

# The Rnf Complex of *Clostridium ljungdahlii* Is a Proton-Translocating Ferredoxin:NAD<sup>+</sup> Oxidoreductase Essential for Autotrophic Growth

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**ABSTRACT** It has been predicted that the Rnf complex of *Clostridium ljungdahlii* is a proton-translocating ferredoxin:NAD<sup>+</sup> oxidoreductase which contributes to ATP synthesis by an H<sup>+</sup>-translocating ATPase under both autotrophic and heterotrophic growth conditions. The recent development of methods for genetic manipulation of *C. ljungdahlii* made it possible to evaluate the possible role of the Rnf complex in energy conservation. Disruption of the *C. ljungdahlii* *rnf* operon inhibited autotrophic growth. ATP synthesis, proton gradient, membrane potential, and proton motive force collapsed in the Rnf-deficient mutant with H<sub>2</sub> as the electron source and CO<sub>2</sub> as the electron acceptor. Heterotrophic growth was hindered in the absence of a functional Rnf complex, as ATP synthesis, proton gradient, and proton motive force were significantly reduced with fructose as the electron donor. Growth of the Rnf-deficient mutant was also inhibited when no source of fixed nitrogen was provided. These results demonstrate that the Rnf complex of *C. ljungdahlii* is responsible for translocation of protons across the membrane to elicit energy conservation during acetogenesis and is a multifunctional device also implicated in nitrogen fixation.

**IMPORTANCE** Mechanisms for energy conservation in the acetogen *Clostridium ljungdahlii* are of interest because of its potential value as a chassis for the production of biocommodities with novel electron donors such as carbon monoxide, syngas, and electrons derived from electrodes. Characterizing the components implicated in the chemiosmotic ATP synthesis during acetogenesis by *C. ljungdahlii* is a prerequisite for the development of highly productive strains. The Rnf complex has been considered the prime candidate to be the pump responsible for the formation of an ion gradient coupled with ATP synthesis in multiple acetogens. However, experimental evidence for a proton-pumping Rnf complex has been lacking. This study establishes the *C. ljungdahlii* Rnf complex as a proton-translocating ferredoxin:NAD<sup>+</sup> oxidoreductase and demonstrates that *C. ljungdahlii* has the potential of becoming a model organism to study proton translocation, electron transport, and other functions of the Rnf complex in energy conservation or other processes.

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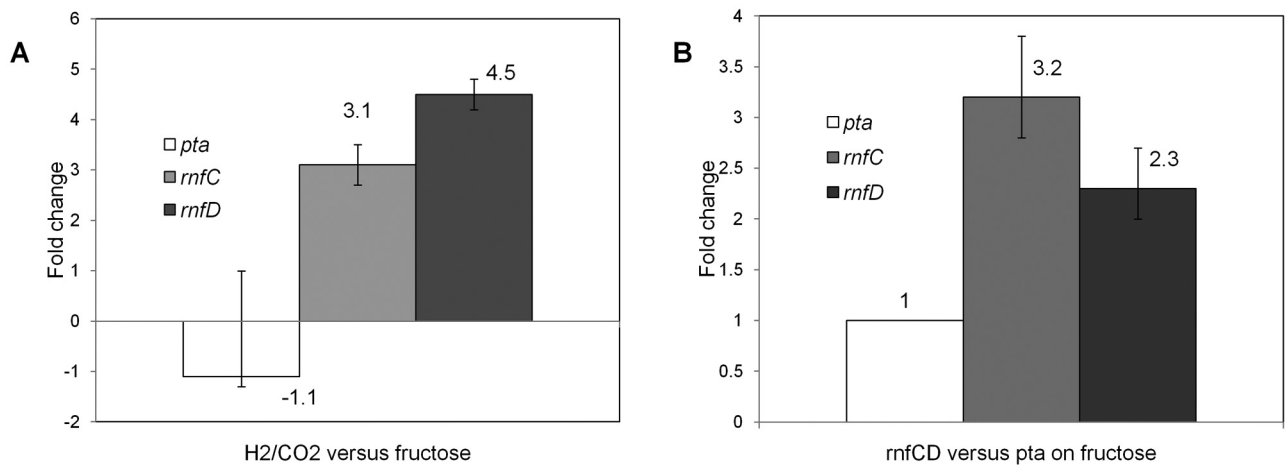
Acetogenesis is a process performed by anaerobic bacteria producing acetate by using CO<sub>2</sub> as an electron acceptor (1–3). Reduction of CO<sub>2</sub> is achieved through the acetyl coenzyme A (acetyl-CoA) pathway also known as the Wood-Ljungdahl pathway (4, 5). Organic compounds such as sugars and inorganic compounds such as H<sub>2</sub> and CO can be used as the electron donor for the reduction of CO<sub>2</sub> (5). In the latter case, the Wood-Ljungdahl pathway and the associated energy conservation mechanisms become the sole mechanism to generate energy for growth (6).

*Clostridium ljungdahlii* is an acetogen capable of growing on syngas, a gas mixture containing mostly CO and H<sub>2</sub> and sometimes CO<sub>2</sub>, to form acetate and ethanol (7). There is interest in *C. ljungdahlii* because of its ability to naturally produce ethanol (7, 8) and 2,3-butanediol (9), as well as for its capacity to express heterologous genes resulting in the production of butanol (10, 11) from syngas. *C. ljungdahlii* can also produce organic compounds from carbon dioxide with electrons derived from an electrode in a process known as microbial electrosynthesis (12).

Analysis of the genomes of *C. ljungdahlii* (10), as well as the

acetogens *Acetobacterium woodii* (13) and *Moorella thermoacetica* (14), has provided insights into the energy conservation mechanisms involved in acetogenesis. Energy conservation in *C. ljungdahlii* during autotrophic growth has been predicted to be exclusively achieved through a proton gradient coupled to ATP synthesis by an H<sup>+</sup>-translocating ATPase (10). The Rnf complex, a membrane ferredoxin:NAD oxidoreductase, is thought to be responsible for generating the proton gradient (1, 10).

Biochemical studies of the Rnf complex of *A. woodii* have indicated that it is a ferredoxin:NAD<sup>+</sup> oxidoreductase coupling Na<sup>+</sup> translocation through the membrane with ATP generation by the Na<sup>+</sup>-dependent F1F0 ATPase (15–18). However, there has been no definitive demonstration *in vivo* that the Rnf complex functions as an energy conservation-associated sodium ion/proton pump during acetogenesis. The recent development of a genetic system in *C. ljungdahlii* permitting gene deletion (19) opens such a possibility for functional *in vivo* studies. The purpose of this study was to determine whether the *C. ljungdahlii* Rnf complex functions as a proton translocation device involved in energy con-



**FIG 1** Quantitative RT-PCR assay of *C. ljungdahlii* *rnfC*, *rnfD*, and *pta* genes. (A) Fold change in the transcript abundance of *C. ljungdahlii* *rnfC*, *rnfD*, and *pta* on  $H_2$ - $CO_2$  compared to that on fructose. (B) Fold change in the transcript abundance of *rnfC* and *rnfD* compared to *pta* on fructose. Each value is the mean from three replicates. *P* values of *rnfCD* transcript abundance during growth on  $H_2$ - $CO_2$  versus growth on fructose in panel A and *P* values of *rnfCD* transcript abundance versus *pta* in panel B are lower than 0.01.

servation during autotrophic and heterotrophic growth and also to identify other possible functions by characterizing an Rnf-deficient strain.

## RESULTS AND DISCUSSION

**Transcript abundance of the *C. ljungdahlii* Rnf complex.** In order to establish the relative functional importance of the Rnf complex during autotrophic and heterotrophic growth, transcript abundance of the first two genes of *C. ljungdahlii* *rnfCDGEAB* was quantified with quantitative reverse transcription-PCR (qRT-PCR). *rnfC* and *rnfD* were both upregulated when cells were grown on  $H_2$ - $CO_2$  versus fructose (Fig. 1A). In comparison, there was no difference in transcript abundance of *pta*, a gene coding for the phosphotransacetylase, an enzyme converting acetyl-CoA to acetyl-P, which is an important reaction in both autotrophic and heterotrophic growth (10). Although *rnfCD* were upregulated on  $H_2$ - $CO_2$ , the transcript abundance of these two genes was still 2- to 3-fold higher than that of *pta*, even when fructose was the sole electron source (Fig. 1B). These results suggest that the Rnf complex has a more important function during autotrophic growth but still makes a contribution to heterotrophic growth.

**Autotrophic growth and ATP generation in the absence of the Rnf complex.** The *rnf* operon was disrupted by single-crossover integration resulting in the absence of *rnfA* and *rnfB* transcripts (Table 1). *rnfA* codes for a putative integral membrane protein with no clear function, whereas *rnfB* codes for a membrane-associated polyferredoxin accepting electrons from ferredoxin and transferring them to membrane domains of the Rnf complex (1, 6).

Inactivation of this portion of the Rnf complex completely inhibited growth on  $H_2$ - $CO_2$  (Fig. 2A). Inactivation of the Rnf complex also completely inhibited ATP synthesis in cell suspensions energized with  $H_2$  (Fig. 2B). This is consistent with the prediction that the Rnf complex is necessary for energy conservation during autotrophic growth in *C. ljungdahlii* (10).

Attempts to construct a plasmid expressing *C. ljungdahlii* *rnfAB* failed, making complementation unfeasible. *rnfAB* appears to be toxic for *Escherichia coli*. However, the single-crossover muta-

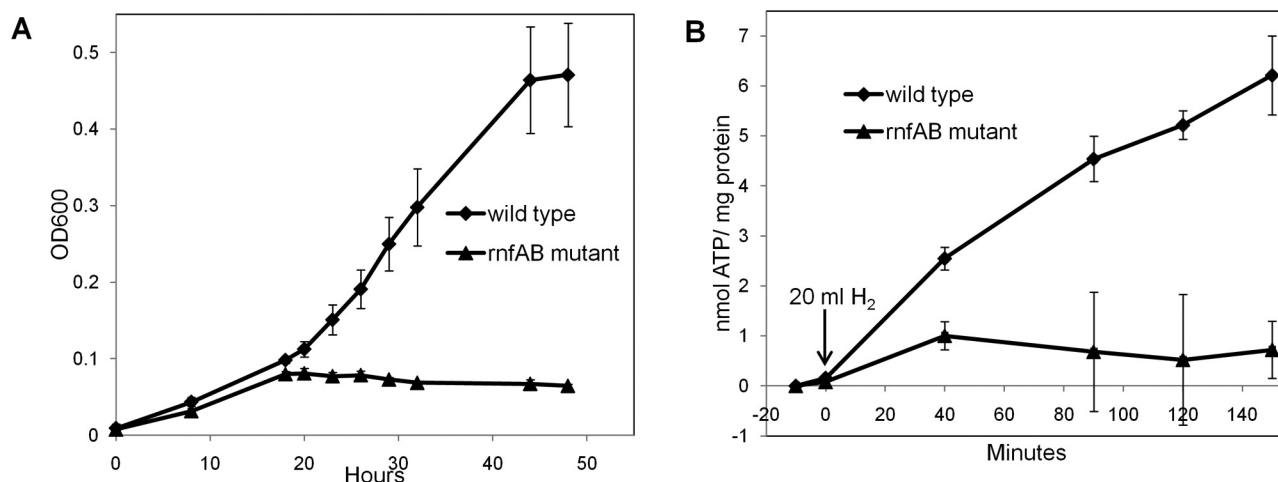
tion of the *rnf* operon is unstable, and the disrupting plasmid is expected to come out of the chromosome if antibiotic selection pressure is not maintained (20), resulting in a reversion to a fully functional *rnf* operon. When the *rnfAB* single-crossover mutant was grown on  $H_2$ - $CO_2$  without clarithromycin (Fig. 3A), the culture did not grow for the first 95 h, presenting a phenotype comparable to the *rnfAB* mutant in the presence of clarithromycin. However, growth resumed at a doubling time comparable to that of the wild type after this initial 95 h. PCR and quantitative RT-PCR demonstrated that the recovery of the growth capacity on  $H_2$ - $CO_2$  could be attributed to the reconstitution of the wild-type *rnf* operon and resulting *rnfAB* expression (Fig. 3; Table 1). This result indicated that the autotrophic growth inhibition observed in the absence of *rnfAB* expression was not the result of an unwanted mutation elsewhere in the chromosome.

**Autotrophic growth of *C. ljungdahlii* is dependent on an Rnf-driven proton gradient.** Several lines of evidence have suggested that *C. ljungdahlii* establishes a proton gradient which is converted into ATP during autotrophic growth rather than relying on a sodium ion gradient like *A. woodii* (3, 10, 17, 21). The genome of *C. ljungdahlii* encodes only an  $H^+$ -translocating AT-

**TABLE 1** Fold change in transcript abundance of *rnfCDGEAB* genes in the *rnfAB* mutant in the presence or not of clarithromycin compared to the wild type

Gene	Fold change of <i>rnfAB</i> mutant: <sup>a</sup>	
	With clarithromycin	Without clarithromycin
<i>rnfC</i>	1.0 (-1.2/1.2)	1.7 (1.4/2.1)
<i>rnfD</i>	1.0 (-1.3/1.3)	1.4 (1.0/2.0)
<i>rnfG</i>	1.7 (1.3/2.3)	1.8 (1.5/2.2)
<i>rnfE</i>	3.3 (1.7/6.3)	1.7 (1.4/2.1)
<i>rnfA</i>	ND	1.6 (1.3/1.9)
<i>rnfB</i>	ND	1.6 (1.3/1.9)

<sup>a</sup> Cultures were grown autotrophically with  $H_2$ - $CO_2$ . Each value is the mean of three replicates. Fold change  $\pm$  standard deviation is in parentheses. ND, no transcript of *rnfA* and *rnfB* was detected. The *rnfAB* mutant was grown under clarithromycin (5  $\mu$ g/ml) selective pressure. The *rnfAB* mutant was transferred 4 times in the absence of clarithromycin.

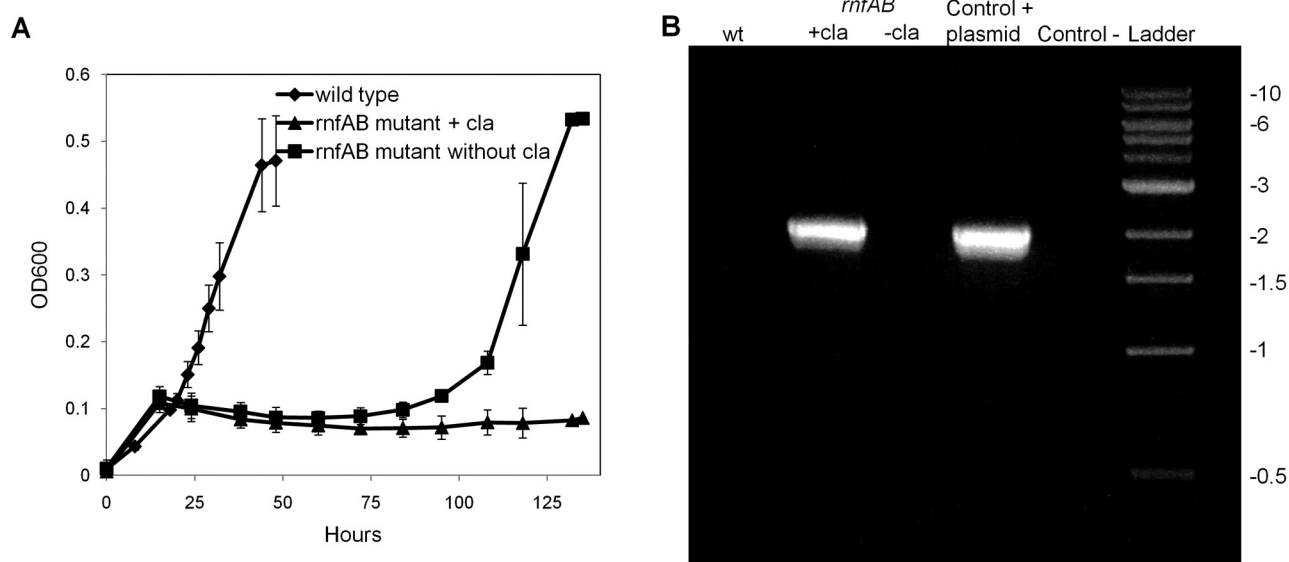


**FIG 2** Autotrophic growth and intracellular ATP synthesis. (A) Growth curves of *C. ljungdahlii* wild type and *rnfAB* mutant with  $H_2$  as the electron donor and  $CO_2$  as the electron acceptor. (B) Intracellular ATP synthesis by wild-type and *rnfAB* mutant cell suspensions. At time 0, 20 ml of  $H_2$  was added to the headspace containing an atmosphere of 80%  $N_2$ -20%  $CO_2$ . Each value is the mean from three replicates.

Pase, and growth was not inhibited by a reduction of the  $Na^+$  concentration in the growth medium (10). When *C. ljungdahlii* was grown with  $H_2$ - $CO_2$  in the presence of the protonophore 3,3',4',5-tetrachlorosalicylanide (TCS) or the sodium-specific ionophore ETH2120, both known to be active in acetogens and/or *Clostridium* spp. (17, 22, 23), TCS completely inhibited growth, whereas ETH2120 had no effect (Fig. 4). These results indicate that a proton gradient is essential for energy conservation.

To determine whether the Rnf complex could be responsible for the formation of the proton gradient necessary for autotrophic

growth, the membrane potential ( $\Delta\Psi$ ), the  $\Delta pH$ , and the proton motive force (PMF) were measured in cell suspensions of the wild type and the *rnfAB* mutant. Cells were grown on fructose, washed, and resuspended in medium without electron donor. At time 0, when 20 ml of  $H_2$  was added to the headspace in the presence of  $CO_2$ , the  $\Delta\Psi$  and PMF were relatively high, presumably generated by the consumption of energy sources stored in the cell during growth on fructose (Fig. 5A to C). However, at 10 min after the addition of  $H_2$ , the  $\Delta\Psi$  dramatically collapsed in the *rnfAB* mutant, whereas it was only slightly diminished in the wild type. The



**FIG 3** Disruption and reconstitution of the *rnf* operon. (A) Growth curves of *C. ljungdahlii* wild type, the *rnfAB* mutant with clarithromycin (5  $\mu g/ml$ ), and the *rnfAB* mutant without clarithromycin when  $H_2$  is the electron donor and  $CO_2$  is the electron acceptor. Each value is the mean from three replicates. (B) DNA gel showing PCR results using 1 primer annealing to *rnfC* and 1 primer annealing to the clarithromycin resistance cassette with the wild type (lane wt), the *rnfAB* mutant under clarithromycin (5  $\mu g/ml$ ) pressure (lane *rnfAB* + cla), and the *rnfAB* mutant after 4 transfers on  $H_2$ - $CO_2$  without clarithromycin (lane *rnfAB* - cla). The positive control is the suicide plasmid used to disrupt the *rnf* operon by single crossover (lane control + plasmid). The negative control is a no-template PCR (lane control -). The numbers on the left indicate the band sizes in kilobases for the NEB 1-kb ladder used as a marker (lane ladder). Genomic DNA was used as the template for PCRs, except where indicated otherwise.

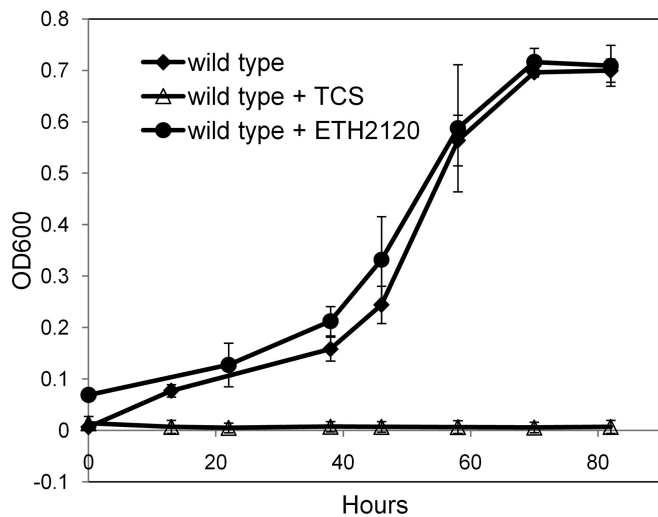


FIG 4 Effect of protonophore and ionophore on *C. ljungdahliae* autotrophic growth. Growth curves of wild type in the presence of 5  $\mu\text{M}$  TCS (protonophore) or 20  $\mu\text{M}$  ETH2120 (sodium-specific ionophore). Each value is the mean from three replicates.

$\Delta\text{pH}$  also completely collapsed in the *rnfAB* mutant but not in the wild type, where it increased significantly at time 100 (Fig. 5B). This result demonstrates that the *C. ljungdahliae* Rnf complex plays an important role in pumping protons out of the cell membrane for energy conservation during autotrophic growth. The collapse of the  $\Delta\Psi$  and the  $\Delta\text{pH}$  resulted in the elimination of the PMF when the Rnf complex was disrupted (Fig. 5C), which explained the absence of autotrophic growth and the lack of ATP synthesis.

**Heterotrophic growth, ATP generation, and proton gradient without the Rnf complex.** Disruption of the Rnf complex also resulted in a growth defect when fructose was the sole electron donor (Fig. 6A). Doubling time during heterotrophic growth of the *rnfAB* deletion mutant was 5 h, 51 min  $\pm$  17 min, whereas doubling time of the wild type was 3 h, 56 min  $\pm$  17 min (mean  $\pm$  standard deviation;  $n = 3$  replicates). ATP synthesis was also significantly reduced in the absence of a functional Rnf complex (Fig. 6B). The *rnfAB* mutant had 24.7%  $\pm$  5.3 less intracellular ATP than the wild type 90 min after 10 mM fructose was added to the cell suspensions.

When cell suspensions were energized with 10 mM of fructose, there was no difference in  $\Delta\Psi$  between the wild type and the *rnfAB*-deficient mutant (Fig. 7A). However, absence of the Rnf complex clearly had an impact on the  $\Delta\text{pH}$  because at times 10 and 100, the  $\Delta\text{pH}$  of the wild type was significantly higher than that of the *rnfAB* mutant (Fig. 7B). At time 100, the reduction of the  $\Delta\text{pH}$  caused a substantial diminution of the PMF in the *rnfAB*-deficient mutant compared to that in the wild type. The reduction of the PMF when the Rnf complex is absent is in agreement with the increase in doubling time and the lower intracellular ATP pool during heterotrophic growth.

When fructose is the electron donor, the bulk of the intracellular ATP pool is generated by the substrate-level phosphorylation associated with glycolysis and the conversion of acetyl-P to acetate (10). As demonstrated here, the Rnf complex of *C. ljungdahliae* also contributes to ATP synthesis during heterotrophic growth and is doing so by generating a proton gradient. The reduced ferredoxin

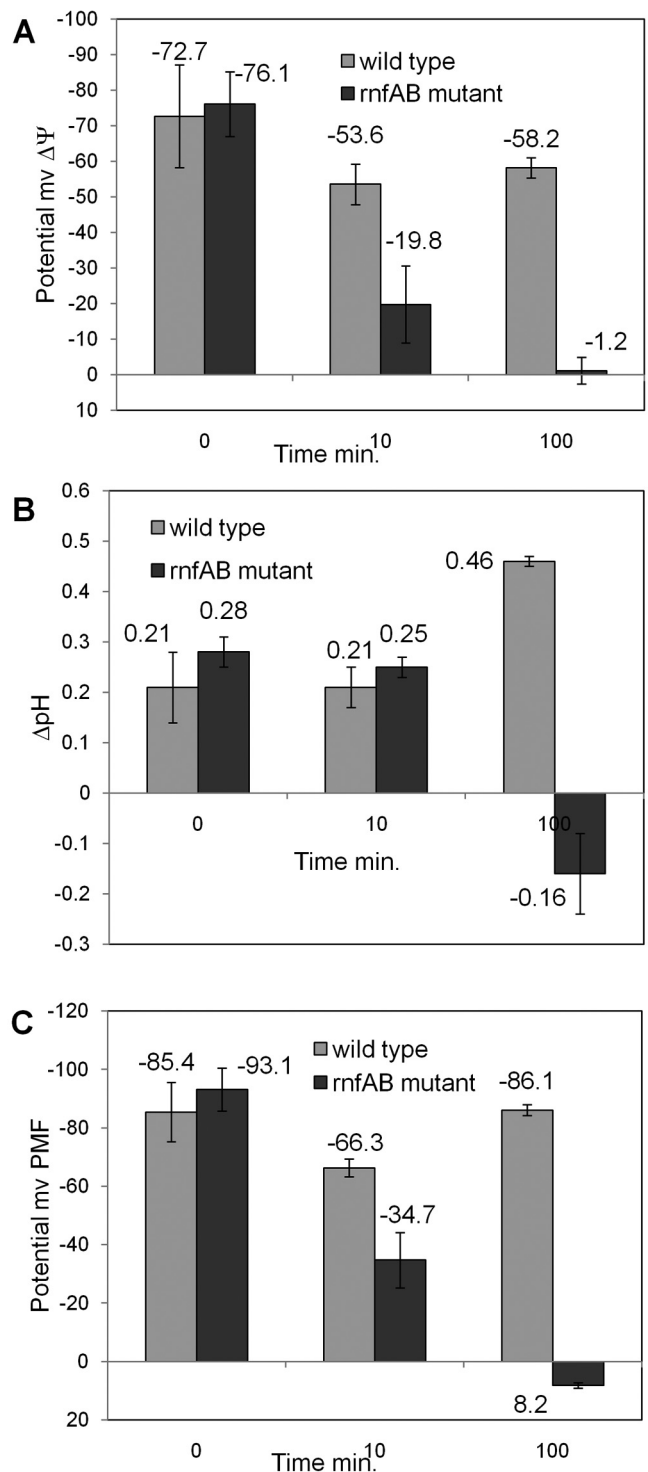
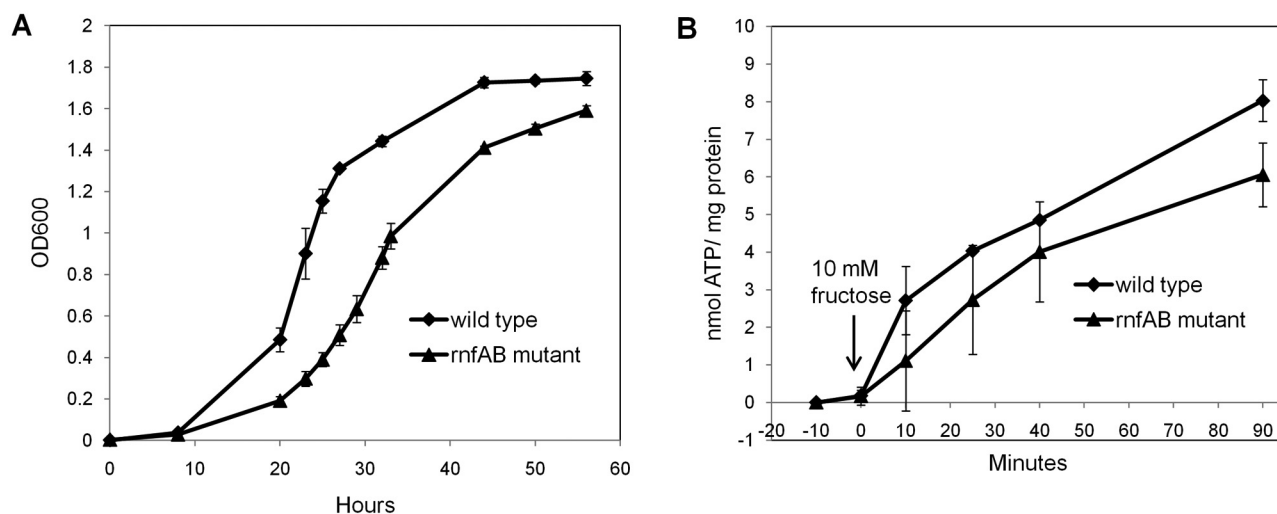


FIG 5  $\Delta\Psi$ ,  $\Delta\text{pH}$ , and PMF with  $\text{H}_2$  as the electron donor.  $\Delta\Psi$  (A),  $\Delta\text{pH}$  (B), and PMF (C) in *C. ljungdahliae* wild-type and *rnfAB* mutant cell suspensions. At time 0, 20 ml of  $\text{H}_2$  was added to the headspace containing an atmosphere of 80%  $\text{N}_2$ -20%  $\text{CO}_2$ . Each value is the mean from three replicates.

required for the formation of an Rnf-dependent proton gradient could be generated by several potential metabolic processes. Electrons necessary for the reduction of ferredoxin might be bifurcated from a highly exergonic reaction catalyzed by the



**FIG 6** Heterotrophic growth and intracellular ATP synthesis. (A) Growth curves of *C. ljungdahlii* wild type and *rnfAB* mutant with fructose as the electron donor. (B) Intracellular ATP synthesis by wild-type and *rnfAB* mutant cell suspensions. At time 0, 10 mM fructose was added. Each value is the mean from three replicates.

methylene-tetrahydrofolate (THF) reductase found in the Wood-Ljungdahl pathway:  $\text{CH}_2 = \text{THF} + \text{NADH} + \text{H}^+ \rightarrow \text{CH}_3\text{-THF} + \text{NAD}^+$  (24). In this model, a second NADH must provide the second electron necessary for the formation of reduced ferredoxin and  $\text{CH}_3\text{-THF}$ . The resulting  $\text{NAD}^+$  is expected to be regenerated by the Rnf complex after electrons from reduced ferredoxin have been collected to generate a proton gradient (10).

Oxidation of  $\text{H}_2$  produced during growth on fructose might also provide an additional source of reduced ferredoxin. An [Fe-Fe]hydrogenase from *A. woodii* was shown to drive endergonic ferredoxin reduction by coupling reduction of the ferredoxin to the exergonic reduction of  $\text{NAD}^+$  with  $\text{H}_2$  using electron bifurcation (25). Genes coding for homologues of the subunits of this hydrogenase are found in *C. ljungdahlii*: Clju\_c14700 to Clju\_c14720.

**The Rnf complex and nitrogen fixation.** Reverse electron flux from NADH driven by the Rnf complex to reduce ferredoxin is thought to be responsible for providing electrons to the nitrogenase of *Rhodobacter capsulatus* (26, 27) and *Pseudomonas stutzeri* (28). The Rnf complex has also been found to participate in the transcriptional and/or posttranslational regulation and in the maturation process of the nitrogenase of *Azotobacter vinelandii* (29) and of *Azoarcus* sp. strain BH72 (30).

The genome of *C. ljungdahlii* contains 4 copies of *nifH*, a gene coding for the dinitrogenase reductase, and 2 copies of *nifDK*, two genes coding for the subunits of the dinitrogenase (10). As expected, *C. ljungdahlii* can grow in the absence of fixed nitrogen in a medium containing fructose as the electron donor (Fig. 8). After two transfers in the absence of fixed nitrogen, growth of the *rnfAB* mutant was completely inhibited (Fig. 8). This phenotype indicates that the Rnf complex of *C. ljungdahlii* has functions beyond energy conservation during autotrophic growth and suggests that proton translocation by the Rnf complex might be reversible, as previously proposed (1).

**Implications.** This study demonstrates *in vivo* that the *C. ljungdahlii* Rnf complex is the only proton pump responsible for generating the PMF necessary for ATP synthesis during au-

totrophic growth on  $\text{H}_2\text{-CO}_2$ . It also shows that the Rnf complex participates in energy conservation processes during heterotrophic growth on fructose by recycling reducing power to form a proton gradient coupled to the production of a portion of the intracellular ATP pool. This is consistent with previous predictions based on analysis of the *C. ljungdahlii* genome (10).

Thus, *C. ljungdahlii* has a completely novel way to conserve energy for an acetogen: the formation of a proton gradient by Rnf, an  $\text{H}^+$ -motive ferredoxin:NAD oxidoreductase. Other acetogens, like *A. woodii*, have an Rnf complex that functions as an  $\text{Na}^+$ -motive ferredoxin:NAD oxidoreductase capable of generating a sodium ion potential across the membrane (13, 15–18), whereas *M. thermoacetica* and several other acetogens contain cytochromes and quinones (14, 31, 32) thought to generate a proton gradient during acetogenesis (5, 33–35). Like *A. woodii* (13), the *C. ljungdahlii* genome (10) is devoid of genes coding for the biosynthesis of quinones or cytochromes.

It also appears that the Rnf complex of *C. ljungdahlii* has an additional function in nitrogen fixation and that the electron flux might be reversible. This is consistent with the apparent versatility of the Rnf complex in other organisms, which has been associated with other energy conservation processes and proton gradient-dependent sugar transport (1, 36) that require electron flux from reduced ferredoxin to  $\text{NAD}^+$ , as well as with phenomena such as nitrogen fixation (26, 27) and oxidative stress response (37) that require electron flux from NADH to oxidized ferredoxin.

With the development of a robust genetic system permitting gene deletion studies (19), it is now possible to study the role of the Rnf complex in energy conservation or other processes and to characterize the mechanism by which the Rnf complex translocates protons and transports electrons. For example, further studies are warranted to establish the specific function of each Rnf subunit and the role of the associated cofactors. Understanding the functioning of the *C. ljungdahlii* Rnf complex will also provide useful information in the effort to increase the production of valuable chemical commodities from syngas (9, 10) and by electrosynthesis (12).

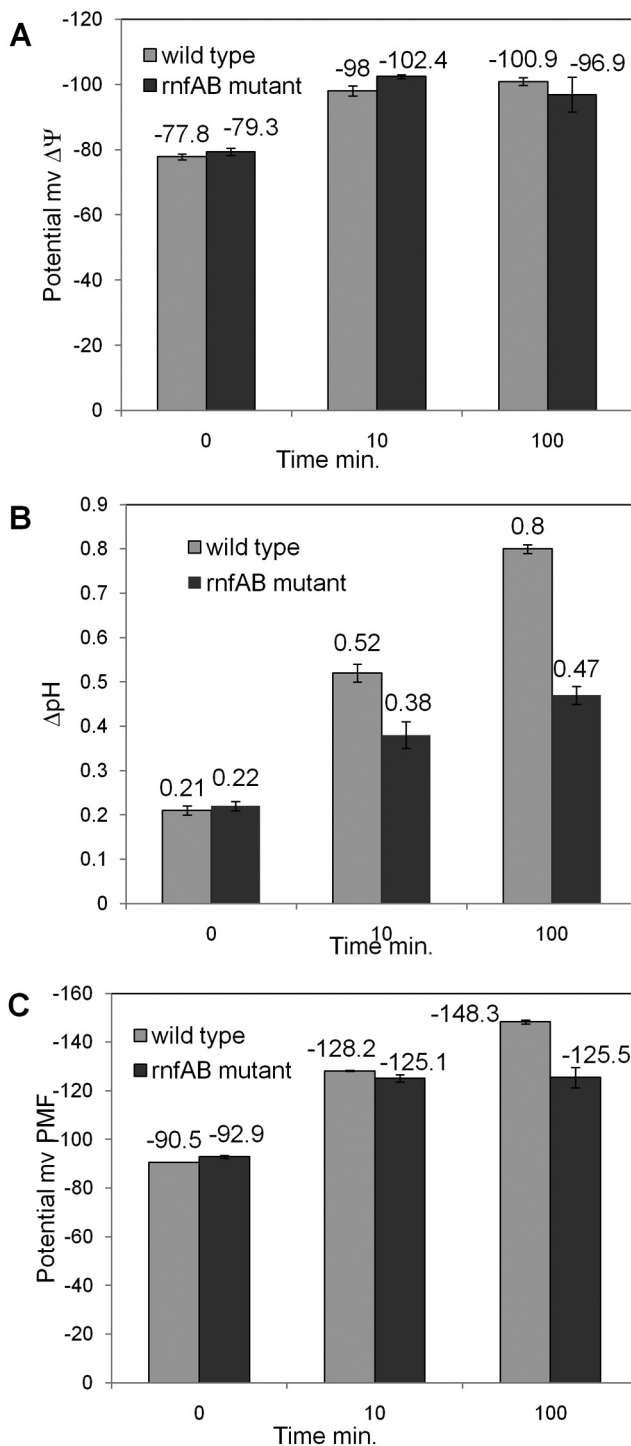


FIG 7  $\Delta\Psi$ ,  $\Delta$ pH, and PMF with fructose as the electron donor.  $\Delta\Psi$  (A),  $\Delta$ pH (B), and PMF (C) in *C. ljungdahlii* wild-type and *rnfAB* mutant cell suspensions. At time 0, 10 mM fructose was added. Each value is the mean from three replicates.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used are listed in Table S1 in the supplemental material. *C. ljungdahlii* strains were routinely cultured anaerobically ( $N_2$ - $CO_2$ , 80:20) at 37°C in DSMZ medium 879. Genetic manipulations were done with YTF (yeast

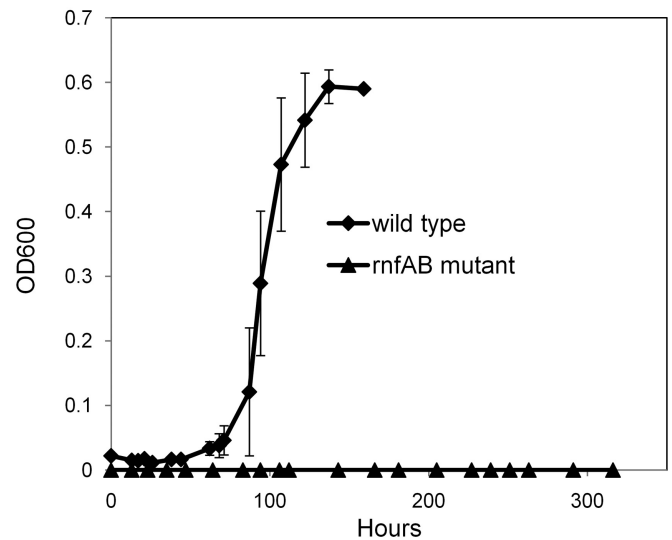


FIG 8 Nitrogen-fixing growth. Growth curves of *C. ljungdahlii* wild type and the *rnfAB* mutant in the absence of fixed nitrogen. Fructose is the electron donor, and the atmosphere contains 80%  $N_2$ -20%  $CO_2$ . Each value is the mean from three replicates.

extract, 1%; Bacto tryptone, 1.6%; sodium chloride, 0.4%; fructose, 0.5%; pH 6) liquid and solid media. For growth studies under heterotrophic conditions, 0.5% fructose was added to DSMZ medium 879. For growth studies under autotrophic conditions, *C. ljungdahlii* strains were cultivated under a pressurized atmosphere (ca. 1.4 atm) of  $H_2$ - $CO_2$  (80:20). To confirm the nature of the electrochemical gradient, 5  $\mu$ M TCS (protonophore) or 20  $\mu$ M ETH2120 (sodium-specific ionophore) was added to *C. ljungdahlii* cultures grown with  $H_2$ - $CO_2$ . To study nitrogen-fixing growth, sources of fixed nitrogen were omitted from the DSMZ medium 879 with 0.5% fructose. *Escherichia coli* was cultivated in Luria-Bertani medium (38). Appropriate antibiotics were added when necessary.

**Construction of the *rnfAB* mutant.** Sequences for all primers used for construction of the *rnfAB* mutant are listed in Table S2 in the supplemental material. The *rnfAB* mutant was constructed as described previously (19). The genome sequence of *C. ljungdahlii* DSM13528 (ATCC 55383) is accession number NC\_014328 in GenBank. Genomic DNA was extracted with the Epicenter MasterPure DNA purification kit (EPICENTRE Biotechnologies, Madison, WI). Plasmids were extracted using the QIAprep Spin miniprep kit (Qiagen, Valencia, CA). The high-fidelity Jump-Start AccuTaq LA DNA polymerase (Sigma-Aldrich, St-Louis, MO) was used in every PCR. Primer pair ClrnfDupBHI/ClrnfDupAvrII was used to amplify 1.03 kb upstream of the *rnfD* coding sequence, and primer pair ClrnfDdnAvrII/ClrnfDdnXhoI was used to amplify 1.02 kb downstream of *rnfD*, with *C. ljungdahlii* genomic DNA as the template. The PCR amplified 5' and 3' flanking regions were ethanol precipitated (38), digested with AvrII (NEB, Beverly, MA), ligated with the T4 DNA ligase (NEB), and cloned into pCR2.1-TOPO with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), resulting in pCR2.1*rnfD*dup+dn. The clarithromycin resistance cassette was amplified from pCL1 (19) using the primer pair Cl-claupXbaI/Cl-claXbaI. The clarithromycin resistance cassette was XbaI digested (NEB) and ligated into the AvrII site of pCR2.1*rnfD*dup+dn, resulting in pCR2.1*rnfD*::*Clar*. The sequence of the construction was verified by Sanger sequencing.

pCR2.1*rnfD*::*Clar* was transformed into *E. coli* strain NEB Express (NEB), and plasmid DNA was then extracted for electroporation into *C. ljungdahlii* as described previously (19). One colony grew after the plating of *C. ljungdahlii* electroporated with the suicide plasmid. PCR validation indicated that a single-crossover event occurred in which pCR2.1*rnfD*::*Clar* was integrated in the *rnf* operon (Fig. 3B), resulting in the loss of *rnfAB* expression, as verified by qRT-PCR (Table 1).

**qRT-PCR.** Sequences from all primers used for quantitative RT-PCR are listed in Table S2 in the supplemental material. Total RNA was extracted with the RNeasy minikit (Qiagen) from midlog cultures grown with  $\text{H}_2$ - $\text{CO}_2$  or fructose. cDNA was generated with the enhanced avian first-strand synthesis kit (Sigma-Aldrich) using random primers. The SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and the ABI 7500 real-time PCR system were used to amplify and to quantify PCR products from *rnfC*, *rnfD*, *rnfG*, *rnfE*, *rnfA*, *rnfB*, and *pta*. Transcript abundance of these genes was normalized with the housekeeping gene *rpoA* constitutively expressed under the tested conditions. Relative levels of transcript abundance of the studied genes were calculated with the  $2^{-\Delta\Delta\text{CT}}$  method (39). Quantitative RT-PCR was done in triplicate for each sample in every experiment. Statistical analysis was performed using Student's *t* test. A *P* value of  $<0.01$  was considered to be statistically significant.

**Cell suspensions.** *Clostridium ljungdahlii* cells were grown on fructose until mid- to late-log phase, harvested by centrifugation, and washed 2 times in 879 medium without an electron donor, yeast extract, resazurin, and vitamins (basal medium; pH 5.9). Washed cell pellets were resuspended in basal medium under an  $\text{N}_2$ - $\text{CO}_2$  (80:20) atmosphere to get a protein concentration of approximately 1 mg/ml as measured by the bicinchoninic acid assay (Sigma-Aldrich). At time 0, 10 mM of fructose was added to the cell suspension or 20 ml of  $\text{H}_2$  was injected into the headspace of a 27-ml pressure tube containing 10 ml of cell suspension.

**Measurement of ATP.** The level of intracellular ATP was measured with a colorimetric ATP assay kit according to the manufacturer's instructions (Abcam, San Francisco, CA). Cells were pelleted and lysed in the ATP assay buffer. After centrifugation to remove insoluble materials, the supernatant was added to a 96-well plate and combined with the reaction mix provided in the kit. Absorbance was read at 550 nm using an Eon microplate spectrophotometer (Bio-Tek, Winooski, VT).

**Measurement of  $\Delta\Psi$ ,  $\Delta\text{pH}$ , and PMF.** Cells were separated from the medium by the silicone oil centrifugation method, and the  $\Delta\Psi$ ,  $\Delta\text{pH}$ , and PMF were measured as described previously (40, 41). Intracellular water space (2.3  $\mu\text{l}/\text{mg}$  protein) was estimated by incubating cells with 4  $\mu\text{Ci}/\text{ml}$  of  $3\text{H}_2\text{O}$  (Sigma-Aldrich) and 1  $\mu\text{Ci}/\text{ml}$  of [ $^{14}\text{C}$ ]taurine (ARC, St-Louis, MO). Total pellet volume was measured by  $3\text{H}_2\text{O}$ , whereas [ $^{14}\text{C}$ ]taurine was used as an extracellular marker (22). To measure the  $\Delta\Psi$ , cells were incubated 5 min at 33°C with 0.07  $\mu\text{Ci}/\text{ml}$  of [ $^3\text{H}$ ]tetraphenylphosphonium ([ $^3\text{H}$ ]TPP $^+$ ) (ARC). [ $^3\text{H}$ ]TPP $^+$  uptake was quantified, and the  $\Delta\Psi$  was calculated with the Nernst equation. Nonspecific binding of [ $^3\text{H}$ ]TPP $^+$  was measured by incubating cell suspensions in the presence of nigericin (5  $\mu\text{M}$ ) and valinomycin (5  $\mu\text{M}$ ) to negate the  $\Delta\Psi$ . To measure the  $\Delta\text{pH}$ , cells were incubated 5 min at 33°C with 0.04  $\mu\text{Ci}/\text{ml}$  of [ $^{14}\text{C}$ ]benzoate (Moravick Biochemicals, Brea, CA). The distribution of [ $^{14}\text{C}$ ]benzoate across the cell membrane was measured, and the  $\Delta\text{pH}$  was calculated with the Henderson-Hasselbalch equation. Uptake of [ $^{14}\text{C}$ ]benzoate was corrected as described previously (22). The PMF was calculated by the following equation:  $\text{PMF} = \Delta\Psi - z\Delta\text{pH}$  (42).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00406-12/-/DCSupplemental>.

TABLE S1, DOCX file, 0.1 MB.

TABLE S2, DOCX file, 0.1 MB.

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