

Review

An overview of the ferredoxin NAD⁺ reductases used for energy conservation in various anaerobic microorganisms

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Abstract: In the context of the development of bioprocesses for the production of biofuels and bulk chemicals, microbial cells are rationally engineered to produce such molecules at high yield and titres in order to develop new biological methods that satisfy economic constraints. The redox and energetic balances of such strains play crucial roles in performance. Processes performed in strict anaerobes have a limited amount of energy available compared to that in aerobic organisms. This energy is obtained through fermentation and/or ion gradient-driven phosphorylation. Such anaerobic organisms have developed energy conservation mechanisms to increase ATP yields. This paper presents the properties of one of these mechanisms catalysed by the Rnf complex, an ion-translocating membrane complex with a ferredoxin NAD⁺ oxidoreductase activity. The Rnf complex performs the transfer of electrons from reduced ferredoxin to NAD⁺ coupled with an ion-motive transport. Ferredoxin is a common electron carrier for anaerobic bacteria and, with NAD⁺, is involved in several pathways of interest for the production of biofuels. This complex was first identified in *Rhodobacter capsulatus* and found to be involved in nitrogen fixation. It was then found to be involved in energy conservation in multiple anaerobic organisms, from acetobacteria such as *Acetobacterium woodii* to sulfate-reducing bacteria such as *Desulfovibrio alaskensis* and autotrophic bacteria such as *Clostridium ljungdahlii* and *Clostridium aceticum*. The Rnf complex triggers two types of ion transports: it can be either a sodium or a proton transporter. Both of these transports create a gradient of ions, generating a membrane potential that is then used by ATPase to produce ATP and thus serving as an energy conservation mechanism. In this review, the available information on the Rnf complex from genetic organization up to its *in vivo* and *in vitro* activities in several microorganisms is summarized, with a special focus on the proton-motive Rnf complex.

Keywords: Rnf complex; energy conservation; ferredoxin NAD⁺ reductase; anaerobic metabolism

1. Introduction

Energy availability and redox balance play a crucial role in rational strain engineering. During aerobic processes, respiration using oxygen as an electron acceptor provides most of the vital energy to sustain cell life. Some facultative bacteria can use alternative electron acceptors, such as NO_3^- , NO_2^- and others, but for strict anaerobes, fermentation and/or ion gradient-driven phosphorylation are the only means to produce energy. The amount of energy produced is generally between 0.3 and 4 moles of ATP per mole of hexoses for strictly anaerobic organisms such as homoacetogenic bacteria[1], which is much lower than the energy produced by aerobic organisms (up to 38 moles of ATP per mole of glucose). Strict anaerobes have developed mechanisms for energy conservation to overcome this limitation: their ATP yield is increased by coupling their metabolic pathways to the generation of transmembrane ion gradients. This gradient of ions is coupled to ATPase to produce ATP by a chemiosmotic mechanism. Some of these mechanisms are well known, such as the fumarate reductase system[2], but others were discovered recently. Among these newly discovered energy conservation systems[3], the energy-converting hydrogenase (Ech) couples hydrogen production to ATP production. Another is the Rnf complex, a ferredoxin: NAD^+ oxidoreductase membrane complex that catalyses the electron transfer from reduced ferredoxin to NAD^+ . It also provides energy to translocate ions across the cytoplasmic membrane, which leads to ATP synthesis by a membrane-bound ATP synthase.

The Rnf complex (**R**hodobacter **n**itrogen fixation) was first identified in *Rhodobacter capsulatus* for its involvement in nitrogen fixation[4,5]. The complex was found to participate in energy conservation in several organisms, such as acetogenic bacteria during autotrophic growth[6]. Breakthroughs concerning the Rnf complex are summarized in Figure 1 below.

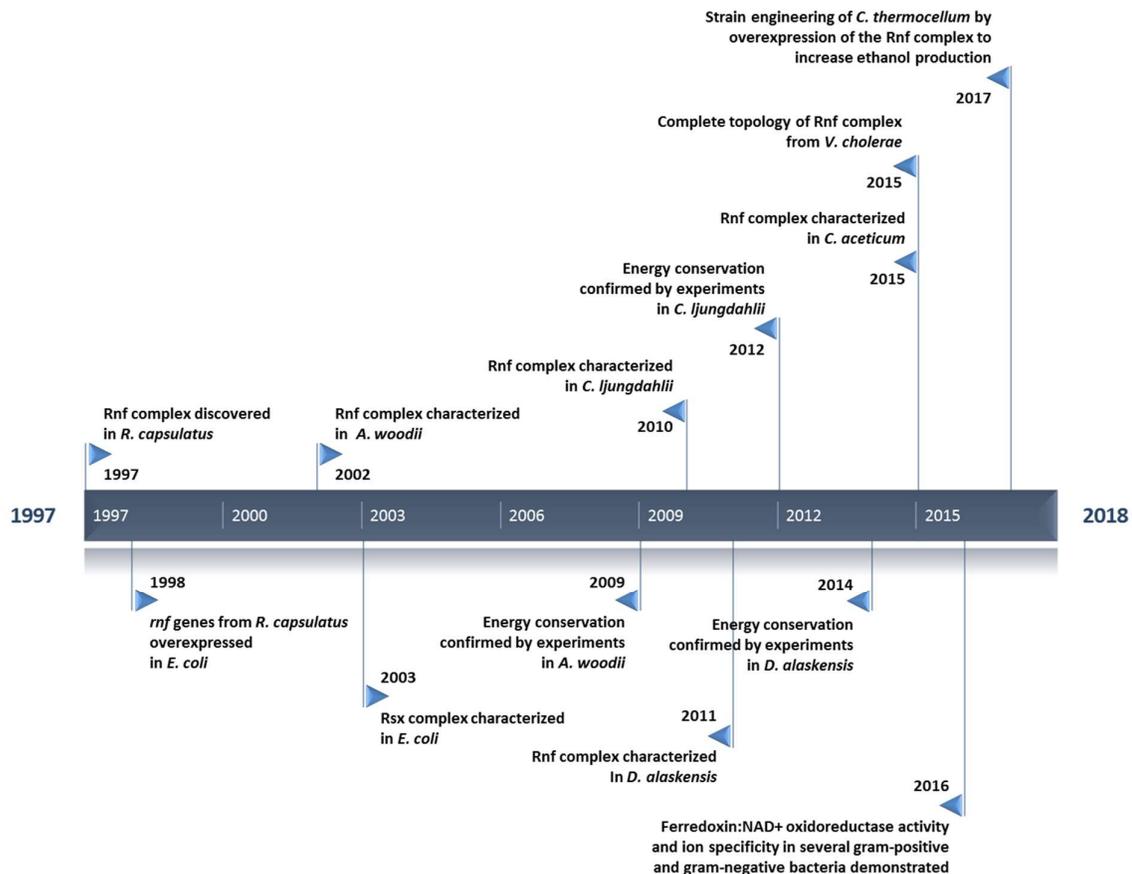


Figure 1. Timeline of the history of the Rnf complex

The Rnf complex was the key to understanding the energetic balance of acetogens during autotrophic growth on inorganic gases such as CO, H₂ and CO₂. Inorganic gases are potential sources of carbon and energy for acetogenic bacteria through the Wood-Ljungdahl pathway (WLP) for the production of acetate[7]. The energetic balance of this pathway[8] remained a mystery due to the absence of a net synthesis of ATP by substrate phosphorylation to sustain autotrophic growth[6], until the recent discovery and characterization of the Rnf complex[9–11].

Among acetogenic bacteria, three organisms are well-studied model energy conservation mechanisms: *Moorella thermoacetica*, *Acetobacterium woodii*[12] and *Clostridium ljungdahlii*. These three organisms have the ability to use syngas as a carbon source[13], but there are some variations in their mechanisms of energy conservation during autotrophic growth[14].

- *M. thermoacetica*, previously called *C. thermoaceticum*, is a gram-positive moderate thermophile: this organism was the key to elucidating the WLP and is able to grow on H₂+CO₂ and CO as carbon sources.
- *A. woodii* is a gram-positive chemolithoautotrophic bacterial species that produces acetate as its main product and uses H₂+CO₂ as well as fructose, glucose, lactate and other compounds as carbon sources.
- *C. ljungdahlii* is a gram-positive chemolithotrophic organism that produces acetate, ethanol and 2,3-butanediol through the WLP.

Both *A. woodii* and *C. ljungdahlii* have a Rnf complex to ensure ATP production, but some variations in the enzymes in the WLP[15] distinguish them. The WLP from *A. woodii* produces 0.3 moles of ATP per mole of acetate by generating an electrochemical Na⁺ potential through the Rnf complex to sustain autotrophic growth. The difference between *A. woodii* and *C. ljungdahlii* is that the first uses a Na⁺ gradient for chemiosmotic ATP synthesis, whereas the second uses a H⁺ gradient. The production of ethanol through the WLP by *C. ljungdahlii* also leads to the production of ATP. For example, *Clostridium autoethanogenum*, a close relative of *C. ljungdahlii*[16], produces 0.14 to 1.5 moles of ATP per mole of ethanol depending on the H₂ partial pressure in the syngas mixture[17]. The third studied model, *M. thermoacetica*, uses an energy-converting hydrogenase (Ech) to ensure ATP production but lacks an Rnf complex and thus cannot be used for its characterization. These new discoveries in acetogenic bacteria for the coupling of WLP with the generation of transmembrane ion gradients have led to the modification of their bioenergetic classification. Previously, they were sorted into two classes: cytochrome containing and Na⁺ independent. A new bioenergetic classification was proposed by K. Schuchmann and V. Müller in 2014[6], 'Rnf-acetogens' and 'Ech-acetogens', with two subclasses depending on whether the complex uses Na⁺ or H⁺ to generate the transmembrane ion gradient leading to ATP synthesis. Few organisms, such as rumen butyrylvibrios[18], are reported in the literature with the combination of both Ech and Rnf complex to ensure energy conservation.

The objective of this review is to present the current state of the art on Rnf complexes with a particular focus on proton-motive Rnf complexes. Most of the knowledge available about the Rnf complex concerns the sodium-motive complex, but studies of the proton-motive complex are on the rise. First, we will discuss the general properties of the Rnf complex: the relationships between structure and function, thermodynamics, and complex activity. Then, we will present examples of organisms that use the proton-motive Rnf complex.

2. General presentation of the Rnf complex

The Rnf complex (Figure 2) is a ferredoxin:NAD⁺ oxidoreductase that couples an electron transport chain with an ionic channel[3]. There are two types of ion transporters[19] for the Rnf complex: sodium transporters[12,20] and proton transporters[21]. Both of these transports create a gradient of ions, generating a membrane potential that is then used by ATPase[22,23] to produce ATP. This translocation of ions is generally coupled with the exergonic reaction of ferredoxin reduction, with NAD⁺ as electron acceptor[24,25].

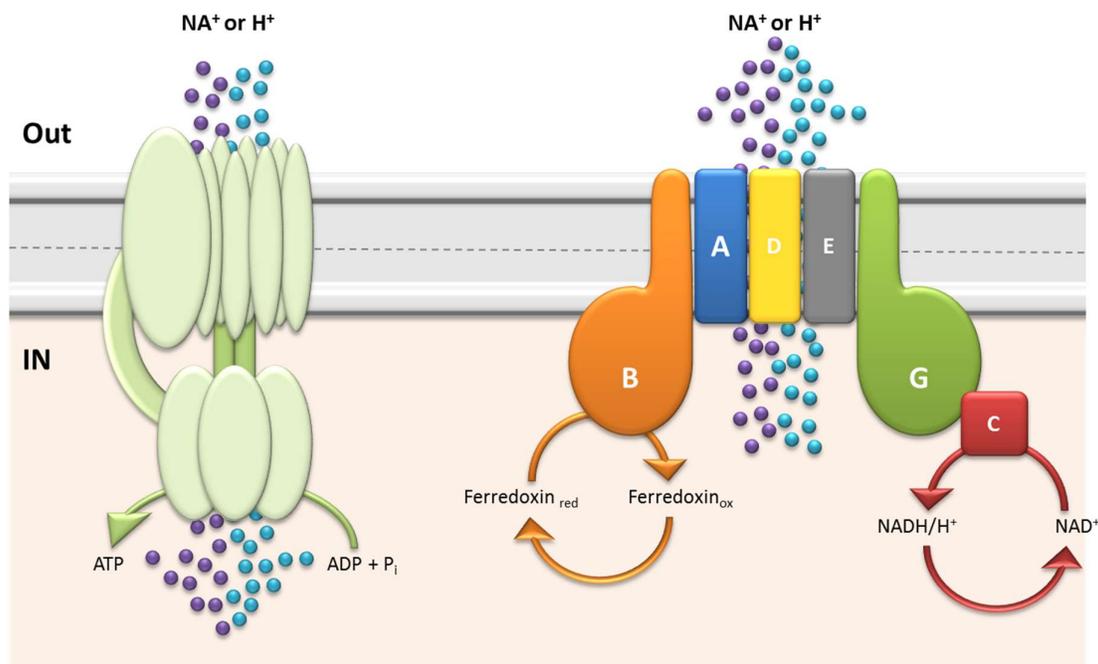


Figure 2. Representation of the Rnf complex coupled to ATPase. Adapted from M.Köpke et al. [26]

Ferredoxin is a common electron carrier containing iron-sulfur clusters (Figure 3) that is found in various organisms, from plant chloroplasts to bacteria and archaea.

The characteristics of ferredoxin differ among organisms:

- The molecular mass of ferredoxin varies from 6 kDa to 12 kDa.
- The number of [FeS] clusters can be one, two or more.
- The structure of the clusters can be [2Fe-2S] or [4Fe-4S].

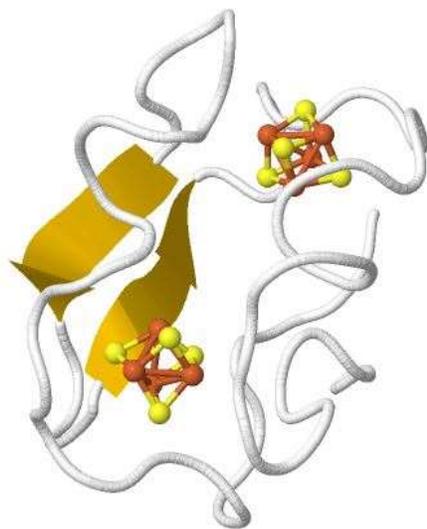


Figure 3. Representation of a 2[4Fe-4S] ferredoxin from *C. pasteurianum* (PDB identification : 1CLF)[27]

The function of this electron carrier is maintained: ferredoxin generally carries only one electron at a time and has a lower reduction potential (E_0'), near -402 mV, than nicotinamides (-320 mV)[28]. Ferredoxin is found as a co-factor in various anaerobic pathways of interest for biofuels and bulk chemical production, such as butanol production in *Clostridia sp.*[29]. Mechanisms that increase the availability of cofactors, such as the Rnf complex, are of special interest for metabolic engineering.

2.1 Energetic properties of Rnf complex

There are two possible mechanisms for ATP synthesis[30]: substrate-level phosphorylation (SLP) and the chemiosmotic mechanism. Only the latter is used by the Rnf complex.

In the SLP mechanism, the free energy from exergonic chemical reactions is directly coupled to the phosphorylation of ADP, leading to ATP synthesis. This free energy needs to be above -31.8 kJ/mol to phosphorylate ADP. However, very few reactions are capable of liberating such a high level of energy, which limits the number of reactions leading to ATP synthesis[31].

The chemiosmotic mechanism involves two steps: first, an electrochemical ion gradient is generated across the membrane, and then, ATP synthesis occurs through ATPases[2]. According to thermodynamics analyses, approximately -20 kJ/mol is required to phosphorylate ADP using an ATPase[31,32]. The Rnf complex uses the chemiosmotic mechanism to produce ATP by translocating Na^+ or H^+ , depending on the organism. The free energy allowing this translocation comes from the electron transfer from reduced ferredoxin ($E_0' = -450$ mV) to NAD^+ ($E_0' = -320$ mV). For example, the electron transfer from the Rnf complex of *A. woodii* generates approximately -25 kJ/mol of free energy[6], and *A. woodii* needs 11.5 to 9.1 kJ/mol with a membrane potential of approximately -180 mV to translocate one Na^+ across the membrane. According to these values[33], each oxidation of ferredoxin allows 1 to 2 Na^+ to cross the membrane. In the worst case scenario (only 1 ion translocated), 3 moles of ferredoxin must be oxidized to produce 1 mole of ATP (Figure 4), and in the best case (2 Na^+ translocated), only 1.5 moles of ferredoxin need to be oxidized to produce 1 mole of ATP.

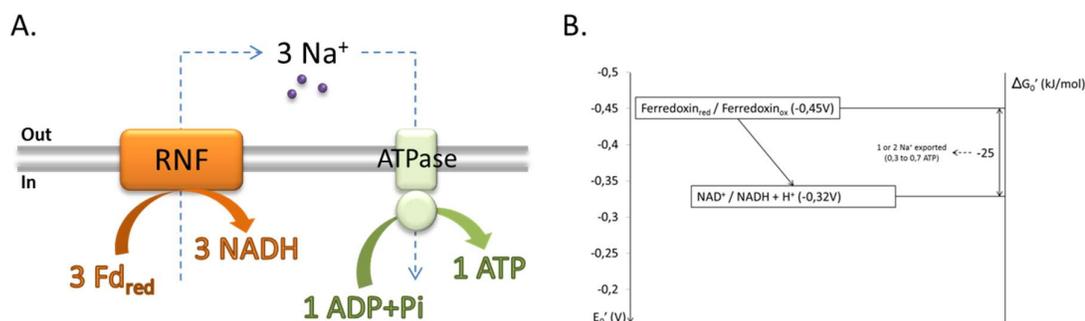


Figure 4. Representation of the oxidation of reduced ferredoxin to NAD^+ by the Rnf complex of *A. woodii*. (A) Schema of the reaction with one sodium translocated per ferredoxin oxidized. (B) Energetic diagram. * Fd_{red}: reduced ferredoxin.

The metabolism of organisms using the WLP pathway, such as *A. woodii*, is entirely designed to operate at a high ratio of reduced ferredoxin to NAD^+ . This ability allows autotrophic growth by generating ATP with the Rnf complex or another ferredoxin oxidoreductase, such as the Ech hydrogenase mentioned above.

The role of the Rnf complex among ferredoxin: NAD^+ oxidoreductases was investigated through enzymatic assays to monitor the activity of these reactions in different organisms.

2.2 Ferredoxin: NAD^+ oxidoreductase activity assays

Recently, ferredoxin: NAD^+ oxidoreductase activity was evaluated *in vitro* on the membranes of several organisms [19] and on inverted vesicles for additional information on the ion specificity[25]. Ferredoxin was purified from *Clostridium pasteurianum* to perform this enzymatic assay. Rnf activity was recently demonstrated in *Clostridium tetanomorphum*, *C. ljungdahlii*, *Bacteroides fragilis*, and *Vibrio cholera*. The first Rnf oxidoreductase activity was demonstrated in *A. woodii*, which was used as a control for the subsequent measurements. *C. tetanomorphum*, *B. fragilis* and *A. woodii* showed Na^+ -dependent activity, whereas the activity in *C. ljungdahlii* and *V. cholerae* was independent of Na^+ addition. The strongest activity was found in *C. tetanomorphum*, a strictly anaerobic organism using glutamate as its carbon source[20], with 900 ± 29 mU/mg of activity. This activity is 18-fold higher

than in *A. woodii*, which was the best characterized organism for Rnf complex activity so far. A significant activity reduction of 431 ± 16 mU/mg is observed for *C. tetanomorphum* in the absence of Na^+ , showing the Na^+ dependence of the activity. *B. fragilis* Δrnf mutant was also constructed for this study, and a reduction of 89% in the ferredoxin:NAD⁺ oxidoreductase activity was measured compared to that of the native strain. These results show the importance of the Rnf complex for energy conservation in *B. fragilis*.

Even if the Rnf complex is mainly described as oxidizing ferredoxin, the substrate may be replaced, and the reaction may also work the other way: *in vitro* assays on the Rnf complex from *Acidaminococcus fermentans*, which is a Na^+ translocating complex, have shown the possibility of replacing ferredoxin with flavodoxin as the electron donor to NAD⁺[34]. Moreover, evidence has shown that the Rnf complex activity in *A. woodii* is reversible and could also import ions into the cell and cause ferredoxin reduction [24].

2.3. Structure-function relation of the Rnf complex

The Rnf complex (Figure 2 mentioned before) consists in 6 subunits, RnfA, RnfB, RnfC, RnfD, RnfE and RnfG. The genes expressing each subunit are expressed in a single operon, in various orders depending on the organism. After bioinformatics analyses, three clusters were built according to the possible gene organization in the operon (Table 1 below).

Table 1. Cluster families of the Rnf complex with a non-exhaustive list of examples.

Cluster type	Cluster schema	Related organisms
<i>rnfABCDGE</i>		<i>R. capsulatus</i> * – <i>E. coli</i> – <i>V. fischeri</i> – <i>V. cholerae</i> – <i>P. stutzeri</i> – <i>A. fermentans</i>
<i>rnfCDGEAB</i>		<i>A. woodii</i> – <i>E. limosum</i> – <i>D. alaskensis</i> ** – <i>C. ljungdahlii</i> – <i>C. tetanomorphum</i> – <i>C. kuyveri</i> – <i>C. acetivum</i> – <i>C. thermocellum</i>
<i>rnfBCDGEA</i>		<i>B. fragilis</i> – <i>B. vulgatus</i> – <i>C. limicola</i>

R. capsulatus* : *rnfABCDGEH*. *D. alaskensis* : *rnfCDGEABF*.

The first cluster, *rnfABCDGE*, includes *R. capsulatus*, *E. coli*, *V. fischeri*, *V. cholerae* and *P. stutzeri*. The second cluster, *rnfCDGEAB*, includes *A. woodii*, *E. limosum* and *Clostridia* sp. such as *C. ljungdahlii*, *C. tetanomorphum*, *C. kuyveri* and *C. ultunense*[35]. The last cluster, *rnfBCDGEA*, includes *B. fragilis*, *B. vulgatus* and *C. limicola*. Despite the variety in gene order and length, all the genes of the Rnf complex maintain their functions between species. RnfB and RnfC are catalytic subunits. The RnfA, RnfD, RnfE and RnfG subunits are involved in electron transfer and ion transport.

2.4 Subunit properties

The properties of the Rnf subunits were mainly deduced from bioinformatics analyses. It was reported in the literature that the *rnf* genes were difficult to clone for overexpression, making research in heterologous organisms or the mutant complementation difficult[21]. Some genes were successfully cloned independently, and further analyses were performed. The available knowledge on each subunit is summarized below, using *A. woodii* (*rnfCDGEAB*) as a model organism with additional information from other organisms when data are available.

For *A. woodii*, the subunit RnfC is a soluble protein of 48.7 kDa covalently bound to the membrane protein RnfG of the Rnf complex[36]. Bioinformatics analyses have shown a cysteine pattern (C-XX-C-XX-C-XXX-C-P) specific to a [4Fe4S] centre and a binding site for NADH. The [4Fe4S] centre was experimentally demonstrated in RnfC from *R. capsulatus* using EPR spectroscopy

correlated with spectrophotometric absorption and iron content[37]. The subunit RnfC seems to be the exit point of electrons transferred through the Rnf complex from ferredoxin to NAD^+ . A schematic representation of the electron transfer from ferredoxin to NAD^+ inside the Rnf complex is depicted in Figure 5 below.

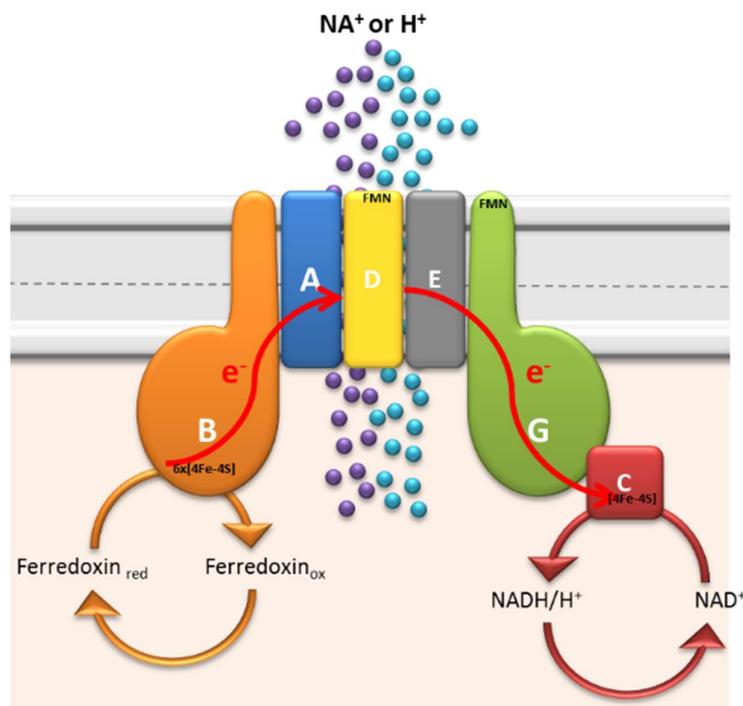


Figure 5. Schematic representation of the structure and function of the Rnf complex

The subunit RnfD in *A. woodii* is a 35 kDa transmembrane protein. Bioinformatics analyses have shown 6 to 9 transmembrane helices and an FMN binding site. The FMN was found to be covalently attached to RnfD in *V. cholera* at threonine 187[38,39]. *In vivo*, in *V. cholera*, this FMN is located in the periplasm. Its functions remain unknown. This FMN binding site in RnfD is largely conserved in all organisms expressing a Rnf complex.

The subunit RnfG is a 22.8 kDa protein with a soluble part, a membrane anchor[36] and a hydrophobic domain of 30 amino acids on the N-terminal side. An FMN was experimentally demonstrated to be covalently bound to RnfG[39] in the subunit in *V. cholerae* at threonine 175[38]. An FMN was also demonstrated to be covalently bound to RnfG in the archaeon *Methanosarcina acetivorans* at threonine 166[40]. This FMN is also located in the periplasm of *V. cholerae*. During the attempt at the heterologous expression of RnfG from *T. maritima* in *E. coli*, no FMN was found to be covalently bound; an enzyme from *T. maritima* that ensures the proper binding of FMN on RnfG may be missing.

The subunit RnfE is a 21.6 kDa membrane protein. Bioinformatics analyses have predicted 6 transmembrane helices anchored in the membrane and detected no co-factor binding site or known pattern for an ionic channel. A fusion between RnfE and PhoA was constructed to understand the orientation of RnfE in the membrane[41]. This fusion confirmed all 6 transmembrane helices and showed that both the C-terminus and the N-terminus are located in the cytoplasm.

The subunit RnfA has a similar structure to RnfE and is a 21.4 kDa membrane protein with 6 transmembrane helices. In contrast to RnfE, the fusion of RnfA and PhoA has shown that both the C-terminal and N-terminal are located in the periplasm[41]. Hypotheses have been proposed about the symmetrical orientation of RnfE and RnfA[41,42]: this orientation could play an important role in the transfer of electrons or in the creation of an ionic channel.

The subunit RnfB is a 36.6 kDa protein kDa[37] with a soluble part and a membrane anchor with a hydrophobic domain of 30 amino acids at the N-terminus. Bioinformatics analyses have shown 6 [4Fe4S] centres: 4 of them have the classic cysteine pattern (C-XX-C-XX-C-XXX-C-P), and 2 of them are not typical, with proline replaced by arginine. These [4Fe4S] centres from RnfB seem to be responsible for the binding between ferredoxin and the Rnf complex; they were demonstrated in *M. acetivorans*[40] and suggest that RnfB is the entry point of electrons into the Rnf complex.

During the past decade, the comprehension of the involvement of the Rnf complex in energy conservation has drastically increased for acetogenic organisms such as *A. woodii*. However, organisms with the proton-motive Rnf complex were characterized even more recently than those with the sodium-motive Rnf complex.

3. Role of the Rnf complex in several organisms

3.1. Role of the Rnf complex in *Clostridium ljungdahlii* metabolism

C. ljungdahlii is a strictly anaerobic homoacetogenic bacterium that uses the energy conservation mechanism of the Rnf complex during autotrophic growth on inorganic gas. Its Rnf complex was first identified by sequence homology with the Rnf complex of *A. woodii* (operon organization: *rnfCDGEAB*), and its functionality and *in vivo* role were then confirmed by experiments[21,43]. Detailed information on *C. ljungdahlii* is summarized in Table 2 at the end.

Strain engineering was performed to understand the involvement of the Rnf complex in *C. ljungdahlii* metabolism. The impact of the inactivation of the *rnf* operon was evaluated by inactivating the transcription of *rnfA* and *rnfB* using a suicide plasmid integrated into the chromosome by single crossover:

- In heterotrophic growth using fructose as carbon source, the inactivation of the Rnf complex significantly reduces the intra-cellular level of ATP, even though other pathways are used by the cell to maintain energized membranes. The doubling time is approximately 5 h 51 min when *rnf* is inactivated, compared to 3 h 56 min for the wild type.
- Likewise, in autotrophic growth on syngas, the proton-motive force is entirely dependent on the Rnf functionality: the inactivation of the Rnf complex results in a complete absence of proton gradient or ATP synthesis. The mutant lacking Rnf activity is unable to grow in autotrophic conditions.

These experimental results in cells were confirmed afterwards with *in vitro* inverted membrane vesicles of *C. ljungdahlii* [19], which resulted in an activity of 256 ± 7 mU/mg for the transfer of electrons from reduced ferredoxin to NAD⁺.

The nature of the ion translocated by Rnf in this organism was confirmed: the transmembrane potential is converted to ATP by H⁺-ATPase, thanks to an efflux of protons instead of sodium ions. Moreover, the protonophore TCS stimulates the Rnf activity, while the sodium ionophore ETH2120 has no effect on Rnf complex activity[19], which supports the interpretation of an Rnf complex using H⁺ as coupling ion.

3.2. Role of the Rnf complex in *Clostridium aceticum* metabolism

Clostridium aceticum is a strictly anaerobic acetogenic endospore-forming organism that uses the Rnf complex coupled with ATPase to maintain its energy balance. Detailed information on *C. aceticum* is summarized in Table 2.

The Rnf complex that was identified by bioinformatics analyses in *C. aceticum*[44] has the following operon organization: *rnfCDGEAB*. This organization is the same as in *C. ljungdahlii*, and this Rnf complex belongs to the same cluster, along with other *Clostridia* sp. and *A. woodii*.

Clostridium aceticum is distinguished by the presence of a cytochrome C in its genome, which is unusual: *C. aceticum* is the first acetogenic Clostridia organism known to contain both Rnf complex and cytochrome C. The functionality of this cytochrome C was determined *in vitro*[44], but no gene for the biosynthesis of either quinones or Ech hydrogenase was found.

The ion specificity of this Rnf complex was demonstrated by experiments with a Na⁺ ionophore and a protonophore[45]: the results showed that the Rnf complex from *C. aceticum* uses a proton gradient to generate ATP through the WLP, using CO as a carbon source in autotrophic culture. The Rnf complex coupled to ATPase seems to be the only energy conservation system in *C. aceticum*[44].

Na⁺ dependence was also demonstrated during this study[45], but for growth only, not for energy conservation. The optimum concentration for autotrophic growth with CO as a substrate was found to be between 60 and 90 mM Na⁺.

3.3. Role of the Rnf complex in *Desulfovibrio alaskensis* metabolism

Desulfovibrio alaskensis is an anaerobic sulfate-reducing bacterium (SRB). The G20 strain was fully sequenced, and no conservation-related cytoplasmic hydrogenase was detected. Detailed information on *D. alaskensis* is summarized in Table 2 at the end. This result indicated that an energy conservation mechanism other than hydrogen cycling was necessary to sustain sulfate respiration[46]. The Rnf complex from *D. alaskensis* G20, also known as *Desulfovibrio desulfuricans* G20, was characterized to determine the importance of Rnf in this energy conservation mechanism. The Rnf operon in this strain has the following structure: *rnfCDGEABF*[46,47].

This Rnf complex belongs to the same cluster as those of *A. woodii* and *Clostridia* sp. but it has an additional gene in the operon, *rnf F*, that encodes “a Flavin transferase that catalyses the transfer of the FMN moiety of FAD and its covalent binding to the hydroxyl group of a threonine residue in a target flavoprotein”(UniProt). RnfF might be necessary to have the proper cofactors attach to the subunits D and G: subunits RnfD and G require the FMN covalently bound to this threonine[38]. However, as explained before, during the attempt at the heterologous expression of RnfG from *T. maritima* in *E. coli*, no FMN was found to be covalently bound. The RnfF or equivalent from *T. maritima* might be necessary to enable the proper cofactors to bind to subunits D and G.

No evidence is available concerning the function of this FMN, yet all studies highlight its importance.

Experimental results on this organism revealed that Rnf mutants achieved a lower proton-motive force than the parent strain[48]. Evidence[49] also showed an insensitivity to a Na⁺ ionophore and high sensitivity to a protonophore during the reduction of sulfate. According to these results, the Rnf complex from *D. alaskensis* was characterized as generating a proton gradient.

3.4. Physiological properties of Rnf in *Vibrio cholerae*

Vibrio cholera is a facultative anaerobe bacteria for which the implication of the Rnf complex in energy conservation remains unclear. Detailed information on *V. cholerae* is summarized in Table 2. To date, the Rnf complex of *Vibrio cholerae* possesses the most advanced and detailed topological information[38,50]: for most organisms, the Rnf complex topology was deduced from bioinformatics analyses alone, but for *V. cholerae*, the bioinformatics analysis was also correlated with experimental results from fusion with reporter groups, such as PhoA/alkaline phosphatase and green fluorescent protein (GFP)[51]. The details for each subunit of this organism are available in the “subunit properties” section of this review. The *rnf* operon has the following structure: *rnfABCDGE*. It belongs to the same cluster as those of *E. coli* and *R. capsulatus*.

Due to the cluster relation and the genetic homology with *E. coli*, the Rnf from *V. cholerae* was expected to be involved in the reduction of superoxide through the oxygen-sensing protein SoxR[52]. However, a recent enzymatic assay seems to invalidate this hypothesis: ferredoxin:NAD⁺ oxidoreductase activity was measured on the membrane of *V. cholerae*[19], and the electron transfer from reduced ferredoxin to NAD⁺ reached a rate of 7.1 ± 0.7 mU/mg. This activity was independent of the presence of Na⁺, suggesting that the Rnf complex generates a proton gradient. The same measurement was performed on *E. coli* membrane, and no activity was detected[19]. Although there is no evidence of the involvement of Rnf from *V. cholerae* in energy conservation, these results show a difference between the Rnf complexes from *V. cholerae* and *E. coli*.

In conclusion, although the topology of the Rnf complex from *V. cholerae* is well known, and it shares homology with the Na⁺-translocating NADH:quinone oxidoreductase (Na⁺-NQR) from *V. cholerae*[53,54], only hypotheses are available regarding its function.

3.4. Physiological properties of “the Rnf complex-like” R_{sox} in *E. coli*

E. coli is a well-studied gram-negative, facultative anaerobic organism that may not need the Rnf complex for energy conservation. Detailed information on *E. coli* is summarized in Table 2 at the end. In *E. coli*, the Rnf complex is known as the R_{sox} complex, for “Reducer of SoxR” [52]. SoxR is an important regulator of the genes involved in the reduction of superoxide and nitrogen monoxide.

The R_{sox} complex of *E. coli* shows homology with the Rnf complex of *Rhodobacter capsulatus*[52]. Bioinformatics analyses have shown hydrophobic domains in R_{soxA}, R_{soxD} and R_{soxE}, which are predicted to be membrane proteins. The topology of R_{soxA} and R_{soxE} was also confirmed by experiments, and the inversion in the membrane of these two closely related proteins was demonstrated[41,42]. The subunits R_{soxB} and R_{soxG} seem to be anchored in the membrane, with hydrophobic domains and soluble parts. The subunit R_{soxC} has the profile of a soluble protein. Analyses also showed the characteristic patterns of 4Fe4S centres in the subunits R_{soxB} and R_{soxC} and of an NADH binding site in R_{soxC}. *In vitro* assays have shown the ability for R_{soxC} to perform NADPH-dependent reduction of cytochrome c when SoxR is missing.

Due to the lack of a Na⁺ ATPase in *E. coli*, we can assume that this R_{sox} complex translocates protons across the membrane. To date, however, no evidence of this translocation has been experimentally demonstrated.

Measurements of ferredoxin:NAD⁺ oxidoreductase activity were conducted on washed *E. coli* membrane, and no activity was detected[19]. This result allows two hypotheses:

- the R_{sox} complex from *E. coli* does not have ferredoxin:NAD⁺ oxidoreductase activity, or
- the reduced ferredoxin from *C. pasteurianum* used for this enzymatic assay, a [4Fe-4S] ferredoxin, is not compatible with the R_{sox} complex of *E. coli*, which has a [2Fe-2S] ferredoxin[55].

Mutants missing the whole *rsx* operon in Top10 strains were also constructed, and no impact on growth was observed in either aerobic or anaerobic conditions.

In conclusion, the R_{sox} complex from *E. coli* does not seem to be involved in energy conservation as it is in acetogenic bacteria. However, it retains homology with the Rnf complexes from other organisms.

Table 2. Comparison of organisms with a proton-motive Rnf complex

Organism	<i>Clostridium ljungdahlii</i>	<i>Clostridium acetivum</i>	<i>Desulfovibrio alaskensis</i>	<i>Vibrio cholerae</i>	<i>E.coli</i>
Culture	strict anaerobe	strict anaerobe	strict anaerobe	facultative anaerobe	facultative anaerobe
Operon organization	<i>rnfCDGEAB</i>	<i>rnfCDGEAB</i>	<i>rnfCDGEABF</i>	<i>rnfABCDGE</i>	<i>rsxABCDGE</i>
Identification method	bioinformatics analyses + experiments				
Particularity	-	-	gene F in the operon	-	-
Protonophore TCS test	stimulating	stimulating	highly stimulating	no data available	no data available
Sodium ionophore test	no effect	no effect	no effect	no effect	no data available
Proton motive force confirmed	yes	yes	yes	yes	no, but absence of Na ⁺ ATPase

NA ⁺ dependent	no	yes, but not for energy conservation	no	no	no
Ferredoxin:NAD ⁺ oxidoreductase activity	256±7 mU/mg	no data available	no data available	7.1±0.7 mU/mg	no activity detected with [4Fe-4S] ferredoxin
Energy conservation mechanism	Rnf complex	Rnf complex	Rnf complex	no evidence of Rnf involvement	no evidence of Rnf involvement
Presence of cytochrome c	no	yes	yes	yes	yes
Presence of Quinone	no	no	yes	yes	yes

4. Conclusion

The high potential of the Rnf complex for strain engineering was demonstrated as it allows electron transfer from ferredoxin to NAD⁺, leading to a production of NADH, which can be reoxidized for the synthesis of reduced products such as alcohols or chemicals. As an example of a potential application, the Rnf complex was homologously overexpressed in *Clostridium thermocellum*, which is a thermophilic anaerobic organism, to increase ethanol production. This organism naturally produces ethanol and also lactate and acetate from cellulose. The functionality of its Rnf complex was already demonstrated: it operates via a putative translocation of proton[56]. Even though the Rnf complex in this organism is not the best known, bioinformatics-based pathway simulation has shown a relation between Rnf and PPI, which suggests that the oxidation of ferredoxin may be coupled to proton export. The Rnf genes were completely deleted from *C. thermocellum*, which resulted in lower ethanol production. In contrast, when the Rnf genes were overexpressed, the production of ethanol reached a titre of 5.1 g/L, meaning the production increased by 30% when using Avicel as the carbon source[57,58]. To observe these results, hydrogenase had to be deleted from *C. thermocellum*. The result seems to be due to the increase in NADH availability for ethanol production resulting from the increase in Rnf complex activity. As the promoter used for this experiment was the native one, more than a 30% increase in the production of ethanol may be achieved by replacing the native promoter with a constitutive promoter.

Theoretically, in *C. thermocellum* without active Rnf, the yield of ethanol production using cellobiose as carbon source is limited to 2 moles per mole of cellobiose (50% of the maximal theoretical yield). With the Rnf complex, the ethanol yield may reach 4 mol/mol of cellobiose (100% of the maximal theoretical yield).

This possibility is a promising example of the use of an energy conservation mechanism to improve performance in engineered strains. However, the Rnf complex from *C. thermocellum* is not well characterized, and the tools to modify the genome are limited[56]. Understanding the accurate mechanism of the Rnf complex remains a limiting point for its use in metabolic engineering, but during the past decade, impressive improvements have been made in the characterization of the mechanism and physiological properties of the Rnf complex, leading to its first use in *C. thermocellum* to increase ethanol yield[57].

The heterologous expression of the Rnf complex of *C. ljungdahlii* in model organisms offers a good alternative to manipulate the Rnf complex. Several model organisms are described in the literature[59] as producing butanol via a well-known ferredoxin:NADH-dependent pathway from *C. acetobutylicum*. The introduction of the Rnf complex from *C. ljungdahlii* into such organisms may generate the appropriate proton-motive force to produce ATP, enhance the availability of NADH and oxidize ferredoxin for the butanol pathway. In such a case, the yield of butanol and the ATP production would be increased.

The use of Rnf in engineered strains has a promising future, but this complex needs to be investigated in details in more organisms, in order to master this powerful tool for ATP production and redox balance.

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