown Na^+-dependent reversible reduction of NAD^+ with Fd (Fig. 2d) (Equation 4) (98–101). In general, *rnf* genes are widely distributed in bacteria and required in some methanogenic archaea (102).

$$NADH + 2\,Fd_{ox} + \Delta\mu H^{+}/Na^{+} \leftrightarrow NAD^{+} + 2\,Fd_{red} + H^{+} \quad (4)$$

Recently, cryo-EM structures of Rnf from *A. vinelandii* and *Clostridium tetanomorphum* implicate strict electron and proton transfer coupling through multiple flavins and FeS clusters (103, 104). This coupling is controlled by significant conformational changes to combine the endergonic reduction of Fd with the energy available in the H^+/Na^+ motive force.

Many aerobic heterotrophs store electrons in the NAD^+/NADH electron couple with a reduction potential of -320 mV and funnel these electrons to oxidative phosphorylation in the electron transport chain (ETC). In order to produce low potential electrons in Fd, they must balance the use of Fix or Rnf with the production of ATP while maintaining a low oxygen environment. One of the most extensively studied diazotrophs is the obligate aerobic *gamma-proteobacteria*, *A. vinelandii* (105). The ability of *A. vinelandii* to fix nitrogen in fully saturated oxygen media is due to multiple mechanisms; from the creation of an extracellular alginate barrier to limit O_2 diffusion, production of superoxide dismutase and catalase to consume oxygen radicals, and as a final barrier of protection, reversible inactivation, and protection of nitrogenase via the FeSII/Shethna protein (106–109). While the above mechanism provides some level of oxygen protection, the key mechanism that supports nitrogen fixation under high oxygen tensions is respiratory protection, where a terminal oxidase consumes oxygen

at a high rate at the cell surface, preventing the accumulation of oxygen in the cytoplasm (110).

Respiratory protection is facilitated by a unique branch of the ETC, including a decoupled NADH-dehydrogenase II (NDHII) and a quinol terminal oxidase called cytochrome *bd* (111–113). This abbreviated pathway is partially coupled to the membrane, only translocating 1 H^+/e-, allowing for a high respiration rate (113, 114). As a result, accumulation of cytochrome *bd* during high aeration and nitrogen fixation is observed, and knockouts of *bd* oxidase can no longer grow under diazotrophic conditions (115). *A. vinelandii* simultaneously expresses a fully proton coupled ETC under nitrogen-fixing conditions with an NDH-I, *cytochrome* bc_1-oxidase, and cytochrome-*o* oxidases similar to complex I-IV seen in other proteobacteria and mitochondria (Fig. 3a) (115–118). The implementation of respiratory protection occurs at an energetic cost reducing the biomass yield of *A. vinelandii* and is sometimes termed a "wasting" mechanism. It has recently been proposed that this respiratory protection mechanism may be elicited in response to a high carbon and oxygen environment (119–121).

A. vinelandii possesses both Fix and Rnf mechanisms for generating low potential electrons for nitrogen fixation. The *rnf* gene cluster is located upstream from the small *nif* cluster and is regulated by *nifA*, the *nif* gene transcriptional activator. The *fix* gene cluster is not a part of the major or minor *nif* gene clusters in *A. vinelandii* but is upregulated under nitrogen-fixing conditions (122, 123). The Rnf and Fix complexes create partial redundancy for electron transport to nitrogenase, and deletion of either *rnf* or *fix* has little effect on diazotrophic growth, but the double mutant, Δ*rnf1*Δ*fix* is unable to grow diazotrophically (89). Other diazotrophs that encode for Rnf rarely have genes encoding for Fix and vice versa, making *A. vinelandii* unusual in having 2 ways to reduce Fd from NAD^+/NADH (17, 124). Recently our lab has investigated the roles of both Rnf and Fix in differing conditions and has shown that while they are redundant in standard conditions of high carbon and oxygen, Fix is required for efficient growth under low oxygen conditions (125).

Other aerobic heterotrophic diazotrophs form symbiotic relationships with plants that provide carbon and a microaerobic environment in exchange for fixed nitrogen. These organisms, which belong to the *alpha-* and *betaproteobacteria*, are generally called rhizobia and are critical for agriculture (126, 127). Root nodules of leguminous plants house specialized symbiotic rhizobia (bacteroids) within infected plant cells enclosed in plant-derived membranes called the symbiosome membrane, allowing for the exchange of nutrients and protection from oxygen diffusion (128). Rhizobia are a diverse category of bacteria with various hosts with specialized metabolism to support nitrogen fixation. Here, we generalize the production of energy and electron transport to nitrogenase in a nodule environment.

The carbon source for rhizobia is predominately C4-dicarboxylic acids, primarily succinate and malate (129). Dicarboxylates are consumed by an NAD^+-dependent malic enzyme paired with PEP-carboxykinase to produce acetyl-CoA, which can be used for carbon storage or consumption in the TCA cycle (130–132). Rhizobia express FixABCX encoded on the Sym plasmid and immediately upstream and regulated by *nif*A (133, 134). Metabolic changes are required to maintain redox balance during nitrogen fixation, including carbon storage in polyhydroxy-3-butyrate (PHB), lipids, or glycogen (135, 136). Amino acid export from the rhizobium and carbon sources from the plant also regulate redox balance in the cell with dicarboxylates creating a highly reduced environment requiring a higher oxygen demand but increasing the supply of reducing equivalents to nitrogenase (137). This balance between carbon storage, amino acid export, nitrogenase demand, and oxygen demand is critical for efficient ammonia export to the plant. Recent studies have shown that *R. leguminosarum* Δ*fixAB* mutants accumulated an order of magnitude more PHB and glycogen, suggesting balancing the redox homeostasis in the rhizobia by shuttling unused electrons to PHB or glycogen (133).

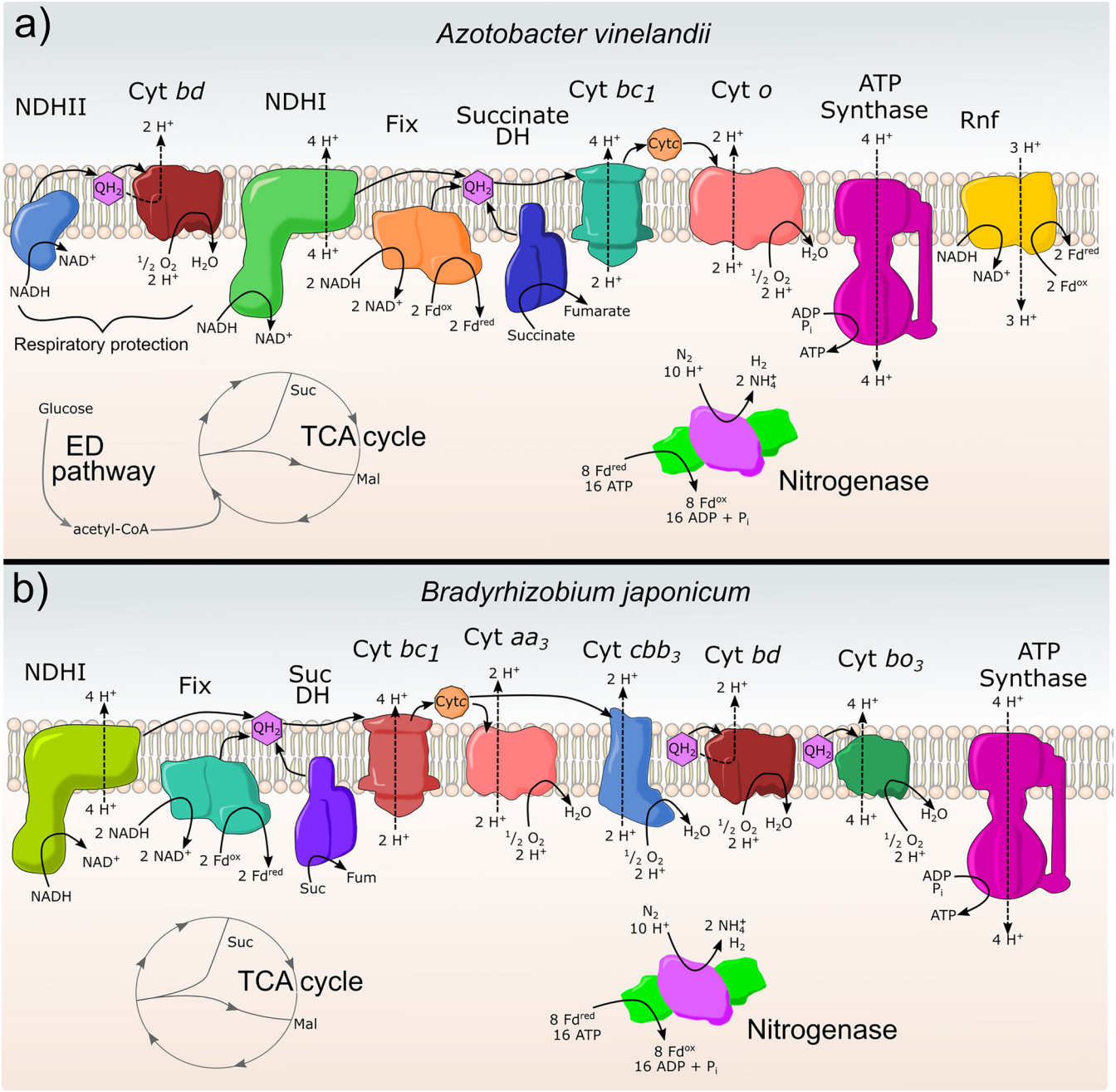

FIG 3 The electron transport systems (ETS) of free-living and symbiotic aerobic diazotrophs. (a) The ETS in *A. vinelandii* can use 2 pathways. The first (left side) is a respiratory protection pathway consisting of an uncoupled type II NADH dehydrogenase (NDHII) and terminal oxidase cytochrome *bd* (Cyt *bd*). The second path is a traditional proteobacterial electron transport chain with full proton coupled complexes: NADH dehydrogenase (NDHI), succinate dehydrogenase (Succinate DH), cytochromes *bc1* (Cyt *bc1*), and terminal oxidase cytochrome *o* (cyt *o*). The production of reduced ferredoxin (Fd) for nitrogenase is also part of the ETS with Fix and Rnf complexes. (b) Electron transport of Rhizobia. A generic look at the electron transport chain of rhizobia primarily based on data from *Bradyrhizobium japonicum*. Carbon enters the bacteroid mostly as organic acids malate (Mal) and succinate (Suc). NADH produced from the TCA cycle is oxidized by NADH dehydrogenase NDHI and produces quinol (QH_2). Succinate dehydrogenase (SucDH) also reduces quinone to QH_2. Electrons for nitrogenase are produced through the enzyme complex Fix which bifurcates electrons from NADH to quinone and ferredoxin (Fd). QH_2 is oxidized by *cytochrome* bc_1 and reduces *cytochrome c* (Cytc). There is a diversity of terminal oxidases in rhizobia: under higher oxygen concentrations, *cytochrome* aa_3 is used, while under lower oxygen conditions (like those present for nitrogen fixation), *cytochrome* cbb_3 is used. Two proton translocating quinol oxidases are also present as cytochrome *bd* and cytochrome bo_3.

Rhizobia also have complex respiratory chains with multiple terminal oxidases (Fig. 3b). Oxygen levels in the nodules are maintained at 10 to 40 nM, protecting nitrogenase from oxygen damage (138). *Bradyrhizobium japonicum* encodes 8 terminal oxidases, including 2 *bd*-type oxidases and 6 heme-copper oxidases, which can be further

divided into 2 quinol oxidases and 4 cytochrome c oxidases (139). Of these, 2 are predominant, the cbb_3-type oxidase is encoded by the *fixNOQP* genes, and the caa_3-type oxidase is encoded by *coxBACF*. The cbb_3-type oxidases have a high affinity for oxygen (K_M = 7 nM) and are essential in symbiosis and transport 0.5 H^+/e- protons across the membrane (140–142). The *cytochrome* caa_3 seems to not be essential under low oxygen concentrations in *B. japonicum* and *R. leguminosarum* but is the most prominent terminal oxidase in aerobic conditions (139, 143, 144).

Recent work has led to the proposal of combining the co-catabolism of succinate and arginine and FixABCX's electron bifurcation mechanism to maintain flux through the electron transport chain (145). The acidification of the symbiosomes may make the driving force of NADH dehydrogenase no longer sufficient, requiring FixABCX as the major NADH regenerating pathway. This proposed path, called CATCH-N, suggests a co-catabolism of 2 equivalents of succinates and 2 arginine leads to the export of 2 alanine and 6 ammonia. This pathway, which nets ~ 2 to 3 ATPs per cycle, depends on using FixABCX as the primary NADH consumer (145). The intricacy of the symbiotic metabolism of plants and rhizobia is still being investigated, but the energy-saving bifurcation of the Fix mechanism allows for maximal ammonia production in highly acidic and low oxygen environments of the symbiosome (146).

ANOXYGENIC PHOTOTROPHS

Purple nonsulfur bacteria (PNSB) are metabolically versatile, growing in aerobic or anaerobic environments as heterotrophs or autotrophs. They can also generate energy through respiration, photosynthesis, and in some cases, fermentation (147, 148). However, PNSB can only fix nitrogen anaerobically in the light, creating ATP through anoxygenic photosynthetic cyclic electron transport and maintaining carbon and electrons supply from organic substrates, such as butyrate (149). To produce ATP, PNSB use light energy absorbed by reaction center 1 (RC1) to reduce quinone to quinol using electrons donated from *cytochrome c*. *Cytochrome bc1* oxidizes quinol and reduces *cytochrome c* while translocating protons to complete the cyclic electron transport. The generated *pmf* can be used to produce ATP and NADH. Generation of NADH is achieved through the reverse reaction of NADH dehydrogenase, and consumption of *pmf* for NADH production is shown to be required for photoautotrophic growth in *R. capsulatus* and *Rhodobacter sphaeroides* (150, 151). During anaerobic phototrophic growth, PNSB must use the Calvin-Benson-Bassham (CBB) cycle as an electron sink, with the benefit of increasing biomass yield (152, 153). However, when cells become nitrogen-limited, nitrogenase becomes the electron sink, and the CBB cycle is no longer needed (154). Therefore, the cyclic electron transport of anoxygenic photosynthesis and reducing environment is ideal for nitrogen fixation.

PNSB have been shown to possess Rnf or Fix as mechanisms for transporting electrons to nitrogenase. In *Rhodopseudomonas palustris* and *R. rubrum*, FixABCX is located directly downstream of the *nif* genes, regulated by *nifA*, and essential for nitrogen fixation (27, 54, 155) (Fig. 4a). In contrast, *R. capsulatus* and *R. sphaeroides* only possess Rnf (92, 149, 156–158) (Fig. 4b). The contrast of 2 different mechanisms for Fd reduction within the same metabolic model of photoheterotrophy offers a potential comparison between Fix and Rnf. Different routes to generate biosynthetic precursors from acetate assimilation also determine the balance of electrons in PNSB. *R. sphaeroids* uses the reductive ethylmalonyl-CoA pathway, while *R. palustris* uses the oxidative glyoxylate shunt (154, 159). In *R. sphaeroids* which relies on Rnf for Fd production, the reductive ethylmalonyl-CoA pathway consumes reductant in the initial assimilation of acetate to malate and negates the use of the CBB cycle as an electron sink (159). In contrast, *R. palustris*, which relies on the Fix complex for Fd production, uses the glyoxylate shunt to produce net NADH and requires an electron sink (160). The contrasting acetate assimilation and Fd production pathways might suggest the co-evolution of Fix and Rnf within corresponding PNSB metabolism. In *R. palustris* the use of Fix means for every Fd produced and utilized as an electron sink in nitrogenase, another pair of electrons is recycled to quinol and, eventually, NADH. The less-reducing environment of *R.*

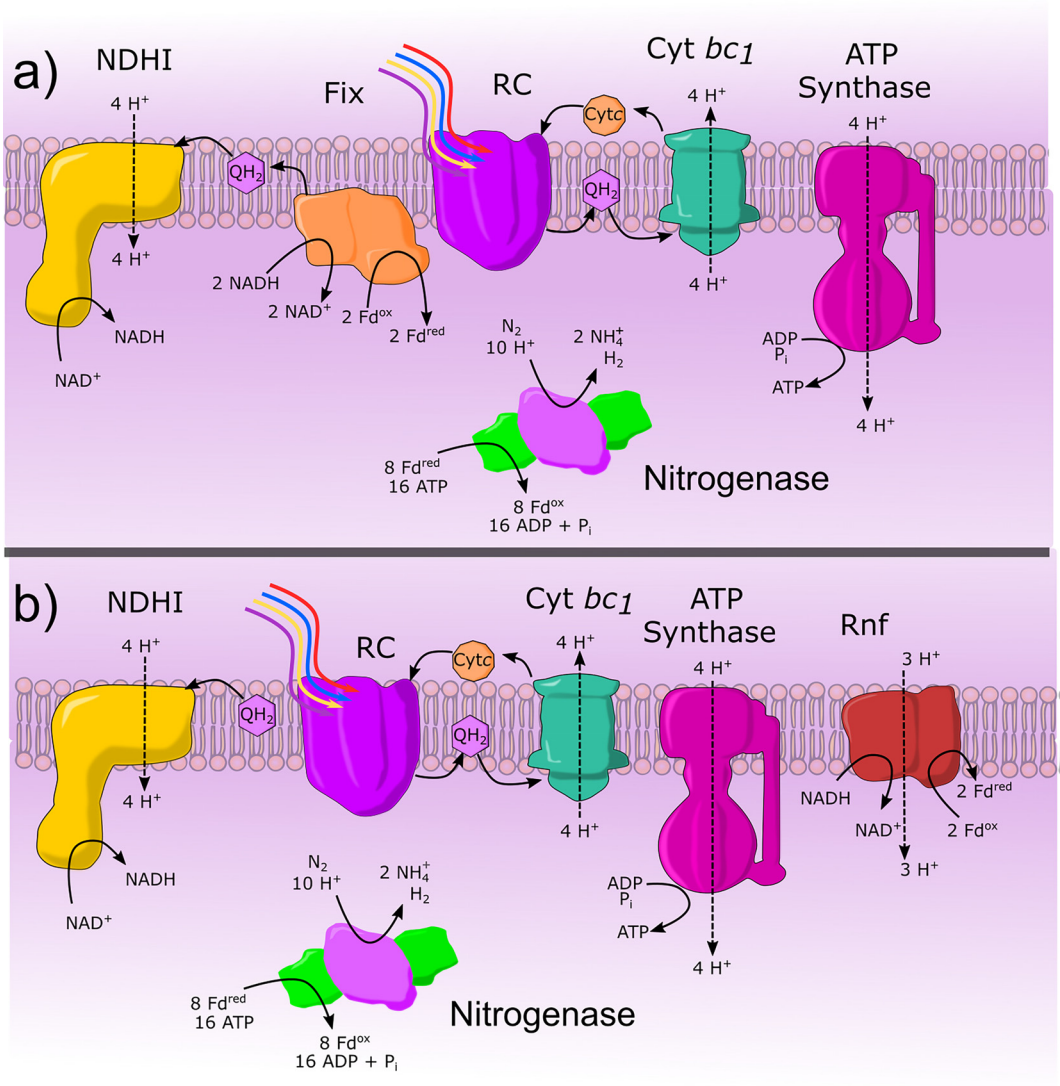

FIG 4 Electron transport to nitrogenase in PNSB. a) A generalization of Fd and ATP production in *R. palustris* and *R. rubrum* with cyclic electron transport between the rection center 1 (RC), which reduces quinone to quinol (QH_2) and oxidation of QH_2 by cytochrome bc_1 and while reducing cytochrome c (Cytc) and translocating protons, Cytc is then oxidized by RC to complete the cycle. NADH is produced by the reverse reaction of NADH dehydrogenase (NDHI). Fix catalyzes the production of Fd in *R. palustris* and *R. rubrum* through electron bifurcation where half of the electrons are directed to the reduction of quinone, either contributing to cyclic electron transport of reduction of NAD^+. b) Fd and ATP production in *R. capsulatus* and *R. sphaeroides* is very similar to the ETC of *R. palustris* and *R. rubrum* but does not contain Fix but only contains Rnf. Rnf consumes *pmf* and competes with ATP synthase and NDH-I for *pmf*.

sphaeroids uses Rnf to consume *pmf*, and there is no corresponding excess reductant. Each enzyme may play a role in balancing electron flow in certain PNSB bacteria and depend on the broader redox homeostasis of the specific metabolism.

OXYGENIC PHOTOTROPHS

While substrate-level reduction of Fd, such as that carried out by PFORs in anaerobic metabolism, is thermodynamically favorable, reducing Fd from NADH is thermodynamically unfavorable. As previously covered, enzymes overcome this by coupling Fd reduction with a thermodynamically favorable reaction, such as using the *pmf* in Rnfs, redistributing exothermic reactions with electron bifurcation in Fix, or the input of light energy in RC/PSI. Reducing Fd directly from NAD(P)H, however, is possible via FNR under high $NADPH/NADP^+$ ratios (Equation 5) (Fig. 2f).

$$NADP^+ + 2\,Fd_{red} + H^+ \leftrightarrow NADPH + 2\,Fd_{ox} \quad (5)$$

FNR was first isolated from pea thylakoids and was shown to be the final step in photosynthetic electron transport from Fd to $NADP^+$ (161, 162). FNRs ubiquity among living organisms and its diversity of cellular roles highlight the requirement for this simple electron transfer mechanism. While thermodynamically favorable in the direction of Fd oxidation and $NAD(P)^+$ reduction, many FNRs serve primarily in the reverse Fd reduction direction (163). So how does FNR overcome the energy barrier of Fd reduction and avoid added input energy mechanisms seen in Rnf, Fix, or PSI?

While there is a diversity of FNRs and well characterized FNRs in photosynthetic electron transfer, we will focus on FNRs involved in nitrogen fixation (see reviews [164, 165] for more on other FNRs). The involvement of FNR in nitrogen fixation is well characterized in the filamentous cyanobacteria *Anabaena* sp. PCC 7120, which forms specialized heterocyst cells that create a microaerobic environment to protect nitrogenase from oxygen. Heterocysts depend on vegetative cells for electrons and carbon, and vegetative cells depend on fixed nitrogen from the heterocysts, making them interdependent (166, 167).

In *Anabaena*, 2 separate transcriptional start sites produce 2 isoforms of FNR from a single gene (*petH*). There is an $\sim$ 34 kDa short FNR (FNR_S) and a longer $\sim$ 46 kDa FNR (FNR_L), which contains an extra N-terminal domain (168, 169). This N-terminal domain allows interaction with cyanobacteria light-harvesting complexes called phycobilisomes (PBS), where interaction in the FNR_L-PBS complex increases the activity toward $NADP^+$ reduction (170–172). In contrast, the FNR_S isoform accumulates in the heterocyst under nitrogen and Fe-deficient conditions and is not associated with the thylakoid membrane (173). The cellular localization suggests specific roles for the isoforms in either the forward reaction to reduce $NADP^+$ for FNR_L or the reverse reaction of Fd reduction for FNR_S. While subcellular localization and protein interaction may help facilitate the reversibility of the reaction, other studies have shown that cofactor environment and protein surface residues tune the reduction potential of the FMN cofactor (reviewed in reference [165]). While tuning the cofactors allows for reversibility, reaction rates still play a significant role in the overall directionality of the reactions. Within the heterocyst, the $NADP^+$/NADPH ratio is usually found in a reductive state of $\sim$ 0.01, powered by the fast glucose-6-phosphate dehydrogenase and oxidative pentose phosphate pathway (OPP) (32). *In vitro* studies with NADPH, FNR, oxidized Fd, and nitrogenase showed maximal nitrogenase activity with a ratio of $\sim$ 0.01 $NADP^+$/NADPH (174, 175). The reduction potential of Fd also affects the overall favorability and kinetics of the reaction. Heterocysts have a specific Fd (HtFd), expressed during nitrogen starvation, that has a higher reduction potential at -351 mV than its vegetative counterpart (VFd) at -384 mV (Fig. 2f) (176). The HtFd is important for nitrogen fixation, but other redundant electron carriers supplement electron transport when deleting the HtFd gene (177). Overall, at equilibrium, the reduction of Fd directly from NADPH is thermodynamically unfavorable; however, cell physiology, cofactor environment, and cellular localization can modulate the reaction parameters to overcome the thermodynamic barriers.

Heterocyst metabolism specializes in protecting nitrogenase from oxygen while maintaining ATP and Fd for nitrogen fixation. First, photosystem II and its water oxidizing activity is limited, while PSI is upregulated, and cyclic electron transport is used to create *pmf* for ATP synthesis (178). Carbon fixation through rubisco is not active, and heterocysts do not contain carboxysomes (179, 180). Nitrogen metabolism also shifts as the glutamine oxoglutarate aminotransferase enzymes are not expressed in heterocysts, leaving glutamine synthetase to incorporate ammonia from nitrogenase and transport glutamine back to the vegetative cells (181). An increase in respiration also occurs through 2 specialized *cytochrome c*-type terminal oxidases (182).

Energy in the form of carbohydrates, in particular sucrose, is imported to the heterocysts from the photosynthetic vegetative cells and metabolized through the OPP (183). Deriving the right balance of low potential electrons and ATP for nitrogen

fixation from the OPP pathway requires delivering electrons from NADPH to Fd, as well as to linear electron transport in the thylakoid membrane to produce ATP. Original experiments in crude extracts showed that nitrogenase activity could be measured with the addition of $NADP^+$, glucose-6-phosphate, and a renewable ATP source, demonstrating the use of the OPP pathway to produce Fd (184). In addition, nitrogenase was active both in the light and the dark but had higher activity in the light, indicating the possibility of directly reducing nitrogenase through NADPH by FNR in the dark and the thylakoid membrane supporting nitrogen fixation under light conditions (184, 185). Within the ocean environment, nitrogen fixation can occur at night, even if at a much lower rate (185, 186). Thylakoid membrane reduction of Fd also contributes to nitrogenase as dibromothymoquinone (DBMIB) which blocks the plastoquinone reduction of Cytb6/f, inhibits nitrogen fixation (187). DBMIB inhibition suggests that NADPH electrons enter linear electron transport at plastoquinone, and that PSI can produce Fd for nitrogenase.

Thylakoid membranes of cyanobacteria contain an NAD(P)H:quinone oxidoreductase named NDH-1, similar to the complex I found in mitochondria (188, 189). The NDH-1 complex, along with ATP synthase and PSI complexes, are more abundant in heterocysts cells than in vegetative cells (190, 191). Recently, Fd has been implicated as the electron donor to NDH-1 as cyanobacterial NDH-1 lacks NADH binding subunits and should be considered a ferredoxin:plastoquinone oxidoreductases (192–194). This new insight places Fd as the central electron carrier in heterocysts where Fd produced either by FNR or PSI is responsible for nitrogen fixation, linear electron transport, and terminal oxidase activity (Fig. 5).

While heterocysts maintain a microaerobic environment through morphological adaptations, consumption of excess O_2 within the heterocysts is required for nitrogenase protection. Flavodiiron protein (FDP), which reduces O_2 directly to H_2O using Fd as an electron source, is responsible for light-induced O_2 uptake in heterocysts of *Anabaena* sp. PCC 7120 (195). Multiple terminal oxidases contribute to oxygen consumption, including the heme-copper-type *cox1* and *cox2* genes encoding 2 aa_3-type terminal oxidases and a *cox3* gene cluster encoding a quinol oxidase. During nitrogen fixation, only *cox2* and *cox3* are expressed in heterocysts along with a *cytochrome* c_6 that delivers electrons specifically to the caa_3-type Cox2 terminal oxidase and is required for nitrogen fixation (196). Uptake hydrogenase (Hup) also plays a crucial role in maintaining redox status in the cell. Although they do not directly produce Fd in the heterocyst, Hups recycle H_2 from nitrogenase back into quinone (197). Heterocysts must maintain the flux of electrons to nitrogenase and through the respiratory chain to produce ATP. Then, electrons also must be prioritized in terminal oxidases and FDPs to protect nitrogenase from any excess oxygen. The total balance of these reactions has not been fully investigated.

More work is needed to elucidate the electron transport mechanism to nitrogenase in heterocysts, but a main electron transport network can be proposed. First, carbon from vegetative cells enters as sucrose and is hydrolyzed to hexoses or hexose phosphates, then metabolized through the OPP pathway to create NADPH, and this NADPH reduces Fd using the FNR_S isoform. From here, reduced Fd can be delivered directly to nitrogenase or used in linear electron flow to produce *pmf* and ATP. Electrons in reduced Fd can also be used to consume O_2 and protect nitrogenase through multiple terminal oxidases and FDPs, which limits O_2 in the cytoplasm (Fig. 5) (195, 196). As stated in the section above, FNR_S activity primarily depends on the NADPH/$NADP^+$ and Fd^{red}/Fd^{ox} ratios. Maintaining high NADPH and low reduced Fd concentrations could also be controlled by the thylakoid membranes and varying terminal electron acceptors (198).

TRANSFER OF NITROGENASE TO NON-DIAZOTROPHIC ORGANISMS

As shown above, bacterial metabolisms have been able to adapt their metabolisms to support the strict criteria of nitrogenase. These complex adaptations require specific electron transport enzymes but also a total rebalance of metabolism and redox state. Efforts

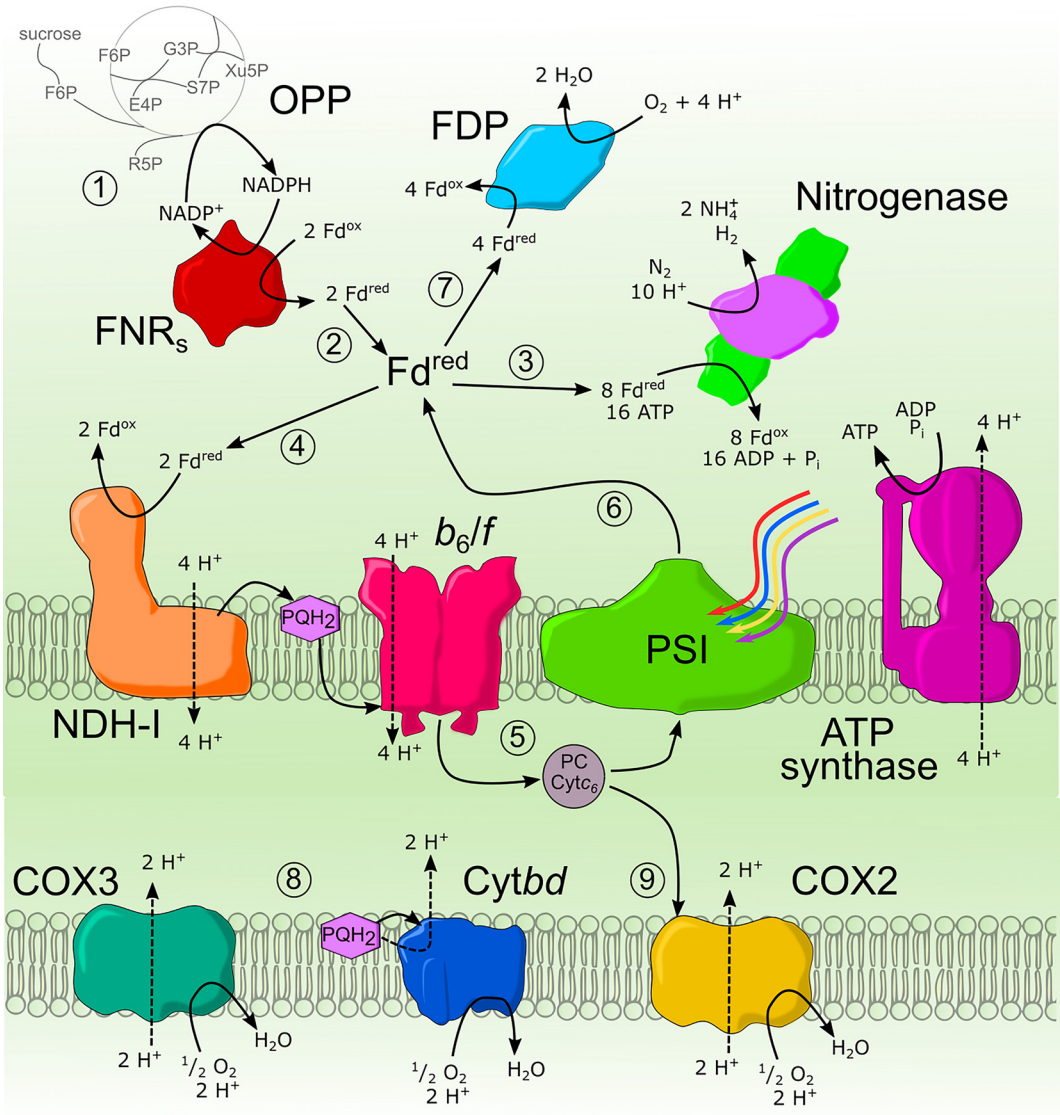

FIG 5 Electron transport in heterocyst of *Anabaena* sp PCC 7120. (1) Carbon enters the heterocyst from the vegetative cells, usually as sucrose, entering the oxidative pentose phosphate (OPP) pathway through fructose-6-phosphate (F6P) to produce NADPH. (2) NADPH is oxidized by the short isoform of ferredoxin:NADPH oxidoreductase (FNR_S). Reduced Fd then has multiple paths; the main pathway (3) would be the reduction of the Fe protein in nitrogenase for nitrogen fixation. Another path (4) for Fd is the cyclic electron transport starting by reducing plastoquinone to plastiquinol (PQH_2) in the ferredoxin:plastoquinone oxidoreductase (NDH-1). Plastiquinone is oxidized by *cytochrome* b_6/f translocating protons across the membrane, and plastocyanin (PC) or *cytochrome* c_6 ($CytC_6$) is reduced (5). Finally, photosystem 1 (PSI) oxidizes PC and uses energy from photons to reduce Fd (6), completing the cyclic electron transport and producing *pmf* for ATP synthase to produce ATP. Heterocysts have a diversity of terminal oxidases capable of reducing O_2 from multiple electron sources. First, flavodiiron proteins (FDP) can reduce O_2 directly from Fd (7). Second, 3 terminal oxidases are found in the thylakoid membrane of heterocysts. First, ubiquinol-oxidase *bd* (Cyt*bd*) and Cox3 (8), while less characterized, are not essential for nitrogen fixation but contribute to respiration. Cox2 (9) exclusively accepts electrons from $Cytc_6$ and translocate protons for ATP synthase.

to transfer nitrogenase into non-diazotrophic organisms have become a promising strategy to overcome the disadvantages of industrial nitrogen fertilizers in agriculture (199–201). Significant advances have been made in synthesizing nitrogenase subunits or its biosynthetic enzymes in yeast, plant mitochondria, and non-diazotrophic cyanobacteria (202–205). In addition to the challenges of synthesizing a working nitrogenase in non-diazotrophic organisms, the metabolic context around nitrogenase must be considered. Yang et al. tested plant FNRs and Fd's ability to reduce nitrogenase in an *E. coli* construct. The authors first showed that *Klebsiella oxytoca* PFOR reduced chloroplast and root-plastid Fds, and these reduced plant Fds could support nitrogenase activity *in vivo* (206).

Further, they tested the ability of plant FNRs and plant Fd to supply nitrogenase with electrons and saw a restoration of ~ 45% of nitrogenase activity compared to *K. oxytoca* PFOR and Fld. This review highlights that many mechanisms can provide low potential reducing equivalents for nitrogen fixation, but each mechanism is adapted for a specific role in each diazotroph. Total physiology, such as maintenance of $NADP^+$/NADPH ratios, Fd reduction potentials, and oxygen protection mechanisms, must be considered if the goal is to support nitrogen fixation with plant FNRs in plant organelles such as root plastids.

Conclusion. The reduction of N_2 by nitrogenase is one of the most energy-intensive and complex reactions in biology. A large amount of energy is required, and nitrogenase's susceptibility to oxygen damage limits its potential in aerobic environments. Even with the intense evolutionary pressure of nitrogen limitation, life has not been able to reduce N_2 through any other enzymatic mechanism, nor has it made the requirements for the nitrogenase reaction conditions less stringent. Diazotrophs utilize specific electron transport enzymes depending on the mode of metabolism to supply low potential electrons for nitrogenase catalysis such as substrate-level Fd oxidoreductases, hydrogenases, photosystem I or other light-driven reaction centers, electron bifurcating Fix complexes, proton motive force-driven Rnf complexes, and Fd:NAD(P)H oxidoreductases. Despite the diversity in pathways and enzymes, each diazotroph has the same criteria of maintaining a microaerobic environment, producing low potential electrons, and providing a high amount of ATP to maximize flux to nitrogenase.

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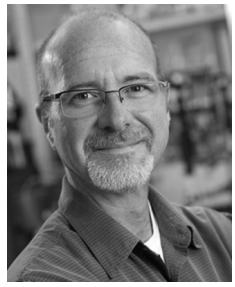

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