REVIEW

Biochemistry, evolution and physiological function of the Rnf complex, a novel ion-motive electron transport complex in prokaryotes

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Abstract Microbes have a fascinating repertoire of bioenergetic enzymes and a huge variety of electron transport chains to cope with very different environmental conditions, such as different oxygen concentrations, different electron acceptors, pH and salinity. However, all these electron transport chains cover the redox span from $NADH + H^+$ as the most negative donor to oxygen/H_2O as the most positive acceptor or increments thereof. The redox range more negative than -320 mV has been largely ignored. Here, we have summarized the recent data that unraveled a novel ion-motive electron transport chain, the Rnf complex, that energetically couples the cellular ferredoxin to the pyridine nucleotide pool. The energetics of the complex and its biochemistry, as well as its evolution and cellular function in different microbes, is discussed.

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The acetogenic bacterium *Acetobacterium woodii*, a paradigm for an organism that has based its bioenergetics on a sodium ion current

Acetogenic bacteria such as *A. woodii* are strictly anaerobic and defined by their ability to use CO_2 as an electron acceptor that is reduced to acetate. Acetogens are characterized by a special pathway for CO_2 reduction, the acetyl-CoA pathway, also named the Wood–Ljungdahl pathway [1–3]. Electrons for CO_2 reduction are derived from various donors such as sugars (fructose, glucose; oxidation via the Embden–Meyerhof pathway), alcohols such as propanediol or methanol, methoxy groups from lignin-derived aromatic compounds such as ferulate, or from molecular hydrogen [4–7]. The pathway of carbon dioxide reduction is coupled to energy conservation via a chemiosmotic mechanism, but the enzymes involved are obscure [8]. However, some acetogens have cytochromes, and a proton-motive electron transport chain is assumed in those whereas others do not have cytochromes. For the latter group, *A. woodii* is a bioenergetic paradigm. It couples carbon dioxide reduction to acetate production with the generation of an electrochemical sodium ion gradient across the membrane that is then used to drive ATP synthesis [9–13] and flagella rotation [9, 14], but again, the enzyme(s) involved is/are unknown [15].

Recently, some light was shed on the mystery of bioenergetics in acetogens. It turned out that instead of CO_2 acetogens can also use alternative electron acceptors such as nitrate (*Moorella thermoacetica*) [16, 17] or arylacrylates such as caffeate (*A. woodii*) [18]. The enzymology and bioenergetics of caffeate respiration in *A. woodii* was unraveled very recently and led to the discovery of a novel Na^+-translocating, electron-transferring ferredoxin:NAD^+ oxidoreductase with similarity to the Rnf complex present

in a wide variety of prokaryotes. The enzymology of caffeate respiration is briefly summarized below; for detailed reviews, the reader is referred to [8, 19].

Enzymology of caffeate respiration in *A. woodii*

Phenylacrylates such as caffeate are derived from lignin degradation and are a major constituent of the global carbon storage in soils [20]. The acetogen *A. woodii* cannot use caffeate as a carbon source but is known to use it as an electron acceptor by reducing the carbon–carbon double bond of caffeate according to:

(eq. 1)

$$+ 2 \, [\text{H}] \longrightarrow$$
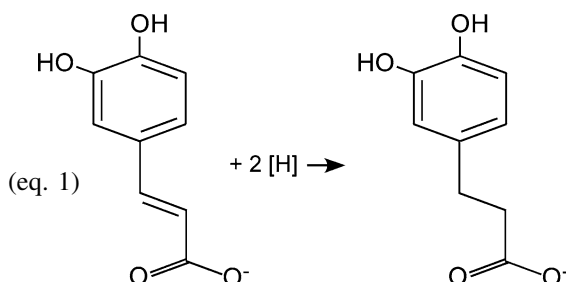
The electrons for caffeate reduction can be derived from various donors such as fructose, methanol or hydrogen [9, 18]. Caffeate reduction with hydrogen as electron donor is coupled to energy conservation in whole cells [18]. Clear evidence for ATP synthesis coupled to caffeate reduction was obtained with resting cells of *A. woodii* in which hydrogen-dependent caffeate reduction was accompanied

by the synthesis of ATP [21], and later it was shown that ATP synthesis occurred by a chemiosmotic mechanism [9]. Most interestingly, like CO_2 reduction, hydrogen-dependent caffeate reduction was strictly Na^+-dependent and ATP synthesis relied on a transmembrane Na^+ gradient. These studies led to the hypothesis that caffeate reduction is coupled to the generation of a transmembrane electrochemical Na^+ gradient that is then used for ATP synthesis by the well-established Na^+ F_1F_O ATP synthase [9–13]. However, the nature of the Na^+-translocating enzyme remained enigmatic until very recently.

To identify the Na^+-translocating enzyme, we have unraveled the entire pathway of electrons derived from the donor, molecular hydrogen, to the acceptor, caffeate (Fig. 1). Based on enzymatic activities, proteomics and also genetic evidence, we have suggested that caffeate is activated to caffeyl-CoA and then reduced to hydrocaffeyl-CoA by NADH via soluble enzymes involving an electron-transferring flavoprotein (Etf) and a caffeyl-CoA reductase/hydrocaffeyl-CoA dehydrogenase. This entire reaction sequence from NADH to caffeyl-CoA was found in the soluble fraction excluding any of these reactions in Na^+ transport [22]. Furthermore, hydrogenase activity in *A. woodii* is cytosolic and the purified enzyme uses ferredoxin as an electron acceptor [23]. This left the electron transfer from reduced ferredoxin (Fd_{red}) to NAD^+ as the only membrane-bound and potentially Na^+-translocating reaction [19, 22]. Indeed, a membrane-bound ferredoxin:NAD^+ oxidoreductase (Fno) activity was found in washed membranes of *A. woodii* [22].

Fig. 1 Model of caffeate respiration in *A. woodii*, showing the flow of electrons from electron donors (fructose or hydrogen) to the acceptor caffeate. Fno/Rnf couples the flow of electrons from reduced ferredoxin (Fd_{red}) to NAD^+ thereby generating a sodium ion gradient across the cytoplasmic membrane. For explanations, see text. *ox* oxidized, *red* reduced
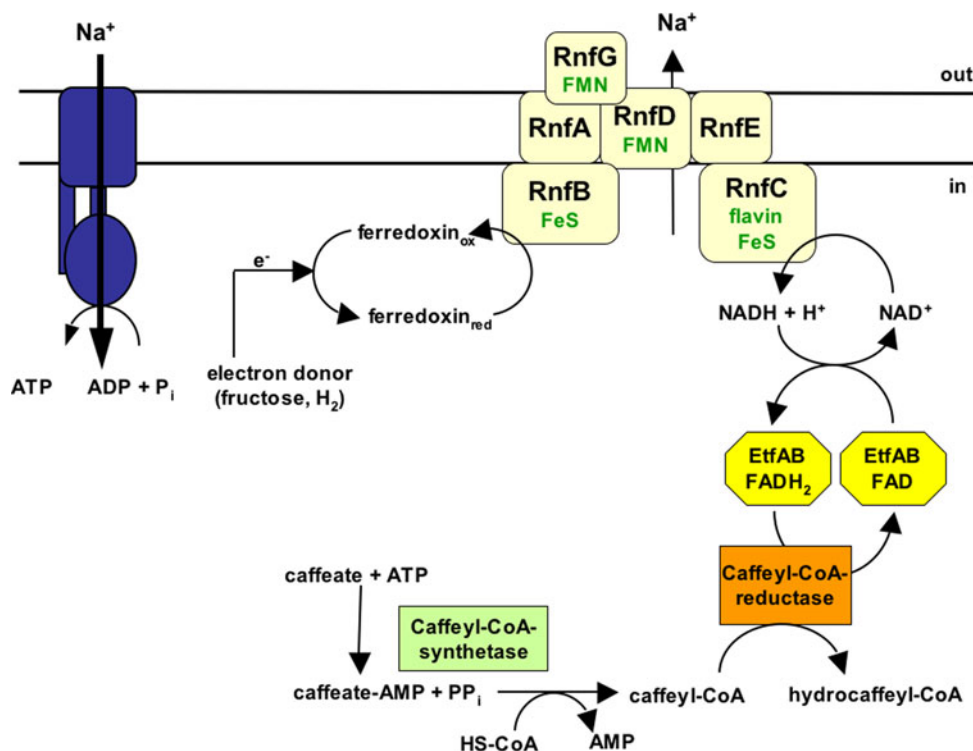
A membrane-bound, Na^+-translocating ferredoxin:NAD^+ oxidoreductase, the Rnf complex

To test whether the Fno activity is coupled to Na^+ translocation, studies with inverted membrane vesicles were performed. These vesicles catalyzed ferredoxin-dependent NAD^+ reduction and electron flow was accompanied by $^{22}Na^+$ transport into the lumen of the vesicles [24]. $^{22}Na^+$ transport was dependent on NAD^+ as electron acceptor and ferredoxin and titanium (III) citrate as electron donor. $^{22}Na^+$ transport was electrogenic and accumulation prevented by the Na^+ ionophore ETH2120 but not by protonophores, indicating a primary event. Addition of ETH2120 during steady state of transport led to a rapid efflux of $^{22}Na^+$ from the vesicles. The data revealed that the ferredoxin:NAD^+ oxidoreductase of *A. woodii* catalyzed a primary Na^+ transport energized by electron flow from ferredoxin to NAD^+ [24].

What is the enzyme catalyzing the Fno activity? We have partially purified an enzyme from *A. woodii* membranes that has Fno activity as well as ferricyanide-dependent NADH oxidation activity. The preparation contained a number of polypeptides [25]. Using N-terminal sequencing, RnfC was identified as one of the major polypeptides. With an antibody directed against RnfG and RnfB, these proteins were also shown to be part of the preparation. Rnf is suggested to be a membrane-bound electron transport complex with some similarity to the Na^+-translocating NADH:ubiquinone oxidoreductase (Nqr) (see below, including Table 2) that is known to couple electron flow from NADH to quinone with the electrogenic translocation of Na^+ outside the cell. Therefore, Rnf was assumed to be a Na^+ pump since its first discovery [26] and was speculated to be the Na^+-translocating coupling site in caffeate respiration of *A. woodii* [22]. With the partially purified complex in hand we searched for inhibitors. Fno activity catalyzed by the partially purified Rnf complex was inhibited by $AgNO_3$, $CuSO_4$, 1,10-phenanthroline, diphenyliodonium chloride, and diphenyleniodonium chloride. At the same time, these inhibitors also abolished Na^+ transport coupled to Fno activity as catalyzed by inverted vesicles [24]. This is consistent with the hypothesis that the Rnf complex catalyzes the observed Na^+-motive ferredoxin:NAD^+ oxidoreductase activity. Although the final proof that Rnf is a sodium pump has to await its functional reconstitution into liposomes, we will, for the sake of clarity, refer in the following to Fno as Rnf.

Genomic organization of the *rnf* genes

The *rnf* genes were first discovered in *Rhodobacter capsulatus* and were shown to be involved in nitrogen fixation (*Rhodobacter nitrogen fixation*). When the *rnf* genes were published for the first time only five genes were described to be in one cluster, *rnfABCDE*, and an additional gene, *rnfF*, was detected outside this cluster [26]. It was also noted that the *rnfABCDE* cluster is transcribed in the opposite direction to other genes of the *nif* region A such as *rnfF*. Later corrections were made to the published DNA sequence of *R. capsulatus* leading now to seven genes in the Rnf cluster, *rnfABCDGEH* [27]. The role of *rnfH* is not clear since it is not present in most *rnf* clusters. Since the *rnf* genes were first described, homologues have also been found in many prokaryotic and so far in two archaeal genomes. However, the organization of the genes differs and three major clusters can be distinguished that are commonly found in bacteria. Clusters similar to the one found in *R. capsulatus* *rnfABCDGE* are present in *Escherichia coli* [28], *Vibrio fischeri* MJ11 (VFMJ11_0974-0969) *Klebsiella pneumoniae* (KP1_3036-3041), *Haemophilus influenzae* (NTHI1989-1990), *Haemophilus ducreyi* (HD0396-0403), *Shigella dysenteriae* (SD1012_2262-2267), *Pseudomonas stutzeri* [29], *Photobacterium profundum* (CAG20939-20944), *Azotobacter vinelandii* [30], and *Vibrio cholerae* (VC0395_A0538-A0532) (Fig. S1A). Interestingly, in some species (*Alteromonas, Azotobacter, Escherichia, Citrobacter, Erwinia, Marinobacter, Shewanella, Serratia, Photobacterium, Shigella*), downstream of *rnfE* is an endonuclease III (oxidative base excision repair protein) encoded in the same orientation. It has been shown that the gene *nth* encoding for endonuclease III in *E. coli* is co-transcribed with the *rnf* genes [31, and see "Physiological roles of Rnf in different bacteria"]. As can be seen in Fig. S1A, the size of RnfC varies. All RnfC subunits contain the conserved motifs (FeS center, flavin- and NADH-binding site), but some have a longer C-terminus.

The next type of cluster *rnfCDGEAB* that was first described in *Clostridium tetani* [32] is also found in *A. woodii* [25], *Clostridium kluyveri* [33], *Clostridium difficile* (CD1137-1142), *Clostridium botulinum* (CBO0368-0373), *Clostridium phytofermentans* (Cphy_0211-0216), *Clostridium thermocellum* (Cther_0911-0906), *Clostridium novyi* (NT01CX_1579-1584), *Clostridium beijerinckii* (Cbei_2449-2454), *Alkaliphilus oremlandii* (Clos_1775-1770), *Alkaliphilus metalliredigens* QYMF (Amet2280-2285), *Thermanaerobacter pseudethanolicus* (Teth39_2124-2119), *Ruminococcus torques* (RUMTOR_10017-10032) and *Ruminococcus obeum* (RUMOBE_01198-01206) (Fig. S1B). Interestingly, this second variation of the Rnf cluster is similar to the order of the genes of the Nqr cluster of *Vibrio alginolyticus* (nqr*ABCDEF*).

In addition, the variation *rnfBCDGEA* is found in some organisms like *Bacteroides vulgatus* (BVU_3890-3885), *Chlorobium limicola* (Clim_0989-0984), *Chlorobium*

luteolum (Plut_1154-1159), *Parabacteroides* sp. (HMPRE F0619_02359-02355), *Prosthecochloris aestuarii* (Paes_ 1309-1314) and *Porphyromonas uenonis* (PORUE0001_ 0056-0061) (Fig. S1C).

To address the transcriptional organization of the *rnf* genes in *A. woodii*, mRNA was extracted from fructose-grown cells and molecular analyses revealed that all six *rnf* genes are indeed found on a single transcript. The same can be expected for the *rnf* cluster found in other organisms. The genomic organization of the *rnf* genes in one operon suggests that the gene products are in a functional context [25].

Most organisms only have one Rnf cluster. However, in *A. vinelandii*, two *rnf* clusters were found in the genome; *rnf1* expression is part of the nitrogen regulon and *rnf2* expression is independent of the nitrogen source of the growth medium [30]. Also, *Desulfobacterium autotrophicum* HRM2 contains two *rnf* clusters in the genome. Furthermore single *rnf* genes like RnfC homologues that are not part of a complete cluster are found in some genomes.

Another interesting observation is that in some species (*Serratia proteamaculans*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*), the genes *rnfABC* are followed by a gene for a transcriptional regulator *araC*, followed by *rnfDGE*. Furthermore, in two *Thermosipho* species (*T. africanus* and *T. melanesiensis*), *rnfB* is replaced by a gene coding for a putative arsenate or citrate transporter, respectively.

In the archaea *Methanosarcina acetivorans* and *Methanococcoides burtonii*, the cluster is organized as in *C. tetani*. However, upstream of *rnfC* is a gene that encodes a predicted multi-heme *c*-type cytochrome. The predicted protein is membrane-associated and could, therefore, contribute to the Rnf complex of both organisms (Fig. S1D). RT-PCR data showed that the corresponding gene in *M. acetivorans* is indeed co-transcribed with the *rnf* genes [34]. Downstream of *rnfB* is another gene that is co-transcribed with the *rnf* genes. The deduced amino acid sequence shows no identity to known proteins but is predicted to be membrane-integral [34].

Properties of the Rnf complex

Rnf subunits of *R. capsulatus* physically interact with each other [35]. An antibody directed against RnfC co-immunoprecipitated with the target antigen and at least five other proteins, one of which was identified as RnfB. RnfC and two unknown polypeptides co-immunoprecipitated using the antibody against RnfB. It was further shown that depletion of one subunit causes destabilization of the complex [36]. A *rnfA* mutant of *R. capsulatus* had almost no RnfB and only a little RnfC, while a *rnfB* mutant had reduced amounts of RnfC, and a *rnfC* mutant had almost no

detectable amounts of RnfB. This was evidence that the presence of RnfA was required for stable existence of both RnfB and RnfC, indicating that these proteins actually form a complex [35, 36].

To date, integrated data on the biochemistry of the Rnf complex are still lacking. The first native purifications of Rnf complexes were mentioned for *Clostridium tetanomorphum* and *Fusobacterium nucleatum* in review papers [37, 38]. The complex was purified from membranes of *C. tetanomorphum*, enriched 30-fold and is composed of six subunits. N-terminal sequencing showed that RnfC, RnfD, RnfG and RnfE were part of the preparation [37]. Proteins with a molecular mass corresponding to those of RnfG and RnfD were found to be fluorescent under UV-illumination indicating the presence of FMN in these subunits. A phosphodiesterase treatment of the denatured protein increased fluoresence intensity but removed the fluorescent band of RnfG, indicating the release of the flavin that might be connected to the protein via phosphodiester linkage as has been shown for NqrC [37, 39]. The purified complex also had a NADH-dependent ferricyanide reduction activity [37].

So far, the subunit stoichiometry in the complex remains unknown, although preliminary experiments performed in our laboratory indicate a molecular mass of the complex of 186 kDa that would argue for each subunit being present as a single copy in the complex.

In summary, the biochemical analysis of the complex is still in its infancy and, therefore, most of the properties of the complex are deduced from bioinformatics analyses of the single subunits. The properties of the subunits are summarized in Table 1.

Properties of the subunits

RnfC

RnfC of *A. woodii* is predicted to have a molecular mass of 48.7 kDa. Hydropathy analyses predict that RnfC is soluble although Western blotting analysis with antibodies directed against RnfC showed that it is membrane-bound in *A. woodii* [25], suggesting a tight association with the membrane-integral Rnf subunits. It was shown by immunological studies that RnfC of *R. capsulatus* is only membrane-associated, since a fraction was released from the membrane by alkaline treatment [36]. Cysteine motifs (C-XX-C-XX-C-XXX-C-P) typical for coordination of 4Fe4S clusters were described in the amino acid sequence of RnfC [26]. The heterologously produced RnfC of *R. capsulatus* indeed harbored at least one FeS cluster, as determined by EPR spectroscopy and an absorbance spectrum with a maximum at 419 nm and a shoulder at 330 nm [27].

Table 1 Properties of Rnf subunits of *A. woodii*

	Subunit					
	RnfC	RnfD	RnfG	RnfE	RnfA	RnfB
Size (bp)	1,332	960	624	591	588	1,002
Mass (kDa)	48.7	35	22.8	21.6	21.4	36.6
Predicted localization	Soluble	Membrane integral	Membrane associated	Membrane integral	Membrane integral	Membrane associated
TMH[a]	0	6–9	1	6	6	1–2
Experimental localization	Membrane	Membrane	Membrane	Membrane	Membrane	Membrane
Cofactors predicted	FeS Flavin	FMN	FMN	–	–	FeS
Cofactors experimentally found	FeS[b]	FMN[c]	FMN[c]	–	–	FeS[b]

[a] *TMH* Number of transmembrane helices deduced from the amino acid sequence

[b] Experimental data come from *R. capsulatus* [27]

[c] Experimental data come from *V. cholerae* [47]

The presence of flavins in Rnf subunits was first suggested by Kumagai et al., and conserved amino acids similar to proposed FMN-binding sites were found in RnfC [36, 40, 41]. So far, the presence of flavin in RnfC has not been shown experimentally. Conserved residues with strong similarity to proposed NADH binding sites in mitochondrial complex I [42, 43], bacterial NAD^+-reducing hydrogenases [44–46], and bacterial complex I [40, 41] are present in RnfC, indicating that it may bind NADH. This is also supported by a $\beta\alpha\beta$-fold in the conserved region of RnfC [36].

RnfD

A. woodii RnfD is predicted to have a molecular mass of 35 kDa and 6–9 transmembrane helices supporting that RnfD is a membrane-integral protein (Fig. S2). The purified Rnf complex of *C. tetanomorphum* contains a fluorescent subunit with the same retention time in an SDS PAGE as RnfD [37], and Backiel et al. presented biochemical evidence for the presence of FMN in RnfD from *V. cholerae*. They produced and purified RnfD of *V. cholerae* and the protein showed fluorescence under UV-illumination and a typical flavin spectrum. Mass spectrometry analysis showed that FMN is covalently attached to threonine-187 in RnfD [47]. This conserved threonine was also detected in the amino acid sequences of RnfD of *A. woodii* at position T157 as well as in others such as *Vibrio harveyi*, *V. fischeri* and *V. alginolyticus* [25], indicating that FMN is conserved across disparate phylogenetic groups. Topological analyses (reporter fusions and computer predictions) indicated that FMN in *V. cholerae* RnfD is located in the periplasm [47]. This is not only in contrast to Nqr where all the cofactors are located on the

cytoplasmic side of the membrane [48] but also very unusual. However, additional biochemical data are required to confirm the cellular localization of the flavin binding site in RnfD and to hypothesize on its biological significance.

RnfG

The deduced protein has a molecular mass of 22.8 kDa and is mainly hydrophilic but has a stretch of 30 hydrophobic amino acids at the N-terminus, which led to the conclusion that this subunit is anchored in the membrane (Fig. S3), which was verified experimentally [25]. The purified Rnf complex of *C. tetanomorphum* contains a fluorescent subunit with the identical SDS gel migration as RnfG [37]. Treatment of the complex with phosphodiesterase removed this band, indicating that the flavin is attached to the protein via phosphodiester linkage [37]. Backiel et al. [47] presented biochemical evidence for the presence of FMN in RnfG of *V. cholerae*. The protein showed fluorescence under UV-illumination and a spectrum typical of flavins. FMN is covalently attached to threonine-175 in RnfG of *V. cholerae* [47]. This residue is conserved at position T185 in *A. woodii* RnfG [25]. Topological analysis (reporter fusions and computer predictions) indicated that the FMN in RnfG is also situated in the periplasmic space, again as opposed to Nqr where its RnfG homologue is localized on the cytoplasmic side of the membrane [48].

The structure of RnfG has been solved from *Thermotoga maritima* in the course of a structural genome project [Protein Data Bank (PDB) ID: 3DCZ, Joint Center for Structural Genomics (JCSG)]. Interestingly, the *T. maritima* RnfG homologue did not contain the flavin cofactor when heterologously produced in *E. coli*.

RnfE

RnfE of *A. woodii* is a membrane-integral protein with a molecular mass of 21.6 kDa. Hydropathy analysis suggests six transmembrane helices for RnfE of *A. woodii* (Fig. S4) with no conserved cofactor binding sites. For membrane topology, see next section on RnfA.

RnfA

A. woodii RnfA is likely to be membrane-integral (six transmembrane helices) (Fig. S5) with no recognized cofactor binding sites. Topological analyses with PhoA fusions of *R. capsulatus* RnfA confirmed its membrane localization, and it was shown that it spans the chromatophore membrane with its odd-numbered hydrophilic regions exposed to the periplasm [36]. The membrane topology of RnfE and RnfA of *E. coli* has been addressed experimentally by PhoA fusions to the homologous *E. coli* proteins YdgQ and Orf193 [49]. These studies revealed that both proteins have six transmembrane helices each but have evolved opposite orientations in the membrane. RnfA is proposed to have N- and C-termini reaching into the periplasm, whereas in RnfE both termini are in the cytoplasm. It is proposed by the authors that this leads to an unusual symmetric complex in the membrane which might have interesting implications for the function in electron transfer or ion channel formation [49] within the Rnf complex.

RnfB

RnfB is predicted to be 36.6 kDa and mainly hydrophilic but has a stretch of 30 hydrophobic amino acids at the N-terminus to anchor to the membrane through one or two transmembrane helices ([27, 36]; Fig. S6). RnfB contains six cysteine motifs typical for six 4Fe4S clusters. Starting at positions C_{172}, C_{246} and C_{279}, the motif C-XX-C-XX-C-XXX-C-P is present (Fig. S6). Starting at positions C_{138}, C_{217} and C_{310}, conserved cysteines are found but they do not form the classical motif, although deviations of the motif have been described [50]. Also, two cysteine clusters contain arginine instead of proline, but substitutions of proline by other amino acids do not seem to alter electron transfer rates [51]. *R. capsulatus* RnfB purified from *E. coli* had a red-brown color, and EPR analysis, absorption spectroscopy and iron content determination suggest the presence of one 2Fe2S cluster [27]. Recombinant RnfB might aggregate in *E. coli* and fold improperly. A reconstitution of the holoprotein was not successful and it is speculated that RnfB alone might not be stable but only as part of the complex [27]. It was also shown that, when cells of *R. capsulatus* were grown under iron limitation, the amount of RnfB was reduced significantly (4-fold less) indicating an iron-dependent regulation of the *rnf* genes in *R. capsulatus* [27]. Since RnfB contains FeS clusters, it is also termed polyferredoxin.

## Pathways of electron and ion flow in Rnf

The Rnf complex of *A. woodii* catalyzes oxidation of Fd_{red} with concomitant reduction of NAD^+ [22, 24]. RnfB is soluble and has a "polyferredoxin"-type feature and, therefore, is presumably the entry point in the cytoplasm for electrons derived from ferredoxin oxidation. The membrane-attached RnfC has an NADH binding site and is, most likely, the exit point for electrons and therefore should be located on the cytoplasmic side. RnfG is anchored to the membrane and the cofactor FMN is predicted to be on the periplasmic side [47]. Nothing is known about the relative topology of the three membrane integral subunits A, D and E. Since RnfE and RnfA do not contain binding sites for cofactors, their role in electron transport is unknown and therefore they might just stabilize the complex or be part of the ion translocation domain.

In summary, we suggest that electrons derived from Fd_{red} enter the complex at RnfB and travel via the other subunits to RnfC. There, the electron pair is generated at the flavin site to reduce NAD^+ to $NADH + H^+$ (Fig. 2). If the predicted topology of subunits is correct, then it is obvious that additional electron carriers have to be present to allow passage from RnfB to the membrane-bound subunits (RnfA, RnfD, and RnfE), to the periplasm (RnfG) and back to the cytoplasm (RnfC).

Electron flow from RnfB to RnfC in *A. woodii* is accompanied by the electrogenic transport of sodium ions out of the cell into the medium and thus establishing an electrochemical sodium ion potential that is used to drive endergonic reactions such as ATP synthesis or flagella rotation [24]. How this is achieved is unknown, but a conformational pump mechanism is most likely since Na^+ cannot be transported by a classical redox loop-type mechanism. In line with this argument, the membrane-integral subunits RnfA, RnfD and RnfE may be involved in ion pumping and their pumping action energized by electron flow. The principle may be the same as used by complex I of the respiratory chain of mitochondria or bacteria [52]. Such a mechanism has the additional charm that it would also allow for proton transport and, as we shall discuss later, proton transport may indeed be true for Rnf complexes from other organisms.

So far, only speculations can be made on subunits or amino acids that are involved in Na^+ binding and/or transport. The membrane-embedded subunits RnfA, RnfD and RnfE might be major players in binding sodium ions

Fig. 2 Topology model of Rnf subunits. Rnf subunits are indicated. N- and C-termini are as indicated. *Fd* ferredoxin, *red* reduced, *ox* oxidized. For explanations, see text
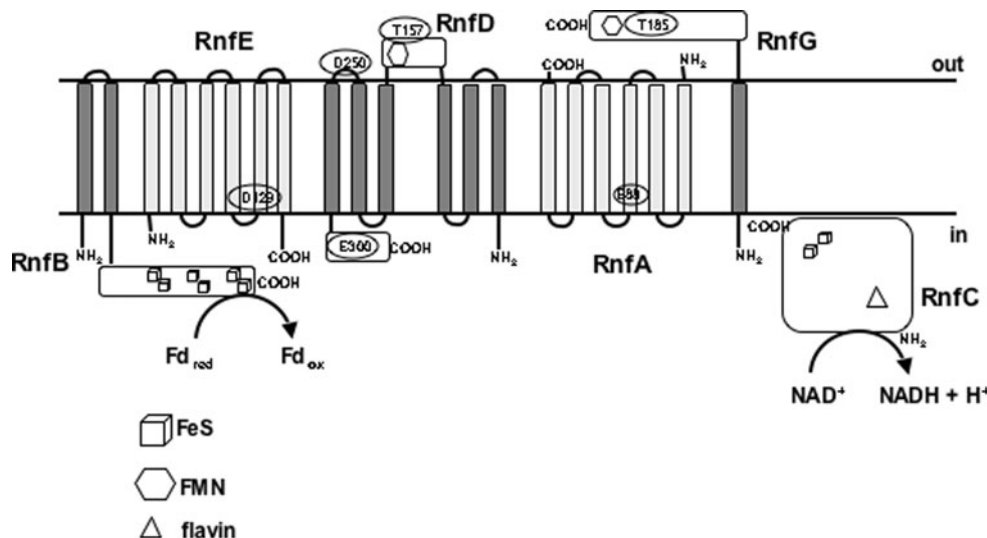
and forming a channel to transport the ion across the membrane. Recently, acidic residues in the corresponding Nqr subunits of *V. cholerae* have been found to be important for sodium binding (NqrB-D397, NqrD-D133 and NqrE-E95) [53]. Substitutions of these amino acids for alanine had a large effect on Nqr activity and on the K_m value for Na^+ (increase of 9-fold). All three mutations abolished the ability to form a membrane potential ($\Delta\psi$). Other mutants (NqrB-E28, NqrB-E144, NqrB-D346 and NqrD-D88) showed altered turnover but no change in K_m for Na^+, indicating that these subunits are not directly involved in Na^+ binding but in forming the channel [53]. It has been shown that, in *V. cholerae*, some of the acidic residues in Nqr are also conserved in the corresponding Rnf subunits of this organism [53]. Alignments of homologous subunits of Nqr revealed that NqrB-D397 is changed to glutamate in RnfD of *A. woodii* (RnfD-E300). NqrB-D346 is also conserved in RnfD at position D250 (Fig. S7). In the topology model, these amino acids are not part of a transmembrane helix but are situated on the opposite sides of the membrane (Fig. 2). NqrD-D133 is conserved in RnfE of *A. woodii* at position D129 (Fig. S8). The topology model shows that RnfE-D129 lies in transmembrane helix 5 (Fig. 2). NqrE-E95 is also conserved in RnfA of *A. woodii* at position E88 (Fig. S9). The topology model predicts that RnfA-E88 lies within helix 3 (Fig. 2). In conclusion, all the predicted Na^+ binding sites of Nqr are conserved in Rnf, whereas only one of the amino acids that may be part of the sodium channel in Nqr is conserved in Rnf. Additional residues certainly play a role in binding and transport of Na^+.

From the data available to date, it is not possible to identify the Na^+ or H^+ binding site in Rnf. They are known, however, in ATP synthase. In the *c* ring rotor of the F_O motor, the Na^+ binding site is sandwiched in between two *c* subunits by five amino acids [54]. At least three residues (Q…ES(T)) are highly conserved in Na^+-ATP synthases [54–58]. This motif is not found in Rnf or in Na^+-motive decarboxylases or Na^+-motive methyltransferases. However, it is important to note that one residue of the Na^+-binding motif is sufficient and essential for H^+ transport [59–61].

Energetics and reversibility of the Rnf complex

As outlined above, so far only three Rnf complexes have been (partially) purified. All are from anaerobic bacteria and have ferredoxin:NAD^+ oxidoreductase activity. The difference in redox potential between ferredoxin ($E^{0\prime} = -500$ to -420 mV) and NADH ($E^{0\prime} = -320$ mV) [62] is equivalent to approximately -20 to -35 kJ/mol. Assuming an electrochemical ion potential of around -200 mV across the cytoplasmic membrane, the free energy change of this reaction would allow for the translocation of one to two ions across the membrane. A lower electrochemical ion potential would increase the number of ions that can be pumped. At any rate, if one assumes the energetically most unfavorable value of one ion exported per mol of ferredoxin oxidized, 3 mol of Fd_{red} must be oxidized to yield the three ions required by the ATP synthase to make 1 mol of ATP.

The Rnf complex described in *A. woodii* uses the energy released by the exergonic electron transfer from Fd_{red} to NAD^+ for outward Na^+ transport and thus the generation of an electrochemical Na^+ gradient across the cytoplasmic membrane. However, bacterial electron transport chains are largely reversible and often the physiology of the bacterial cell requires reverse electron flow [63]. This is, for example, encountered in chemolithoautotrophs that grow by oxidation of an electron donor such as nitrite that is too electropositive

to allow direct reduction of NAD^+ required for biosynthetic reactions. In this case, reverse electron flow drives energetic uphill transport of electrons to the redox level of NAD^+ [64–68]. A reversal of the ferredoxin-dependent NAD^+ reduction is essential in many metabolic scenarios (see examples below). Ferredoxin is used as a low potential reductant not only in anaerobes, but also in aerobic bacteria that may use reverse electron flow driven by $\Delta\mu Na^+$ or $\Delta\mu H^+$ to overcome the energy barrier in the endergonic reduction of ferredoxin with NADH as reductant. Ferredoxin is then used as electron donor for, for example, nitrogenase or regulatory proteins (see below). Anaerobes that grow autotrophically have to produce Fd_{red} with very low redox potential from H_2 as a reductant for anabolic reactions, i.e. acetyl-CoA carboxylation to pyruvate or formylmethanofuran reduction (in methanogenic archaea). Thus, mechanistically, ferredoxin:NAD^+ oxidoreductase activity is freely reversible and may only be controlled by the magnitude of the thermodynamic parameters. In addition, it may be possible that the direction of reaction is also controlled by intrinsic properties of the proteins involved.

Evolution of the Rnf complex

Ferredoxin as "high energy" intermediate in electron transfer reactions

Ferredoxin plays a very central role in the energy metabolism of many anaerobic bacteria and archaea and has evolved as an electron carrier very early in life history [69]. However, its role in the bioenergetics of anaerobes was underestimated for a long time, and only recently did the hypothesis arise that anaerobes do even use the potential difference between ferredoxin at −500 to −420 mV and pyridine nucleotides at −320 mV to establish an ion gradient, which drives endergonic reactions such as ATP synthesis. This is equivalent to one-third to one-half of an ATP. Enzymes such as the pyruvate:ferredoxin

oxidoreductase, pyruvate:formate lyase [70, 71], hydrogenase [23], CO dehydrogenase [72] and some formate dehydrogenases [73, 74] use ferredoxin as an electron acceptor. Therefore, enzymes must be present that connect the cellular ferredoxin pool to that of reduced pyridine nucleotides. Apart from Rnf, another membrane-bound ferredoxin-dependent oxidoreductase has been described, the Ech hydrogenase (Fig. 3).

Ech and complex I of the respiratory chain, two evolutionary related, energy conserving complexes

The energy converting hydrogenase (Ech) is part of the family of the NiFe-hydrogenases and is found in bacteria as well as in archaea [75]. The biochemically most thoroughly studied enzyme is from the archaeon *Methanosarcina barkeri*, and its biochemical function and physiological role has been described in a review [76]. Briefly, the enzyme from *M. barkeri* has six subunits encoded by the *echABCDEF* operon. Two of the subunits (A and B) are apparently membrane-integral and have no cofactors bound whereas the others are located on the cytoplasmic side of the membrane, have FeS centers, and catalyze the oxidation of Fd_{red} coupled to the reduction of protons to hydrogen. As outlined above, this reaction is exergonic and assumed to be the driving force for vectorial ion transport across the membrane. Back in 1986, Bott et al. [77] demonstrated that CO oxidation (which is coupled to ferredoxin reduction) is accompanied by the generation of a membrane potential, and later, evidence was presented that this reaction drives outward proton translocation [78]. Recently, direct experimental evidence for ion (proton) transport coupled to proton reduction with ferredoxin as reductant was obtained [79].

As discussed above for Rnf, Ech is a reversible enzyme. The methanogenic archaeon *M. barkeri* grows chemolithoautotrophically on $H_2 + CO_2$ that are converted to methane [80, 81]. The first step in methanogenesis from $H_2 + CO_2$, the formation of formylmethanofuran, requires

Fig. 3 Model of Ech and Rnf complexes. The potential difference between electron donor and acceptor is used to build up a transmembrane ion potential that is then used by ATP synthase to synthesize ATP. *Fd* ferredoxin, *ox* oxidized, *red* reduced
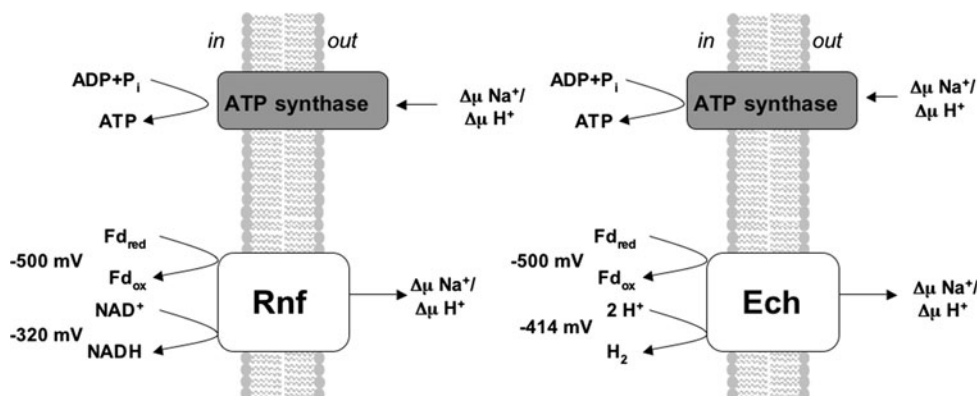
ferredoxin as electron carrier and is highly endergonic with hydrogen as reductant. The energy barrier is overcome by reverse electron flow from hydrogen to ferredoxin catalyzed by Ech hydrogenase and driven by the electrochemical ion gradient across the membrane. Other endergonic reactions that require Fd_{red} are CO_2 reduction to CO, mediated by acetyl-CoA synthase/CO dehydrogenase, and the carboxylation of acetyl-CoA to pyruvate, mediated by the pyruvate:ferredoxin oxidoreductase. Both reactions are essential for CO_2 fixation into biomass. The reverse reaction, oxidation of Fd_{red} with concomitant release of hydrogen coupled to the generation of a proton or sodium motive force is encountered during methanogenesis from acetate or CO [82].

Direct evidence for proton transport coupled to the Ech reaction is available [79]. However, protons as well as sodium ions, depending on the organism, may be used as coupling ion. Some methanogens with their documented sodium bioenergetics may use Na^+. The same may be true for *Pyrococcus furiosus* that has hydrogenases of the Eha- and Ehb-type [80, 83] and a Na^+-driven A_1A_O ATP synthase [84]. Others like *Rhodospirillum rubrum* or *Carboxydothermus hydrogenoformans* that have no documented sodium bioenergetics may use the proton motive force [76].

With the first sequences available for hydrogenases, it was immediately evident that hydrogenases (including Ech) and complex I are evolutionary related and evolved from a common ancestor. The evolution of hydrogenases and complex I was discussed recently in excellent reviews [76, 85–88]. The membrane-bound Ech subunits EchA and EchB are highly homologous to Nuo subunits. EchA is homologous to NuoL, NuoM and NuoN, and EchB is homologous to NuoH [76]. These subunits form the catalytic core of complex I. However, it is interesting to note that neither complex I nor Ech show any homology to subunits of Rnf, indicating two different lines of evolution that resulted in different solutions to the same problem.

Rnf and Nqr, two evolutionary related, energy conserving complexes

When the *rnf* genes of *R. capsulatus* were described for the first time, database searches revealed that some subunits of the at that time already established Na^+-translocating Nqr were the closest homologues. Nqr was first described in the facultative anaerobic marine bacterium *V. alginolyticus* by Tokuda and Unemoto [89]. This bacterium is dependent on Na^+ for solute transport and flagella rotation and, therefore, a primary sodium ion pump that generates a sodium ion potential was assumed. Elegant experiments using whole cells, inverted vesicles and purified enzymes identified this novel type of energy conserving NADH:quinone oxidoreductase as not being related to complex I in this organism

[90]. Nqr feeds electrons into the electron transport chain and it was shown unequivocally by different methods that this electron transfer reaction is coupled to vectorial, electrogenic Na^+ transport out of the cell [91, 92]. That was the first example of a Na^+-pumping NADH:quinone oxidoreductase, an enzyme later also found in several other bacteria [93, 94].

Nqr is an enzyme complex with an approximate molecular mass of 200 kDa consisting of six subunits (NqrA-F) encoded by the *nqr* operon. The complex channels electrons from NADH to quinone and thereby translocates sodium ions out of the cell [91, 92]. Three subunits (NqrB, NqrD, NqrE) are membrane-bound, NqrA is hydrophilic, and NqrC and NqrF are connected to the membrane via transmembrane helices. NqrF contains the NADH-binding site, the binding site for FAD and iron-sulfur clusters and is therefore characterized as the electron entry site [52, 93]. The two subunits NqrB and NqrC contain covalently bound FMN that is attached by an ester bond through a threonine residue of the protein [39, 95]. NqrB is suggested to contain part of the quinone binding site. Also, the presence of a riboflavin was experimentally shown for the Nqr complex [96]. In addition to flavins and iron-sulfur clusters, the Nqr complex contains a tightly bound ubiquinone-8 [52, 91, 93, 97, 98].

It is important to note that the mode of action, their physiological roles and also the electron input and output modules are quite different in Rnf and Nqr. Rnf mediates electron transfer from ferredoxin to NAD^+, but Nqr from NADH to quinone. Energetically, Rnf is located "upstream" of Nqr and covers redox spans between -500 and -320 mV, whereas Nqr covers the -320 to $+90$ mV [99] region (Fig. 4). RnfC is the electron output module and does not share significant sequence similarity with any of the Nqr subunits, but like NqrF, the electron input module of Nqr, it contains an NADH binding site, flavin and FeS clusters [100]. Therefore, it is considered the functional homologue of NqrF. RnfB is a polyferredoxin-type protein and the electron input module of Rnf. Apparently, there is no functional homologue in Nqr. The only subunits with significant sequence similarity are the membrane-bound subunits and a FMN-containing subunit (Table 2). RnfD is similar to NqrB, RnfG to NqrC, RnfE to NqrD, and RnfA to NqrE. In addition, there is modest sequence identity between RnfE and NqrE and RnfA and NqrD.

Phylogenetic analyses of Rnf genes

Phylogenetic analyses of RnfD revealed that *A. woodii* RnfD groups with other clostridia (*Clostridium*, *Thermoanaerobacter*, *Eubacterium*) and *Thermanaerovibrio*. The RnfDs from proteobacteria are a monophyletic group, except for members of the sulfate-reducing bacteria, while

Fig. 4 The redox span used for energy conserving reactions in biology and the enzymes used. Rnf and Ech cover the most reduced range from −500 to −320 mV, complex I and Nqr the range from −320 to +90 mV, complex III from +90 to +240 mV, complex IV from +240 to +816 mV. For the sake of clarity, the region from −320 to +816 mV is shown by only four respiratory enzyme complexes. Please note that there are a couple of enzymes that work in this region. *Fd* ferredoxin, *red* reduced, *UQ* ubiquinone
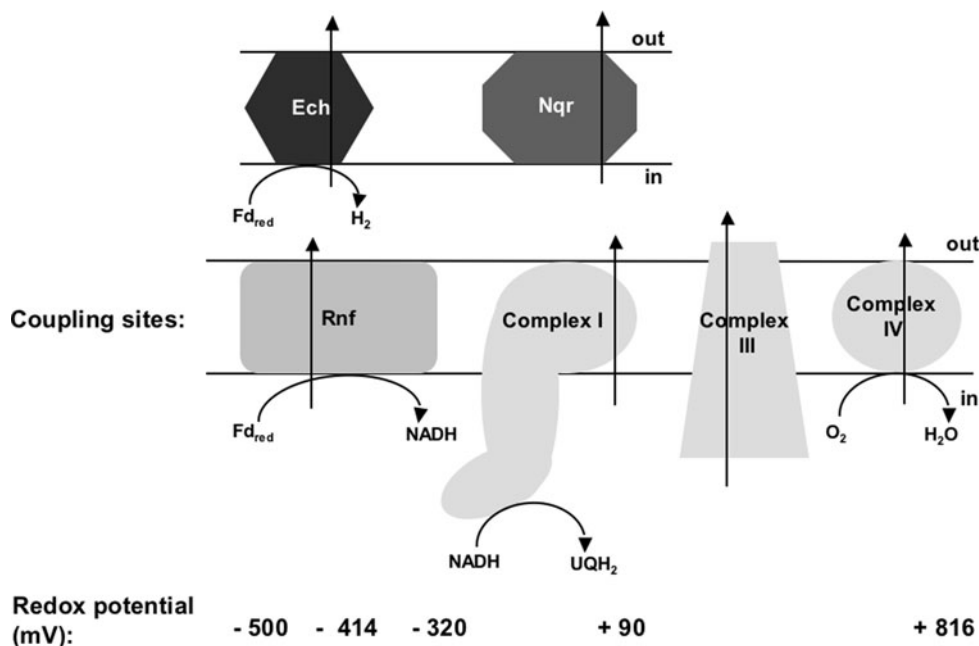

Table 2 Sequence identity of *A. woodii* Rnf subunits to *V. alginolyticus* Nqr subunits

	RnfD (%)	RnfE (%)	RnfA (%)	RnfG (%)
NqrB	27			
NqrD		37	20	
NqrE		24	45	
NqrC				18

ClustalW was used for alignments and score determination

the Thermotogaceae also form a robust cluster. The phylogenetic affinities for other phyla (Archaea, Fusobacteria, Dictyoglomi, Spirochaetes, Bacteroidetes/Chlorobi) are less straightforward. The genome of the clostridium *Natranaerobius thermophilus* stands out since it has four copies of the *rnfD* gene with amino acid percent identities among each other in the range from 30 to 62% suggesting it might have different functions or regulations to adapt to its unique environment (Fig. 5). In the case of RnfC, the proteobacteria also group together, except for the sulfate reducers. *A. woodii* RnfC is closest to *Thermoanaerobacter* as well as other clostridia (Fig. 6). Both RnfD and RnfC were not only found in multiple copies in the genomes of clostridia, especially in the case of *Natranaerobius*, but also had greater sequence diversity when compared to other phylogenetic groups.

Distribution of *rnf* genes

A search of gene sequences deposited in databases revealed that 154 organisms contain *rnf* but no *nqr* operons (1,026 genomes; January 2010). It is noteworthy to discriminate between the presence of single, *rnf*-like genes and the presence of the entire *rnf* operon in the genome, since, for example, *rnfC*-like genes can be found in addition to the one in the *rnf* operon in another genomic context. In contrast, only 31 genomes contain the *nqr* operon alone, mostly strains in the families Chlamydiaceae and Neisseriaceae. In addition, 109 genomes have both Nqr and Rnf, most in the family enterobacteria, *Shewanella* spp. and Pasteurellaceae. The majority of enterobacteria, however, only contain the *rnf* operon.

As shown above, the *rnf* genes are widely distributed and are present in different phylogenetic and trophic groups, for example in chemolithoautotrophs, photolithoautotrophs and chemoorganoheterotrophs. There is also no correlation to salinity, pH or temperature of the environment since the genes are also present in a number of extremophiles. They are found in aerobes, facultative aerobes and anaerobes, but there is a very strong bias towards anaerobic life style. Only a handful of strictly aerobic species have the *rnf* genes, most being obligate intracellular symbionts like *Buchnera* spp, in contrast to more than 150 facultative anaerobic/anaerobic *rnf*-containing species (see Tables 3 and 4). This certainly reflects the essential role of *rnf* genes in energy conservation in strict anaerobes and may suggest a role of *rnf* in anaerobic metabolism in the facultative anaerobes. However, at least for enterobacteria, ferredoxin is not known to play a major role in fermentation or anaerobic respiration which questions the role of Rnf in energy metabolism. Instead, a role of *rnf* in keeping a certain redox potential in the cell and oxygen stress response (see below) is suggested [28].
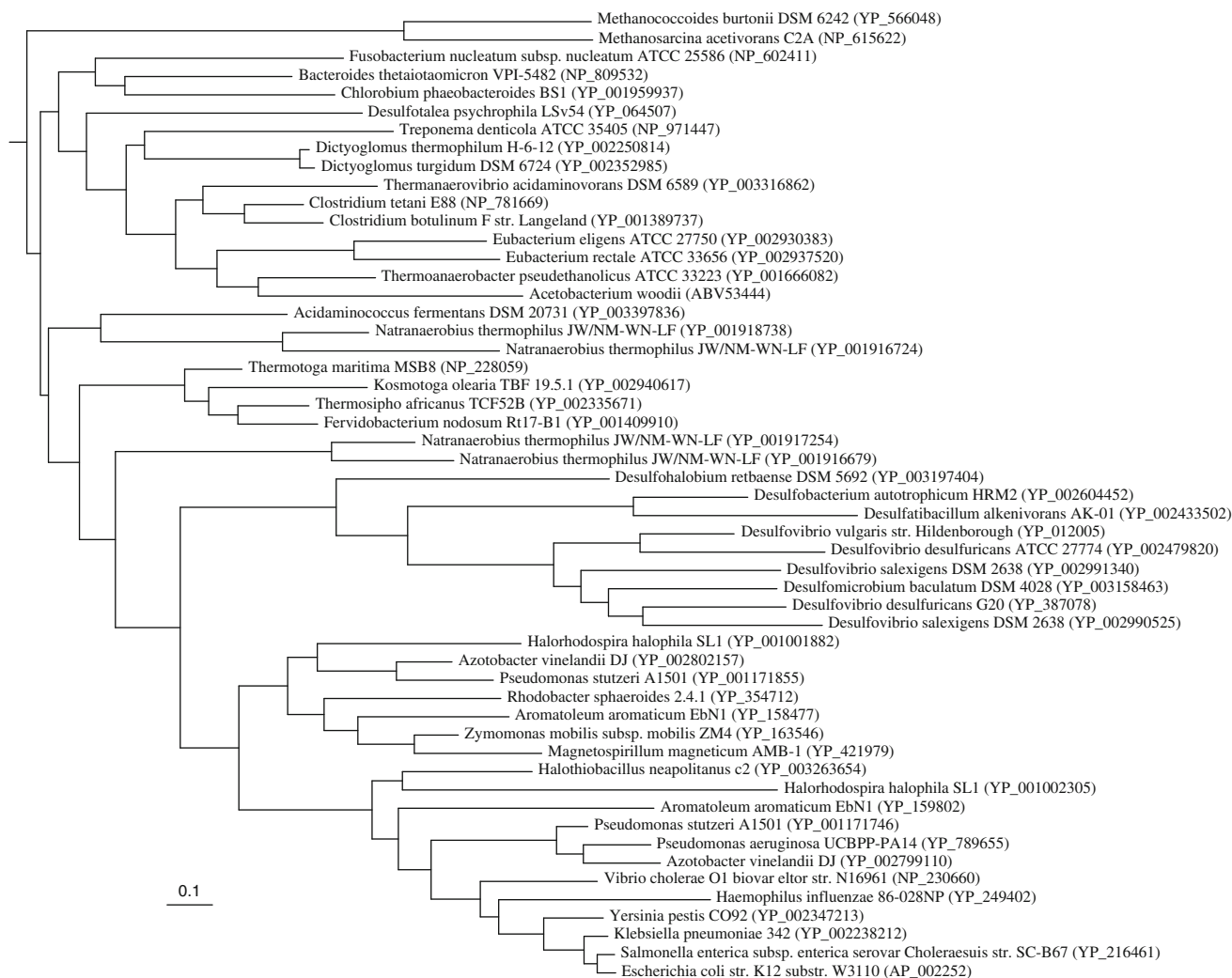
Fig. 5 Maximum likelihood tree of the RnfD homologs. The tree contains representative sequences from the phyla where RnfD is found (Proteobacteria, Firmicutes, Methanosarcinaceae, Fusobacteria, Dictyoglomi, Spirochaetes, Bacteroidetes/Chlorobi, Synergistales, Thermotogales). The sequence of the NqrB homolog of *Pseudomonas aeruginosa* (accession number NP_251688) served as the outgroup. *Number* between *parentheses* is the Genbank accession number. *Scale bar* represents the number of substitutions per site

Another interesting observation is the presence of *rnf* genes in a number of pathogenic bacteria (Table 5). Again, this may be for different reasons. The clostridia are fermenting organisms that produce butyrate. As described below, Rnf is a key enzyme in electron flow and energy conservation in the fermentation pathway. Whether or not the Rnf complex is involved in pathogenicity as a virulence factor itself or part of a sensory/regulatory circuit is a highly interesting question that needs to be addressed in the future. This question strikes another, more general one: is the sodium ion circuit across the membrane involved in induction of virulence factors [101]? Indeed, studies performed with *V. cholerae* suggest an intimate linkage of the sodium ion potential to the expression of virulence factors. As mentioned above, *V. cholerae* contains both, Nqr and Rnf. Nqr is the site of entry for electrons into the electron transport chain, whereas the role of Rnf is unknown. Nqr

mutants have an elevated expression of ToxT, a regulator for the expression of virulence genes [101, 102].

Evolution of the coupling ion used by Rnf

So far, the Rnf complex of *A. woodii* is the first Rnf complex for which ion transport has been demonstrated experimentally. There, the coupling ion is Na^+, and this was actually expected since this anaerobe uses exclusively Na^+ in its bioenergetics, i.e. as coupling ion for ATP synthesis, flagella rotation and solute symport [8, 14, 15, 19, 103]. The same may be true for others, for example *C. tetani* or *V. cholerae* that have established Na^+ bioenergetics. However, Rnf complexes are widespread in prokaryotes and found in organisms that are neither known nor suggested to have Na^+-based bioenergetics such as, for
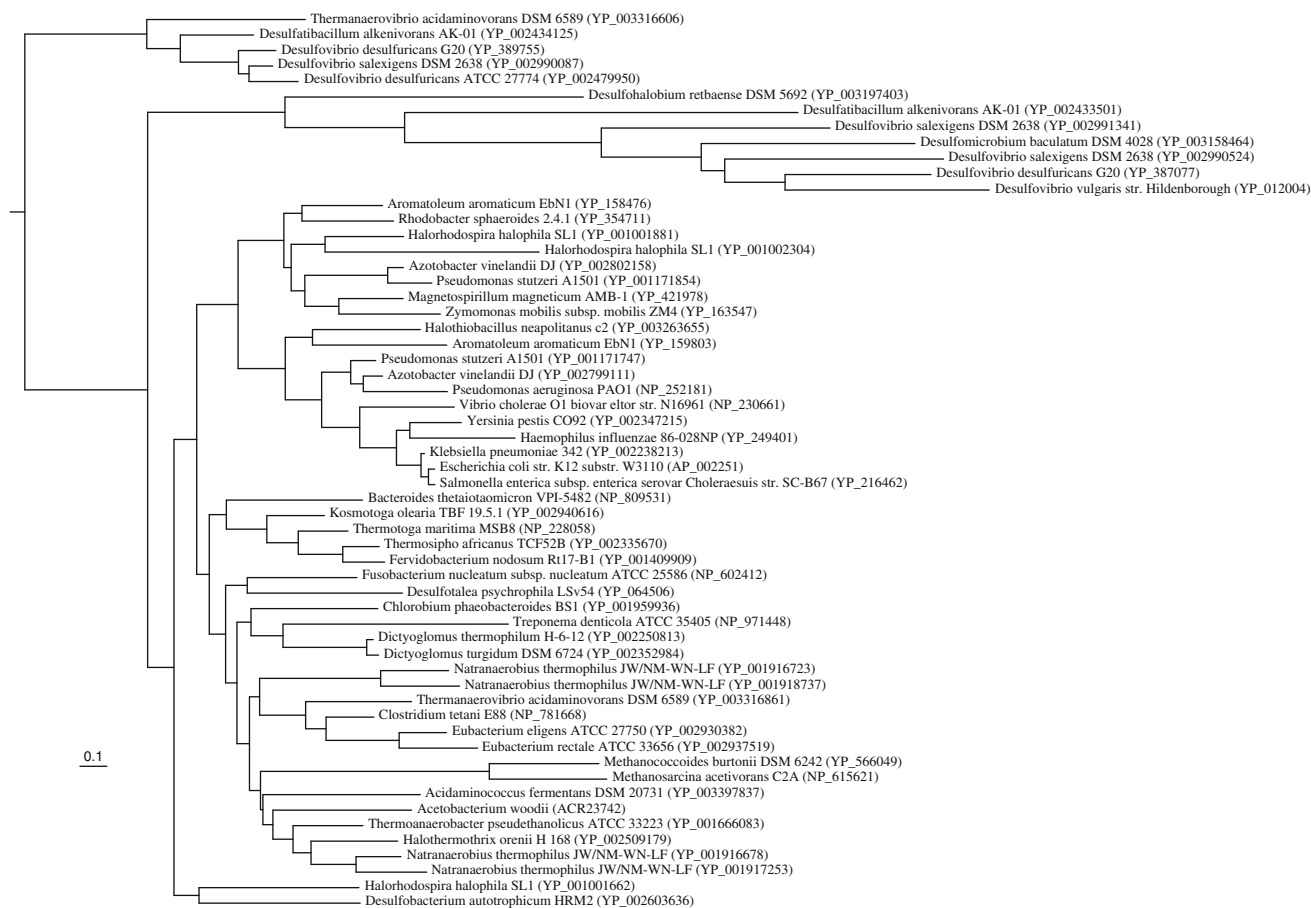
Fig. 6 Maximum likelihood tree of the RnfC homologs from representative sequences. The sequence of *Desulfatibacillum alkenivorans* AK-01 (accession number YP_002433501) served as the outgroup. *Number* between *parentheses* is the Genbank accession number. *Scale bar* represents the number of substitutions per site

example, *R. capsulatus* or *E. coli*. In these organisms, protons are likely to be the coupling ions in Rnf. Moreover, acetogenic bacteria were classified bioenergetically into the H^+-dependent subgroup that has cytochromes and the cytochrome-free, Na^+-dependent subgroup [8, 104]. However, it may turn out that some Na^+-independent acetogens without cytochromes have a proton-motive Rnf complex. Indeed, based on genome sequence data and a non-observed Na^+-dependence of growth, it is speculated that the acetogen *Clostridium ljungdahlii* has a H^+-coupled Rnf complex [105]. Therefore, the old bioenergetic differentiation of acetogens may no longer be valid. Instead, we would have two groups based on the type of electron transfer reactions: one which has cytochromes and the other which has Rnf. Both groups may turn out to contain H^+ as well as Na^+ pumping specimens. Actually, the Rnf complex is the first electron transport complex in bioenergetics that may use both, sodium ions or protons, depending on the organism. This has been suggested for complex I [106–108] and IV [109] of the bacterial electron transport chain but not yet shown unequivocally. Furthermore, there is no H^+ translocating Nqr known.

Physiological roles of Rnf in different bacteria

Rhodobacter capsulatus

The *rnf* genes were first discovered in the non-sulfur, photosynthetic purple bacterium *R. capsulatus* [26]. Insertion and deletion mutants of *R. capsulatus* were created focusing on genes in the *nifA* region to unravel novel genes involved in nitrogen fixation. Indeed, six novel genes *rnfABCDEF* involved in nitrogen fixation were discovered by analyzing diazotrophic growth. The in vivo and in vitro nitrogenase activities were reduced in a *ΔrnfE* mutant (1.4% in vivo, 26.3% in vitro compared to wild-type). The mutants *ΔrnfE*, *ΔrnfC*, *ΔrnfA* and *ΔrnfF* contained significantly reduced amounts of nitrogenase (only 30% compared to wild-type) as shown by Western blotting analysis [26]. The same mutants had higher growth yields in the presence of metronidazole than the wild-type suggesting that these mutants contained lower amounts of Fd_{red} [26]. Metronidazole leads to toxic derivatives in presence of high amounts of ferredoxin [110, 111]. Later, Saeki and Kumagai [112] demonstrated that Rnf is

Table 3 Aerobes and facultative anaerobes containing *rnf*

Acholeplasma laidlawii	*Magnetococcus* sp. *MC1*	*Shewanella denitrificans*
Actinobacillus pleuropneumoniae	*Magnetospirillum magneticum*	*Shewanella frigidimarina*
Actinobacillus succinogenes	*Magnetospirillum magnetotacticum*	*Shewanella halifaxensis*
Aeromonas hydrophila	*Mannheimia succiniciproducens*	*Shewanella loihica*
Aeromonas salmonicida	*Marinobacter algicola*	*Shewanella* sp. *MR4*
Aliivibrio salmonicida	*Marinobacter aquaeolei*	*Shewanella MR7*
Alkalilimnicola ehrlichei	*Marinobacter* sp.	*Shewanella oneidensis*
Alcanivorax borkumensis	*Marinomonas* sp. MWYL1	*Shewanella pealeana*
Alteromonas macleodii	*Methylococcus capsulatus*	*Shewanella piezotolerance*
Aromatoleum aromaticum	*Nitrosococcus oceani*	*Shewanella putrefaciens*
Azotobacter vinelandii	*Pasteurella dagmatis*	*Shewanella sediminis*
Buchnera aphidicola	*Pasteurella multocida*	*Shewanella W3181*
Buchnera sp.	*Photobacterium damselae*	*Shewanella woodyi*
Cellvibrio japonicus	*Photobacterium profundum*	*Shigella boydii*
Citrobacter koseri	*Photobacterium* sp.	*Shigella dynsenteriae*
Citrobacter rodentium	*Photorhabdus luminescens*	*Shigella flexneri*
Colwellia psychrerythraea	*Proteus mirabilis*	*Shigella sonnei*
Dechloromonas aromaticum	*Pseudoalteromonas atlantica*	*Sodalis glossinidius*
Enterobacter 638	*Pseudoalteromonas haloplanktis*	*Symbiobacterium thermophilum*
Enterobacter sakazakii	*Pseudomonas aeruginosa*	*Thioalkalivibrio HL_EbGR7*
Erwinia carotovora	*Pseudomonas mendocina*	*Thiobacillus denitrificans*
Erwinia pyrifoliae	*Pseudomonas stutzeri*	*Thiomicrospira crunogena*
Erwinia tasmaniensis	*Psychromonas ingrahamii*	*Vibrio cholerae*
Escherichia coli	*Rhodobacter capsulatus*	*Vibrio fischeri*
Escherichia fergusonii	*Rhodobacter sphaeroides*	*Vibrio harveyi*
Haemophilus ducreyi	*Saccharophagus degradans*	*Vibrio parahaemolyticus*
Haemophilus influenzae	*Salmonella enterica*	*Vibrio splendidus*
Haemophilus parasuis	*Salmonella typhi*	*Vibrio vulnificus*
Haemophilus somnus	*Salmonella typhimurium*	*Yersina enterocolitica*
Hahella chejuensis	*Serratia proteamaculans*	*Yersina pestis*
Halorhodospira halophila	*Shewanella amazoniensis*	*Yersina pseudotuberculosis*
Idiomarina loihiensis	*Shewanella ANA3*	*Zymomonas mobilis*
Idiomarina baltica	*Shewanella baltica*	
Klebsiella pneumoniae	*Shewanella benthica*	

essential for nitrogen fixation in the light but also has an essential role in nitrogen fixation during anaerobic growth in the dark with dimethylsulfoxide as alternative electron acceptor. Furthermore, overproduction of the *rnf* operon in *R. capsulatus* resulted in higher levels of nitrogenase activity (50–100%) supporting once again the role of Rnf in nitrogen fixation in this organism [35]. In summary, it is concluded that the Rnf system in *R. capsulatus* could use the electrochemical ion potential generated by the photosynthetic apparatus to drive a reverse electron flow to reduce ferredoxin which serves as the ultimate donor for the dinitrogenase reductase. Therefore, the Rnf complex of *R. capsulatus* was suggested as a new electron transfer system that links known electron transport systems in the membrane to the nitrogenase system in the cytoplasm [26].

This function, production of "low redox" electrons, may be a general theme for the function of Rnf in aerobic organisms.

Pseudomonas stutzeri

Upstream of the *fdx* and *nifENX* genes that are all involved in nitrogen fixation in *P. stutzeri*, a *rnf* gene cluster (*rnfABCDGEH*) was detected. *rnf* insertion mutants were created in this organism and were found to display strongly reduced nitrogenase activities [113], as was shown for *R. capsulatus rnf* mutants. Therefore, also in *P. stutzeri*, Rnf could drive reverse electron flow from NADH generated by substrate oxidation to the reduction of ferredoxin that then delivers electrons to dinitrogenase reductase.

Table 4 Anaerobes containing Rnf

Alkaliphilus oremlandii	Clostridium thermocellum	Petrotoga mobilis
Alkaliphilus metalliredigens	Desulfatibacillum alkenivorans	Porphyromonas endodontalis
Acetobacterium woodii	Desulfobacterium autotrophicum	Porphyromonas gingivalis
Azoarcus BH72	Desulfotalea psychrophila	Porphyromonas uenonis
Bacteroides fragilis	Dethiobacter alkaliphilus	Prosthecochloris aestuarii
Bacteroides thetaiotaomicron	Dichelobacter nodosus	Prosthecochloris vibrioformis
Bacteroides vulgatus	Dictyoglomus thermophilum	Ruminococcus obeum
Brachyspira hyodysenteriae	Dictyoglomus furgidum	Ruminococcus torques
Chlorobium limicola	Fervidobacterium nodosum	Syntrophobacter fumaroxidans
Chlorobium luteolum	Finegoldia magna	Syntrophus aciditrophicus
Chlorobium phaeobacteroides	Fusobacterium nucleatum	Thermoanaerobacter pseudoethanolicus
Chloroherpeton thalassium	Halothermothrix orenii	Thermoanaerobacter X514
Clostridium beijerinckii	Methanococcoides burtonii[a]	Thermosipho africanus
Clostridium botulinum	Methanosarcina acetivorans[a]	Thermosipho melanesiensis
Clostridium difficile	Natranaerobius thermophilus	Thermotoga lattingae
Clostridium kluyveri	Parabacteroides distasonis	Thermotoga maritima
Clostridium novyi	Parabacteroides johnsonii	Thermotoga neapolitana
Clostridium perfringens	Parabacteroides merdae	Thermotoga petrophila
Clostridium phytofermentans	Parabacteroides sp.	Thermotoga RQ2
Clostridium tetani	Pelobacter carbinolicus	Treponema denticola
Clostridium tetanomorphum	Pelodictyon luteolum	

[a] Archaea

Table 5 Pathogens containing Rnf

Actinobacillus pleuropneumoniae	Fusobacterium nucleatum	Salmonella typhimurium
Aeromonas hydrophila	Haemophilus ducreyi	Serratia proteamaculans
Aeromonas salmonicida	Haemophilus influenzae	Shewanella loihica
Bacteroides fragilis	Haemophilus parasuis	Shewanella putrefaciens
Bacteroides thetaiotaomicron	Haemophilus somnus	Shigella boydii
Bacteroides vulgatus	Klebsiella pneumoniae	Shigella dysenteriae
Brachyspira hyodysenteriae	Mannheimia succiniciproducens	Shigella flexneri
Citrobacter koseri	Pasteurella multocida	Shigella sonnei
Clostridium botulinum	Photorhabdus luminescens	Vibrio cholerae
Clostridium difficile	Porphyromonas gingivalis	Vibrio parahaemolyticus
Clostridium perfringens	Proteus mirabilis	Vibrio splendidus
Clostridium tetani	Pseudoalteromonas atlantica	Vibrio vulnificus
Dichelobacter nodosus	Pseudoalteromonas haloplanktis	Yersina enterocolitica
Enterobacter sakazii	Pseudomonas aeruginosa	Yersina pestis
Erwinia carotovora	Pseudomonas mendocina	Yersina pseudotuberculosis
Escherichia coli	Pseudomonas stutzeri	
Escherichia fergusonii	Salmonella enterica	
Finegoldia magna	Salmonella typhi	

Escherichia coli

In E. coli, a system homologous to Rnf has been found and called Rsx (reducer of SoxR). The rsx locus contains the genes rsxABCDGE [28]. SoxR is a 17-kDa transcription factor that senses oxidant levels (mainly superoxide and nitric oxide) by its FeS center and activates oxygen defense systems. Oxidized SoxR increases synthesis of SoxS which activates target gene expression and SoxR is reduced upon removal of oxidative stress. Rnf/Rsx might maintain this
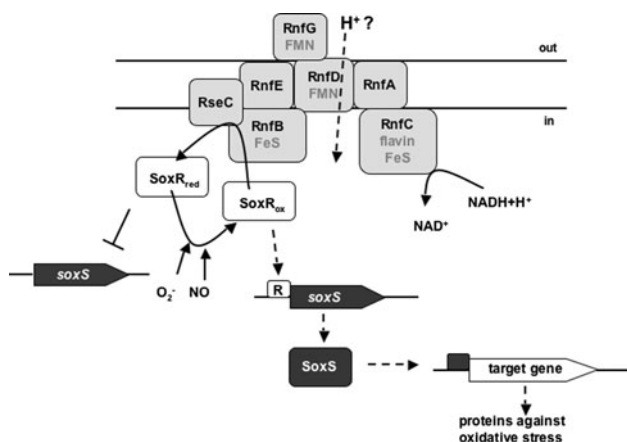
Fig. 7 Model of proposed electron flow in *E. coli*. Electrons deriving from NADH are channeled through the complex to keep SoxR in its reduced state. When oxidized SoxR activates target genes against oxidative stress. For explanation, see text. *red* reduced, *ox* oxidized

reduced state during aerobic growth. A random insertional mutation screen for constitutive SoxS expression phenotype in the absence of oxidants revealed that the *rnf/rsx* genes (*rnf/rsx*A–E) were affected. The redox level of SoxR was significantly decreased in these *rnf/rsx* mutants [28] indicating that Rnf keeps SoxR reduced. Therefore, in contrast to the *Rhodobacter/Pseudomonas* system where Rnf transfers electrons to nitrogenase, in *E. coli* it is involved in oxidative stress response and transfers electrons to SoxR. As depicted in Fig. 7, it was believed that SoxR has a much more negative redox potential than NADH and the energy barrier is overcome by the proton-motive force as driving force. However, experimental evidence points to a more electropositive potential of -285 mV for SoxR [114]. How this fits into the picture remains to be established.

Azotobacter vinelandii

A. vinelandii is a nitrogen-fixing bacterium and contains two *rnf* clusters that are differentially regulated [30]. *rnf2* is constitutively expressed regardless of the nitrogen source. *rnf1* and *nif* expression was found to be activated 10-fold after 6 h of ammonium deprivation. Deletion mutants in either *rnf1* or *rnf2* and a double mutant $\Delta rnf1/rnf2$ were constructed. It was shown that the nitrogen fixation activity was reduced in all mutants. Seven hours after nitrogenase derepression (ammonium-free growth conditions), the activity was 15 ($\Delta rnf1$), 35 ($\Delta rnf2$) and 5% ($\Delta rnf1/rnf2$) of the level of the wild-type. Furthermore, the *nifHDK* (nitrogenase) expression was analysed by reporter assays using β-galactosidase. Both single mutants showed lower β-galactosidase activity than the wild-type and lower protein levels after 3 h of nitrogenase derepression. After 7 h, the protein levels reached wild-type levels but the activity

was still impaired. It was further shown that *rnf* mutants accumulate a Fe-deficient and inactive form of dinitrogenase reductase (NifH) which could explain the reduced activities. Therefore, Curatti et al. [30] suggest that the Rnf complex (Rnf1 and Rnf2) in *A. vinelandii* is required for early expression of *nifHDK* (nitrogenase) during nitrogenase derepression and stable accumulation of the 4Fe4S cluster in NifH. In contrast to *R. capsulatus*, the data do not support a role of Rnf in electron transport to nitrogenase in *A. vinelandii*.

Vibrio cholerae

V. cholerae is a facultative anaerobic chemo-organoheterotrophic bacterium pathogenic to human but it is also able to persist in the absence of the human host [115]. Like other *Vibrio* strains, it does not have complex I of the respiratory chain (NADH dehydrogenase) but the Na^+-motive Nqr. In addition, it has a Rnf complex that has been analyzed biochemically [47]. Its role may be as in aerobes to produce "low redox" electrons to reduce cellular components involved in signaling, stress response, or other physiological activities. However, the function of Rnf is unknown but it would be interesting to see expression profiles of Rnf under different growth conditions and in planktonic versus host-associated cells.

Clostridia

The Rnf complex was discovered in a number of clostridial genomes: *C. tetani*, *C. tetanomorphum*, *C. kluyveri*, *C. botulinum*, *C. beijerinckii*, *C. difficile*, *C. novyi*, *C. perfringens*, *C. phytofermentans* and *C. thermocellum*. The Rnf complex in *C. tetani* is involved in electron flow during butyrate fermentation (Fig. 8). Fermentation of sugars occurs via glycolysis to pyruvate, which is then oxidized by pyruvate:ferredoxin oxidoreductase to carbon dioxide, acetyl-CoA and Fd_{red}. Two mol of acetyl-CoA are converted to acetoacetyl-CoA which is then converted via hydroxybutyryl-CoA and crotonyl-CoA to butyryl-CoA and subsequently to butyrate. The Fd_{red} generated by the pyruvate:ferredoxin oxidoreductase is thought to be reoxidized by the Rnf complex that drives export of ions (Na^+/H^+) from the cytoplasm, thus establishing an ion gradient across the membrane that can be used for ion (Na^+/H^+)-dependent symporters to take up sugars and amino acids into the cell. Whether Rnf of *C. tetani* generates a Na^+ or H^+ potential remains to be established. In contrast to *Clostridium acetobutylicum* and *C. perfringens*, *C. tetani* does not harbor the genes for an F_1F_o ATP synthase. *C. tetani* has the genes for a V-type ATPase and another ATPase that is annotated as archaeal "Na^+-V-type" [32, 116]. However, both do not have the conserved
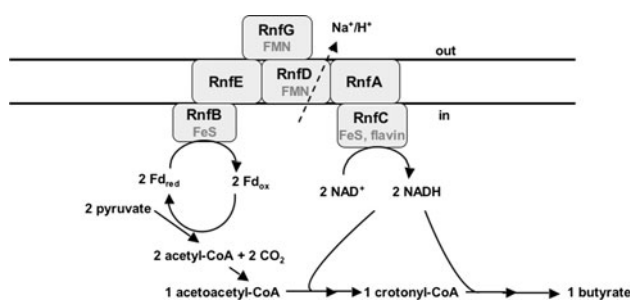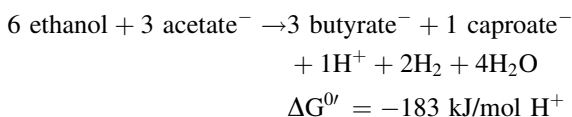
Fig. 8 Model of proposed electron flow in *C. tetani*. For explanation, see text. *Fd* ferredoxin, *red* reduced, *ox* oxidized. *C. tetani* is a peptolytic *Clostridium* that preferentialy ferments amino acids and the degradation of many amino acids results in the formation of pyruvate

Na^+-binding motif of ATPases and both have a *c* subunit with one or no ion binding in four transmembrane helices suggesting a role in ATP hydrolysis and making a function in ATP synthesis unlikely. Therefore, the ion gradient established by the Rnf complex is not used as driving force for ATP synthesis but, most likely, substrate transport. Whether Rnf uses H^+ or Na^+ remains to be addressed experimentally.

Rnf is also found in the glutamate-fermenting *C. tetanomorphum*. Glutamate is oxidized to pyruvate and acetate via 3-methylaspartate. Pyruvate is then converted to butyrate as described above. The function of Rnf in this organism might be equivalent to that in *C. tetani*. Rnf is an Na^+-pumping enzyme accepting electrons from Fd_{red} (derived from pyruvate oxidation or crotonyl-CoA reduction) and generating NADH for butyrate synthesis [37].

Clostridium kluyveri ferments ethanol and acetate according to:

$$6 \text{ ethanol} + 3 \text{ acetate}^- \rightarrow 3 \text{ butyrate}^- + 1 \text{ caproate}^-$$
$$+ 1H^+ + 2H_2 + 4H_2O$$
$$\Delta G^{0\prime} = -183 \text{ kJ/mol } H^+$$

The mechanism of H_2 formation from NADH via ferredoxin could not be explained for a long time since this reaction is endergonic. The reductant for crotonyl-CoA and acetoacetyl-CoA reduction is NAD(P)H that derives, for example, from glycolysis. In contrast to acetoacetyl-CoA reduction ($E^{0\prime} = -240$ mV), reduction of crotonyl-CoA ($E^{0\prime} = -10$ mV) with NADH + H^+ is highly exergonic. Recently, it was demonstrated for *C. kluyveri* that the energy released during crotonyl-CoA reduction with NADH + H^+ as reductant is used to drive the endergonic reduction of ferredoxin via a process termed "electron bifurcation" which offers a mechanism for H_2 production in *C. kluyveri* [117, 118]. A butyryl-CoA dehydrogenase/Etf complex has been described that is able to bifurcate electrons via FAD (bound to Etf) from the oxidant NADH. Of the two electrons from NADH, one proceeds to the more positive electron

acceptor crotonyl-CoA, which is converted to butyryl-CoA, while the other is transported in the opposite direction to the more negative acceptor ferredoxin that is used for H_2 production. Another NADH delivers the second electron to complete the reduction of each acceptor. This way, the endergonic reduction of ferredoxin is driven by the exergonic reduction of crotonyl-CoA. The bifurcation is accomplished by the prosthetic group FAD, which is reduced to its hydroquinone form by NADH. One electron is then transferred to the next FAD and further to ferredoxin, whereas the other is used by the dehydrogenase [118]. The same principle is known for the bc_1 complex of the respiratory chain. The complex oxidizes ubihydroquinone and transfers electrons to cytochrome *c*. The electrons from hydroquinone are bifurcated; one electron is transferred via a Rieske FeS cluster to cytochrome *c*, while the second electron is transferred via hemes to quinone, which is reduced to hydroquinone. This reaction is strictly coupled and is completed by the bifurcation of the second pair of electrons from another hydroquinone. In total, half the electrons are recycled from another hydroquinone, back to the quinone, thus the Q-cycle leads to a doubling of the proton-motive efficiency [119]. This sort of direct coupling in which the downhill transport of one electron drives the uphill transport of another is probably seen more often in anaerobes. Examples are the bifurcating hydrogenase of *Thermotoga maritima* [120] and the heterodisulfide reductase in Methanothermobacter [80].

Fd_{red} is used by Rnf that is encoded in the genome of *C. kluyveri* to pump ions across the cytoplasmic membrane while generating NADH [33]. In Fig. 9, a simplified version of the metabolism of *C. kluyveri* is shown. Ethanol is oxidized to acetyl-CoA. Through a cycle, the electrons are transferred to the butyryl-CoA dehydrogenase/Etf complex and butyrate is formed. At the end, the Fd_{red} is split and used by hydrogenase to produce hydrogen and by Rnf to generate an ion gradient. The coupling ion of Rnf is unknown at present, but since the ATP synthase of *C. kluyveri* has no conserved Na^+ binding site, one would expect a H^+-based Rnf complex.

Syntrophus aciditrophicus and other synthrophic bacteria

Syntrophus aciditrophicus is a syntrophic bacterium that grows on fatty acids, benzoate, cyclohexane, carboxylate, cyclohex-1-ene carboxylate and crotonate in coculture with methanogens or sulfate reducers [121]. Degradation of substrates with H_2 production is

thermodynamically unfavorable (propionate $\Delta G^{0\prime} = + 76.1$ kJ/mol, butyrate $\Delta G^{0\prime} = +48.6$ kJ/mol, benzoate $\Delta G^{0\prime} = +70.1$ kJ/mol), therefore the syntrophic partner (methanogen) is needed that uses the produced H_2 as a

Fig. 9 Metabolism of *C. kluyveri*. For the sake of simplicity, only the formation of butyrate and H_2 is shown. Reactions involved in caproate formation are analogous (butyryl-CoA condenses with acetyl-CoA). Ten mol NADH are consumed by the butyryl-CoA-DH complex. They are bifurcated to produce 5 mol butyryl-CoA and 5 mol Fd_{red}. Fd_{red} is either used by hydrogenase to produce H_2 or by Rnf to produce NADH and thereby generating an ion gradient. For further explanation, see text. *[H]* reducing equivalents, *Fd* ferredoxin, *red* reduced, *ox* oxidized
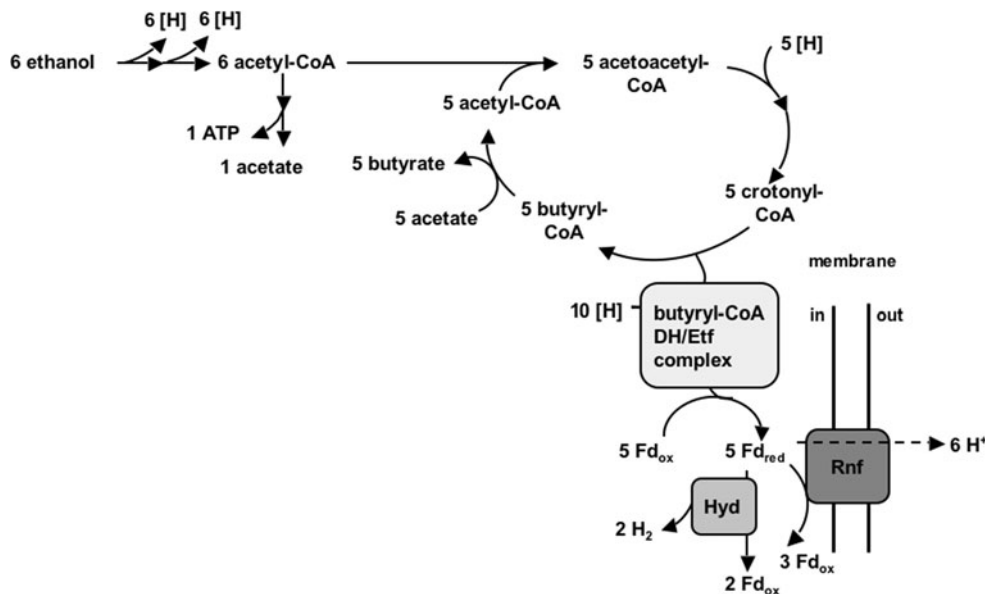
substrate and thus lowers the hydrogen partial pressure in the ecosystem. Mechanistically, the syntrophic bacterium must have a way to overcome the energy barrier between $NADH + H^+$ (generated during the oxidation reaction) and Fd_{red} (the ultimate precursor of hydrogen). The genome sequence of *S. aciditrophicus* revealed a couple of membrane-bound FeS proteins and oxidoreductases as well as a *rnf* gene cluster. The latter is assumed to be the key enzyme in the bioenergetics of the syntrophic metabolism since it provides the cell with the precursor of hydrogen. The driving force for NADH-dependent ferredoxin reduction by Rnf is assumed to be an Na^+ gradient established by a glutaconyl-CoA decarboxylase.

Desulfobacterium autotrophicum and other sulphate reducers

Desulfobacterium autotrophicum is a chemolithoautotrophic sulfate-reducing bacterium that can conserve energy by sulfate reduction to sulfide with H_2 as reductant [122, 123]. Uptake of sulfate is an energy-dependent process and, in addition, sulfate is activated at the expense of 2 mol ATP prior to reduction. Sulfate reduction to sulfide requires eight electrons, provided by periplasmic hydrogenases. This results in eight scalar protons that would allow for the synthesis of 2.6 mol ATP (considering 3 H^+/ATP). Clearly, additional mechanisms for the generation of a proton (ion) motive force must exist. This is where Rnf comes into play. Electron flow to sulfate could be carried out by a soluble, ferredoxin-reducing hydrogenase and the Rnf complex which would lead to NADH production, the reductant for sulfate. This would allow for additional ATP synthesis by an Rnf-mediated ion motive force [124].

Methanosarcina acetivorans

Like anaerobic bacteria, the anaerobic methanogenic archaea have developed mechanisms to conserve energy from the redox difference of ferredoxin and the central electron carrier, F_{420}. However, it should be noted that the genomes of only two methanogens contain *rnf* genes (*M. acetivorans* and *M. burtonii*). *M. acetivorans* produces methane from acetate according to:

$$Acetate + H_2O \rightarrow CO_2 + CH_4$$
$$\Delta G^{0\prime} = -31 \text{ kJ/mol [80]}$$
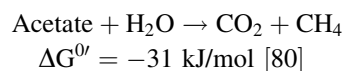
Methanogenesis from acetate has the lowest $\Delta G^{0\prime}$ of any methanogenic pathway and the free energy change may not even allow the synthesis of 1 mol ATP. However, methanogens have developed highly efficient mechanisms to conserve energy [82]. Acetate is oxidized by CO dehydrogenase/acetyl-CoA synthase to an enzyme-bound carbonyl group and an enzyme-bound methyl group [125, 126]. The methyl group is transferred to tetrahydromethanopterin. The next step is a methyl group transfer from methyltetrahydromethanopterin to coenzyme M. This reaction is catalyzed by a membrane-bound methyltransferase composed of a hydrophobic membrane translocator and a hydrophilic, corrinoid-containing subdomain that catalyzes the methyl transfer. The energy liberated by the methyl transfer reaction is used to pump Na^+ out of the cell [127, 128]. The next step, methane formation, is catalyzed by a soluble enzyme. Interestingly, this enzyme uses coenzyme B (CoB) as a reductant for methane formation thereby generating a disulfide of coenzyme M (CoM) and CoB, the so-called heterodisulfide. This is used as an electron acceptor for anaerobic respiration. Electrons for

Fig. 10 Model of involvement of Rnf in metabolism of *M. acetivorans*. A possible way of electron transport is shown. Note that other ways of electron transport are possible (see "Physiological roles of Rnf in different bacteria"). For explanation, see text. *MP* methanophenazine, *Cdh* CO-dehydrogenase/acetyl-CoA synthase, *Hdr* heterodisulfide reductase, *Cyt c* cytochrome *c*, *CoM* coenzyme M, *CoB* coenzyme B, H_4SPT tetrahydrosarcinapterin. *Fd* ferredoxin, *ox* oxidized, *red* reduced
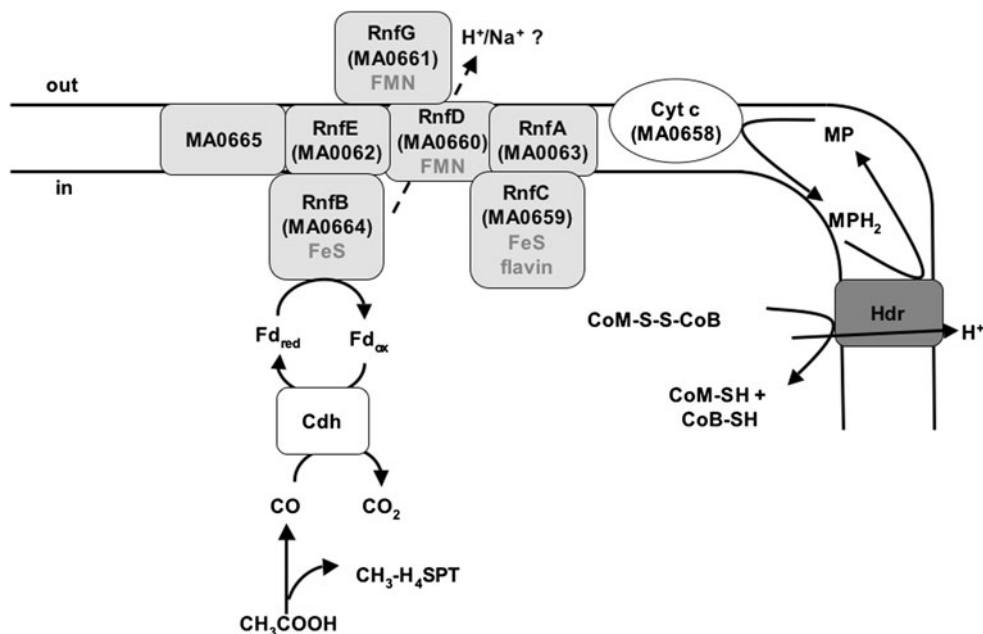
the reduction derive from oxidation of enzyme-bound CO which yields Fd_{red}. The electron transport chain involves methanophenazine as electron carrier and an ion-motive heterodisulfide reductase [80, 129]. The question is whether there is an additional coupling site between ferredoxin and methanophenazine. In a proteomic study, Rnf was detected at a higher concentration (2.5- to 3.5-fold) in acetate-grown cells than in methanol-grown cells [130]. Therefore, it was speculated that Rnf presents a coupling site in acetate metabolism of *M. acetivorans* (Fig. 10). Due to the recognized role of Na^+ bioenergetics in methanogens, it is speculated that Rnf is a sodium ion pump in *M. acetivorans* but again, one should consider that the ion specificity is not known, and also not for the heterodisulfide reductase of *M. acetivorans*. Whether Rnf is indeed an ion (Na^+) pump in *M. acetivorans* needs to be addressed experimentally. Moreover, if F_{420} is the electron acceptor of the Rnf complex, there may be an additional coupling site in between F_{420} and the methanophenazine. Most interestingly, the Rnf complexes of *M. acetivorans* and *M. burtonii* are the only Rnf complexes so far known that contain a cytochrome *c*. Therefore, one may hypothesize that the cytochrome *c* is involved in F_{420} reduction. Moreover, it may also be possible that F_{420} is not the electron acceptor of the Rnf complex, but that the electrons are channeled into the membrane via cytochrome *c*. Moreover, it may also be possible that the cytochrome *c* has a completely different function such as channeling electrons into the system from yet unknown electron donors.

During growth on trimethylamine or other methylated C_1-compounds, one-quarter of the methyl group is oxidized to CO_2. One step of the oxidation pathway, the oxidation of formyl-methanofuran to CO_2, is exergonic and coupled to the reduction of ferredoxin. Fd_{red} may be oxidized through

Rnf, either with concomitant reduction of F_{420}, the central electron carrier of methanogens, or directly with reduction of the heterodisulfide [80]. In any case, Rnf might be used to generate an electrochemical ion potential. Like *M. acetivorans*, *M. burtonii* is a member of the cytochrome-containing Methanosarcinales [80]. It does not grow on acetate but on methanol [131], and *rnf* and cytochrome *c* genes are present in its genome. The function of Rnf may have to do with the electron flow during methyl group oxidation as in *M. acetivorans*.

Conclusion

The Rnf complex is a novel ion-motive electron transport chain widely distributed in prokaryotes that deserves further attention. Its physiological role has been demonstrated in *A. woodii* as the key bioenergetic enzyme in caffeate respiration. Indeed, in this type of respiration, it is the only coupling site. The same may be true for acetogenesis. In some other anaerobes, it is likely to have the same function, and in aerobes, it may be involved driving reverse, endergonic electron flow. However, future work has to concentrate on defining the physiological role of the Rnf complex in the different bacteria and archaea. Moreover, the biochemical analyses is in its infancy and nothing is known about how electron flow is coupled to ion transport. These are the challenging tasks for future work.

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