FEMS Microbiology Ecology, 92, 2016, fiw084

doi: 10.1093/femsec/fiw084
Advance Access Publication Date: 21 April 2016
Minireview

MINIREVIEW

A survey of the energy metabolism of nodulating symbionts reveals a new form of respiratory complex I

Mauro Degli Esposti[1,2,*] and Esperanza Martinez Romero[1]

[1]Center for Genomic Sciences, UNAM, Cuernavaca, 62210, Mexico and [2]IIT, Genoa 16163, Italy

*Corresponding author: Italian Institute of Technology, Nanotoxicology, Via Morego, 30, Genoa 16163, Italy. Tel: 0039 010 71781987; E-mail: mauro.degliesposti@iit.it

One sentence summary: This review examines in depth fundamental aspects underlying the efficiency of nitrogen fixation in nodulating symbionts of plants that have not been considered before.

Editor: Gerard Muyzer

ABSTRACT

A limiting factor in agriculture is the availability of nitrogen in the soil, which may be compensated by biological N_2 fixation catalysed by bacteria. Most biological N_2 fixation occurs in root nodules of plants that respond to bacterial infection by establishing symbiosis with selected prokaryotes. The plants provide energy metabolites and a microoxic environment to the bacterial symbionts to facilitate their capacity of N_2 fixation. Despite enormous advances in the molecular genetics of this symbiosis between plants and nodulating bacteria, we still do not understand fundamental aspects which determine the efficiency of N_2 fixation in the nodules, and therefore the capacity to biologically enrich agricultural soils. Here we provide an overview of the central features of the energy metabolism that sustains N_2 fixation, with emphasis on the bacterial respiratory chain supplying the electrons and ATP required for the nitrogenase reaction. We discuss common trends and specific variations in the integrated process of respiratory electron transport and N_2 fixation. Among such variations we introduce green complex I, an ancient version of NADH:ubiquinone oxidoreductase that is present in several Rhizobiaceae and may facilitate N_2 fixation.

Keywords: nitrogen fixation; nodulating symbionts; TCA cycle; respiratory complex I; ubiquinone

INTRODUCION

Fixation of atmospheric N_2 is an essential biological process carried out by nitrogenases (Young 2005) that convert gaseous N_2 to ammonia (NH_3 or NH_4^+) in a variety of bacteria collectively known as diazotrophs (Bothe *et al.* 2010; Ormeño-Orrillo, Hungria and Martinez-Romero 2013). The great majority of these prokaryotes fix N_2 under anaerobic or microoxic conditions because their nitrogenase enzyme is inactivated by oxygen (Hoffman *et al.* 2014). Nitrogenase catalyses a complex redox reaction requiring two molecules of ATP per each electron and proton transferred to N_2 for producing NH_3, releasing H_2 as a by-product (Bothe *et al.* 2010; Hoffman *et al.* 2014). Soluble ferredoxins or similar low potential redox proteins, e.g. flavodoxins (Shah, Stacey and Brill 1983), function as electron donors to nitrogenase (Bothe *et al.* 2010; Hoffman *et al.* 2014) and are reduced by different metabolic systems (Fig. 1). These systems together with proton-pumping ATP synthase supply electrons and ATP to the nitrogenase reaction at the expense of other redox and ATP-dependent functions of the cell. Hence, N_2 fixation imposes a considerable energetic cost to diazotrophic organisms.

Several bacteria have evolved mutualistic associations with plants that provide the metabolic fuel and microoxic conditions to efficiently sustain N_2 fixation, a reaction that they rarely carry out as free-living organisms (Dixon and Kahn 2004; Hirsch and Mauchline 2015). These organisms belong to a few groups of proteobacteria (Martínez-Romero 2009; Martínez-Romero *et al.* 2010; Hirsch and Mauchline 2015) that are generally called rhizobia after the genus *Rhizobium*, which originally was found in

Received: 3 February 2016; **Accepted:** 18 April 2016
© FEMS 2016. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

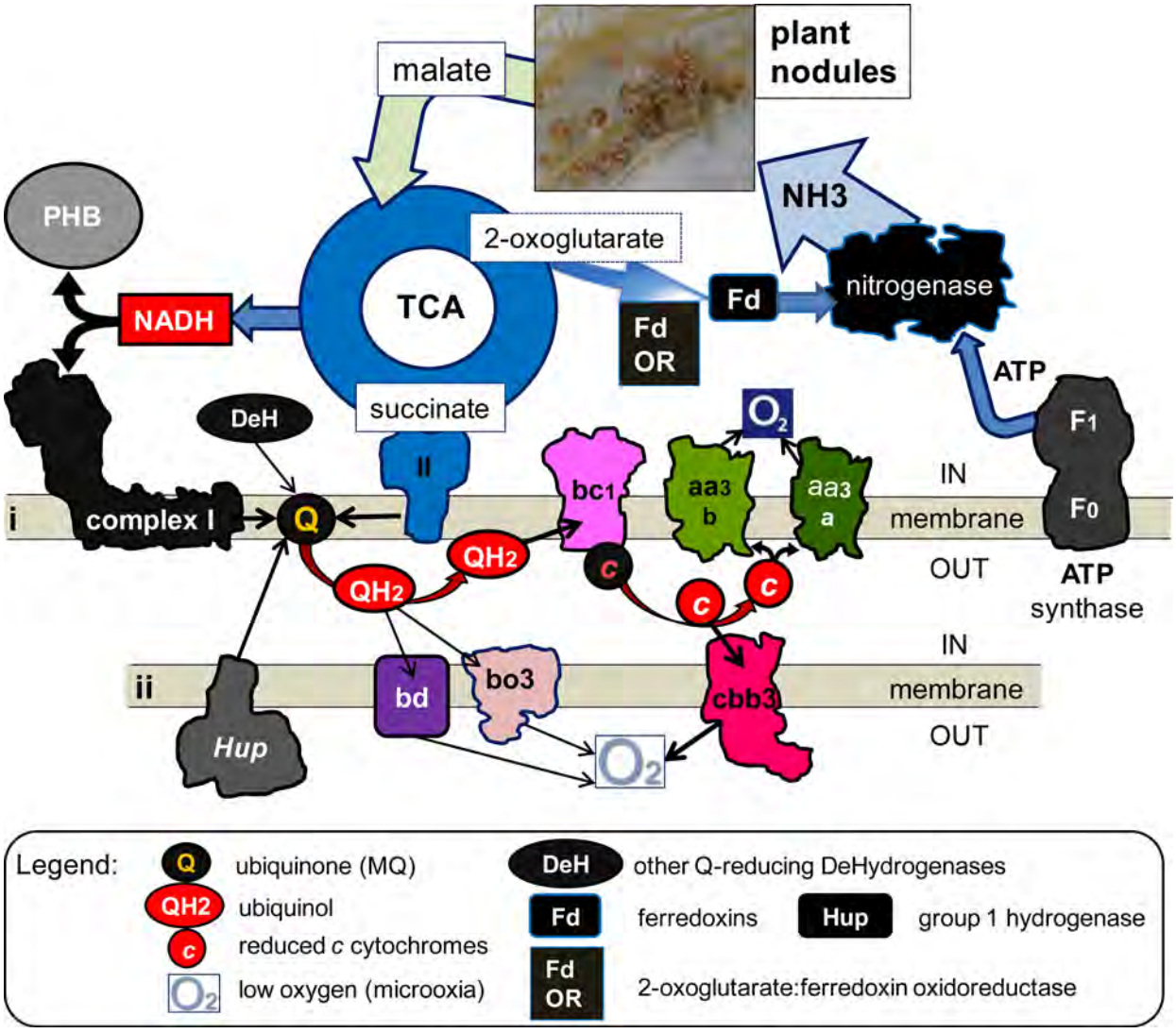

Figure 1. The energy metabolism and respiratory chain of nodulating symbionts. The illustration presents a general overview of the bioenergetic enzyme complexes of rhizobial respiratory chains and is organized in two sets. Set **i** contains the connection between the TCA cycle and the respiratory chain via complex II (succinate dehydrogenase), as well as respiratory complexes that are common to aerobic proteobacteria and mitochondria, with two different aa3-type cytochrome *c* oxidases: COX operon types b and a—for instance type a-III in *S. fredii* NGR234 (Li *et al.* 2013; cf. Degli Esposti *et al.* 2014). Set **ii** contains Q-reducing group 1 hydrogenase (Vignais and Billoud 2007), usually called *Hup* hydrogenase (Leyva *et al.* 1990), and three terminal oxidases that react with low concentrations of oxygen: bd-type and bo3-type quinol oxidase (Li *et al.* 2013; cf. Degli Esposti *et al.* 2014; Youard *et al.* 2015) and cbb3-type cytochrome *c* oxidase, which is strongly overexpressed in both nodulating symbionts and microaerobic conditions (Pessi *et al.* 2007; Vercruysse *et al.* 2011). The potential bypass of the TCA cycle that occurs in both *Frankia* and *Bradyrhizobium* with the upregulation of 2-oxoglutarate:ferredoxin oxidoreductase (FdOR, see the text) is indicated by the pale blue arrow. The electron carriers that accumulate as a result of bacterial adaptation to the microaerobic conditions and metabolic changes in the nodules are highlighted in white over a red background: NADH and ubiquinol from the TCA cycle and reduced cytochrome *c* from electron transport maintained via the bc_1 complex (and possibly other cytochrome *c* reducing enzymes such as alcohol dehydrogenases, not shown). The enzyme complexes are represented with silhouettes defining the contour of the published crystal structures in relation to the lipid membrane, rendered in grey. This information is missing for bd-type ubiquinol oxidase, which is therefore represented by a rectangle. IN indicates the cytoplasmic side and OUT the periplasmic side of the bacterial membrane. Malate is indicated as the predominant metabolic fuel provided by the plant host to the bacterial symbionts (Udvardi and Poole 2013). PHB, poly-hydroxy-butyrate.

association with root nodules of leguminous plants (Quispel 1988). Nodules are specialized structures formed in response to bacterial infection of plant roots, in which these bacteria become capable of fixing nitrogen after undergoing differentiation into symbiotic bacteroids (Haag *et al.* 2013; Udvardi and Poole 2013; Czernic *et al.* 2015). The process of nodulation is intimately linked to symbiotic N_2 fixation in specific associations between bacteria and plants, which are responsible for a large proportion of the enrichment of N compounds in the soil (Dixon and Kahn 2004; Haag *et al.* 2013; Hirsch and Mauchline 2015; Yates *et al.* 2015). Before the introduction of chemical fertilizers, legumes were used in crop rotation to increase nitrogen in the soil, a procedure which recently has become more important as a result of the increase in 'organic' agriculture all over the world and the environmental and biological impact of chemical fertilizers (Hirsch and Mauchline 2015; Yates *et al.* 2015). Consequently, there is increasing interest in nodulating bacterial symbionts and their efficiency of fixing N_2 in the field (Yates *et al.* 2015; http://www.n2africa.org, accessed 14 December 2015).

Concerted efforts by researchers and producers are directed to find suitable matches between bacteria and plant hosts in diverse agricultural environments (Martínez-Romero 2009)

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

and also under stress conditions imposed by soil erosion and climate changes (Zahran 1999; Hungria and Vargas 2000; Hirsch and Mauchline 2015; Yates *et al.* 2015). Unfortunately, results obtained under controlled laboratory conditions do not easily translate into increased efficiency of N_2 fixation in the field (Burgos *et al.* 1999). Currently, there is abundant genomic information on rhizobia (Ormeño-Orrillo *et al.* 2012; Althabegoiti *et al.* 2014; Rogel *et al.* 2014; Siqueira *et al.* 2014; Kumar *et al.* 2015; Remigi *et al.* 2016) but the biochemical characterization of genetic diversity is scant, particularly in regard to the bioenergetic and metabolic systems that efficiently sustain the nitrogenase reaction (outlined in Fig. 1).

Different levels of nitrogen-fixing capabilities have been found among distinct rhizobia strains in legume symbioses (Martínez-Romero 2009) and some of those having superior levels have been chosen for use as inoculants in agricultural fields to improve crop yields (Hungria *et al.* 2000, 2006; Hungria, Campo and Mendes 2003; Yates *et al.* 2015). The molecular genetic basis for different levels of N_2 fixation by rhizobia is understood only in a few cases, but not in depth. For example, some strains that are capable of recycling H_2 (Baginsky *et al.* 2002; Brito *et al.* 2005) have been found to present higher N_2-fixing capabilities (Baginsky *et al.* 2005). After works published in the pregenomics era (de Hollander and Stouthamer 1980; Miller *et al.* 1988; O'Brian and Maier 1989; Soberón, Aguilar and Sánchez 1993; Delgado, Bedmar and Downie 1998), there are no recent reviews on the respiratory chain of rhizobia, a crucial aspect of N_2 fixation, although the topic is covered to some extent in Geddes and Oresnik (2014) and Youard *et al.* (2015). To fill this knowledge gap, we present here an in-depth overview of the energy metabolism and respiratory chain of nodulating symbionts that encompasses currently available information on genomics and transcriptomics, defining common trends and specific variations in the integrated pathways of energy metabolism and N_2 fixation.

The respiratory chain and N_2 fixation in diazotrophs

The earliest studies on the bioenergetics of N_2 fixation focused on proteobacterial diazotrophs including *Klebsiella pneumoniae* and *Azotobacter vinelandii* (for a review, see Poole and Hill 1997). The information accumulated with these organisms indicated that the TCA cycle and the reoxidation of its products by the respiratory chain provide the fundamental supply of electrons and ATP required by the nitrogenase reaction (for a review, see Poole and Hill 1997; Dunn 1998; Poole and Allaway 2000). Such essential concepts are summarized in Fig. 1, which illustrates the key link between the TCA cycle and the low potential electron carriers that directly feed electrons to nitrogenase, ferredoxins or flavodoxins. For instance, *K. pneumoniae* possesses an alternative form of pyruvate dehydrogenase, pyruvate:flavodoxin oxidoreductase (Schmitz, Klopprogge and Grabbe 2002), which is typical of anaerobic bacteria using a reverse version of the TCA (rTCA; Campbell *et al.* 2009). Bypassing the step feeding into the TCA cycle, this enzyme reduces a flavodoxin that then transfers electrons directly to the nitrogenase enzyme under anaerobic conditions (Shah, Stacey and Brill 1983; Hill and Kavanagh 1980) and is also present in environmental *Klebsiella* taxa (Martínez-Romero *et al.* 2015). An important and often overlooked result of the above bypass is the diminished substrate flow into the TCA cycle with consequent limited production of NADH, which would normally accumulate under microaerobic conditions due to the block of respiratory activities (see below). Moreover, *Klebsiella* possesses two terminal oxidases that reoxidize ubiquinol even at very low levels of oxygen, therefore attenuating the ubiquinol overload deriving from enhanced activity of Q-reducing enzymes (Fig. 1). These are the ubiquinol oxidases of the bd- and bo3-type shown in set ii of Fig. 1, which constitute the typical terminal oxidases of proteobacteria adapted to microaerobic environments such as the guts of animals (Chouaia *et al.* 2014).

2-Oxoglutarate:ferredoxin oxidoreductase (FdOR, Fig. 1) is an alternative enzyme for the 2-ketoglutarate dehydrogenase step of the TCA cycle which is also typical of anaerobic organisms (Baughn *et al.* 2009; Campbell *et al.* 2009). This enzyme could bypass the ketoglutarate dehydrogenase step of the TCA cycle that is most sensitive to the accumulation of NADH and often rate limiting in the whole cycle (Green and Emerich 1997; Dunn 1998; Poole and Allaway 2000), producing the most common reductants for the nitrogenase reaction, ferredoxins (Fig. 1). The same step of the TCA cycle is alternatively bypassed by another enzyme, succinate semialdehyde dehydrogenase, in nodulating symbionts of the *Bradyrhizobium* genus (Green *et al.* 2000; reviewed by Udvardi and Poole 2013). The activity of this enzyme does not produce reduced ferredoxins and thus may simply alleviate the accumulation of NADH generated by a fully functional TCA cycle under oxygen-limited conditions. Our survey on the transcriptomics of nodulating symbionts has uncovered the upregulation of FdOR enzymes in the nodulating actinomycete, *Frankia alni* (Fig. 2A; cf. Alloisio *et al.* 2010). This enzyme is also present in duplicate copies in the genome of related organisms such as the *Frankia* symbiont of *Datista glomerata* (Persson *et al.* 2015; Louis Tisa, pers. comm.) and, albeit at a lower level, in *Bradyrhizobium diazoefficiens* USDA 110 (Table S1, Supporting Information; cf. Pessi *et al.* 2007). Hence, the bypass of the TCA cycle that can directly reduce ferredoxins is present in ancestral nodulating symbionts of the *Frankia* genus.

Being obligate aerobes or microaerophiles, Rhizobiaceae have lost another ferredoxin reductase system that is expressed in (facultatively) anaerobic bacteria, namely the NADH:ferredoxin oxidoreductase described by Klugkist, Haaker and Veeger (1986). This activity, which is associated with the cytoplasmic membrane where the respiratory complexes are plugged in, predominantly derives from the expression of the RnfABCDGE complex, an Na^+-translocating ferredoxin:NAD^+ oxidoreductase complex that is also present in fermenting anaerobic bacteria (Biegel *et al.* 2011). The complex feeds electrons to nitrogenase in the photosynthetic diazotroph, *Rhodobacter capsulatus* (Schmehl *et al.* 1993) as well as in the N_2-fixing endophyte *Azoarcus* (Krause *et al.* 2006). In *A. vinelandii*, the NAD(P)H:oxidoreductase activity now attributed to the Rnf complex in relation to N_2 fixation (Klugkist, Haaker and Veeger 1986; Haaker and Veeger 1977) gave rise to the concept of 'respiratory protection' of the nitrogenase enzyme by the redox activity of respiratory complexes. This concept generated the paradox of N_2 fixation dependence upon a respiratory chain that needs to work under conditions of low oxygen to avoid inactivation of nitrogenase (Bertsova, Bogachev and Skulachev 1998; for a review, see Poole and Hill 1997).

The respiratory chain of nodulating symbionts and its problems

Rhizobiaceae are aerobic bacteria that can adapt to the microaerophilic conditions of root nodules (Encarnación *et al.* 1995). Indeed, they lack the NADH:ferredoxin oxidoreductase capacity of feeding electrons to nitrogenase via the RnfABCDGE complex of *R. capsulatus* (Schmehl *et al.* 1993; for a review,

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

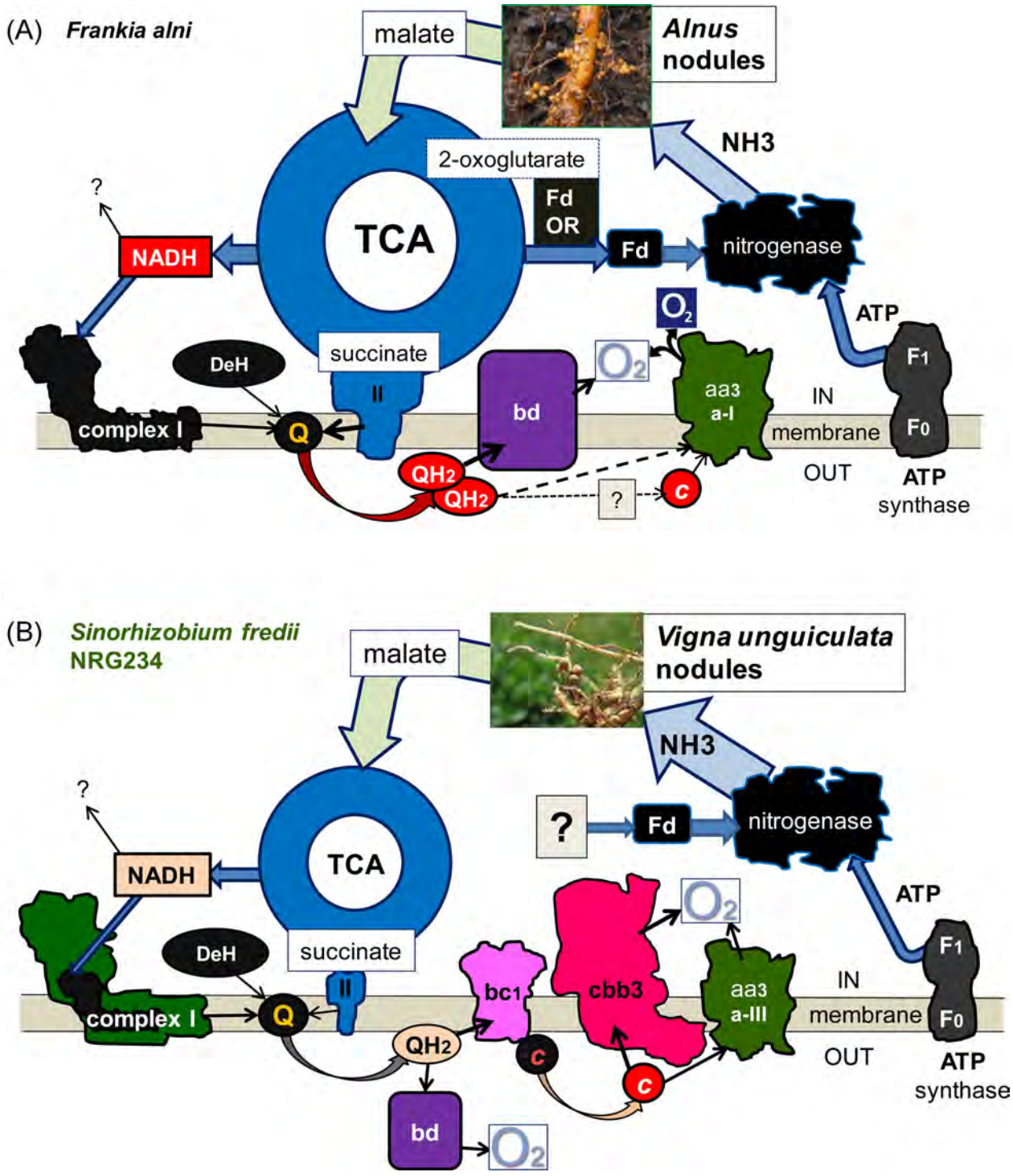

Figure 2. Modified respiratory chain in two diverse nodulating symbionts with different inputs from the TCA cycle. **(A)** The actinomycete *F. alni* nodulates roots of trees such as *Alnus* (alder) but does not differentiate into bacteroids as do rhizobia. Its respiratory chain lacks the bc_1 complex and both aa3-type and cbb3-type cytochrome *c* oxidases, as well as the bo3-type quinol oxidase (Alloisio *et al.* 2010). A COX operon type a-I oxidase is however present and may cooperate in the reoxidation of ubiquinol, given the ubiquinol oxidase capacity of such ancestral oxidases (Gao *et al.* 2012). Excess ubiquinol is predominantly reoxidized by the bd-type oxidase that is strongly upregulated in nodulating symbionts (Alloisio *et al.* 2010). During symbiosis, *Frankia* upregulates the entire TCA cycle, which also feeds electrons to nitrogenase via a bypass mediated by overexpressed FdOR (cf. Fig. 4). These changes in respiratory chain are also present in ancestral *Frankia* symbionts (Persson *et al.* 2015). **(B)** The Rhizobiaceae, *S. fredii* NGR234 nodulating *Vigna unguiculata* shows instead a downregulation of the TCA cycle (Li *et al.* 2013), which attenuates the problem of excess NADH, as well as ubiquinol overload. This organism possesses two different forms of complex I that may reoxidize excess NADH (Li *et al.* 2013; Spero *et al.* 2015): the standard NUO14 enzyme of α-proteobacteria and green complex I. While other Rhizobiaceae show a transient upregulation of the bd-type ubiquinol oxidase in the early phase of bacteroid differentiation (Karunakaran *et al.* 2009), *S. fredii* maintains this upregulation after 3 or more weeks of symbiosis (Li *et al.* 2013), thereby alleviating ubiquinol overload. Conversely, the combined action of upregulated cbb3-type and COX operon type a-III alleviates the excess of reduced *c*-type cytochromes, some of which are also overexpressed in bacteroids (Li *et al.* 2013). Contrary to *Frankia* and other Rhizobiaceae, *S. fredii* does not have hydrogenases (Table 1; cf. Li *et al.* 2013). Symbols and representations are as in Figs 1 and S1 (Supporting Information).

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

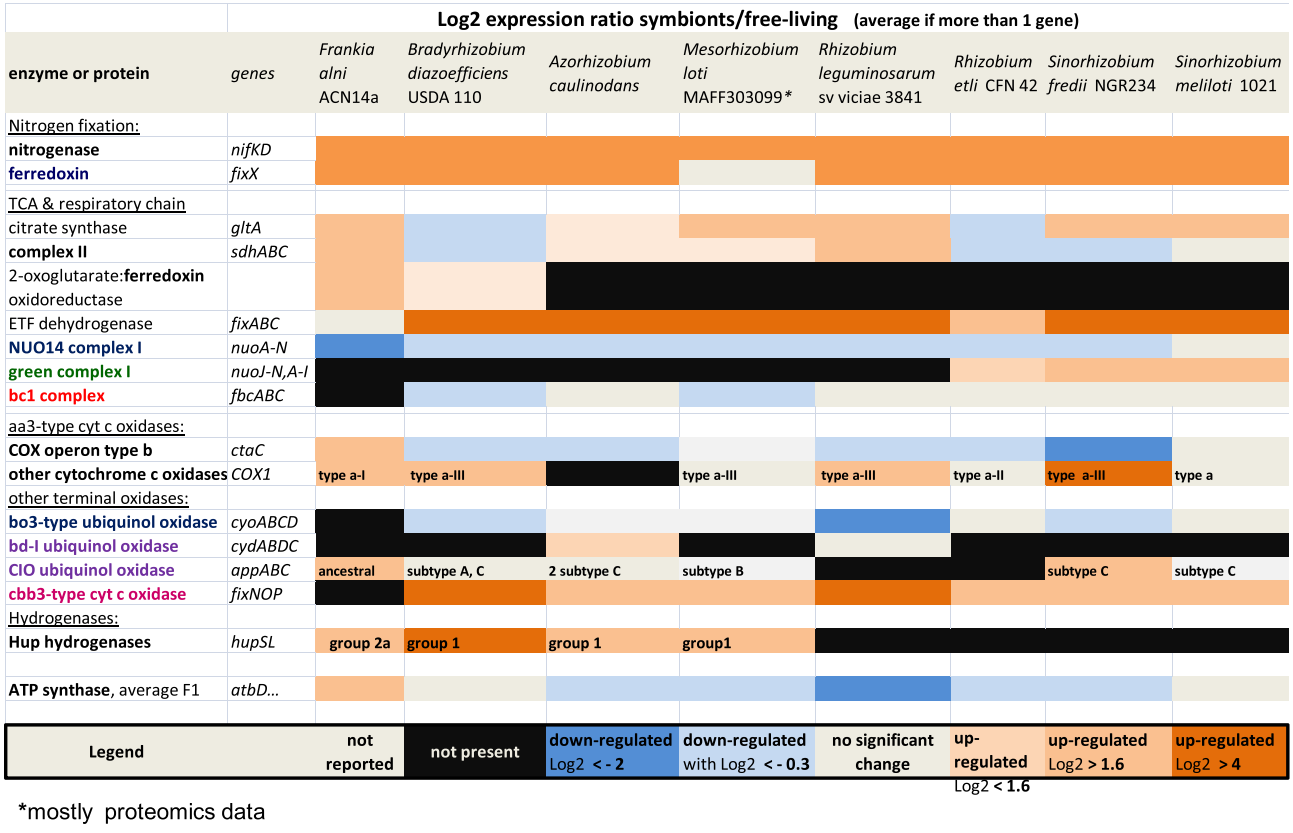

Figure 3. Differential expression of genes for energy metabolism in nodulating symbionts. The presence or absence of bioenergetic systems was verified as described previously (Degli Esposti *et al.* 2014, 2015). Note that the results for *Mesorhizobium loti* MAFF303099 predominantly derive from proteomic analysis (*, Nambu *et al.* 2015) and are complemented with transcriptomics data from Peng *et al.* (2014). For other organisms, data were extracted from the following works of transcriptomics: *F. alni* ACN14a (Alloisio *et al.* 2010), *B. diazoefficiens* USDA 110 (Pessi *et al.* 2007), *A. caulinodans* ORS 571 (Tsukada *et al.* 2009), *Rhizobium leguminosarum* sv. viciae 3841 (Karunakaran *et al.* 2009), *R. etli* CFN 42 (Vercruysse *et al.* 2011), *S. fredii* NGR234 (Li *et al.* 2013) and *S. meliloti* 1021 (Capela *et al.* 2006). Of note, the recent transcriptomic data of *Frankia* symbiont of *D. glomerata* indicate upregulation of complex I but not of hydrogenases (Persson *et al.* 2015). This *Frankia* has two different operons for bd-type ubiquinol oxidase, for which no transcriptomic results are reported (Persson *et al.* 2015), and also two operons for 2-oxoglutarate:ferredoxin oxidoreductase like *F. alni* ACN14a. Conversely, *B. diazoefficiens* (japonicum) has a second operon of bo3-type ubiquinol oxidase plus a ba3-type cytochrome oxidase for which expression changes have not been documented (Pessi *et al.* 2007; cf. Youard *et al.* 2015). Results of differential expression between symbionts (N-fixing) and free-living bacteria are expressed as Log2 values and are the average of the indicated genes. Quantitative values are represented in a heat map with the colour coding detailed in the legend at the bottom of the table. Of note, the downregulation of ATP synthase is unlikely to significantly reduce the rate of ATP production in symbionts because this enzyme is normally present in large excess with respect to the proton pumping redox enzymes of the respiratory chain plugged into the same cytoplasmic membrane, as in mitochondria.

see Biegel *et al.* 2011). The initial characterization of the respiratory chain and bioenergetic capacity of nodulating rhizobia followed that of free-living diazotrophs and indicated a similar respiration-supported activity of nitrogenase (Laane *et al.* 1979; de Hollander and Stouthamer 1980; Miller *et al.* 1988). Thus, the respiratory chain of rhizobia is considered to provide both the electrons and the ATP required for N_2 fixation within bacteroids (Miller *et al.* 1988). However, it remains obscure how respiratory complexes reduce soluble ferredoxins or other low potential reductants for nitrogenase (Poole and Allaway 2000; Udvardi and Poole 2013). This problem is indicated by the question mark upstream to ferredoxin in the scheme for the respiratory chain of *Sinorhizobium fredii* NGR234 shown in Fig. 2B (see also Figs S1 and S2, Supporting Information, for other rhizobia).

Many rhizobia have a succinate semialdehyde dehydrogenase enzyme that does not seem to provide a bypass to the TCA cycle as in *B. diazoefficiens* (Prell *et al.* 2009; Udvardi and Poole 2013). Consequently, these nodulating symbionts face three consecutive problems of electron carrier accumulation in their energy metabolism, indicated in red colour in Figs 1 and 2: (1) excess of NADH produced upstream or within the TCA cycle; (2) ubiquinol overload in the membrane, contributed by complex I reoxidizing NADH, succinate dehydrogenase of the TCA cycle and other membrane-associated dehydrogenase that reduce Q, for instance electron transport flavoprotein dehydrogenase, which is constantly upregulated in nodulating symbionts, being encoded by *fix* genes (Figs 2 and 3, Table S1, Supporting Information); (3) accumulation of reduced *c* cytochromes (either soluble cyt c_2 or membrane-anchored cyt c_{552}, cf. Table S1, Supporting Information) due to the high turnover and normal expression of the bc_1 complex that reoxidizes ubiquinol (Youard *et al.* 2015).

With the exception of *Frankia* organisms, which do not have cbb3-type terminal oxidases (Fig. 2A), nodulating symbionts regularly overexpress their cbb3 oxidase to levels almost comparable to those of the nitrogenase enzyme (Figs 3 and 4), thereby alleviating the downstream accumulation of electrons in *c* cytochromes. To which extent this may reduce the excess of NADH and ubiquinol overload derived from the altered energy metabolism of nodulating symbionts depends on the different combinations of respiratory systems that are present in the genome of diverse rhizobia (Figs 1 and 2 and S2 and S3, Supporting Information). For example, several nodulating symbionts express cytochrome *c* oxidases other than the classical COX related

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

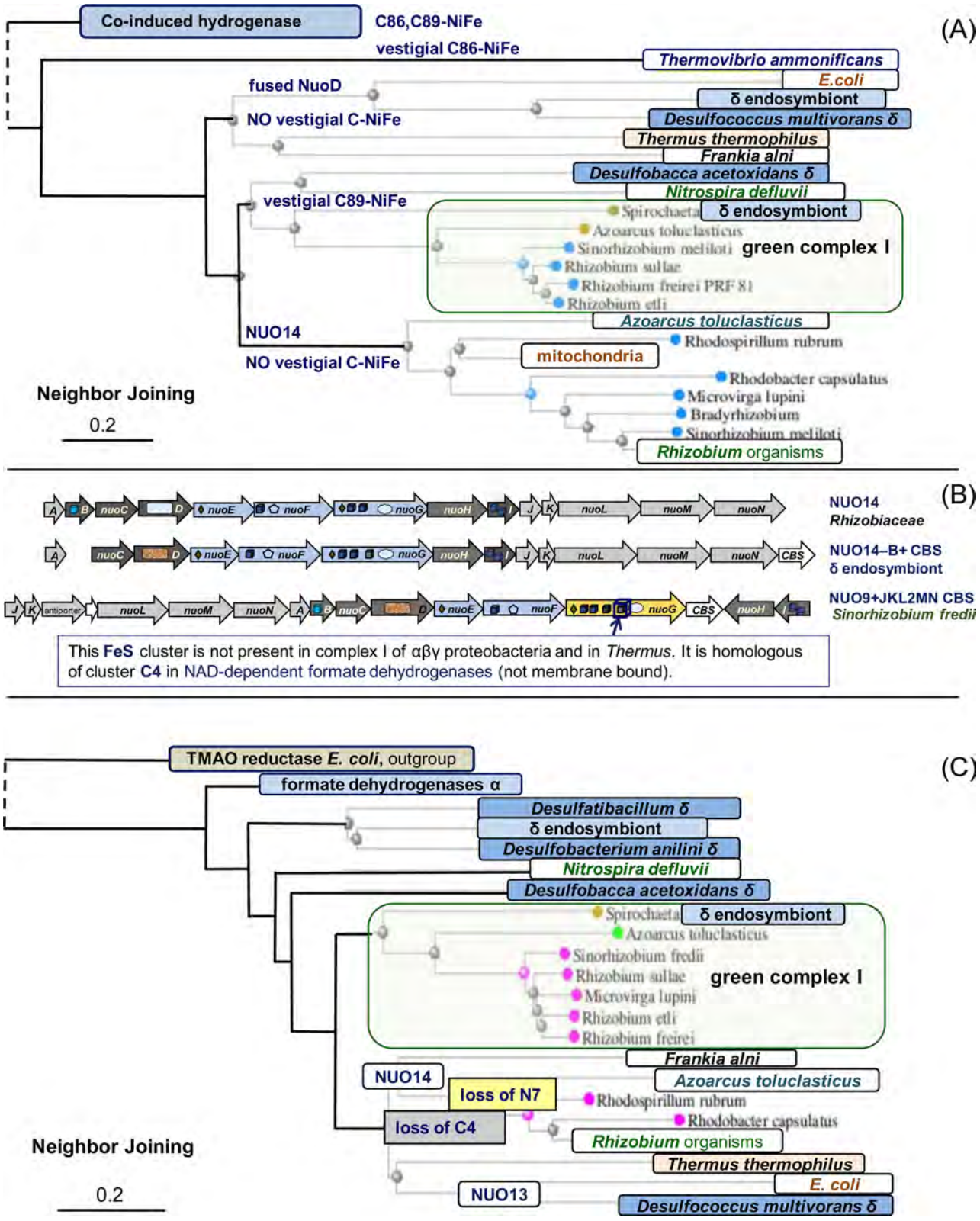

Figure 4. Phylogeny and gene cluster of green complex I. **(A)** Phylogenetic tree of the NuoD subunit of complex I from bacteria including all nodulating symbionts examined here and also eukaryotic mitochondria. **(B)** Sequence of the gene clusters of different operons of bacterial complex I. The genes and redox groups are represented as previously reported (Degli Esposti 2016). Note the distinctive presence of a protein of ca. 150 amino acids having a tandem CBS domain, hence a Bateman domain (Baykov, Tuominen and Lahti 2011), within the gene cluster of green complex I of Rhizobiaceae, or at the end of the cluster in the purported Spirochaeta organism most likely belonging to a delta proteobacteria endosymbiont of termites (see the text). **(C)** Phylogenetic tree of the NuoG subunit of complex I from various bacteria including all nodulating symbionts examined here, as in A. Similar trees have been obtained with maximum-likelihood methods.

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

to the mitochondrial enzyme, in particular those of COX operon type a-II and a-III (Figs 2B and 3 and S1, Supporting Information). These enzymes are likely to have high to intermediate affinity for oxygen (Degli Esposti *et al*. 2014) and therefore can diminish the accumulation of reduced *c* cytochromes under conditions of N_2 fixation (Figs 1 and 2, S1 and S2, Supporting Information). The observation that rhizobia preferentially (over) express COX operons of type a instead of bo3-type oxidases (Fig. 3) suggests that the accumulation of reduced *c* cytochromes is more critical than ubiquinol overload in the physiology of bacteroids, despite the lower yield in generating membrane potential by reoxidizing reduced cytochrome *c* than reoxidizing ubiquinol (Degli Esposti *et al*. 2014). In any case, it is extremely unlikely that electron transfer to oxygen is coupled to reactions supplying redox equivalents to nitrogenase, which can be reduced only by proteins having a redox potential lower than that of NADH (Klugkist, Haaker and Veeger 1986; Tichi, Meijer and Tabita 2001).

How do rhizobia feed electrons to nitrogenase?

Early studies suggested that NADH supplied by the TCA cycle could provide the electrons required for the nitrogenase reaction (Haaker and Veeger 1977; Klugkist, Haaker and Veeger 1986). However, this possibility is in principle contradicted by the unfavourable redox gap between NAD(P)H and low potential ferredoxins or flavodoxins that directly reduce nitrogenase (–340 mV vs –400 mV and below; cf. Klugkist, Haaker and Veeger 1986; Poole and Allaway 2000). Moreover, the available genomes of rhizobia do not code for the abovementioned rTCA enzymes or the Rnf complex that can directly reduce ferredoxins or flavodoxins. Therefore, we need to look at other enzymes that are present in rhizobial genomes and can react with ferredoxins via their low potential redox groups, for instance formate dehydrogenase (Hartmann, Schwanhold and Leimkühler 2015) and group 4 hydrogenases (Vignais and Billoud 2007).

Most rhizobia possess the NAD^+-dependent formate dehydrogenase originally discovered in *Ralstonia* (Oh and Bowien 1998), which is homologous to the NADH-reacting module of respiratory complex I (Degli Esposti *et al*. 2015). This soluble redox enzyme contains low potential iron-sulphur (Fe-S) clusters that can in principle react with ferredoxins in the electron flow from the substrate formate (Hartmann, Schwanhold and Leimkühler 2015). Formate is a by-product of the catabolism of amino acids and TCA metabolites that is also present in bacteroids (Poole and Allaway 2000; Udvardi and Poole 2013). However, transcriptomic data indicate that the operon of NAD^+-dependent formate dehydrogenase is clearly downregulated under N_2-fixing conditions (Table S1, Supporting Information, and data not shown). Moreover, non N_2-fixing members of the Rhizobiaceae family such as *Agrobacterium* also contain the enzyme. Consequently, formate dehydrogenase is not associated with the process of N_2 fixation and probably cannot supply electrons to the nitrogenase reaction.

Group 4 hydrogenases related to archaean *ehr* have been found among diazotrophs and nodulating symbionts (Marreiros *et al*. 2013). These enzymes are membrane bound and lack canonical cysteine ligands to the Ni-Fe cluster that forms the catalytic core of hydrogenases (Vignais and Billoud 2007; Sprecher, Gittings and Ludwig 2012; Marreiros *et al*. 2013). *Azorhizobium caulinodans* contains the prototypic enzyme coded by the *Hyq* operon, which is essential for N_2 fixation under diazotrophic conditions. We have found that the catalytic subunits of *Hyq* hydrogenase, often annotated with different gene names (i.e. *HypE*), are distributed among various Rhizobiaceae, especially of the *Mesorhizobium* and *Rhizobium* genera (Table 1). Structurally, *Hyq* proteins resemble catalytic and integral membrane subunits of respiratory complex I, suggesting evolutionary relationships (Marreiros *et al*. 2013), as well as potential functional similarities between the two enzymes (Sprecher, Gittings and Ludwig 2012).

However, the *HyqG* homologue of the catalytic NuoD subunit of complex I lacks all the residues known to bind ubiquinone in the crystal structure of complex I (Baradaran *et al*. 2013; Degli Esposti 2016). Hence, it is very unlikely that *Hyq* hydrogenase may transport electrons from H_2 to Q as proposed earlier (Sprecher, Gittings and Ludwig 2012). Instead, it might use ferredoxins as potential acceptors for electron transfer from H_2, in analogy with homologous archaean enzymes (Hedderich 2004; Marreiros *et al*. 2013). We have thus looked at the transcriptomics of *A. caulinodans* (Tsukada *et al*. 2009), which uniquely possesses the *Hyq* hydrogenase operon among the organisms with available expression data in nodulating symbionts (Fig. 3 and Table 1). Intriguingly, the genes of its operons are downregulated over 4-fold in nodulating symbionts versus free-living bacteria (Tsukada *et al*. 2009). It is therefore difficult to envisage a role for *Hyq* hydrogenase in feeding electrons to nitrogenase, even if its H_2 oxidoreductase activity may facilitate N_2 fixation by removing hydrogen, a by-product of the nitrogenase reaction (Sprecher, Gittings and Ludwig 2012), as in the case of group 2a hydrogenases which are overexpressed in *F. alni* symbionts (Alloisio *et al*. 2010; Constant *et al*. 2011). Under symbiotic conditions, group 1 hydrogenase (also called *Hup* for H_2 uptake; Leyva *et al*. 1990; Vignais and Billoud 2007) is likely to be more important than *Hyq* in fulfilling this function.

Systematic analysis of various genes annotated as 'ferredoxin reductase' in rhizobial genomes has revealed the consistent upregulation of one membrane-associated enzyme in N_2-fixing symbionts, the *mocRDEF* operon for rhizopine oxygenase (Bahar *et al*. 1998; cf. Karunakaran *et al*. 2009; Li *et al*. 2013; Nambu *et al*. 2015). However, this system reduces a Rieske Fe-S protein with high redox potential that shuttles electrons between the NADH-ferredoxin reductase and the desaturase components of the operon, as in the related toluene 4-monooxygenase (Elsen *et al*. 2007). Hence, the reductase component of the rhizopine oxygenase may only relieve the excess of NADH in nodulating symbionts.

In sum, the survey of the properties and differential expression of enzymes potentially reducing ferredoxin has indicated that such enzymes are not responsible, in all likelihood, for feeding electrons to nitrogenase in rhizobial bacteroids, thereby leaving a big question mark on the electron supply to nitrogenase (cf. Fig. 2B). However, the same survey has indicated the intriguing recurrence of a second operon of complex I, green complex I, which is coded in several genomes of rhizobia (Table 1) and appears to be upregulated during N_2 fixation (Fig. 3 and S3, Supporting Information).

The phylogenesis and properties of green complex I

During a recent evolutionary analysis of respiratory complex I (Degli Esposti 2016), a restricted group of proteobacteria emerged as a separate clade in the phylogenetic trees of key subunits of the enzyme (Fig. 4). The group includes a single β-proteobacterium, *Azoarcus toluclasticus* (which does not form nodules in plants), and about 70 α-proteobacteria, predominantly of the Rhizobiaceae family (Fig. 4 and Table 1). All these organisms present two different operons for respiratory complex I: a NUO14 coding for the standard 14 subunits of

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

Table 1. Phylogenetic distribution of hydrogenases and green complex I among rhizobia. Operons of the enzyme complexes that are missing one or more subunits are considered incomplete. The distribution of the menaquinol (MQ) methyltransferase has been considered after the work of Xie *et al.* (2011).

Organisms ad strains, with notes	*Hyq* Hydrogenase	*Hup* Hydrogenase	green complex I	MQ methyl-transferase	NOTES
Azorhizobium caulinodans ORS 571	YES	yes			transcriptome
Mesorhizobium amorphae	YES	yes		yes	
Mesorhizobium sp. LNHC232B00, LNHC221B00, LNHC252B00, LSJC280B00, URHC0008, WSM3224, ORS332	YES				
Mesorhizobium opportunistum	YES				
Mesorhizobium alhagi CCNWXJ12	YES				
Mesorhizobium loti USDA 3471	YES				
Mesorhizobium plurifarium (3 strains)	YES				
Mesorhizobium huakuii 7653R				yes	
Mesorhizobium loti MAFF303099				yes	transcriptome
Mesorhizobium sp. L48C026A00 & WSM2561			YES	yes	
Rhizobium leguminosarum sv. trifolii TA1, SRDI565, **WSM2012**[a,b]	YES		YES	yes	
Rhizobium leguminosarum sv. viciae UPM1131 & **Ps8**[a,b]	YES		YES	yes	
Rhizobium sp. CF142	YES		YES		
Rhizobium mesoamericanum STM3625 & **STM6155**[b]	YES		YES		
Rhizobium sullae[a,b]	YES		YES	yes	superior fixer c
Rhizobium freirei PRF 81[b]	YES	yes	YES		superior fixer c
Rhizobium etli sv mimosae str. Mim1	YES	Yes, incomplete	YES		superior fixer c
Rhizobium etli str. IE4771		yes	YES		
Rhizobium mongolense		yes	YES	yes	
Rhizobium gallicum sv. gallicum R602		yes	YES	yes	
Rhizobium leguminosarum sv. viciae UPM1137, GB30, Vh3 and **Vc2**[a,b]		yes	YES	yes	
Rhizobium leguminosarum sv. trifolii CC283b[a,b]		yes	YES	yes	
Rhizobium leguminosarum sv. trifolii CB782 & **WSM2297**[a,b]			YES		
Rhizobium leguminosarum sv. viciae 248, UPM1137 and **TOM**[a]			YES	yes	
Rhizobium leguminosarum sv. phaseoli CCGM1[b]			YES		
Rhizobium leguminosarum strain OV483[a,b]			YES		
Rhizobium etli sv. phaseoli str. IE4803			YES	yes	
Rhizobium grahamii CCGE 502			YES	yes	
Rhizobium phaseoli CNPAF512[b]			YES	yes	
Rhizobium etli sv. phaseoli CFN 42[b]			YES		transcriptome
Rhizobium phaseoli sv. phaseoli Ch24-10[b]			YES		
Rhizobium giardinii			YES		
Rhizobium alamii			YES		
Rhizobium sp. OR191, LPU83, CF258, CF122, YR295, Pop5, IBUN, 42MFCr.1, OV201, CF048, CF097			YES		
Rhizobium leguminosarum strain OV152[a,b]			Yes, incomplete		
Sinorhizobium medicae WSM419[a]			YES		
Sinorhizobium meliloti L5-30, RU11/001, strain RMO17, AK83, AK75, AK11, AK58, 2011, A0643DD, AE608H,A0641M, C0438LL, 1A42, MVII-I, CIAM1775, Mlalz-1, GVPV12, **CCNWSX0020**[a,b]			YES		
Ensifer adhaerens			YES		
Ensifer adhaerens (Sinorhizobium morelense)			YES		
Sinorhizobium meliloti BL225C, SM11, 4H41, **Rm41**[a,b]			YES		
Sinorhizobium meliloti 1021[a,b]			YES		transcriptome
Ensifer sp. BR816, **WSM1721**[b]		Yes, incomplete	YES		
Ensifer sp. USDA 6670 & **TW10**[b]			YES		
Sinorhizobium sp. PC2			YES		
Sinorhizobium fredii NGR234			YES		transcriptome
Sinorhizobium fredii 45436, CCBAU 05557, CCBAU 83643, CCBAU 83666, **CCBAU 8375**[b]			YES		
Sinorhizobium fredii CCBAU 05631, CCBAU 83643, CCBAU 83666, CCBAU 83753, NGR234, USDA 257, **HH103**[b]			YES		
Sinorhizobium fredii GR64[b]	YES	yes	YES		
Sinorhizobium arboris		yes	YES		
Sinorhizobium americanum CCGM7		yes	YES		superior fixer c
Ensifer sojae[b]			YES		
Sinorhizobium meliloti ORF-378 & **1A4**[a,b]			Yes, incomplete		
Microvirga sp. JC119 - Methylobacteraceae			YES		
Microvirga lupini - Methylobacteraceae			YES		
Bosea sp. LC85 - Bradyrhizobiacae			Yes, incomplete		
Leisingera sp. ANG-M1 - Rhodobacterales			Yes, incomplete		
Azoarcus toluclasticus - beta proteobacteria			Yes, incomplete		
Moritella sp. PE36 - gamma proteobacteria			Yes, incomplete		
Spirochaeta sp. JC202 - actually delta endosymbiont			Yes, incomplete		

a Terminal bacteroid differentiation
b Host is an extensive agriculture crop with economic value
c superion N fixation capacity with respect to related organisms

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

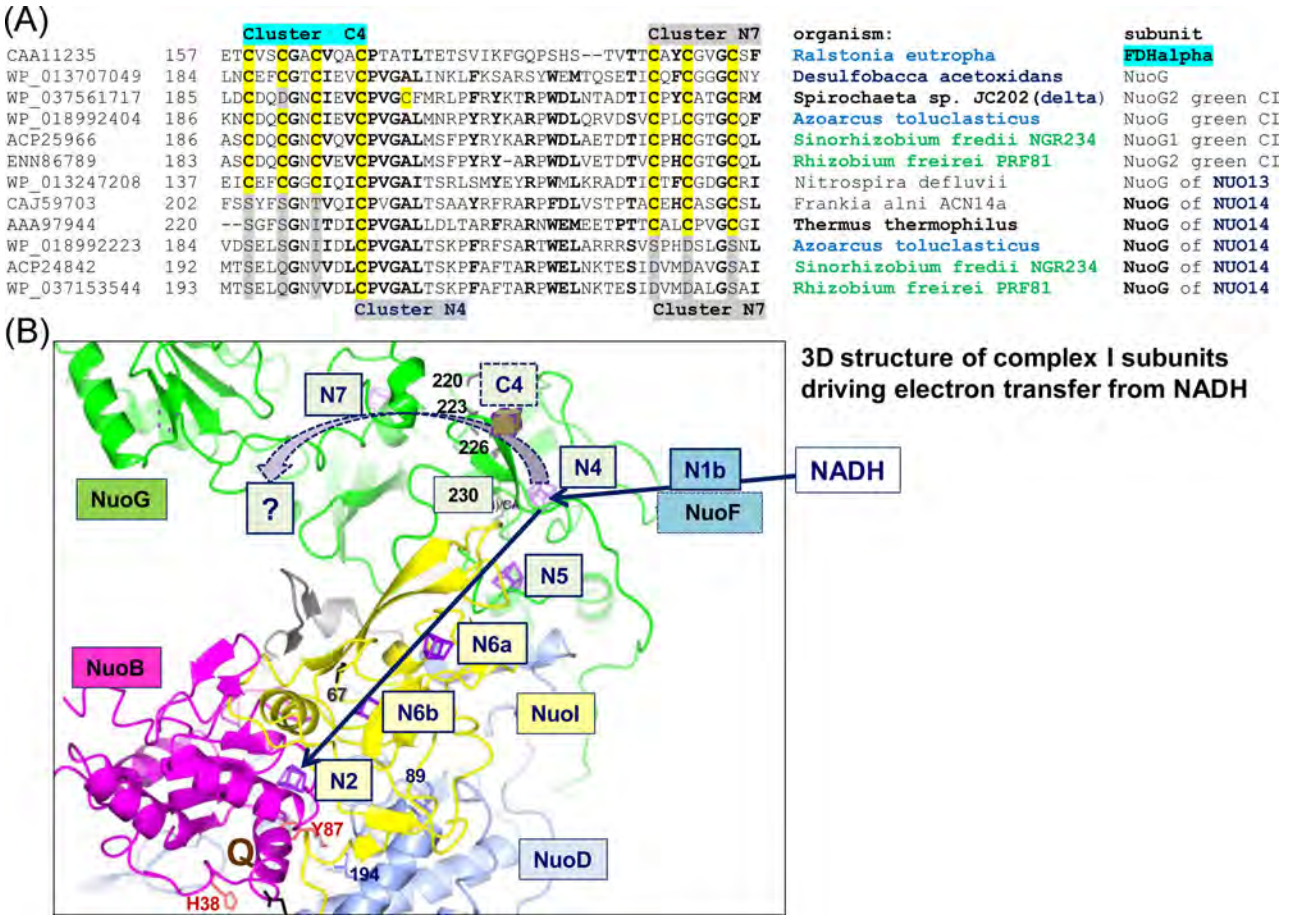

Figure 5. Molecular differences in electron transfer subunits of green complex I. **(A)** The alignment of the N-terminal region of the NuoG subunit of various bacterial organisms highlights (in yellow) the conserved cysteine residues that function as ligands for Fe_4S_4 clusters C4 and C5 (Oh and Bowien 1998). C5 corresponds to cluster N7 in bacterial complex I (Sazanov and Hinchliffe 2006). Residue C230 is involved in the binding of cluster N4 in bacterial and mitochondrial complexes that have lost cluster C4, as indicated at the bottom of the alignment. **(B)** The 3D model of the hydrophilic domain of *Thermus* complex I (Baradaran *et al.* 2013) focuses on the electron transport sequence from cluster N1b reduced by NADH to ubiquinone (Q). The ribbon rendering of the proteins NuoG, NuoI, NuoB and NuoD shows conserved residues involved in Q-binding (in red) and the cysteine residues that are uniquely conserved in green complex I (blue for those in NuoD and black for all the others). The iron–sulphur clusters are indicated by the blue boxes and the dashed arrow shows the alternative electron pathway that may be provided by cluster C4 effectively bridging cluster N4 with cluster N7, which remains isolated from the main electron pathway in *Thermus* complex I (Baradaran *et al.* 2013). In bacterial formate dehydrogenases alpha subunits, the homologous cluster C4 forms an integral part of the electron pathway from the reducing substrate formate to the NAD+ acceptor via the Mo-containing cofactor (Oh and Bowien 1998; Jormakka *et al.* 2002). The question mark indicates an unknown acceptor for the pathway from cluster N4 to cluster N7 via cluster C4.

α-proteobacteria (Dupuis *et al.* 1998; Yagi *et al.* 1998) and another with a peculiar rearrangement of the gene order and including a CBS domain protein (Bateman 1997) (Fig. 4B). After analysis of the phylogenetic distribution and composition of this second operon, we realized that it constitutes a conserved genomic element in approximately one-half of known organisms of the *Rhizobium* and *Sinorhizobium* genera, but not in other taxa of the same Rhizobiaceae family that do not fix nitrogen such as *Agrobacterium* (Table 1 and data not shown). Contrary to the NUO14 operon of complex I, green complex I is invariably upregulated in the nodulating symbionts (Fig. 3) of the organisms that possess it in their genome (Table 1).

In evolutionary terms, green complex I is more ancient than the NUO14 enzyme because its Q-binding NuoD subunit contains vestigial cysteine ligands of the Ni-Fe group such as C89 (following the numeration of *Thermus* complex I—Baradaran *et al.* 2013; cf. Degli Esposti 2016). This subunit derives from the homologous large subunit of Ni-Fe hydrogenases, retaining cysteine ligands of the Ni-Fe group in some ancestral organisms (Fig. 4A; cf. Degli Esposti 2016). Moreover, the deepest branch in the phylogenetic trees of either the NuoD (Fig. 4A) or the NuoG (Fig. 4C) subunit is occupied by a bacterium reported to be affiliated to the Spirochaetae—*Spirochaeta* sp. JC202 (Tushar *et al.* 2015)—but most likely belonging to the class of δ-proteobacteria, as our analysis of bioenergetic proteins indicates. This is supported by the consideration that *Spirochaeta* sp. JC202 has been found in the gut of termites (Tushar *et al.* 2015), in which it is easy to confound a δ-proteobacterial symbiont (Sato *et al.* 2009) with a spirochete in the absence of the proper reference organism (Rosenthal *et al.* 2013). Indeed, the genome of the bacterium purportedly annotated as *Spirochaeta* sp. JC202 contains features that are typical of δ-proteobacteria such as the fusion of the NuoD, NuoC and NuoB subunit of complex I, which is characteristic of sulphate-reducing organisms of this class (Fig. 4A; cf. Göker *et al.* 2011; Marreiros *et al.* 2013; Spero *et al.* 2015). These δ-proteobacteria seem to have the most ancestral form of the NuoG subunit originating from the large (alpha) subunit of the abovementioned NAD^+-dependent formate dehydrogenase (Fig. 4C; cf. Oh and Bowien 1998; Degli Esposti 2016). In the δ-proteobacterial symbiont corresponding to *Spirochaeta* sp. JC202,

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

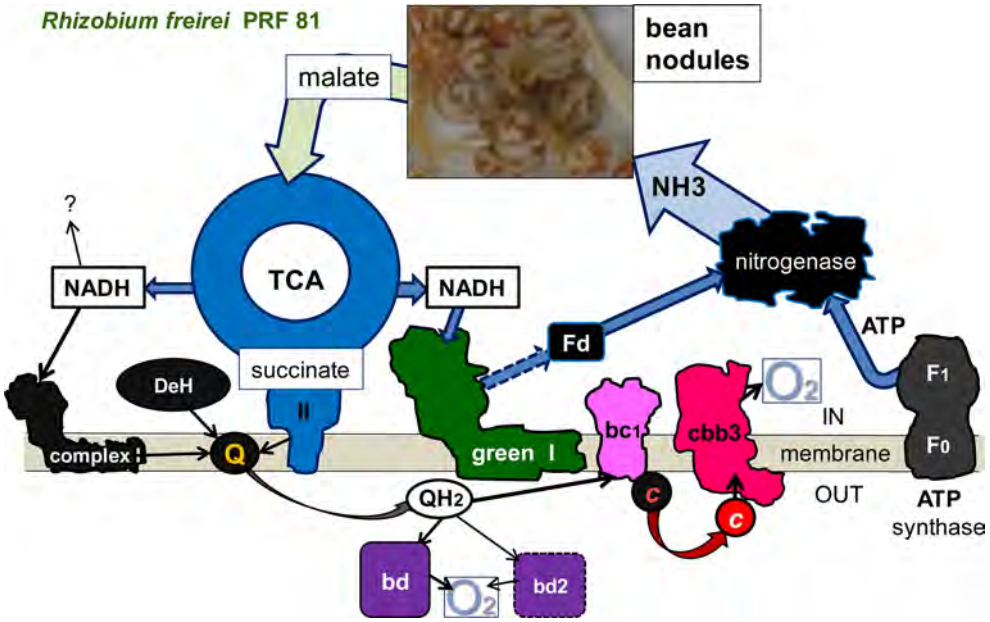

Figure 6. Deduced energy metabolism in nodulating symbionts of *Rhizobium freirei* and hypothetical role of green complex I. The energy metabolism and respiratory chain of nodulating symbionts of *R. freirei* PRF 81 has been deduced from genomic data (Ormeño-Orrillo *et al.* 2012). The diagram also illustrates the possible function of green complex I expressed in this organism (Table 1), namely a bridging redox link between the TCA cycle and nitrogenase via a NADH-ferredoxin oxidoreductase reaction that may be catalysed via the additional Fe-S clusters present in the NuoG subunit of green complex I (cf. Fig. 5). *Rhizobium freirei* contains two operons for CIO-type bd oxidases (Degli Esposti *et al.* 2015), similarly to *Frankia* symbiont of *D. glomerata* (Persson *et al.* 2015).

the second operon of complex I shows a gene sequence comparable to that of green complex I but with a CBS protein at the end and the absence of the catalytic NuoB subunit (Fig. 4B). Portions of the same gene sequence are present in organisms that have an incomplete green complex I, for example the Bradyrhizobiaceae *Bosea* sp. LC85 or the rhizobiaceae *S. meliloti* ORF-378 (Table 1). This suggests gradual erosion of an ancestral operon for complex I (cf. Price, Arkin and Alm 2006) that presumably originated within a clade of δ-proteobacteria specialized for microoxic symbiotic environments such as that of termite hindgut. Only a few extant organisms might have maintained green complex I for its possible role in the adaptation to similar microoxic conditions associated with established symbiosis. Additional sequence analysis strongly suggests that green complex I did not arise from a duplication of the other operon of complex I as in the case of the cbb3 oxidase of rhizobia (cf. Karunakaran *et al.* 2009; Li *et al.* 2013).

The most prominent difference between green complex I and its cognate operon present in all aerobic proteobacteria including chizobia is the much longer NuoG subunit (by almost 200 residues), which retains the cysteine ligands of two Fe_4S_4 clusters that are present in the Fdsa (alpha) subunit of NAD^+-dependent formate dehydrogenase: cluster C4 and cluster C5 (Fig. 5A; cf. Oh and Bowien 1998). The latter corresponds to cluster N7 originally characterized in *Escherichia coli* complex I (Pohl *et al.* 2007) and subsequently found in the structure of *Thermus* complex I (Sazanov 2015). This cluster is located too far from the nearest redox group, cluster N4 in complex I corresponding to cluster C3 in Fdsa (Oh and Bowien 1998; Sazanov and Hinchliffe 2006), to be involved in effective electron transfer (Sazanov 2015). Besides the NuoG subunit of green complex I, the simultaneous presence of the signatures of both cluster C4 and N7 (C5) is observed in delta and epsilon proteobacteria (data not shown). Hence, green complex I may fulfil a different redox function than that normally carried out by respiratory complex I, presumably similar to that of the NuoEF subunits retained in the hydrogenosomes of anaerobic eukaryotes such as *Trichomonas* (Stairs, Leger and Roger 2015). In hydrogenosomes, this minimal complex I system transfers electrons from TCA-produced NADH to ferredoxin, which is subsequently reoxidized by a Fe–Fe hydrogenase that evolves H_2 as nitrogenase (Stairs, Leger and Roger 2015)—indeed, this hydrogenase is structurally related to nitrogenase (Mulder *et al.* 2010). We propose that green complex I may fulfil a similar function in nodulating symbionts, as illustrated in Figs 5B and 6. The question mark near cluster N7 in Fig. 5B indicates an unknown acceptor, which may well be a ferredoxin (Fig. 6) as in hydrogenosomes (Stairs, Leger and Roger 2015). Indeed, ancestral forms of complex I do react with ferredoxins or similar small redox proteins (Weerakoon and Olson 2008; Degli Esposti 2016; Ma and Ogawa 2015).

CONCLUSIONS AND PERSPECTIVES

We have presented here an updated overview of the energy metabolism of nodulating symbionts that focuses on major issues surrounding the integration of this metabolism with the process of N_2 fixation, which is beneficial to the host plant as well as to agriculture. The major unresolved problem remains how rhizobia supply electrons to the nitrogenase reaction (question mark in Fig. 2B, S1 and S2, Supporting Information). Our survey indicates that ancestral nodulating symbionts of the *Frankia* genus may have resolved this problem by overexpressing an alternative enzyme of the TCA cycle that directly reduces ferredoxins, which then supply electrons to nitrogenase (Figs 1 and 2A). Except for some Bradyrhizobiaceae (Fig. 3 and Table S1, Supporting Information), rhizobia have lost such an enzyme bypassing the TCA cycle, but have acquired another bioenergetic system that may also reduce ferredoxins, green complex I (Fig. 6). This hypothetical role of green complex I provides new perspectives not only to characterize another version of the

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

respiratory complex, but also to understand the physiological and biochemical links between the respiratory chain and N_2 fixation in rhizobia. While green complex I operon has been lost by several nodulating organisms, it has been retained in rhizobia showing superior N_2 fixation capacity, as shown in Table 1. Therefore, it is possible that the function of green complex I increases the efficiency of N_2 fixation in the nodules (cf. Galibert *et al.* 2001), with consequent beneficial effect in nitrogen enrichment of agricultural soil. We are now exploring such a new perspective by studying selected rhizobia.

Our survey highlights the general trends and specific variations in the respiratory chain of nodulating symbionts that can be deduced from the abundant genomic information which is now available, thereby producing a step forward in filling the widening gap between genetic information and phenotypic (biochemical and agriculturally relevant) characterization of rhizobia. Ultimately, Nature has followed various ways to establish a mutually beneficial symbiosis between nodulating bacteria and plants that fits diverse ecological situations, but does not necessarily enhance the energy-demanding process of N_2 fixation beyond levels that are compatible with survival and fitness. It is up to us scientists to understand the general patterns underlying this evolution and then exploit its features so as to enhance the efficiency in N_2 fixation for the benefit of agriculture and the environment.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

ACKNOWLEDGEMENTS

We thank Luis Servin and Lucero Y. Rivera-Najera for assistance in some aspects of this work, Louis Tisa (University of New Hampshire, USA) and Mike Dunn, Otto Geiger and Diego Gonzalez-Halphen (Universidad Nacional Autonoma de Mexico) for helpful discussion.

FUNDING

This work was supported by funds from Consejo Nacional de Ciencia y Tecnologia (CONACyT), in particular sabbatical proposal No: 263876 for MDE.

Conflict of interest. None declared.

REFERENCES

Alloisio N, Queiroux C, Fournier P *et al.* The Frankia alni symbiotic transcriptome. *Mol Plant Microbe In* 2010;**23**:593–607.

Althabegoiti MJ, Ormeño-Orrillo E, Lozano L *et al.* Characterization of *Rhizobium grahamii* extrachromosomal replicons and their transfer among rhizobia. *BMC Microbiol* 2014;**14**:6.

Baginsky C, Brito B, Imperial J *et al.* Diversity and evolution of hydrogenase systems in rhizobia. *Appl Environ Microb* 2002;**68**:4915–24.

Baginsky C, Brito B, Imperial J *et al.* Symbiotic hydrogenase activity in *Bradyrhizobium* sp. (*Vigna*) increases nitrogen content in *Vigna unguiculata* plants. *Appl Environ Microb* 2005;**71**:7536–8.

Bahar M, de Majnik J, Wexler M *et al.* A model for the catabolism of rhizopine in Rhizobium leguminosarum involves a ferredoxin oxygenase complex and the inositol degradative pathway. *Mol Plant Microbe In* 1998;**11**:1057–68.

Baradaran R, Berrisford JM, Minhas GS *et al.* Crystal structure of the entire respiratory complex I. *Nature* 2013;**494**:443–8.

Bateman A. The structure of a domain common to archaebacteria and the homocystinuria disease protein. *Trends Biochem Sci* 1997;**22**:12–3.

Baughn AD, Garforth SJ, Vilchèze C *et al.* An anaerobic-type alpha-ketoglutarate ferredoxin xidoreductase completes the oxidative tricarboxylic acid cycle of Mycobacterium tuberculosis. *PLoS Pathog* 2009;**5**:e1000662.

Baykov AA, Tuominen HK, Lahti R. The CBS domain: a protein module with an emerging prominent role in regulation. *ACS Chem Biol* 2011;**6**:1156–63.

Bertsova YV, Bogachev AV, Skulachev VP. Two NADH:ubiquinone oxidoreductases of Azotobacter vinelandii and their role in the respiratory protection. *Biochim Biophys Acta* 1998;**1363**:125–33.

Biegel E, Schmidt S, González JM *et al.* Biochemistry, evolution and physiological function of the Rnf complex, a novel ionmotive electron transport complex in prokaryotes. *Cell Mol Life Sci* 2011;**68**:613–34.

Bothe H, Schmitz O, Yates MG *et al.* Nitrogen fixation and hydrogen metabolism in cyanobacteria. *Microbiol Mol Biol R* 2010;**74**:529–51.

Brito B, Baginsky C, Palacios JM *et al.* Biodiversity of uptake hydrogenase systems from legume endosymbiotic bacteria. *Biochem Soc Trans* 2005;**33**:33–5.

Burgos PA, Castellanos J, Mora Y *et al.* Field inoculation of common bean (*Phaseolus vulgaris* L.) with high efficiency *Rhizobium* strains. In: Martínez E, Hernández G (eds). *Highlights of Nitrogen Fixation Research*. New York: Kluwer Academic/Plenum Publishers, 1999, 255–7.

Campbell BJ, Smith JL, Hanson TE *et al.* Adaptations to submarine hydrothermal environments exemplified by the genome of Nautilia profundicola. *PLoS Genet* 2009;**5**:e1000362.

Capela D, Filipe C, Bobik C *et al.* Sinorhizobium meliloti differentiation during symbiosis with alfalfa: a transcriptomic dissection. *Mol Plant Microbe In* 2006;**19**:363–72

Chouaia B, Gaiarsa S, Crotti E *et al.* Acetic acid bacteria genomes reveal functional traits for adaptation to life in insect guts. *Genome Biol Evol* 2014;**6**:912–20.

Constant P, Chowdhury SP, Hesse L *et al.* Genome data mining and soil survey for the novel group 5 [NiFe]-hydrogenase to explore the diversity and ecological importance of presumptive high-affinity H(2)-oxidizing bacteria. *Appl Environ Microb* 2011;**77**:6027–35.

Czernic P, Gully D, Cartieaux F *et al.* Convergent evolution of endosymbiont differentiation in dalbergioid and inverted repeat-lacking clade legumes mediated by nodule-specific cysteine-rich peptides. *Plant Physiol* 2015;**169**:1254–65.

Degli Esposti M. Genome analysis of structure-function relationships in respiratory complex I, an ancient bioenergetic enzyme. *Genome Biol Evol* 2016;**8**:126–47.

Degli Esposti M, Chouaia B, Comandatore F *et al.* Evolution of mitochondria reconstructed from the energy metabolism of living bacteria. *PLoS One* 2014;**9**:e96566.

Degli Esposti M, Rosas-Pérez T, Servín-Garcidueñas LE *et al.* Molecular evolution of cytochrome bd oxidases across proteobacterial genomes. *Genome Biol Evol* 2015;**7**:801–20.

de Hollander JA, Stouthamer AH. The electron transport chain of Rhizobium trifolii. *Eur J Biochem* 1980;**111**:473–8.

Delgado MJ, Bedmar EJ, Downie JA. Genes involved in the formation and assembly of rhizobial cytochromes and their role in symbiotic nitrogen fixation. *Adv Microb Physiol* 1998; **40**:191–231.

Dixon R, Kahn D. Genetic regulation of biological nitrogen fixation. *Nat Rev Microbiol* 2004;**2**:621–31.

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

Dunn MF. Tricarboxylic acid cycle and anaplerotic enzymes in rhizobia. *FEMS Microbiol Rev* 1998;**22**:105–23.

Dupuis A, Chevallet M, Darrouzet E *et al*. The complex I from Rhodobacter capsulatus. *Biochim Biophys Acta* 1998; **1364**:147–65.

Elsen NL, Moe LA, McMartin LA *et al*. Redox and functional analysis of the Rieske ferredoxin component of the toluene 4-monooxygenase. *Biochemistry* 2007;**46**:976–86.

Encarnación S, Dunn M, Willms K *et al*. Fermentative and aerobic metabolism in Rhizobium etli. *J Bacteriol* 1995;**177**: 3058–66.

Galibert F, Finan TM, Long SR *et al*. The composite genome of the legume symbiont Sinorhizobium meliloti. *Science* 2001;**293**:668–72.

Gao Y, Meyer B, Sokolova L *et al*. Heme-copper terminal oxidase using both cytochrome c and ubiquinol as electron donors. *P Natl Acad Sci USA* 2012;**109**:3275–80.

Geddes BA, Oresnik IJ. Physiology, genetics, and biochemistry of carbon metabolism in the alpha proteobacterium Sinorhizobium meliloti. *Can J Microbiol* 2014;**60**:491–507.

Göker M, Teshima H, Lapidus A *et al*. Complete genome sequence of the acetate-degrading sulfate reducer Desulfobacca acetoxidans type strain(ASRB2). *Stand Genomic Sci* 2011; **4**:393–401.

Green LS, Emerich DW. Bradyrhizobium japonicum does not require α-ketoglutarate dehydrogenase for growth on succinate or malate. *J Bacteriol* 1997;**179**:194–201.

Green LS, Li YZ, Emerich DW *et al*. Catabolism of α-ketoglutarate by a sucA mutant of Bradyrhizobium japonicum: evidence for an alternative tricarboxylic acid cycle. *J Bacteriol* 2000;**182**:2838–44.

Haag AF, Arnold MF, Myka KK *et al*. Molecular insights into bacteroid development during Rhizobium-legume symbiosis. *FEMS Microbiol Rev* 2013;**37**:364–83.

Haaker H, Veeger C. Involvement of the cytoplasmic membrane in nitrogen fixation by Azotobacter vinelandii. *Eur J Biochem* 1977;**77**:1–10.

Hartmann T, Schwanhold N, Leimkühler S. Assembly and catalysis of molybdenum or tungsten-containing formate dehydrogenases from bacteria. *Biochim Biophys Acta* 2015;**1854**:1090–100.

Hedderich R. Energy-converting [NiFe] hydrogenases from archaea and extremophiles: ancestors of complex I. *J Bioenerg Biomembr* 2004;**36**:65–75.

Hill S, Kavanagh EP. Roles of nifF and nifJ gene products in electron transport to nitrogenase in Klebsiella pneumoniae. *J Bacteriol* 1980;**141**:470–5.

Hirsch PR, Mauchline TH. The importance of the microbial N cycle in soil for crop plant nutrition. *Adv Appl Microbiol* 2015;**93**:45–71.

Hoffman BM, Lukoyanov D, Yang ZY *et al*. Mechanism of nitrogen fixation by nitrogenase: the next stage. *Chem Rev* 2014;**14**:4041–62.

Hungria M, Andrade DS, de O Chueire LG *et al*. Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris* L.) rhizobia from Brazil. *Soil Biol Biochem* 2000;**32**:1515–28.

Hungria M, Campo RJ, Mendes IC. Benefits of inoculation of the common bean (*Phaseolus vulgaris*) crop with efficient and competitive *Rhizobium tropici* strains. *Biol Fert Soils* 2003; **39**:88–93.

Hungria M, Franchini JC, Campo RJ *et al*. Nitrogen nutrition of soybean in Brazil: contributions of biological N_2 fixation and N fertilizer to grain yield. *Can J Plant Sci* 2006;**86**:927–39.

Hungria M, Vargas MAT. Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crop Res* 2000;**65**:151–64.

Jormakka M, Törnroth S, Byrne B *et al*. Molecular basis of proton motive force generation: structure of formate dehydrogenase-N. *Science* 2002;**295**:1863–8.

Karunakaran R, Ramachandran VK, Seaman JC *et al*. Transcriptomic analysis of Rhizobium leguminosarum biovar viciae in symbiosis with host plants Pisum sativum and Vicia cracca. *J Bacteriol* 2009;**191**:4002–14.

Klugkist J, Haaker H, Veeger C. Studies on the mechanism of electron transport to nitrogenase in Azotobacter vinelandii. *Eur J Biochem* 1986;**155**:41–6.

Krause A, Ramakumar A, Bartels D *et al*. Complete genome of the mutualistic, N2-fixing grass endophyte Azoarcus sp. strain BH72. *Nat Biotechnol* 2006;**24**:1385–91.

Kumar N, Lad G, Giuntini E *et al*. Bacterial genospecies that are not ecologically coherent: population genomics of Rhizobium leguminosarum. *Open Biol* 2015;**5**:140133.

Laane C, Krone W, Konings WN *et al*. The involvement of the membrane potential in nitrogen fixation by bacteroids of Rhizobium leguminosarum. *FEBS Lett* 1979;**103**:328–32.

Leyva A, Palacios JM, Murillo J *et al*. Genetic organization of the hydrogen uptake (hup) cluster from Rhizobium leguminosarum. *J Bacteriol* 1990;**72**:1647–55.

Li Y, Tian CF, Chen WF *et al*. High-resolution transcriptomic analyses of Sinorhizobium sp. (fredii) NGR234 bacteroids in determinate nodules of Vigna unguiculata and indeterminate nodules of Leucaena leucocephala. *PLoS One* 2013; **8**:e70531.

Ma W, Ogawa T. Oxygenic photosynthesis-specific subunits of cyanobacterial NADPH dehydrogenases. *IUBMB Life* 2015;**67**:3–8.

Marreiros BC, Batista AP, Duarte AM *et al*. A missing link between complex I and group 4 membrane-bound [NiFe] hydrogenases. *Biochim Biophys Acta* 2013;**1827**:198–209.

Martínez-Romero E. Coevolution in Rhizobium-legume symbiosis? *DNA Cell Biol* 2009;**28**:361–70.

Martínez-Romero E, Silva-Sanchez J, Barrios H *et al*. Draft genome sequences of Klebsiella variicola plant isolates. *Genome Announc* 2015;**3**:e01015–15.

Martínez-Romero JC, Ormeño-Orrillo E, Rogel MA *et al*. Trends in rhizobial evolution and some taxonomic remarks. In: Pontarotti P (ed.). *Evolutionary Biology - Concepts, Molecular and Morphological Evolution*. Heidelberg: Springer, 2010, 301–15.

Miller RW, McRae DG, Al-Jobore A *et al*. Respiration supported nitrogenase, activity of isolated Rhizobium meliloti bacteroids. *J Cell Biochem* 1988;**38**:35–49.

Mulder DW, Boyd ES, Sarma R *et al*. Stepwise [FeFe]-hydrogenase H-cluster assembly revealed in the structure of HydA(DeltaEFG). *Nature* 2010;**465**:248–51.

Nambu M, Tatsukami Y, Morisaka H *et al*. Quantitative time-course proteome analysis of Mesorhizobium loti during nodule maturation. *J Proteomics* 2015;**125**:112–20.

O'Brian MR, Maier RJ. Molecular aspects of the energetics of nitrogen fixation in Rhizobium-legume symbioses. *Biochim Biophys Acta* 1989;**974**:229–46

Oh JI, Bowien B. Structural analysis of the fds operon encoding the NAD+-linked formate dehydrogenase of Ralstonia eutropha. *J Biol Chem* 1998;**273**:26349–60.

Ormeño-Orrillo E, Hungria M, Martinez-Romero E. Dinitrogen-fixing prokaryotes. In: Rosenberg E, DeLong EF, Lory S *et al*. (eds). *The Prokaryotes. Prokaryotic Physiology and Biochemistry*. Berlin-Heidelberg: Springer, 2013, 427–51.

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016

Ormeño-Orrillo E, Menna P, Almeida LG *et al*. Genomic basis of broad host range and environmental adaptability of Rhizobium tropici CIAT 899 and Rhizobium sp. PRF 81 which are used in inoculants for common bean (Phaseolus vulgaris L.). *BMC Genomics* 2012;**13**:73.

Peng J, Hao B, Liu L *et al*. RNA-Seq and microarrays analyses reveal global differential transcriptomes of mesorhizobium huakuii 7653R between bacteroids and free-living cells. *PLoS One* 2014;**9**:e93626.

Persson T, Battenberg K, Demina IV *et al*. Candidatus Frankia datiscae Dg1, the actinobacterial microsymbiont of Datisca glomerata, expresses the canonical nod genes nodABC in symbiosis with its host plant. *PLoS One* 2015;**10**:e0127630.

Pessi G, Ahrens CH, Rehrauer H *et al*. Genome-wide transcript analysis of Bradyrhizobium japonicum bacteroids in soybean root nodules. *Mol Plant Microbe In* 2007;**20**:1353–63.

Pohl T, Bauer T, Dörner K *et al*. Iron-sulfur cluster N7 of the NADH:ubiquinone oxidoreductase (complex I) is essential for stability but not involved in electron transfer. *Biochemistry* 2007;**46**:6588–96.

Poole P, Allaway D. Carbon and nitrogen metabolism in Rhizobium. *Adv Microb Physiol* 2000;**43**:117–63

Poole RK, Hill S. Respiratory protection of nitrogenase activity in Azotobacter vinelandii-roles of the terminal oxidases. *Biosci Rep* 1997;**17**:303–17.

Prell J, Bourdès A, Karunakaran R *et al*. Pathway of gamma-aminobutyrate metabolism in Rhizobium leguminosarum 3841 and its role in symbiosis. *J Bacteriol* 2009;**191**:2177–86.

Price MN, Arkin AP, Alm EJ. The life-cycle of operons. *PLoS Genet* 2006;**2**:e96.

Quispel A. Hellriegel and Wilfarth's discovery of (symbiotic) nitrogen fixation hundred years ago. In: Bothe H, de Bruijn FJ, Newton WE (eds). *Nitrogen Fixation: Hundred Years After: Proceedings of the 7th International Congress on N Nitrogen Fixation*. Kohln: Gustav Fischer, 1988, 3–12.

Remigi P, Zhu J, Young JP *et al*. Symbiosis within Symbiosis: evolving nitrogen-fixing legume symbionts. *Trends Microbiol* 2016;**24**:63–75.

Rogel MA, Bustos P, Santamaría RI *et al*. Genomic basis of symbiovar mimosae in *Rhizobium etli*. *BMC Genomics* 2014;**15**:575.

Rosenthal AZ, Zhang X, Lucey KS *et al*. Localizing transcripts to single cells suggests an important role of uncultured deltaproteobacteria in the termite gut hydrogen economy. *P Natl Acad Sci USA* 2013;**110**:16163–8.

Sato T, Hongoh Y, Noda S *et al*. Candidatus Desulfovibrio trichonymphae, a novel intracellular symbiont of the flagellate Trichonympha agilis in termite gut. *Environ Microbiol* 2009;**11**:1007–15.

Sazanov LA. A giant molecular proton pump: structure and mechanism of respiratory complex I. *Nat Rev Mol Cell Bio* 2015;**16**:375–88.

Sazanov LA, Hinchliffe P. Structure of the hydrophilic domain of respiratory complex I from Thermus thermophilus. *Science* 2006;**311**:1430–6.

Schmehl M, Jahn A, Meyer zu Vilsendorf A *et al*. Identification of a new class of nitrogen fixation genes in Rhodobacter capsulatus: a putative membrane complex involved in electron transport to nitrogenase. *Mol Gen Genet* 1993;**241**: 602–15.

Schmitz RA, Klopprogge K, Grabbe R. Regulation of nitrogen fixation in Klebsiella pneumoniae and Azotobacter vinelandii: NifL, transducing two environmental signals to the nif transcriptional activator NifA. *J Mol Microb Biotech* 2002;**4**:235–42.

Shah VK, Stacey G, Brill WJ. Electron transport to nitrogenase. Purification and characterization of pyruvate:flavodoxin oxidoreductase. The nifJ gene product. *J Biol Chem* 1983;**258**:12064–8.

Siqueira AF, Ormeño-Orrillo E, Souza RC *et al*. Comparative genomics of *Bradyrhizobium japonicum* CPAC 15 and *Bradyrhizobium diazoefficiens* CPAC 7: elite model strains for understanding symbiotic performance with soybean. *BMC Genomics* 2014;**15**:420.

Soberón M, Aguilar GR, Sánchez F. Rhizobium phaseoli cytochrome c-deficient mutant induces empty nodules on Phaseolus vulgaris L. *Mol Microbiol* 1993;**8**:159–66.

Spero MA, Aylward FO, Currie CR *et al*. Phylogenomic analysis and predicted physiological role of the proton-translocating NADH:quinone oxidoreductase (complex I) across bacteria. *MBio* 2015;**6**:e00389–15.

Sprecher BN, Gittings ME, Ludwig RA. Respiratory membrane endo-hydrogenase activity in the microaerophile Azorhizobium caulinodans is bidirectional. *PLoS One* 2012;**7**:e36744.

Stairs CW, Leger MM, Roger AJ. Diversity and origins of anaerobic metabolism in mitochondria and related organelles. *Philos T Roy Soc B* 2015;**370**:20140326.

Tichi MA, Meijer WG, Tabita FR. Complex I and its involvement in redox homeostasis and carbon and nitrogen metabolism in Rhodobacter capsulatus. *J Bacteriol* 2001;**183**:7285–94.

Tsukada S, Aono T, Akiba N *et al*. Comparative genome-wide transcriptional profiling of Azorhizobium caulinodans ORS571 grown under free-living and symbiotic conditions. *Appl Environ Microb* 2009;**75**:5037–46.

Tushar L, Sravanthi T, Sasikala C *et al*. Draft Genome Sequence of Spirochaeta sp. Strain JC202, an Endosymbiont of the Termite (Isoptera) Gut. *Genome Announc* 2015;**3**:e01481–14.

Udvardi M, Poole PS. Transport and metabolism in legume-rhizobia symbioses. *Annu Rev Plant Biol* 2013;**64**:781–805.

Vercruysse M, Fauvart M, Beullens S *et al*. A comparative transcriptome analysis of Rhizobium etli bacteroids: specific gene expression during symbiotic nongrowth. *Mol Plant Microbe In* 2011;**24**:1553–61.

Vignais PM, Billoud B. Occurrence, classification, and biological function of hydrogenases: an overview. *Chem Rev* 2007;**107**:4206–72.

Weerakoon DR, Olson JW. The Campylobacter jejuni NADH:ubiquinone oxidoreductase (complex I) utilizes flavodoxin rather than NADH. *J Bacteriol* 2008;**190**:915–25.

Xie F, Cheng G, Xu H *et al*. Identification of a novel gene for biosynthesis of a bacteroid-specific electron carrier menaquinone. *PLoS One* 2011;**6**:e28995.

Yagi T, Yano T, Di Bernardo S *et al*. Procaryotic complex I (NDH-1), an overview. *Biochim Biophys Acta* 1998;**1364**:125–33.

Yates R, Howieson J, De Meyer SE *et al*. High-quality permanent draft genome sequence of Rhizobium sullae strain WSM1592; a Hedysarum coronarium microsymbiont from Sassari, Italy. *Stand Genomic Sci* 2015;**10**:44.

Youard ZA, Abicht HA, Mohorko E *et al*. A plethora of terminal oxidases and their biogenesis factors in Bradyrhizobium japonicum. In: de Bruijn FJ (ed.). *Biological Nitrogen Fixation*, Vol. 1. Hoboken, New Jersey, USA: Wiley-Blackwell, 2015, 293–305.

Young JPW. The phylogeny and evolution of nitrogenases. In: Palacios R, Newton W (eds). *Genomes and Genomics of Nitrogen-fixing Organisms. Volume* **3** of the series Nitrogen Fixation: Origins, Applications, and Research Progress. Heidelberg: Springer, 2005, 221–41.

Zahran HH. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol Mol Biol R*1999;**63**:968–89.

Downloaded from http://femsec.oxfordjournals.org/ by guest on November 25, 2016