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The path of electron transfer to nitrogenase in a phototrophic alpha-proteobacterium

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Summary

The phototrophic alpha-proteobacterium, *Rhodopseudomonas palustris*, is a model for studies of regulatory and physiological parameters that control the activity of nitrogenase. This enzyme produces the energy-rich compound H_2, in addition to converting N_2 gas to NH_3. Nitrogenase is an ATP-requiring enzyme that uses large amounts of reducing power, but the electron transfer pathway to nitrogenase in *R. palustris* was incompletely known. Here, we show that the ferredoxin, Fer1, is the primary but not sole electron carrier protein encoded by *R. palustris* that serves as an electron donor to nitrogenase. A flavodoxin, FldA, is also an important electron donor, especially under iron limitation. We present a model where the electron bifurcating complex, FixABCX, can reduce both ferredoxin and flavodoxin to transfer electrons to nitrogenase, and we present bioinformatic evidence that FixABCX and Fer1 form a conserved electron transfer pathway to nitrogenase in nitrogen-fixing proteobacteria. These results may be useful in the design of strategies to reroute electrons generated during metabolism of organic compounds to nitrogenase to achieve maximal activity.

Introduction

Biological nitrogen fixation is catalyzed by the enzyme nitrogenase, which reduces nitrogen gas (N_2) to ammonia (NH_3). H_2 is also formed as a product of nitrogenase activity. Nitrogenase is restricted to Euryarchaeota among the Archaea but is widely distributed among Bacteria and is particularly widespread among the proteobacteria (Boyd *et al.*, 2011a, 2015). Nitrogen fixation requires large amounts of ATP and electrons as shown in the following equation:

$$N_2 + 8H^+ + 16ATP + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (1)$$

In all microbes studied, the direct electron donor to nitrogenase is either a ferredoxin (Fd) or a flavodoxin (Fld). Fds coordinate FeS clusters, whereas Flds use a flavin mononucleotide (FMN) to mediate electron transfer. The Fld, NifF, has been shown to be the sole electron donor to nitrogenase in the gamma-proteobacterium, *Klebsiella pneumoniae*, but in other bacteria, Fds or a combination of Fds and Flds have been implicated in electron transfer to nitrogenase (St. John *et al.*, 1975; Roberts *et al.*, 1978; Klipp *et al.*, 1989; Martin *et al.*, 1989; Jouanneau *et al.*, 1995; Gennaro *et al.*, 1996; Edgren and Nordlund, 2005; 2006; Yang *et al.*, 2016; Pence *et al.*, 2017). Most nitrogen-fixing bacteria encode several Fds and Flds and the extent to which some are preferred over others *in vivo* is not well explored (Poudel *et al.*, 2018).

Here, we sought to address the question of preferred electron donor to nitrogenase in the phototrophic alpha-proteobacterium *R. palustris*. *R. palustris* has served as a model organism for *in vivo* studies of production of energy-rich compounds including H_2 and more recently, methane, by nitrogenase (Gosse *et al.*, 2010; Fixen *et al.*, 2016; Zheng *et al.*, 2018). This organism encodes the electron-transfer complex FixABCX, which bifurcates electrons from NADH to generate reduced quinone and reduced Fd or Fld (Edgren and Nordlund, 2006; Ledbetter *et al.*, 2017). Mutants with deletions in *fixC* or *fixX* are unable to grow under nitrogen-fixing conditions, suggesting that FixABCX is required for electron transfer to nitrogenase (Huang *et al.*, 2010). Genomic analyses indicate that *R. palustris* encodes multiple Fd proteins and one Fld (Larimer *et al.*, 2004).

Here, we show that in *R. palustris* the Fd, Fer1 (RPA4631), is the primary but not sole electron donor utilized during nitrogen fixation, and a flavodoxin, FldA

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(RPA2117), is an important electron donor for nitrogen fixation under low-iron conditions. We present a model in which FixABCX is capable of reducing both Fd and Fld for the reduction of nitrogenase. Using comparative genomics, we present evidence that FixABCX from many nitrogen-fixing proteobacteria likely interacts with specific Fds and Flds that are phylogenetically related to those that we found to be important for nitrogenase activity in *R. palustris*.

Results

The FixABCX complex is required for nitrogen fixation in R. palustris

Two components of the FixABCX complex, FixC (RPA4603) and FixX (RPA4602), were previously shown to be required for nitrogen fixation by *R. palustris* CGA009 (Huang *et al*., 2010). To determine if the other two components, FixA (RPA4605) and FixB (RPA4604), are also required, we constructed in-frame deletion mutants of *fixA* and *fixB*. As expected, neither of these mutants grew under nitrogen-fixing conditions, but both grew as well as the wild-type parent when ammonium was supplied as a nitrogen source (Fig. 1 and Table 1). Growth of the *R. palustris fixA* or *fixB* mutant was restored under nitrogen-fixing conditions by complementation with the corresponding wild type allele *in trans* (Fig. 1A). This indicates that, like FixC and FixX, FixA and FixB are required for nitrogen-fixation by *R. palustris* and that FixABCX is the only enzyme used to generate low potential electrons for nitrogenase in this organism.

Fer1 is the primary electron donor to nitrogenase

In vitro studies with FixABCX from *Azotobacter vinelandii* showed that this complex bifurcates electrons from NADH to generate reduced quinone and reduced Fd or Fld as the primary electron donor to nitrogenase (Ledbetter *et al*., 2017). *R. palustris* has three Fd genes in its *nif* gene cluster (*rpa4602-rpa4634*): *fdxB* (*rpa4612*), *ferN* (*rpa4629*) and *fer1* (*rpa4631*), all of which are upregulated under nitrogen-fixing conditions (Oda *et al*., 2005) (Supporting Information Fig. S1). RNA-seq analysis indicates that Fer1 is the most highly expressed (4Fe-4S) Fd in *R. palustris* under nitrogen-fixing conditions (Supporting Information Fig. S1). Two other (4Fe-4S) Fds that are encoded by *R. palustris* were not differentially expressed under nitrogen-fixing conditions. One of these, *badB* (*rpa0662*), functions in anaerobic benzoate degradation (Boll and Fuchs, 1998), and the other, *fdxA* (*rpa1733*), has no known function.

We constructed in-frame deletion mutations in each of the nitrogenase gene cluster-associated Fd genes in *R. palustris* cells and found that the *fer1* deletion mutant had a growth defect under nitrogen-fixing conditions (Fig. 1 and

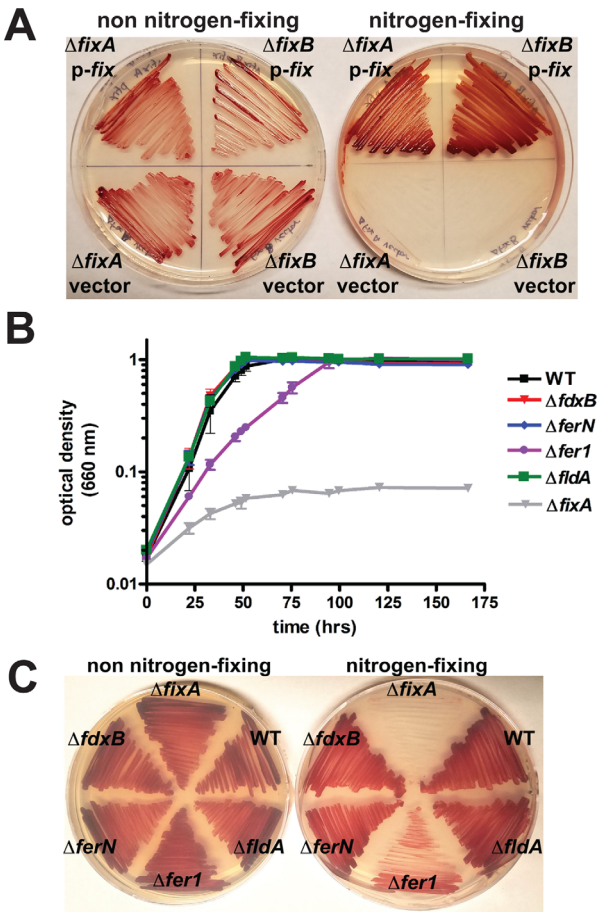

Fig. 1. FixABCX and the ferredoxin, Fer1, are important for nitrogen fixation in *R. palustris*.
A. *R. palustris fixA* or *fixB* deletion mutants grew on minimal medium with ammonium sulfate (non-nitrogen-fixing) but were unable to grow on minimal medium without ammonium sulfate (nitrogen-fixing). Growth was restored on nitrogen-fixing medium by complementation with p-*fix*, a plasmid encoding the corresponding wild type allele *in trans*.
B. Growth of WT *R. palustris* or *R. palustris* Δ*fdxB*, Δ*ferN*, Δ*fer1* or Δ*fldA* mutants in nitrogen-fixing medium. Data are from three independent cultures and error bars represent one standard deviation.
C. Growth phenotypes of single mutants in Fd or Fld genes on minimal medium with ammonium sulfate (non-nitrogen-fixing) or on minimal medium without ammonium sulfate (nitrogen-fixing).

Table 1). As expected, this mutant also had reduced *in vivo* nitrogenase activity (Fig. 2A). To determine if this was due to impaired electron transfer to nitrogenase, as opposed to the possibility that Fer1 was playing role in nitrogenase assembly, we assayed *in vitro* nitrogenase activity. Unlike the *in vivo* assay, which uses whole cells, the *in vitro* assay uses cell lysates with dithionite added as an electron donor. If the primary role of Fer1 in nitrogen fixation is to donate electrons to nitrogenase, then dithionite should be able to circumvent the requirement for Fer1 *in vitro*. As shown in Fig. 2B, *in vitro* nitrogenase activity was not

Table 1. Growth rates of *fix*, ferredoxin (*fdxB*, *ferN* and *fer1*) and flavodoxin (*fldA*) deletion mutants under non-nitrogen-fixing and nitrogen-fixing conditions.

	Doubling time (h)[a]			
Genotype	Non-N_2-fixing	N_2-fixing	Non-N_2-fixing (low iron)[b]	N_2-fixing (low iron)
WT	7.3 ± 0.1	12 ± 0.7	12.5 ± 1.4	40 ± 1.5
Δ*fixA*	7.3 ± 0.3	> 100	12 ± 0.7	> 100
Δ*fixB*	7.2 ± 0.2	> 100	ND	ND
Δ*fixX*	7.2 ± 0.2	> 100	ND	ND
Δ*fdxB*	8.7 ± 0.7	11 ± 2.1	ND	ND
Δ*ferN*	7.9 ± 0.1	15 ± 1.6	ND	ND
Δ*fer1*	7.3 ± 0.2	18 ± 0.4	11 ± 1.8	56 ± 6.4
Δ*fer1* pBBRMCS-5	8.0 ± 0.3	22 ± 4.0	ND	ND
Δ*fer1* p-*fer1*	8.2 ± 0.2	12 ± 2.5	ND	ND
Δ*fldA*	7.4 ± 0.1	11 ± 1.8	11 ± 0.9	50 ± 5.5
Δ*fdxB ferN*	7.5 ± 0.4	14 ± 0.6	ND	ND
Δ*fdxB fer1*	8.2 ± 0.5	19 ± 1.7	ND	ND
Δ*ferN fer1*	8.2 ± 0.3	22 ± 1.4	ND	ND
Δ*fldA fer1*	7.4 ± 0.3	20 ± 0.3	ND	> 100
Δ*fdxB ferN fer1*	7.9 ± 0.1	21 ± 1.0	ND	ND
Δ*fldA ferN fer1*	7.6 ± 0.4	> 100	ND	> 100

ND, not determined.
a. All doubling times are the averages from three independent experiments and are shown in hours ± standard deviation.
b. Nitrogen-fixing medium with $FeSO_4$ omitted.

impaired and actually increased in the Δ*fer1* mutant compared to wild-type cells, indicating that Fer1 functions as a direct electron donor to nitrogenase and is not involved in another function that might affect nitrogenase activity, such as metallocluster biosynthesis. Increased nitrogenase activity in the *in vitro* assay was likely due to the increased levels of nitrogenase observed in the *R. palustris* Δ*fer1* mutant (Fig. 2C). As shown in Fig. 2C, all strains with a deletion in *fer1* had increased levels of NifH, which is most likely a response to the nitrogen limitation experienced by these mutants.

The growth defect observed for the *R. palustris* Δ*fer1* mutant was not as severe as the growth defects of the *fixA*, *fixB* or *fixX* deletion mutants suggesting that another Fd may be able to compensate for Fer1. To test this possibility, *R. palustris* cells with deletions in *fer1* and *ferN* or *fer1* and *fdxB* were constructed and grown under nitrogen-fixing conditions. As shown in Fig. 3 and Table 1, the *fer1 ferN* double deletion mutant exhibited a more pronounced growth defect than the *fer1* single deletion mutant. *R. palustris* cells with all three Fds deleted showed a growth defect similar to that of the *fer1 ferN* double deletion mutant (Table 1). This indicates that FerN but not FdxB is functionally redundant with Fer1. This is perhaps not surprising since FerN shares 69% amino acid identity with Fer1.

R. palustris encodes one flavodoxin, FldA. The flavodoxin, NifF, in *K. pneumoniae*, *A. vinelandii* and *Rhodobacter capsulatus* has been shown to be involved in electron transfer to nitrogenase (Nieva-Gómez *et al.*, 1980; Bennett *et al.*, 1988; Martin *et al.*, 1989; Yakunin *et al.*, 1993; Gennaro *et al.*, 1996), and we wondered if FldA could be involved in electron transfer to nitrogenase in *R. palustris*. The *fldA* gene is outside of the *nif* gene cluster in *R. palustris*, and FldA is not an ortholog of NifF (Pérez-Dorado *et al.*, 2013) (Supporting Information Fig. S1). However, expression of *fldA* is up-regulated more than fourfold under nitrogen-fixing conditions (Oda *et al.*, 2005; Supporting Information Fig. S1). As shown in Fig. 1 and Table 1, a *R. palustris* Δ*fldA* mutant did not have a growth defect under nitrogen-fixing conditions, but as FerN, FldA is functionally redundant with Fer1 (Fig. 3 and Table 1). A *R. palustris fer1 fldA* double deletion mutant grew more slowly than a *fer1* mutant when grown under nitrogen-fixing conditions. Moreover, a *fer1 ferN fldA* triple deletion mutant was unable to grow under nitrogen-fixing conditions indicating that there is functional redundancy between the two Fds and Fld (Fig. 3 and Table 1). Collectively, these data suggest that FixABCX can donate electrons to both Fd and Fld *in vivo*.

The role of the electron donor flavodoxin during nitrogen fixation under low iron conditions

It is clear that Fer1 is the major electron donor to nitrogenase and that FerN and FldA are functionally redundant with Fer1. It is unclear, however, whether *R. palustris* encounters conditions in which electron transfer by Fer1 is impaired. One such condition might be iron limitation, as under such conditions, cells would be expected to be

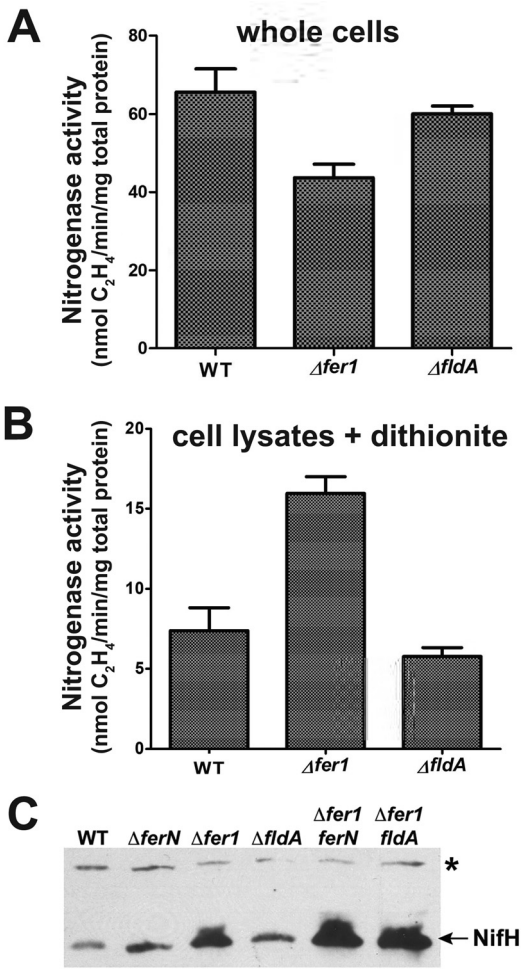

Fig. 2. Fer1 directly donates electrons to nitrogenase.
A. Nitrogenase activity determined using acetylene reduction by whole cells of *R. palustris* WT, Δ*fer1* or Δ*fldA* mutants. Error bars represent the standard error mean from two independent cultures.
B. Nitrogenase activities in *R. palustris* WT, Δ*fer1* mutant or Δ*fldA* mutant cell lysates with dithionite provided as the electron donor. Dithionite circumvents the need for a Fd or Fld to serve as an electron donor. Error bars represent standard error mean from two independent cultures.
C. Levels of NifH, the dinitrogenase reductase component of nitrogenase, in *R. palustris* WT, Δ*ferN*, Δ*fer1*, Δ*fldA*, Δ*ferN* Δ*fer1* or Δ*fer1* Δ*fldA* mutants grown in nitrogen-fixing medium. An equal amount of total protein (8 μg) was loaded per lane. A nonspecific band is marked with an asterisk.

impaired in their ability to synthesize FeS clusters. In some organisms, expression of Fld is induced by iron-limiting conditions and Fld expression is used as a marker for low-iron conditions in marine environments (Laudenbach *et al.*, 1988; La Roche *et al.*, 1996; Achenbach and Genova, 1997).

To test the role of Fer1 and FldA in nitrogen fixation under low-iron conditions, wild-type *R. palustris* and *R. palustris* mutants unable to synthesize Fer1 or FldA were grown in nitrogen-fixing medium in which no exogenous iron was added (low iron). Trace amounts of iron are likely still present in this medium, but as shown in Table 1, omitting exogenous iron from the medium significantly inhibits its growth of *R. palustris*. Under low-iron, non-nitrogen-fixing conditions, the *fldA* deletion mutant grew similar to wild-type, indicating that FldA is not required for growth under low iron, non-nitrogen-fixing conditions (Table 1). However, as shown in Fig. 4A and Table 1, under low-iron, nitrogen-fixing conditions, the *fldA* deletion mutant grew more slowly than the wild-type. Similar to the Δ*fer1* mutant, the Δ*fldA* mutant expressed nitrogenase enzyme that was active *in vitro* with dithionite as an electron donor indicating that an active nitrogenase is synthesized in the Δ*fldA* mutant and FldA is likely directly donating electrons to nitrogenase (Fig. 2B). Although FldA is important for nitrogen fixation under low-iron conditions, it was necessary to construct a *fer1 ferN fldA* triple mutant to completely disrupt growth under low-iron, nitrogen-fixing conditions, which shows that Fer1 and FerN can still contribute to electron transfer even under low-iron conditions (Fig. 4A and Table 1).

A role for flavodoxin under low-iron conditions also suggests that expression of the genes encoding FldA and the Fds may be regulated by iron availability. Using qPCR, relative expression levels of *fldA*, Fd genes and *fixX* were determined in wild-type *R. palustris* grown in nitrogen-fixing medium with no additional iron (0 μM), 2.5 μM iron or

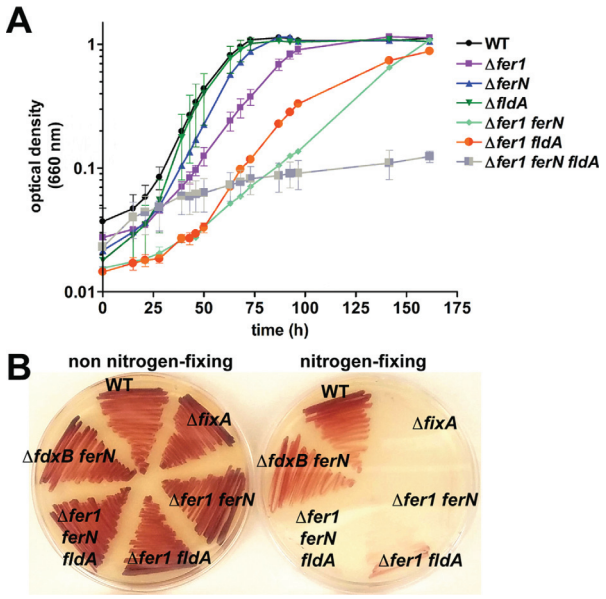

Fig. 3. The ferredoxin, FerN, and the flavodoxin, FldA, also play a role in electron transfer to nitrogenase.
A. Growth of WT *R. palustris* and Δ*ferN*, Δ*fer1*, Δ*fldA* or the triple deletion mutant in nitrogen-fixing medium. Data are from three independent cultures and error bars represent one standard deviation.
B. Growth phenotypes of double mutants in Fd or Fld genes or a triple *ferN fer1 fldA* deletion mutant on minimal medium with ammonium sulfate (non-nitrogen-fixing) or on minimal medium without ammonium sulfate (nitrogen-fixing).

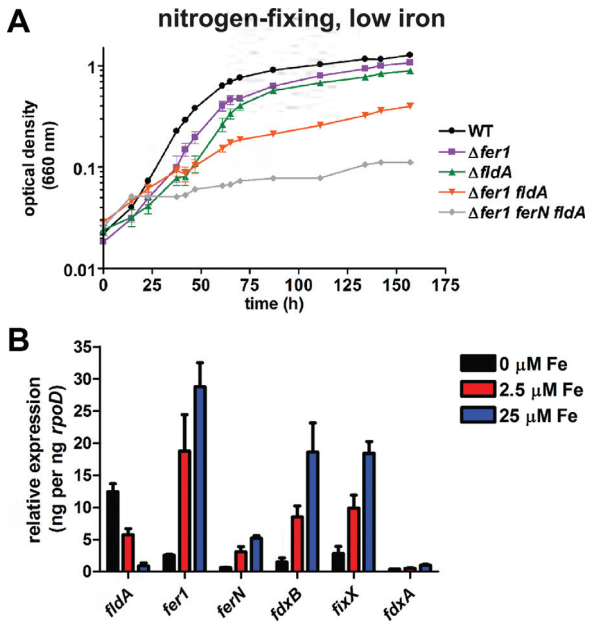

Fig. 4. FldA is an important electron donor under low iron conditions.
A. Growth of *R. palustris* WT and mutant derivatives in nitrogen-fixing medium with no added $FeSO_4$ (low iron conditions). Data are from three independent cultures and error bars represent one standard deviation.
B. Relative transcript levels of genes encoding FldA, FixX and Fds in WT *R. palustris* grown in nitrogen-fixing medium with no added $FeSO_4$ (0 μM) or $FeSO_4$ added to a final concentration of 2.5 or 25 μM.

25 μM iron added. As shown in Fig. 4B, expression of *fldA* was inversely correlated with the amount of iron added to nitrogen-fixing medium. The highest level of *fldA* expression was observed in medium with the lowest iron concentration, and the lowest level of *fldA* expression was observed in medium with the highest iron concentration, whereas the opposite was true for the Fd genes. This indicates that the expression of Fld and Fd in *R. palustris* is regulated, at least in part, by iron availability and is consistent with the observation that FldA plays a more important role in electron transfer under low-iron conditions.

Co-occurrence of Fer1, FerN and FldA homologs with FixABCX

From the above work, we can conclude that FixABCX is the major pathway for electron transfer during nitrogen fixation in *R. palustris* and is likely involved in reducing the Fds, Fer1 and FerN and the Fld, FldA. While this genetic analysis shows a clear functional linkage between FixABCX and Fer1, FerN and FldA in *R. palustris*, we wondered if this is true for other nitrogen-fixing organisms that encode *fixABCX*.

Among the 4,588 available bacterial and archaeal genomes, 359 were identified as being from putative nitrogen-fixing microbes since they encoded for the minimum components needed to form an active nitrogenase (i.e., NifHDKEB; Boyd *et al.*, 2011a,b). Of the 359 genomes, 28.4% (n=102) encode FixABCX (Supporting Information Table S1). The taxonomic distribution of Fer1, FerN and FldA in the 359 genomes was also determined. Additionally, homologs of the *A. vinelandii* Fld, NifF, were determined since NifF is not an ortholog of FldA and has been shown (Nieva-Gómez *et al.*, 1980; Gennaro *et al.*, 1996) and hypothesized (Poudel *et al.*, 2018) to be involved in electron transport to nitrogenase in pathways that do not involve FixABCX. Fer1 and FerN were detected only in proteobacteria with the exception of one Verrucomicrobia genome that encoded FerN (Supporting Information Table S1). Fer1 was identified in 64 genomes whereas FerN was identified in 29 genomes, while 15 genomes encoded both Fer1 and FerN. NifF was identified in 44 genomes, whereas FldA was identified in 60 genomes.

Co-occurrence analysis of gene orthologs is a method that has been used to gain insight into potential molecular-level interactions among proteins (Kim and Price, 2011). Co-occurrence analysis revealed that Fer1, FerN and FldA co-occurred with FixABCX in the genomes of nitrogen-fixing bacteria. Microbes that do not encode FixABCX tend to encode NifF and rarely FldA but not Fer1 or FerN (Fig. 5A and Supporting Information Table S1). As expected, correlation analysis indicates that the distribution of FixABCX is strongly correlated with the distribution of Fer1 and to a lesser extent FerN in genome sequences (Fig. 5B). Taken together, these observations are consistent with the notion that diazotrophs that encode FixABCX and Fer1 likely use Fer1 as the primary electron carrier.

Discussion

Known electron transfer pathways to nitrogenase include pyruvate:ferredoxin/flavodoxin oxidoreductase (PFOR/NifJ), the *Rhodobacter* nitrogen fixation (Rnf) complex, hydrogenases and FixABCX, and a number of nitrogen-fixing organisms can utilize more than one pathway (Edgren and Nordlund, 2006; Herrmann *et al.*, 2008; Khanna and Lindblad, 2015; Poudel *et al.*, 2018; Ledbetter *et al.*, 2017). *R. palustris* does not encode Rnf, but it encodes genes that are annotated as PFOR as well as FixABCX. *R. palustris* also encodes an uptake NiFe-hydrogenase, but strain CGA009, used in these studies, has a defect in expression of the uptake hydrogenase (Rey *et al.*, 2006). Here, we found that FixABCX is required for electron transfer to nitrogenase in *R. palustris*. Neither PFOR nor any other enzyme is able to reduce Fd or Fld sufficiently for *R. palustris* to grow under nitrogen-fixing conditions.

FixABCX uses a process called Flavin-Based Electron Bifurcation (FBEB), to transfer electrons from NADH to the

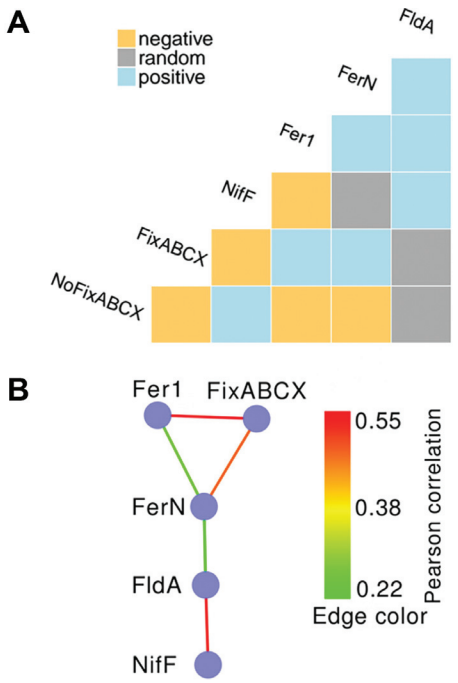

Fig. 5. FixABCX and Fer1 form a conserved electron transfer pathway to nitrogenase in nitrogen-fixing bacteria.
A. Co-occurrence analysis of the distribution of Fds (Fer1 and FerN) and Flds (FldA and NifF) in genomes that encode for Fix (FixABCX) or that do not encode for Fix (no FixABCX). Positive co-occurrence (exceeding the 95% statistical significance threshold for greater than expected co-occurrences) is represented in cyan, negative co-occurrence (actual co-occurrences less than the 5% statistical significance threshold for expected co-occurrences) is represented in yellow, and random (exceeding the 5% statistical significance but below 95% of the threshold for greater than expected co-occurrences) co-occurrence is represented in grey.
B. Network map depicting the correlation among genomes that encode for FixABCX, Fer1, FerN, FldA and NifF. Edges between nodes (depicting specified protein) represent correlations between those nodes or more specifically, those proteins. The force directed organic layout within Cytoscape was used to visualize the correlation in the network.

high potential acceptor, coenzyme Q1, which allows for reduction of a low potential acceptor like Fd (Buckel and Thauer, 2013; Peters *et al.*, 2016). The reduced Fd^{2-} generated via electron transfer from NADH serves as the low potential electron donor to nitrogenase. *In vitro*, Fd can be replaced by the Fld semiquinone during a FBEB event (Chowdhury *et al.*, 2016; Ledbetter *et al.*, 2017). Here, we show *in vivo* evidence that FixABCX from *R. palustris* can also donate electrons to Fd and Fld (Fig. 6).

In *R. palustris*, the Fd, Fer1, is the major electron donor to nitrogenase. Fer1 does not appear to play a major role in cofactor biosynthesis and assembly of nitrogenase. This is also the case for FldA (Fig. 2). A *fer1 ferN fldA* triple deletion mutant exhibited a phenotype similar to Δ*fixA*, Δ*fixB*, Δ*fixC* or Δ*fixX* mutant. This indicates that Fer1, FerN and FldA can each receive electrons from FixABCX (Fig. 6). The functional redundancy shared by Fer1, FerN and FldA suggests that *R. palustris* may encounter conditions in which Fer1 is not functional. Under low-iron conditions, the use of FldA, which relies on flavin rather than FeS clusters for electron transfer, would allow the cell to cope with iron limitation. Indeed, we found that cells that cannot synthesize FldA are impaired in their ability to grow under low-iron, nitrogen-fixing conditions.

FerN and Fer1 are 69% identical, and FerN and Fer1 homologs in *Rhodospirillum rubrum* are involved in direct electron transfer to nitrogenase (Edgren and Nordlund, 2005). These observations support a model in which FerN can act as a direct electron donor to nitrogenase in the absence of Fer1. However, it is possible that the predominant role of FerN is to contribute to nitrogenase activity in some other capacity, such as metallocluster biosynthesis, and functional redundancy between FerN and Fer1 is only relevant under synthetic conditions in which Fer1 or FerN synthesis has been disrupted. We have also shown that the third Fd encoded in the *nif* gene cluster, FdxB, is not functionally redundant with Fer1 or FerN, and it is unclear what role, if any, it plays in nitrogen fixation. Further investigation into the role of FerN and FdxB is needed to understand the physiological function of these Fds.

We suggest that FixABCX and Fer1 form a conserved electron transfer pathway to nitrogenase in other nitrogen-fixing proteobacteria. This hypothesis is supported by co-occurrence analysis of gene homologs, which shows that

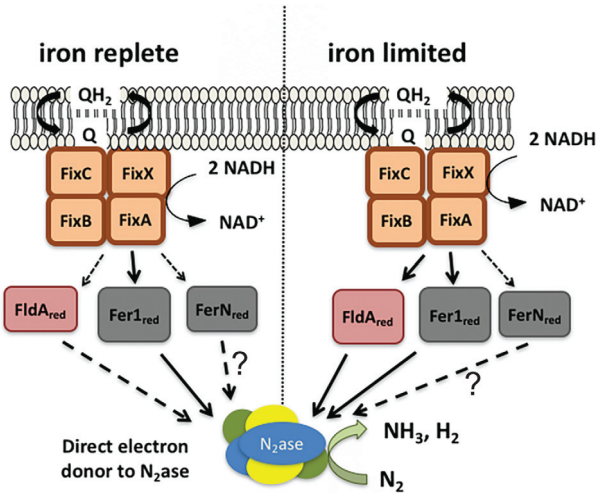

Fig. 6. Electron transfer pathway to nitrogenase in *R. palustris* in iron-replete and iron-limiting conditions.
FixABCX bifurcates electrons from NADH to generate reduced quinone and reduced Fd or Fld. The Fd, Fer1, is the major e^- donor to nitrogenase under iron-replete conditions. Under iron-limiting conditions, expression of the Fld, FldA, increases and FldA acts as a major electron donor to nitrogenase. Solid arrows depict the major route of electron transfer to nitrogenase, while arrows with dotted lines depict possible alternative routes for electron transfer to nitrogenase.

nitrogen-fixing bacteria that encode FixABCX also tend to encode Fer1 and to a lesser extent FerN and FldA. Nitrogen-fixing microbes that do not encode FixABCX and that thereby likely rely on another electron transfer pathway like PFOR or Rnf for generation of reduced Fd or Fld, are more likely to encode NifF. This suggests that there is specificity for which Fd/Fld can interact with FixABCX and nitrogenase.

Fd and Fld act as intracellular 'wires' that connect electron donors and electron acceptors. Understanding the factors that determine Fd or Fld specificity for certain partners could allow new connections to be engineered between different electron donors and acceptors. If electron transfer to nitrogenase is the limiting step for its activity, then rerouting more electrons to this enzyme may help to maximize its activity.

Experimental procedures

Bacteria and growth conditions

All studies were carried out with *R. palustris* wild-type strain CGA009 or its mutant derivatives. Cells were grown aerobically during genetic manipulation on defined mineral medium (PM) (Kim and Harwood, 1991) agar supplemented with 10 mM succinate at 30°C. All *R. palustris* strains were grown anaerobically in PM liquid culture or plates supplemented with 20 mM acetate or in nitrogen-fixing medium (NFM) liquid culture or plates supplemented with 20 mM acetate. NFM is the same as PM but with ammonium sulfate omitted. Liquid growth medium was deaerated and then dispensed into culture tubes in an anaerobic glove box, and the tubes were sealed with rubber stoppers. N_2 gas was provided in the headspace of sealed culture tubes. Plates were incubated in a GasPak™ EZ anaerobe container system with indicator (Becton Dickinson). Anaerobic cultures were incubated at 30°C with light (30 μmol photons m^{-2} s^{-1}) provided from a 60 W incandescent light bulb (General Electric). NFM described above contains 2.5 μM $FeSO_4$. NFM with no added $FeSO_4$ or 25 μM $FeSO_4$ was used for low and high iron conditions. *Escherichia coli* S17-1 was grown in LB medium at 37°C. When appropriate, *R. palustris* was grown with gentamicin at 100 μg ml^{-1}. *E. coli* cultures were supplemented with gentamicin 20 μg ml^{-1}.

Acetylene reduction assay

Both *in vivo* and *in vitro* nitrogenase activities were measured by monitoring the production of ethylene from acetylene by gas chromatography. *Rhodopseudomonas palustris* wild type, Δ*fer1*, Δ*fldA* cells were grown in NFM containing 2.5 μM $FeSO_4$ to an OD_{660} of 0.4–0.6. Half of the culture was transferred to a 16 ml rubber septum-sealed tube containing argon to measure nitrogenase activity of intact cells. The tube was ventilated to atmospheric pressure and acetylene was injected. Tubes (in triplicate) were kept in front of a 60 W lamp and gas phase samples (100 μl) were withdrawn with a Hamilton sample lock syringe from the tube headspace. Ethylene produced by nitrogenase activity was measured over time as previously described (Oda *et al*., 2005).

For the *in vitro* nitrogenase activity, 50 ml of cells were grown to an OD_{600} of 1.1. Cell-free extracts of wild type, Δ*fer1*, Δ*fldA* cells were prepared by bead beating in a degassed 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$ (Buffer A). The supernatant, obtained by centrifugation at 60,000 × *g* for 30 min at 4°C was transferred to an 8 mL serum vial containing an ATP regeneration system (5 mM ATP, 30 mM phosphocreatine, 0.1 mg ml^{-1} creatine phosphokinase, 2 mg ml^{-1} BSA) and 15 mM sodium dithionite in Buffer A under an argon atmosphere. Acetylene was then injected into the serum vial to start the assay. Ethylene production over time was measured using gas chromatography (Oda *et al*., 2005; Yang *et al*., 2012). Total protein concentrations were determined using the Bio-Rad Protein assay kit.

Genetic manipulation of R. palustris. In-frame deletion constructs of *fdxB* (*rpa4612*), *ferN* (*rpa4629*) and *fer1* (*rpa4631*) were prepared by In-Fusion cloning of PCR amplified fragments that included the start codon plus 1 kb upstream from the start codon and the stop codon plus 1 kb downstream from the stop codon of each gene. An in-frame deletion construct of *fldA* (*rpa2117*) was constructed by PCR amplification of its start codon plus 1 kb upstream of the start codon and 1 kb downstream from codon 154. Genomic DNA purified from *R. palustris* CGA009 was used as the template for amplification with Phusion High-Fidelity DNA polymerase (New England Biolabs). The two 1 kb fragments for each gene were incorporated into PstI-digested pJQ200SK using the In-Fusion PCR cloning system (Clontech). All plasmids were mobilized into *R. palustris* by conjugation with *E. coli* S17-1, and double-crossover events for deletions or allelic exchange were achieved using a previously described selection and screening strategy (Rey and Harwood, 2010). All deletions were verified using PCR.

Quantitative PCR (qPCR) analysis. RNA was prepared from cultures grown to an OD_{660} of 0.4–0.6 as described previously (Oda *et al*., 2005). Primers for qPCR are listed in Supporting Information Table S2. Reactions were performed using Ssofast Evagreen Supermix (Bio-Rad) with 1 ng cDNA as template and 500 nM of each primer in a final reaction volume of 25 μL, and cycling parameters were set per optimized cycling conditions for a CFX96 qPCR machine (Bio-Rad). Standard curves for quantification of transcripts were generated using dilutions of *R. palustris* chromosomal DNA from 1×10^{-4} to 10 ng. Transcript levels of the gene tested were normalized to transcript levels of *rpoD*.

Compilation of homologs. Genomes ($n = 359$) that encode for Mo-nitrogenase were compiled. Genomes were screened for FixABCX via BLASTp analysis using FixA (WP_011665895), FixB (WP_011665894), FixC (WP_011665893) and FixX (WP_011665892) from *R. palustris* as queries. Likewise, genomes were screened for homologs of Fds (Fer1 and FerN) and Flds (FldA and NifF) using Fer1 (ACF03601), FerN (ACF03599) and FldA (ACF00922) from *R. palustris* and NifF (AGK18380) from *A. vinelandii* as BLASTp queries. All BLASTp analyses specified 30% sequence identities and 60% sequence coverage.

Statistical and network analysis. A binary table was generated based on the presence and absence of a complete complement of genes coding for FixABCX (Garcia Costas *et al.*, 2017), Fds (Fer1 and FerN) and Flds (FldA and NifF) in the nitrogenase-encoding genomes. The table was then used as an input for co-occurrence analysis using the co-occurrence package in R (Griffith *et al.*, 2016). The binary table was also subjected to Pearson correlation analysis to identify correlations among the distribution of genes encoding the Fds/Flds and FixABCX using the correlation package in Cytoscape (version 3.2.0) (Smoot *et al.*, 2011). The correlation was viewed as a network using the force directed organic layout in Cytoscape.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Taxonomic distribution of Fd, Flds and FixABCX homologs in the genomes of putative nitrogen-fixing microbes.

Table S2. Strains, plasmids and primers used.

Fig. S1. The gene encoding Fer1 is the most highly expressed electron donor under nitrogen-fixing conditions.

A. The *nif* gene cluster in *R. palustris* encodes *fixABCX* (*rpa4602-rpa4605*) and three Fds – *fdxB* (*rpa4612*), *ferN* (*rpa4629*) and *fer1* (*rpa4631*). Gene functions are annotated as described in Oda *et al*. (2005).

B. Genomic context of *fldA* (*rpa2117*).

C. The relative transcript levels measured as reads per kilobase per million uniquely mapped reads (RPKM) are shown for all genes encoding a putative 4Fe-4S Fd or a Fld in *R. palustris* wild-type cells grown photoheterotrophically in minimal medium with ammonium sulfate (non-nitrogen fixing) or without ammonium sulfate (nitrogen fixing).