Membrane Localization, Topology, and Mutual Stabilization of the *rnfABC* Gene Products in *Rhodobacter capsulatus* and Implications for a New Family of Energy-Coupling NADH Oxidoreductases†

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ABSTRACT: The *rnf* genes in *Rhodobacter capsulatus* are unique nitrogen fixation genes that encode potential membrane proteins (RnfA, RnfD, and RnfE) and potential iron−sulfur proteins (RnfB and RnfC). In this study, we first analyzed the localization and topology of the RnfA, RnfB, and RnfC proteins. By activity and immunoblot analysis of expression of translational fusions to *Escherichia coli* alkaline phosphatase, RnfA protein was shown to span the chromatophore membrane with its odd-numbered hydrophilic regions exposed to periplasm. By alkaline treatment of membrane fractions and following immunoblot analysis using antibodies against recombinant proteins expressed in *E. coli*, both RnfB and RnfC proteins were revealed to situate at the periphery of the chromatophore membranes. Second, mutual interaction of the Rnf proteins was analyzed by immunochemical determinations of RnfB and RnfC proteins in *rnf* mutants and their complemented derivatives. The contents in cellular fractions indicated that RnfB and RnfC stabilize each other and that the presence of RnfA is necessary for stable existence of both proteins. These results support a hypothesis that the Rnf products are subunits of a membrane complex. Finally, we detected homologs of *rnf* genes in *Haemophilus influenzae* and *Vibrio alginolyticus* by data base searches and in *E. coli* by cloning of a fragment of an *rnfA* homolog followed by a data base search. Close comparisons revealed that RnfC has potential binding sites for NADH and FMN which are similar to those found in proton-translocating NADH:quinone oxidoreductases and that RnfA, RnfD, and RnfE show similarity to subunits of sodium-translocating NADH:quinone oxidoreductases. We predict that the putative Rnf complex represents a novel family of energy-coupling NADH oxidoreductases.

Biological reduction of molecular nitrogen, *i.e.,* nitrogen fixation, is catalyzed by the enzyme, nitrogenase, that consists of two components, dinitrogenase reductase and dinitrogenase (Dean & Bolin, 1993; Dean & Jacobson, 1992). The former component transfers electrons to the latter with hydrolysis of two ATPs per electron during the catalytic process. In the conventional molybdenum nitrogenase, the dinitrogenase is called MoFe-protein which is an $\alpha_2\beta_2$ tetramer containing the two iron−molybdenum cofactors (FeMo-cos)[1] and two P-clusters, whereas the dinitrogenase reductase is called Fe-protein which is a γ_2 homodimer containing a [4Fe-4S] cluster.

Genetic and biochemical studies to date have revealed that physiological nitrogen fixation requires not only the primary products of the structural genes encoding the nitrogenase components but also a number of other gene products (Arnold *et al.,* 1988; Bishop & Premakumar, 1992; Dean & Jacobson, 1992). Such proteins are required for maturation of the nitrogenase components, for regulation of the enzyme system, and for the electron transport to dinitrogenase reductase. The proteins needed for maturation which includes biosynthesis and insertion of metalloclusters to the apo components are conserved among bacterial species, probably reflecting the conserved structures of the nitrogenase enzyme. The regulatory proteins are also conserved to some extent. In contrast, the proteins that participate in the electron transport pathway(s) to nitrogenase seem to be less conserved and have been less worked out. Both flavodoxins and ferredoxins are believed to be the direct electron donors to dinitrogenase reductases. However, little is known about their reduction *in vivo*, possibly because the electron carriers are also utilized by other metabolic pathways. *Klebsiella pneumoniae* is the only organism in which the pathway has been conclusively determined (Hill & Kavanagh, 1980; Shah *et al.,* 1983). In this organism, a pyruvate:flavodoxin oxidoreductase encoded by *nifJ* donates an electron to a flavodoxin encoded by *nifF*. These two proteins constitute a nonbranched electron pathway specific for nitrogen fixation.

Since Kamen and Gest discovered that *Rhodospirillum rubrum* fixes nitrogen under the phototrophic condition

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[1] Abbreviations: FeMo-co, iron−molybdenum cofactor; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; X-phosphate, 5-bromo-4-chloro-3-indolyl phosphate disodium salt; ORF, open reading frame; Na^+-NDH, Na^+-translocating NADH:quinone oxidoreductase; H^+-NDH, proton-translocating NADH:quinone oxidoreductase; Complex I, mitochondrial proton-translocating NADH:ubiquinone oxidoreductase.

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(Kamen & Gest, 1949), most species of purple non-sulfur photosynthetic bacteria have been demonstrated to be diazotrophic (Madigan *et al.*, 1984; Madigan, 1995). These bacteria are intriguing because they can utilize light to support the energy-requiring nitrogenase reaction. In the photosynthetic reaction center of Rhodospirillaceae species, however, the redox potential of the primary quinone acceptor (Q_A) molecule is too high to achieve direct reduction of ferredoxin or flavodoxin (Knaff, 1978; Prince & Dutton, 1978). A pyruvate:ferredoxin oxidoreductase has been purified from *R. rubrum* and demonstrated to support pyruvate-dependent nitrogenase activity *in vitro* in the presence of ferredoxin (Brostedt & Nordlund, 1991). The amino acid sequence of the enzyme shows homology to that of NifJ in *K. pneumoniae*; however, the disruption of its gene scarcely affected the diazotrophic growth of *R. rubrum* (Lindblad *et al.*, 1996).

Among the members of the family Rhodospirillaceae, *Rhodobacter capsulatus* shows the highest nitrogenase activity (Madigan *et al.*, 1984) and its genes related to nitrogen fixation have been most extensively studied; more than 50 genes are known to be involved in nitrogen fixation by Mo-nitrogenase or/and alternative Fe-only nitrogenase (Kranz & Cullen, 1995; Ludden & Roberts, 1995; Masepohl & Klipp, 1996). It is now believed that the ferredoxin I encoded by *fdxN* is the primary physiological electron donor to Mo-nitrogenase (Jouanneau *et al.*, 1995; Saeki *et al.*, 1991; Schatt *et al.*, 1989; Schmehl *et al.*, 1993) and probably also to the Fe-only nitrogenase (Schüddekopf *et al.*, 1993). By nucleotide sequence, interposon-mutagenesis, and complementation analysis of the genomic region upstream of *fdxN*, we have demonstrated that at least two genes, which are situated in a direction opposite of *fdxN*, are required for nitrogen fixation by Mo-nitrogenase (Saeki *et al.*, 1993). Independently, Schmehl *et al.* have performed nucleotide sequence and interposon-mutagenesis analysis of a broader genomic region and identified six genes required for nitrogen fixation (Schmehl *et al.*, 1993). They designated them *rnfABCDE* and *rnfF* for *Rhodobacter n*itrogen *f*ixation; the *rnfA* and *rnfB* genes correspond to the two genes we identified. Predicted products of *rnf* genes do not show significant homology to known proteins except that products of *rnfB* and *rnfC* each possess a 2[4Fe-4S]-type ferredoxin-like domain. Interestingly, the products of *rnfA*, *rnfD*, and *rnfE* are predicted to be transmembrane proteins, the feature which is unique among proteins related to nitrogen fixation except the oxygen sensor protein FixL in *Rhizobium meliloti* (Lois *et al.*, 1993). The Rnf proteins are expected to form a new electron transfer system that links the known electron transport system in the membrane to the nitrogenase system in cytoplasm (Masepohl & Klipp, 1996). They can be subunits of an energy-consuming ferredoxin reductase.

As a step to gain more insight into the biochemical significance of the *rnf* gene products, we have analyzed the membrane localization and topology of RnfA protein by translational fusion to *Escherichia coli* alkaline phosphatase (PhoA) and determined the localization of the two potential iron−sulfur (Fe-S) proteins, RnfB and RnfC, by immunochemical detection using antibodies against recombinant proteins expressed in *E. coli*. Mutual interactions among these proteins were then studied by their contents in specific mutants and in the complemented derivatives. We have also performed close comparison of *rnf* genes and their homologs in *Haemophilus influenzae*, *Vibrio alginolyticus*, and *E. coli*. The results implied that the Rnf proteins constitute a member of a novel family of energy-coupling NADH oxidoreductases.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The bacterial strains used were listed in Table 1. The *E. coli* strains used were DH5α (Gibco-BRL) for plasmid construction, BL21(DE3) (Studier *et al.*, 1990) and TG1 (Sambrook *et al.*, 1989) for preparation of recombinant proteins, and C600(pDPT51) (Taylor *et al.*, 1983) and S17-1 (Simon *et al.*, 1983) both for conjugative transfer to *R. capsulatus*. They were grown at 37 °C on LB medium (Sambrook *et al.*, 1989) unless otherwise stated. *R. capsulatus* strains were wild-type SB1003 (Yen & Marrs, 1976) and its derivatives. They were cultivated at 30 °C under the dark aerobic conditions on the complex MPYE medium which is the PYS medium (Weaver *et al.*, 1975) supplemented with 30 mM sodium DL-malate or under the illuminated anaerobic conditions on either the $(NH_4)_2SO_4$-enriched medium RCV-A (Saeki *et al.*, 1993), glutamate medium RCV-E (Saeki *et al.*, 1991), or ammonium free medium RCV-NF (Saeki *et al.*, 1991). The capacity to fix nitrogen gas was tested as reported elsewhere (Saeki *et al.*, 1996). To obtain diazotrophically grown cells, the strains were inoculated with 200 mL of RCV-NF medium in a 500 mL flask and placed in an illuminated GasPak anaerobic jar (BBL Microbiology System, Cockeysville, MD) for 16 h. Cells derepressed for *nif* and *rnf* genes in the presence of fixed nitrogen were obtained by growing the strains similarly with RCV-E medium for 16 h. Tetracyclin was used at 10 and 1 μg/mL for *E. coli* and *R. capsulatus*, respectively, and the concentrations of other antibiotics were described previously (Saeki *et al.*, 1991, 1993).

Molecular Genetic Techniques

Recombinant DNA techniques were as reported earlier (Saeki *et al.*, 1991, 1993) or according to Sambrook *et al.* (1989). Nucleotide sequences were determined with an Applied Biosystem DNA sequencer after the thermal cycling reaction with Taq DNA polymerase. Mobilization of plasmids to *R. capsulatus* was accomplished as reported previously (Saeki *et al.*, 1991) using the *E. coli* donor strains C600(pDPT51) and S17-1, respectively, for transferring derivatives of pBR322 and pRK415 (Keen *et al.*, 1988). Interposon mutagenesis to disrupt the chromosomal copy of *rnfB* or *rnfC* in *R. capsulatus* was performed by insertion of a kanamycin resistant gene fragment using gene transfer agents prepared from *R. capsulatus* CB1127 (Young *et al.*, 1989). Correct insertion of interposon was confirmed by Southern hybridization analysis.

Plasmid Construction

The plasmids used and constructed are listed in Table 1. In constructions involving polymerase chain reaction (PCR), the products were subcloned and verified for correct amplification by sequence determination.

Plasmids with rnfA−phoA Fusions (pTFD341−pTFD346; see Figure 1). As a preparative step for making fusion genes, we made a plasmid pKSD300 that had a 3.8 kbp *Hin*dIII-*Apa*LI fragment of the *R. capsulatus rnfA* gene region from

Table 1: Bacterial Strains and Plasmids Used

strains and plasmids	relevant markers and properties	reference
E. coli		
BL21(DE3)	F$^-$ *ompT[lon] hsdS$_B$* (r_B^- m_k^+; and *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene	Studier *et al.*, 1990
C600(pDPT51)	conjugation helper strain for a pBR322 derivative	Taylor *et al.*, 1983
DH5α	*supE*44 $\Delta\lambda$acU169 (ϕ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Gibco-BRL
S17-1	Pro$^-$ Res$^-$ Mod$^+$ *recA*, RP4-TC::Mu::Km::Tn7	Simon *et al.*, 1983
TG1	*supE hsd*Δ5 thiΔ(*lac-proAB*) F′(*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	Sambrook *et al.*, 1989
R. capsulatus		
CB1127	*rif*-10 *crtG*121; GTA overproducer	Young *et al.*, 1989
HK71	*rif*-10; *rnfC*::*neo*	this study
HK72	*rif*-10; *rnfC*::*neo*	this study
KF11	*rif*-10; *rnfB*::*neo*	this study
KF12	*rif*-10; *rnfB*::*neo*	this study
KN227	*rif*-10; *rnfA*::*neo*	Saeki *et al.*, 1993
SB1003	*rif*-10; wild-type	Yen & Marrs, 1976
plasmids		
A75	Km^r; cosmid containing *R. capsulatus* DNA	Saeki *et al.*, 1991
pASE01	Amp^r; fragment of *E. coli rnfA* homolog	this study
pASE02	Amp^r; fragment of *E. coli rnfA* homolog	this study
pFNX1	Amp^r; *rnfAB* and *fdxCN*	Saeki *et al.*, 1993
pFNX2	Amp^r; *rnfAB* and *fdxCN*	Saeki *et al.*, 1993
pET3a	Amp^r; T7 expression vector	Studier *et al.*, 1990
pHKS50	Amp^r; first 70 bp of *rnfC*	this study
pHKS51	Amp^r; *rnfABC*	this study
pHKS52	Amp^r; *rnfC* without ribosome binding site	this study
pHKS53	Amp^r; P_{lac}-*(His)$_6$-rnfC*	this study
pHKS70	Tet^r; *rnfABC*	this study
pHKS71	Tet^r, Km^r; *rnfAB, rnfC::neo*	this study
pHKS72	Tet^r, Km^r; *rnfAB, rnfC::neo*	this study
pKSD300	Amp^r; *rnfA* and *fdxCN*	this study
pKSN220	Amp^r; *rnfAB* and *fdxCN*	Saeki *et al.*, 1993
pQE30	Amp^r; for N-terminal (His)$_6$ tag	QIAGEN Inc.
pQE32	Amp^r; for N-terminal (His)$_6$ tag	QIAGEN Inc.
pQE60	Amp^r; for C-terminal (His)$_6$ tag	QIAGEN Inc.
pTFD341	Tet^r; *rnfA*′192::*phoA*′	this study
pTFD342	Tet^r; *rnfA*′160::*phoA*′	this study
pTFD343	Tet^r; *rnfA*′128::*phoA*′	this study
pTFD344	Tet^r; *rnfA*′103::*phoA*′	this study
pTFD345	Tet^r; *rnfA*′73::*phoA*′	this study
pTFD346	Tet^r; *rnfA*′35::*phoA*′	this study
pTFK11	Amp^r, Km^r; *rnfB::neo*	this study
pTFK12	Amp^r, Km^r; *rnfB::neo*	this study
pTFQ08	Amp^r; *rnfB* coding region	this study
pTFQ09	Amp^r; P_{lac}-*(His)$_6$-rnfB*	this study
pTFQ10	Amp^r; P_{lac}-*rnfB*	this study
pTFQ11	Amp^r; P_{lac}-*rnfB-(His)$_6$*	this study
pTFQ12	Spc^r; P_{puf}-*rnfB-(His)$_6$*	this study
pTFQ13	Spc^r; P_{puf}-*rnfB*	this study
pTFQ14	Amp^r; P_{T7}-*rnfB-(His)$_6$*	this study
pTSV1	Spc^r; *R. capsulatus* expression vector	Saeki *et al.*, 1996
pTSV2	Spc^r; *R. capsulatus* expression vector	this study
pUC118	Amp^r	Sambrook *et al.*, 1989
pUC119	Amp^r	Sambrook *et al.*, 1989
pRK415	Tet^r; broad-host range vector	Keen *et al.*, 1988
pUCKM1	Amp^r, Km^r; nonpolar *neo* cartridge from *Tn*5	Saeki *et al.*, 1991
pUI310	Amp^r; *phoA*′ for translational fusion	Varga & Kaplan, 1989

plasmid pFNX2 (Saeki *et al.*, 1993). In pKSD300, the *Apa*LI site that is situated at the 3′-terminal vicinity of *rnfA* had been blunted before subcloning into the *Hin*dIII-*Hin*cII site of pUC118, and the *Mlu*I site that is situated between the NifA-binding site and the promoter for *rnfABCDE* operon had been blunted and modified to a *Xho*I site by inserting a linker, pCCCCTCGAGAGG. The 1.3 kbp *Eco*RI-*Bam*HI fragment containing *rnfA* was excised from pKSD300 and fused to an *E. coli phoA* gene fragment that had been from pUI310 (Varga & Kaplan, 1989) and modified to have a *Hin*dIII site at its downstream end. The fusion gene fragment was subcloned in the *Eco*RI-*Hin*dIII site of the pRK415 vector to make pTFD341. For construction of other fusions, we synthesized five downstream primers: TFP2, 5′-AAG GAT CCT GCG CCA GCC GTT CGC GCA T-3′; TFP3, 5′-AAG GAT CCA GAT GGC CCT GAA TGT ACA TC-3′; TFP4, 5′-AAG GAT CCA GCG CCT TGT GCA GGT CG-3′; TFP5, 5′-AAG GAT CCC GCA GGA ATT TCA GGT CGA G-3′; and TFP6, 5′-AAG GAT CCG TCT TGC GCG AGA CGC CCA TGA-3′. These primers and a common upstream primer (TM13R, 5′-CAC AGG AAA CAG CTA TGA CC-3′) were used to amplify truncated *rnfA* gene fragments by PCR with pTFD341 as a template. Each fragment was first digested with *Eco*RI and *Bam*HI and then cloned in the *Eco*RI-*Bam*HI site of pUC118 for verification. The truncated *rnfA* fragments were excised by digestion with *Xho*I and *Bam*HI and subcloned in pTFD341 to substitute

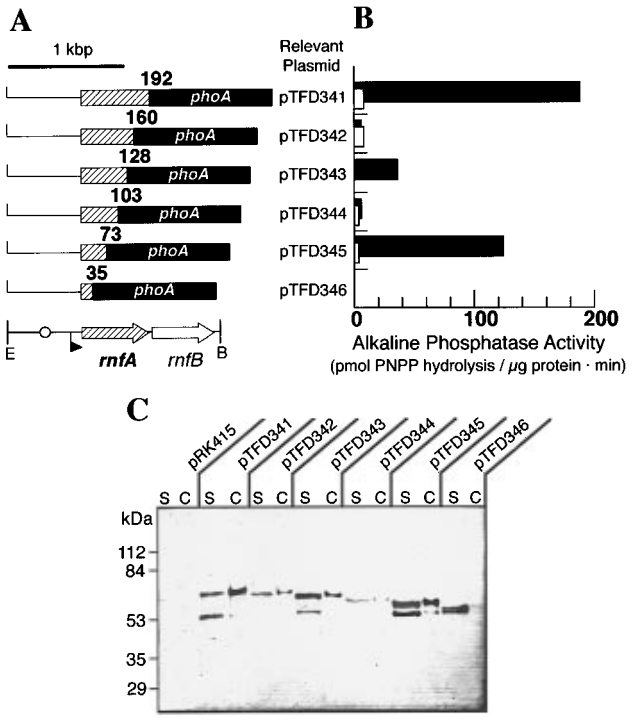

FIGURE 1: Activity and immunoblot analysis of alkaline phosphatase of chromatophore and soluble fractions from diazotrophically grown *R. capsulatus* cells containing the *rnfA*−*phoA* fusion gene. (A) The construction of fusion genes is illustrated together with the physical map of the *rnfA* locus in *R. capsulatus*. Hatched and filled bars indicate *R. capsulatus rnfA* and *E. coli phoA*, respectively. The number at the junction indicates the position of the last residue of the RnfA moiety. The open circle and the arrowhead symbolize the probable NifA-binding site and the potential −24/−12 promoter, respectively. The fragments were subcloned into pRK415 to yield the plasmid used. Abbreviations for the restriction enzymes are E for *Eco*RI and B for *Bam*HI. (B) Filled bars show alkaline phosphatase activity of the chromatophore fraction from *R. capsulatus* cells harboring the indicated plasmid, while open bars show that of the soluble fraction. Values are averages of at least two independent experiments. (C) Soluble (S) and chromatophore (C) fractions from diazotrophically grown cells harboring the indicated plasmid were electrophoresed by the method of Laemmli (1970) with a 10% acrylamide gel. The proteins blotted onto Immobilon-P membrane were reacted with monoclonal antibodies against *E. coli* PhoA protein. A *R. capsulatus* SB1003 derivative that harbored the vector pRK415 was used as a control. Each lane contained 1 μg of proteins.

the corresponding longer fragment to make pTFD342−pTFD346.

Plasmids for Interposon Mutagenesis of rnfB and rnfC (pTFK11, pTFK12, pHKS71, and pHKS72). The plasmid pKSN220 (Saeki *et al.*, 1993) was digested with *Sma*I and ligated with a *Sma*I-digested kanamycin resistant gene (*neo*) cartridge from pUCKM1 (Saeki *et al.*, 1991) in the two possible orientations to generate plasmids pTFK11 and pTFK12 for disruption of *rnfB*.

As preparative steps for disrupting *rnfC*, we first constructed pHKS51 that contained the 2.5 kbp *Eco*RI fragment of the *R. capsulatus rnfABC* gene region from cosmid A75 (Saeki *et al.*, 1991). The *Eco*RI site at a downstream vicinity of *rnfC* was blunted before subcloning into the *Eco*RI-*Hin*cII site of pUC119. The *Eco*RI-*Hin*dIII fragment with *rnfABC* was then excised and ligated with pRK415. The resultant plasmid pHKS70 was digested with *Bam*HI and ligated with a *Bam*HI-digested *neo* cartridge from pUCKM1 to generate pHKS71 and pHKS72 for disruption of *rnfC*.

Plasmids for Expressing RnfB and RnfC Proteins with a Hexahistidine Tag in E. coli (pTFQ11, pTFQ14, pTFQ09, and pHKS53). To obtain the coding region of *rnfB*, we synthesized two primers: NETB1, 5′-TTT TCT AGA TAT CGC AGC CGC CGC TTC CAT-3′; and NETB2, 5′-TTT TCT AGA TCT GGC TGC GGT CTC CGC GCT TG-3′. Using plasmid pFNX2 as a template, the region was amplified and then digested with *Xba*I, blunted, and finally subcloned in the *Sma*I site of pUC18. From a certified clone, the *rnfB* fragment was excised by digestion with *Eco*RV and *Bgl*II. It was subcloned into the pQE60 vector (Qiagen Inc., Chatsworth, CA) that had been previously digested with *Nco*I, blunted, and then digested with *Bgl*II. The resultant plasmid pTFQ11 contained an engineered *rnfB* gene to encode a product with a $(His)_6$ tag at its C terminus [RnfB-$(His)_6$ protein]. We further made a plasmid pTFQ14 to achieve stronger transcription under control of the T7 promoter. The engineered *rnfB* fragment was excised from pTFQ11 by digestion with *Eco*RI and *Hin*dIII, modified appropriately, and subcloned into the pET3a vector (Sambrook *et al.*, 1989) that had been digested with *Xba*I and *Bam*HI, yielding pTFQ14.

Another *rnfB* fragment retaining its own termination codon was amplified by PCR with the primers NETB1 and TM13R using the same template as above. The fragment was digested with *Xba*I and subcloned in the *Xba*I site of pUC118 to make plasmid pTFQ08. From pTFQ08, the *Eco*RV-*Hin*dIII fragment containing *rnfB* was excised and subcloned into the *Sma*I-*Hin*dIII site of the pQE32 vector (Qiagen Inc.). The resultant plasmid pTFQ09 contained an engineered gene to encode the $(His)_6$-RnfB protein.

The first 70 bp fragment of the *rnfC* gene was amplified by PCR with the TM13R primer and another primer NETC1, 5′-TTT GGT ACC ATG AGA CTT CCC TCG AT-3′, using pFNX1 (Saeki *et al.*, 1993) as a template. The obtained fragment was digested with *Kpn*I and *Bam*HI and subcloned in pUC118 to make pHKS50. The remaining portion of the *rnfC* gene was ligated into pHKS50 as a 1.7 kbp *Bam*HI-*Hin*dIII fragment from pHKS51. The resultant plasmid was named pHKS52. A 1.8 kbp *Kpn*I-*Hin*dIII *rnfC* fragment was excised from pHKS52 and ligated with the pQE30 vector (Qiagen Inc.) to generate plasmid pHKS53 that contained an engineered gene to encode the $(His)_6$-RnfC protein.

Plasmids for Expressing rnfB and Its Derivative by the puf Promoter (pTFQ13 and pTFQ12). The *Eco*RV-*Hin*dIII fragment containing *rnfB* was excised from pTFQ08 and subcloned into the pQE60 vector (Qiagen Inc.) that had been previously digested with *Nco*I, blunted, and then digested with *Hin*dIII to make plasmid pTFQ10. The *Eco*RI-*Hin*dIII fragment that contained the engineered *rnfB* gene with the initiation codon and the ribosome binding site from pQE60 was excised from pTFQ10 and subcloned into the *Eco*RI-*Hin*dIII site of the pTSV2 vector that was a derivative of pTSV1 (Saeki *et al.*, 1996) with the original *Bam*HI site modified to *Hin*dIII. The resultant plasmid pTFQ13 contained the *rnfB* gene under control of the *R. capsulatus puf* promoter (Young *et al.*, 1989). Similarly, another *Eco*RI-*Hin*dIII fragment that contained the engineered gene for encoding the $(His)_6$-RnfB protein was prepared from pTFQ11 and subcloned in pTSV2 to generate plasmid pTFQ12.

Plasmids Containing a Fragment of the E. coli rnfA Homolog (pASE01 and pASE02). To obtain a fragment of *rnfA* homolog, we synthesized three degenerate primers

(mixed bases are shown in parentheses or by N which represents all the four bases): NETA1, 5′-GGN TT(AG) TG-(TC) CCN TT(TC) ATG-3′; NETA2, 5′-GGN CTN TG(TC) CCN TT(TC) ATG-3′; and NETA4, 5′-GC(AG) CA(AG) TTN GTN GT(ATG) AT-3′. Using genomic DNA from *E. coli* DH5α as a template, a nucleotide fragment of an expected size was amplified by PCR with the primers NETA2 and NETA4. The fragment was end-blunted and cloned in the *Sma*I site of pUC18 to yield pASE01 and pASE02.

Fractionation of R. capsulatus Cells

R. capsulatus cells were fractionated essentially as described previously (Frenkel & Nelson, 1971) with modifications described below. Manipulations were performed under anaerobic conditions using an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI) or under a flow of pure nitrogen gas, except those for RnfA−PhoA fusion experiments that were performed under aerobic conditions. All buffers used were prepared by repeated degassing and flushing with nitrogen gas. *R. capsulatus* cells were harvested at late log phase from 200 mL of culture and suspended in a 1:10 ratio in 50 mM *N*-morpholinopropanesulfonate-KOH, 100 mM KCl, and 5 mM 2-mercaptoethanol buffer (pH 7.6) (MKM buffer). The suspension was supplemented with phenylmethanesulfonyl fluoride (PMSF) to 1 mM. Then, the cells were usually broken at 5 °C by passing through an Aminco French pressure cell at 18 000 psi. In the RnfA−PhoA fusion experiments, the cells were broken by sonication at 15 kHz. Cell debris was removed by centrifugation at 25000*g* for 30 min. Cell free extracts were further centrifuged at 125000*g* for 1 h. The supernatant was centrifuged again to obtain the "soluble fraction" that contained cytoplasmic and periplasmic fractions. The precipitate was resuspended in MKM buffer containing 10% (w/v) sucrose, placed on a stepwise layer of 25, 35, and 45% (w/v) sucrose in the same buffer, and centrifuged at 4 °C at 80000*g* for 10 h using a Hitachi SRP28SA1 rotor. The upper dark red band at the interface of 25 and 35% (w/v) sucrose density was taken, diluted with MKM buffer containing 10% (w/v) sucrose, and centrifuged again at 125000*g* for 1 h. The resultant precipitate was resuspended with the same buffer and used as the "chromatophore fraction".

Purification of Denatured RnfB and RnfC Proteins with the (His)$_6$ Tag from E. coli

The RnfB-(His)$_6$ protein was expressed in *E. coli* BL21-(DE3) (Studier *et al.*, 1990) harboring the plasmids pTFQ14 and pKY206 (Mizobata *et al.*, 1992); the latter plasmid was for coexpression of GroEL-ES proteins. An overnight preculture was transferred in a 1:100 ratio to NZCYM medium (Sambrook *et al.*, 1989). When they were grown to an OD_{660} of 0.7−0.8, isopropyl D-thiogalactopyranoside (IPTG) was added to 1 mM. After further incubation for 4 h, the cells were harvested and frozen in liquid nitrogen. They were thawed, suspended in a 1:5 ratio of 0.1 M sodium phosphate and 10 mM Tris-HCl buffer (pH 8.0) containing 8 M urea, and incubated for 1 h at room temperature. The suspension was centrifuged to remove cell debris and applied on a Ni−NTA−agarose (Qiagen Inc.) column. The column was developed essentially according to the protocol supplied by the manufacturer. Two peptides with apparent sizes of 26 and 30 kDa were obtained by elution with 0.1 M sodium acetate buffer (pH 5.1) containing 8 M urea. The two were separated by ion-exchange chromatography on a DEAE-Sepharose column using a gradient of NaCl from 0 to 0.4 M in 5 mM sodium phosphate buffer (pH 7.0) containing 4 M urea. The smaller 26 kDa peptide was confirmed to be the RnfB derivative by the determination of its N-terminal 17-amino acid sequence. A typical yield of RnfB-(His)$_6$ peptide was 250 μg from 18 g (wet weight) of *E. coli* cells.

The (His)$_6$-RnfB protein was expressed in *E. coli* TG1 harboring the plasmid pTFQ09. An overnight preculture was transferred in a 1:100 ratio to LB medium supplemented with ferric citrate to 45 μM. When the OD_{660} reached 0.7−0.8, the culture was supplemented with IPTG to 1 mM and further incubated for 2.5 h. At that time, the recombinant product became the most major peptide in the cells. Proteins were extracted from the harvested cells and subjected to chromatography on a Ni−NTA−agarose column by the same manner as described above. The sample eluted with 0.1 M sodium acetate and 8 M urea buffer (pH 5.0) was at least 95% pure. The remaining impurity was removed either by preparative SDS−polyacrylamide gel electrophoresis (SDS−PAGE) or by anion-exchange chromatography on a DEAE-Sepharose column. A typical yield of (His)$_6$-RnfB peptide was 15 mg from 5 g (wet weight) of *E. coli* cells.

The (His)$_6$-RnfC protein was expressed in *E. coli* TG1 harboring pHKS53 and purified by a protocol essentially similar to those for the (His)$_6$-RnfB protein except that cation exchange was performed in the last step. The chromatography was carried out on a Shodex IEC SP-825 column using a gradient of Na_2SO_4 from 0 to 0.3 M in 25 mM sodium acetate buffer (pH 5.0) containing 6 M urea. A typical yield of (His)$_6$-RnfC peptide was 20 mg from 5 g (wet weight) of *E. coli* cells.

Preparation and Purification of Antibodies against RnfB and RnfC Proteins

Antisera against RnfB-(His)$_6$ and (His)$_6$-RnfC proteins were raised in female New Zealand white rabbits. Approximately 500 μg of each antigen was injected in the skin of the back with Freund's complete adjuvant for the initial immunization. After 4 and 6 weeks, booster injections were performed with 200 μg of each protein with Freund's incomplete adjuvant. Serum was obtained after 10 days from the last injection. For affinity purification of antibodies, the (His)$_6$-RnfB and (His)$_6$-RnfC proteins were, respectively, coupled to NHS-activated HiTrap columns (Pharmacia Biotech) according to the manual from the manufacturer. The serum was supplemented with Tris-HCl (pH 8.0) and Tween 20 to 50 mM and 0.1%, respectively, and passed through the corresponding antigen column. The column was first washed with 50 mM Tris-HCl (pH 8.0) containing 0.1% (w/v) Tween 20. The bound antibodies were eluted by 0.1 M sodium citrate buffer containing 0.1% (w/v) Tween 20 (pH 3.5) following immediate neutralization with 1 M Tris-HCl buffer (pH 9.5).

Alkaline Treatment

Alkaline treatment to release peripheral proteins from membrane fractions was performed anaerobically as described above for cellular fractionation. The chromatophore membranes were first suspended in a 1:80 ratio in 0.1 M

disodium carbonate buffer (pH 11.3) containing 5 mM 2-mercaptoethanol. A control experiment was performed simultaneously with MKM buffer. The suspension was incubated at 4 °C for 1 h and then centrifuged at 125000*g* for 1 h. To recover the released protein, the supernatant was supplemented with trichloroacetic acid to a final concentration of 15% (w/v), incubated at 20 °C for 1 h, and centrifuged at 12000*g* for 20 min. The resultant precipitate was washed twice with acetone and dissolved in the appropriate volume of SDS−PAGE sample buffer described below.

Determination of the Alkaline Phosphatase Activity, Protein Amount, and Amino Acid Sequence

PhoA activity was assayed at 28 °C by hydrolysis of *p*-nitrophenyl phosphate in 0.5 M Tris-HCl buffer (pH 8.0) with slight amounts of SDS and chloroform as described previously (Brickman & Beckwith, 1975). The molecular extinction value of *p*-nitrophenol at 420 nm was assumed to be 14 200.

The protein amount was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard. The amino-terminal sequence of a recombinant protein was determined with an Applied Biosystems model 470A peptide sequencer.

SDS−PAGE and Western Blot Analysis

SDS−PAGE was carried out according to Laemmli (Laemmli, 1970) or Kadenbach *et al*. (1983). Samples were mixed with an equal volume of 2× SDS−PAGE sample buffer, which consisted of 125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, and 0.01% (w/v) bromophenol blue, and boiled for 5 min. Following the separation by SDS−PAGE, the proteins were transferred to a polyvinylidene difluoride filter (Immobilon-P, Millipore) and reacted with the purified antibodies. The bound antibodies were detected using PhoA-conjugated goat anti-mouse immunoglobulin antibody or PhoA-conjugated goat anti-rabbit immunoglobulin antibody. Molecular markers were purchased from Bio-Rad.

Sequence Analysis

A homology search was carried out by the BLAST program (Altschul *et al.*, 1990) running at the National Center for Biotechnology Information or at the *E. coli* Databank[2] at the Nara Institute of Technology (Nara, Japan). Homology analysis was also performed with the Gennetyx-Mac software package (SDC Software Co., Tokyo, Japan). Secondary structure prediction was performed by PredictProtein[3] (Rost, 1996) and NNpredict[4] (Kneller *et al.*, 1990). Phylogenetic analysis was performed by the ODEN program package written by Y. Ina at the National Institute of Genetics (Mishima, Japan).

RESULTS

Localization and Topological Analysis of the RnfA Protein. The hydropathy analysis of the predicted amino acid sequence of RnfA protein suggests that the protein may have six membrane-spanning segments (Saeki *et al.*, 1993; Schmehl *et al.*, 1993). To confirm the membrane localization and to analyze the topology of RnfA protein, we have constructed six translational fusions of the *rnfA* gene to the *E. coli phoA* gene (Figure 1A) by site-specific mutagenesis utilizing PCR. The fusions were made at the carboxyl end of each hydrophobic region except the N-terminal region, to minimize the number of fusions according to Boyd *et al.* (1993). The fusion genes were subcloned, together with the upstream DNA region containing the promoter and NifA-binding site for the *rnfABCDE* operon, into a broad host range vector pRK415. The resultant six plasmids, pTFD341−pTFD346, were mated to *R. capsulatus* wild-type strain SB1003. High PhoA activity is expected if the fusion junction is in or near the periplasmic vicinity of RnfA, since the RnfA moiety will act as a signal sequence to direct the PhoA moiety to the periplasm where PhoA is active. In contrast, no or very low activity is expected if the junction is in or near the cytoplasmic vicinity of RnfA, since the PhoA moiety will be retained in cytoplasm where PhoA is inactive.

First, the six *R. capsulatus* strains were examined for expression of PhoA activity using agar plates containing 40 μg/mL 5-bromo-4-chloro-3-indolyl phosphate disodium salt (X-phosphate). On nitrogen free agar plates, the *R. capsulatus* cells harboring either pTFD341, -343, or -345 formed blue colonies under illuminated anaerobic conditions, while those harboring any of the other three plasmids remained brown (data not shown). None of the six strains showed blue color on agar plates containing NH_4^+ which represses expression of *nif* genes.

Second, diazotrophically grown cells of the six strains were fractionated, to chromatophore and soluble fractions. The fractions were examined for PhoA activity (Figure 1B) and for the contents of fusion polypeptides (Figure 1C). Marked PhoA activities were only observed with the chromatophore fractions from the three strains harboring pTFD341, -343, and -345. In immunoblot analysis using the antibodies against *E. coli* PhoA, fusion peptides of expected sizes were detected in all the fractions except the chromatophore fraction from the cells of pTFD346/SB1003. In addition to them, smaller polypeptides were also detected in soluble fractions. They are presumably proteolytic products cleaved in the PhoA portion of the fusion products, since no similar peptides were detected in the control sample from pRK415/SB1003.

These results confirm that RnfA is a polytopic transmembrane protein located in the chromatophore and that its hydrophobic segments alternately span the chromatophore membrane with the odd-numbered hydrophilic regions exposed to periplasm; we have yet no experimental data on the first hydrophilic, *i.e.*, N-terminal, region. The most plausible topological model for RnfA is illustrated in Figure 2. The model is in accordance with the positive inside rule suggested by von Heijne (1992).

Localization of the RnfB Protein. The predicted product of *rnfB* consists of 180 amino acids and has a hydrophobic stretch at the N-terminal region (Saeki *et al.*, 1993; Schmehl *et al.*, 1993). First, localization of the product was investigated with the antibodies raised against a $(His)_6$-tagged RnfB protein expressed in *E. coli*. The RnfB polypeptide was detected in the chromatophore fraction but not in the soluble fraction of the diazotrophically grown cells of the wild-type strain SB1003, and it was not detected in either

[2] *E. coli* Databank at the Nara Institute of Science and Technology (Nara, Japan): URL http://genome4.aist-nara.ac.jp/.

[3] PredictProtein server (Heidelberg, Germany): URL http://www.embl-heidelberg.de/predictprotein/predictprotein.html.

[4] NNpredict server (San Francisco, CA): URL http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html.

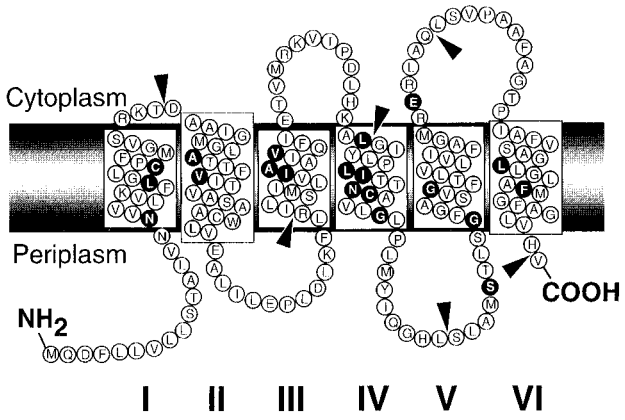

FIGURE 2: Topological model of the RnfA protein. An arrowhead indicates the position where *E. coli* PhoA protein was fused. The residues conserved among 10 homologs (see Figure 8) are shown by inverse contrast.

the chromatophore or soluble fraction from cells grown on the NH_4-containing medium RCV-A (data not shown).

For further confirmation, we created interposon mutants, KF11 and KF12, that had the kanamycin resistant gene inserted at the *Sma*I site in *rnfB*; the former had the drug resistant gene in the same orientation as *rnfB*, while the latter had it in the opposite orientation (Figure 3). These strains did not grow diazotrophically under illuminated anaerobic conditions. This Nif$^-$ phenotype of KF11 was complemented by the plasmid pCP-BH2 that contained *rnfAB* but not *rnfCDE*, whereas the phenotype of KF12 could not be complemented by the same plasmid. Therefore, KF11 was shown to contain a nonpolar mutation, while KF12 contains a polar mutation. To investigate the presence of the RnfB protein, both SB1003 and KF11 cells were grown on the glutamate medium RCV-E under illuminated anaerobic condition and fractionated. Under such conditions, the *nif* and *rnf* genes are derepressed in the presence of fixed nitrogen. The RnfB polypeptide was detected only in the chromatophore fraction of SB1003 cells and not in the similar fraction of KF11 cells (Figure 4A). When the chromatophore fraction from SB1003 cells was further subjected to an alkaline treatment with 0.1 M disodium carbonate buffer (pH 11.3), a portion of the RnfB protein was released from the membrane (Figure 4B). These results revealed that the RnfB protein is a peripheral protein situated at the chromatophore membrane and imply that its N-terminal hydrophobic stretch is not a membrane anchor.

The orientation of the RnfB protein was accessed by the activity and size of a LacZ fusion protein. We had previously observed that SB1003 cells harboring a plasmid pKSN250, which contained a recombinant gene for encoding a fusion product of the first 122 amino acids of RnfB to the *E. coli* LacZ protein, displayed significant β-galactosidase activity (Saeki *et al.*, 1993). Since a β-galactosidase fusion protein is believed to remain active only when it is situated either in the bacterial cytoplasm or on cytoplasmic surface of the membrane, the total proteins of pKSN250/SB1003 cells were analyzed for the size of the fusion product(s) to determine if the active enzyme is a full size fusion product or truncated products with a catalytic moiety. Immunoblot analysis using a monoclonal antibody against *E. coli* LacZ revealed that only fusion peptides of an expected size existed in the cells grown under the *nif*-derepressed condition (data not shown). These results suggest that RnfB is located at the cytoplasmic surface of the chromatophore membrane.

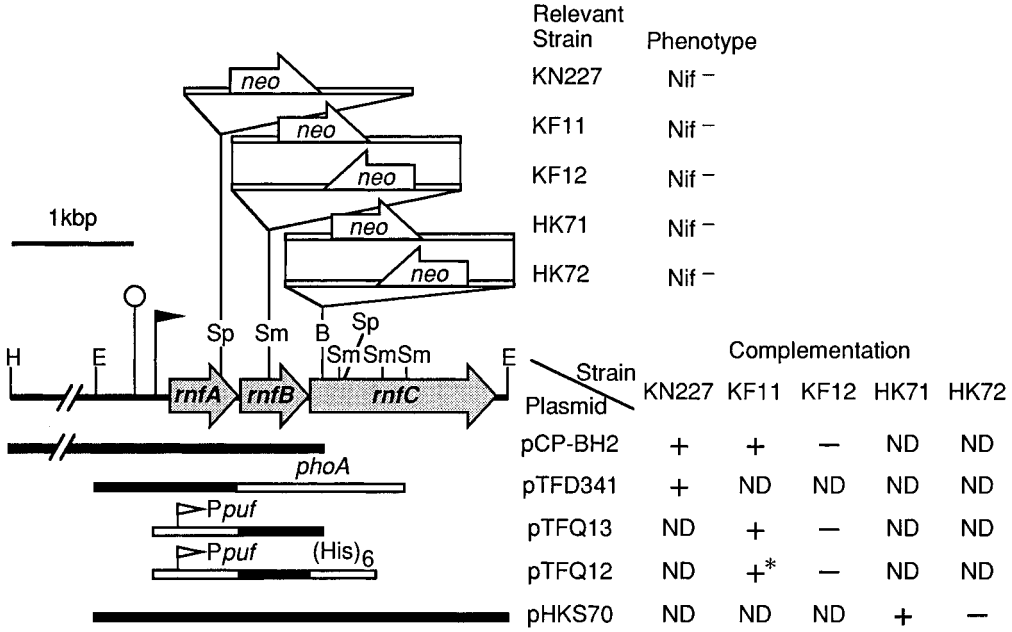

FIGURE 3: Complementation analysis of five *rnf* mutants together with the physical map of their *rnf* loci and the gene fragments used in the complementation experiment. The Nif$^-$ phenotype indicates no growth on nitrogen free agar medium under illuminated anaerobic conditions. The ability of a plasmid to recover the capacity of diazotrophic growth to the rate comparable to that of wild-type SB1003 is indicated with a + while the inability with a −; +* indicates slightly slower growth than that of wild type. ND means not determined. In the physical map, *rnf* genes are symbolized by gray arrows. Gene fragments from other genetic loci or other organisms are symbolized by open bars and open arrows; the *E. coli phoA* gene and synthetic codons for the hexahistidine peptide followed by termination codon are as indicated. The Kanamycin resistant gene (*neo*) is indicated with an open arrow; the *puf* promoter of *R. capsulatus* is indicated with an open arrowhead. H, Sm, and Sp symbolize the restriction enzyme sites for *Hin*dIII, *Sma*I, and *Sph*I, respectively. Other symbols are as described for Figure 1. The strain KN227 and the plasmid pCP-BH2 were previously described. Plasmids pTFQ12 and -13 are pBR322 derivatives, while pTFD341 and pHKS70 are pRK415 derivatives.

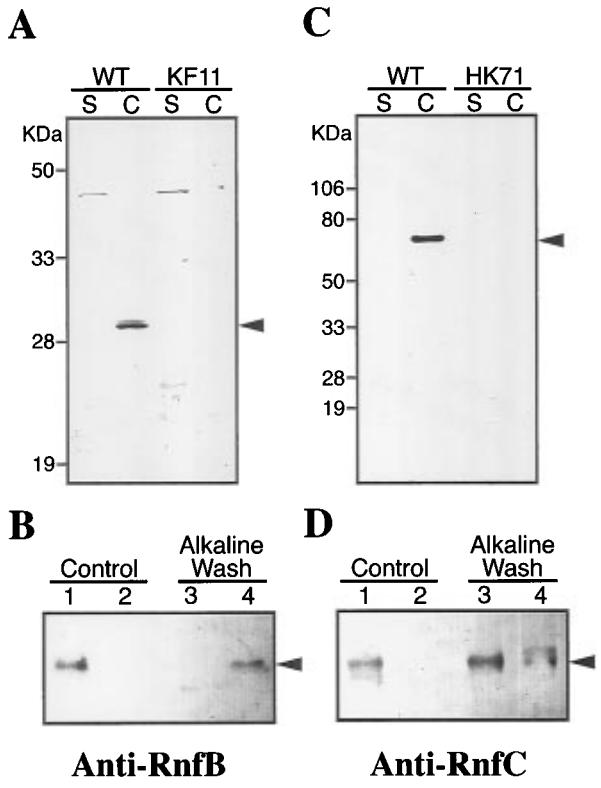

FIGURE 4: Immunoblot analysis of RnfB and RnfC proteins for deducing its localization. The electrophoresis runs were performed on 13% acrylamide gels containing 6 M urea according to the method of Kadenbach *et al*. (1983). Blotted proteins were reacted with the purified antibodies against RnfB (A and B) and RnfC (C and D). (A) Presence of RnfB protein in the chromatophore fraction from wild-type cells. Cells of wild-type SB1003, WT, and *rnfB* mutant KF11 were grown in glutamate−malate medium RCV-E to derepress *nif* and *rnf* genes and fractionated under anaerobic conditions. Soluble and chromatophore fractions were symbolized by S and C, respectively. The lanes contained 20 μg of each protein. The polypeptide at the 47 kDa position seems to be detected by nonspecific reaction, since it was also detected with preimmunized serum. (B) Effects of alkaline treatment on the RnfB protein in chromatophore membranes from wild-type cells. Lane 3 was loaded with an alkaline-treated membrane sample, while lane 4 was loaded with a released protein sample. Lanes 1 and 2 were loaded, respectively, with membrane and released protein samples from the control experiment. Each experiment was started with membranes containing 10 μg of proteins. (C) Presence of RnfC protein in the chromatophore fraction from wild-type cells. Samples from cells of wild-type SB1003 and *rnfC* mutant HK71 were analyzed. (D) Effects of alkaline treatment on the RnfC protein in chromatophore membranes from wild-type cells. Membaranes were treated as described for panel B.

Localization of the RnfC Protein. The predicted product of *rnfC* consists of 519 amino acids and is relatively hydrophilic (Schmehl *et al.*, 1993). Localization of the product was investigated with the antibodies raised against a $(His)_6$-tagged RnfC protein expressed in *E. coli*. The RnfC polypeptide was detected in the chromatophore fraction but not in the soluble fraction of the diazotrophically grown cells of the wild-type strain SB1003, and it was not detected in either the chromatophore or soluble fraction from cells grown on the NH_4-containing medium RCV-A (data not shown).

For further confirmation, we created interposon mutants, HK71 and HK72, that had the kanamycin resistant gene inserted at the *Bam*HI site in *rnfC*; the former had the drug resistant gene in the same orientation as *rnfC*, while the latter had it in the opposite orientation (Figure 3). These strains did not grow diazotrophically. This Nif^- phenotype of HK71

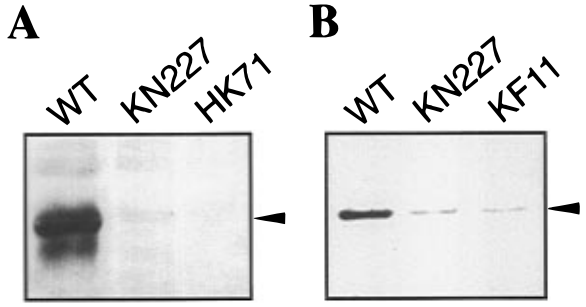

FIGURE 5: Immunoblot analysis of RnfB and RnfC proteins in the chromatophore fractions from cells of various *rnf* mutants derepressed for *nif* and *rnf* genes. The chromatophore fractions from cells grown in glutamate−malate medium RCV-E were analyzed. The conditions of electrophoresis were as described for Figure 4. Lanes were loaded with membranes containing 10 μg of each protein: (A) detection with purified anti-RnfB antibodies and (B) detection with purified anti-RnfC antibodies.

was complemented by the plasmid pHK70 that contained *rnfABC* but not *rnfDE*, whereas the phenotype of HK72 could not be complemented by the same plasmid. Therefore, HK71 was shown to contain a nonpolar mutation, while HK72 contains a polar mutation. To investigate the presence of the RnfC protein, the *nif* and *rnf* genes in both SB1003 and HK71 were derepressed as described above for SB1003 and KF11. The RnfC polypeptide was detected only in the chromatophore fraction of SB1003 cells and not in the similar fraction of the mutant cells (Figure 4C). When the chromatophore fraction from SB1003 cells was further subjected to the alkaline treatment, a portion of the RnfC protein was released from the membrane (Figure 4D). These results revealed that the RnfC protein is a peripheral protein at the chromatophore membrane.

Mutual Stabilization of the rnfABC Gene Products. The above results showing that the RnfA, RnfB, and RnfC proteins locate at the chromatophore membrane seem to support the hypothesis that they associate to form a complex. If the hypothesis is correct, it is assumed that depletion of one particular subunit causes destabilization of the complex. To test this assumption, first, we investigated the contents of RnfB and RnfC proteins in chromatophore fractions from the nonpolar mutants of *rnfA*, *rnfB*, and *rnfC* after derepression of *nif* and *rnf* genes (Figure 5). In *rnfA* mutant strain KN227, we detected almost no RnfB protein and a very slight amount of the RnfC protein. Similarly, a very small amount of RnfC protein was detected in the *rnfB* mutant KF11, and almost no RnfB protein was observed in the *rnfC* mutant HK71. The soluble fractions from the three mutant cells did not contain either the RnfB or RnfC protein (data not shown).

The decrease of the product of *rnfB* that is situated upstream of the disrupted *rnfC* gene in HK71 is consistent with the above assumption. However, the decrease of the *rnfB* and/or *rnfC* products in the other two mutants could have been effects of the insertion mutagenesis that might have caused a partial decrease of the transcription or translation level of the genes situated downstream of the disrupted gene. To test this alternative explanation, we used plasmids, pTFD341, pTFQ13, and pTFQ12, that should express, respectively, only the *rnfA*−*phoA* fusion gene, the authentic *rnfB* gene, and a modified *rnfB* to encode a $(His)_6$-tagged product. The *rnfA* and *rnfB* mutants harboring the corresponding plasmids grew diazotrophically on the RCV-

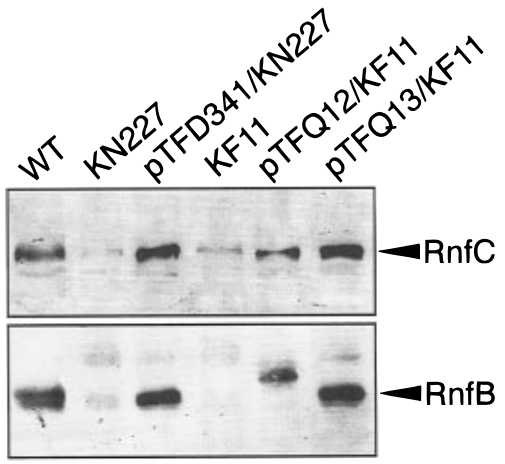

FIGURE 6: Immunoblot analysis of RnfB and RnfC proteins in the chromatophore fractions from cells of nonpolar mutants of *rnfA* and *rnfB* and their complemented derivatives. Analyzed samples were the chromatophore fractions from cells grown in glutamate−malate medium RCV-E. The conditions of electrophoresis were as described for Figure 4. The phenotypes of the strains are shown in Figure 3. Lanes were loaded with membranes containing 20 μg of each protein. Upper (for the presence of RnfC) and lower (for the presence of RnfB) panels were from the same Immobilon-P membrane.

NF plate at a rate comparable to that of wild-type strain SB1003 (Figure 3); it should be noted that the plasmid pTFQ12 with the modified *rnfB* gene complemented the mutant KF11. They were grown on the glutamate medium RCV-E to derepress the *nif* and *rnf* genes and fractionated for the immunochemical analysis (Figure 6). The chromatophore fraction from pTFD341/KN227 cells contained equivalent amounts of RnfB and RnfC proteins compared to those contained in a similar fraction from wild-type cells. A similar increase of RnfC protein was observed in the chromatophore fractions from both KF11 derivatives harboring pTFQ12 and pTFQ13. The soluble fractions from the three transconjugant cells did not contain either the RnfB or RnfC protein (data not shown). These results excluded the alternative possibility and revealed that the presence of the RnfA protein is necessary for stable existence of both RnfB and RnfC proteins.

Detection of Homologs to the rnfABCDE Genes. When the *rnf* genes were identified, no homologs were known. However, the determination of the whole genome sequence[5] of *H. influenzae* (Fleischmann *et al.*, 1995) enabled us to detect a potential homolog of the *rnfABCDE* operon. The six consecutive open reading frames (ORFs), *hi1683*−*hi1688*, in *H. influenzae* encoded proteins similar to the *rnf* gene products with identity values of more than 30% (see below). The Hi1684 and Hi1685 products each possess a 2[4Fe-4S]-type ferredoxin-like domain as do RnfB and RnfC. These indicate that the putative *hi1683*−*hi1688* operon is a homolog of the *rnfABCDE* operon in terms of both products, sequences, and gene organization (Figures 7−9). In addition, our data base search and manual comparison revealed the presence of five potential homologs of RnfA (Figure 8). Two of them are encoded by potential genes *nqrE* and *nqrD* in a marine bacterium *V. alginolyticus* (Figure 7). They had been cloned with the genes encoding the subunits of Na^+-translocating NADH:quinone oxidoreductase (Na^+-NDH) complex (Beattie *et al.*, 1994; Hayashi *et al.*, 1995) and are supposed to encode transmembrane subunits of the complex (Pfenninger-Li *et al.*, 1996). The other two are encoded by *hi0170* and *hi0168* in *H. influenzae*[5] and are supposed to be homologs for the *nqrE* and *nqrD* genes in *V. alginolyticus* (Hayashi *et al.*, 1996). We also found the C-terminal portion of RnfE protein shows similarity to RnfA.

Since *H. influenzae* does not fix nitrogen, there could be other nondiazotrophs that possess homologs of the *rnfABCDE* operon. Focusing on the similarity between RnfA and Hi1683, we made a set of degenerated PCR primers to amplify a portion of the *rnfA* homolog which encodes residues 24−115 of the RnfA protein (Figure 8). A nucleotide fragment was amplified from *E. coli* DH5α genomic DNA, cloned into pUC18 vectors, and sequenced. The obtained sequence of 276 bp seemed to encode a polypeptide similar to the corresponding region of the RnfA protein. Using the sequence as a key for the data base search,

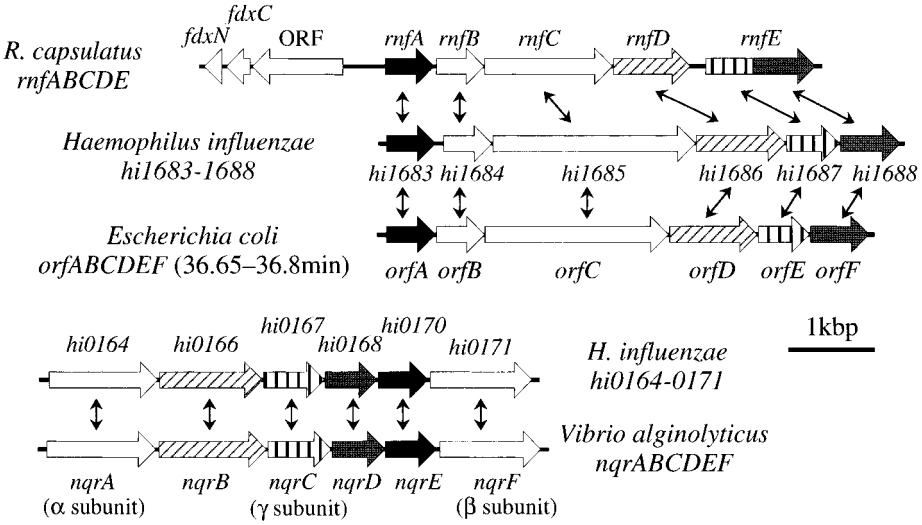

FIGURE 7: Gene organization of the *rnfABCDE* operon and its homologs. Operons with genes or ORFs that show similarity to the members of the *R. capsulatus rnfABCDE* operon are found in *H. influenzae* (Fleischmann *et al.*, 1995), *E. coli* (this study), and *V. alginolyticus* (Beattie *et al.*, 1994; Hayashi *et al.*, 1995). *E. coli* ORFs are tentatively marked as *orfA*−*orfF*. Genes that show similarity to *rnfA*, *rnfD*, the 5′-portion of *rnfE*, and 3′-portion of *rnfE* are indicated, respectively, by black, hatched, striped, and dark gray arrows. RnfA and the N-terminal portion of RnfE are similar to each other as shown below in Figure 8. Lines with arrowheads at both sides indicate close similarity.

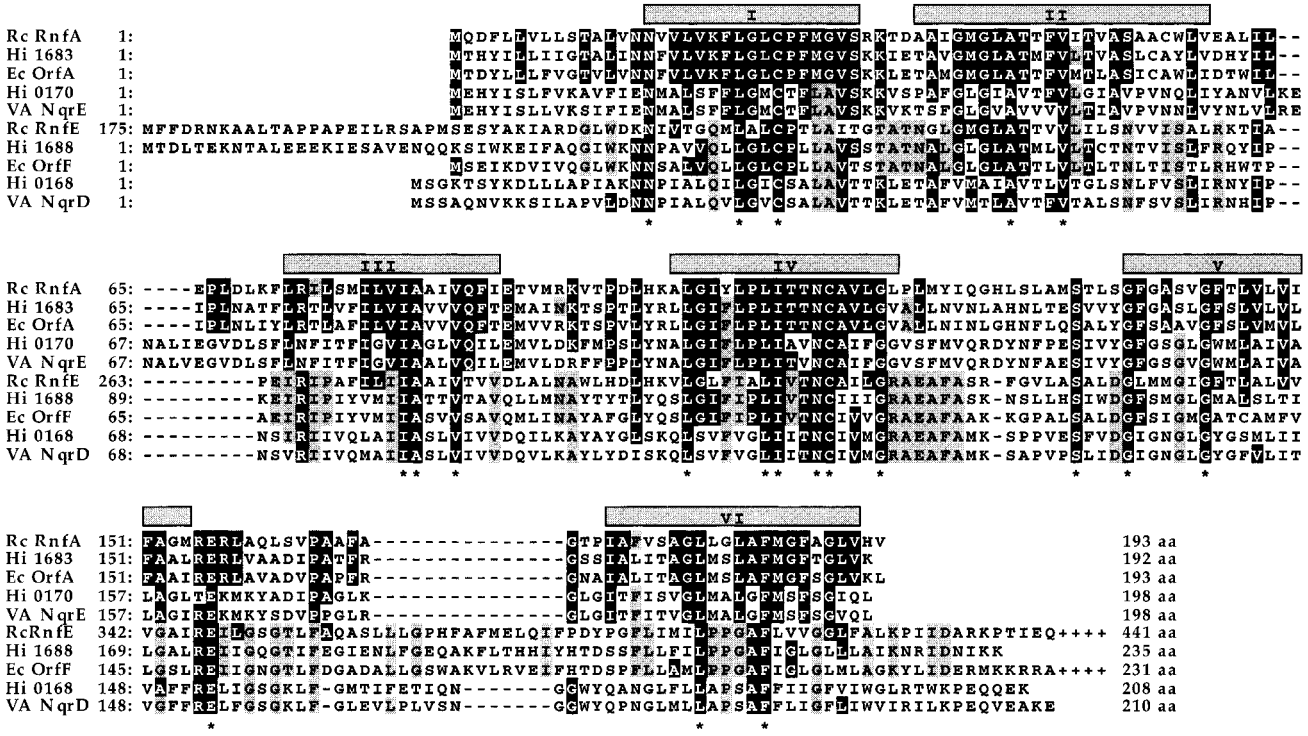

FIGURE 8: Comparison of amino acid sequences of 10 RnfA homologs. Proteins compared are products of genes and ORFs in *H. influenzae*, *E. coli*, and *V. alginolyticus*. The location of each ORF is shown in Figure 7. Residues conserved by RnfA, Hi1683, and OrfA are highlighted by inverse contrast. Among the remaining residues, those conserved by the C-terminal portion of RnfE, Hi1688, and OrfF were shaded. An asterisk below the comparison indicates complete conservation. Numbers at the left side of sequences are amino acid positions. Other symbols are − for a gap introduced to increase similarity and + for an additional sequence to be followed. The gray bars over sequences indicate the six predicted transmembrane segments.

we found that the region at 36.65−36.8 min of the *E. coli* genome contains six ORFs similar to the *rnf* genes (Figure 7). We tentatively call them *orfABCDEF*. Results of close comparison of the products of the three operons together with some products of the two *nqr* operons are summarized in Figures 8 and 9.

All 10 RnfA homologs possess six hydrophobic segments (I−VI in Figure 8) with the potential to span membrane and seem to have a similar topology. Twenty amino acids are completely conserved, including the two Cys residues situated in hydrophobic segments I and IV. A phylogenetic analysis based on the shown comparison suggested that the 10 RnfA homologs are divided into two subgroups and that middle ancestors of the two subgroups diverged from a primeval common ancestor by a gene duplication (data not shown).

In addition to the 2[4Fe-4S]-type ferredoxin domain, the three RnfB homologs were each found to possess an arrangement of the four Cys residues which is supposed to serve as ligands for a [4Fe-4S] cluster in the 55 kDa subunit (AcsC) of the corrinoid/Fe-S protein from *Clostridium thermoaceticum* (Lu *et al.*, 1993) (Figure 9, part i).

The three RnfD homologs were found to have similarity to Hi0166 and NqrB proteins (Figure 9, part iii) with hydrophobic segments conserved. The three homologs of the N-terminal portion of RnfE were found to display similarity to Hi0167 and NqrC proteins (Figure 9, part iv). The orders of the potential homologs of *nqrB*, *nqrC*, and *nqrD* genes are conserved in the five operons compared (Figure 7).

[5] *H. influenzae* Rd Genome Database at the Institute for Genomic Research (Rockville, MD): URL http://www.tigr.org/.

RnfC Homologs Have Potential NADH- and FMN-Binding Sites. Through further data base searches using sequences of the conserved regions among three RnfC homologs, we found that residues 146−188 (in RnfC numbering) show strong similarity to the proposed NADH-binding sites of the NADH-binding subunits of bacterial proton-translocating NADH:quinone oxidoreductase (H^+-NDH) (Yagi, 1993; Yagi *et al.*, 1993), mitochondrial proton-translocating NADH: ubiquinone oxidoreductase (Complex I) (Fearnley & Walker, 1992; Walker, 1992), and bacterial NAD-reducing hydrogenases (Malki *et al.*, 1995; Schmitz *et al.*, 1995; Tran-Betcke *et al.*, 1990) (Figure 9, part ii). Although the sequences in RnfC homologs do not completely conform to the commonly accepted consensus sequence of GXG-XXGXXXG (Scrutton *et al.*, 1990), 10 residues are conserved in the regions of approximately 40 residues among 14 proteins compared and secondary structure prediction indicated that the region of RnfC has a $\beta\alpha\beta$-fold. Therefore, it is likely that the RnfC homologs bind NADH at the region. In addition, we found that residues 243−284 show similarity to the proposed FMN-binding sites (Fearnley & Walker, 1992; Malki *et al.*, 1995; Tran-Betcke *et al.*, 1990; Walker, 1992; Yagi, 1993; Yagi *et al.*, 1993) of the above oxidoreductases. Although the consensus for binding of this nucleotide is less clear, it is possible that RnfC contains FMN.

DISCUSSION

We have experimentally located the RnfA, RnfB, and RnfC proteins at the chromatophore membrane in *R. capsulatus*. To our knowledge, the results are the first experimental evidence for membrane proteins that are specifically required as nonregulatory components for nitrogen fixation;

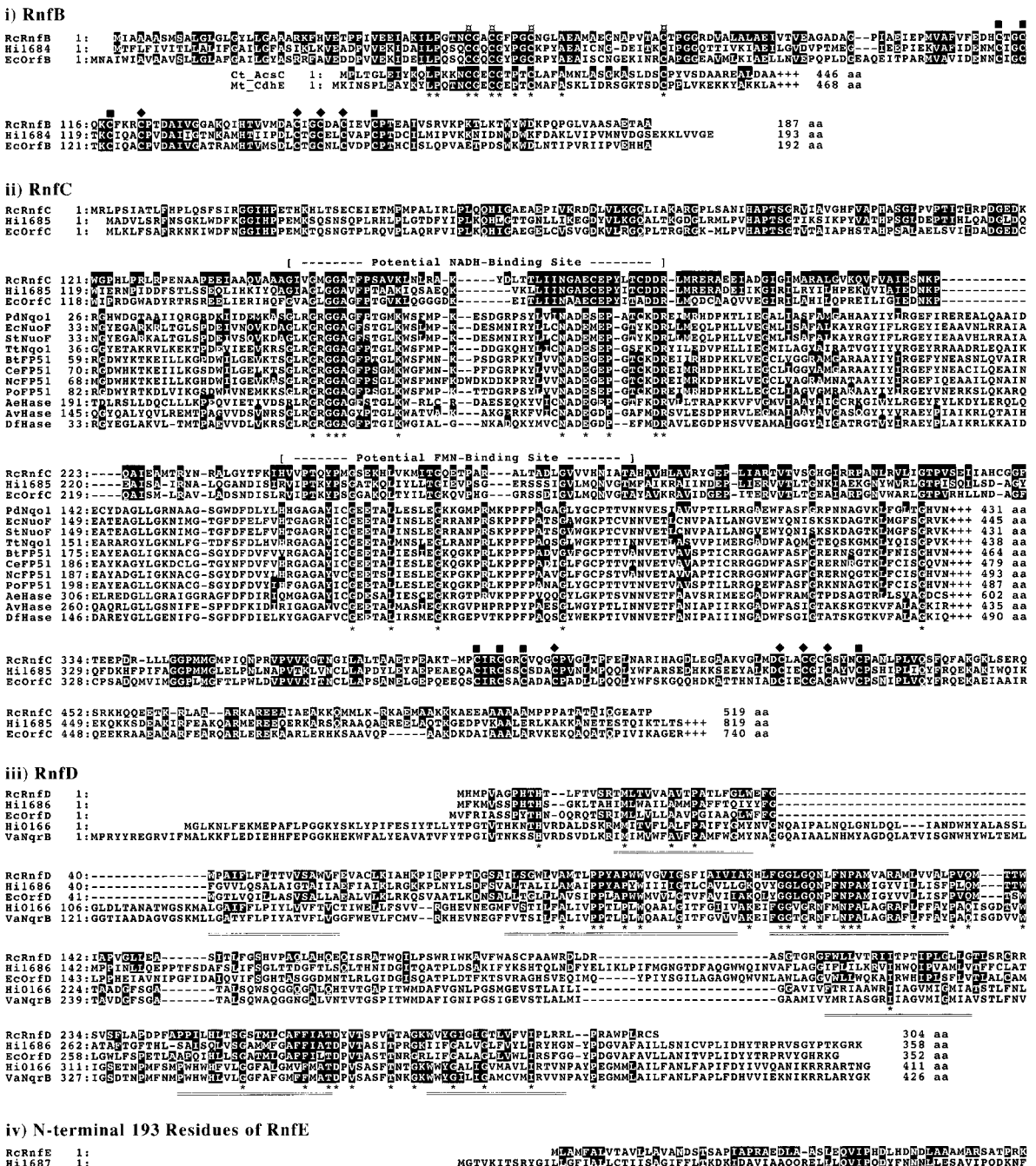

FIGURE 9: Comparison of amino acid sequences of homologs of RnfB, RnfC, RnfD, and the N-terminal portion of RnfE. The residues identical to the corresponding Rnf protein are highlighted by inverse contrast. An asterisk indicates complete conservation among compared counterparts. Numbers at the left side of sequences are amino acid positions. Each set of ◆, ■, and a circle with tick marks indicates Cys residues that might chelate a [4Fe-4S]-type cluster. − shows a gap introduced to increase similarity, while + shows additional sequence to be followed. Potential transmembrane segments of RnfD and its counterparts are double-underlined. Gene locations for compared proteins are shown in Figure 7. Proteins additionally compared are (i) the 55 kDa subunit (Ct_AcsC) of the corrinoid/Fe-S protein from *Clostridium thermoaceticum* (Lu *et al.*, 1993) and the *δ*-subunit (Mt_CdhE) of another corrinoid/Fe-S protein from *Methanosarcina thermophila* (Hochheimer *et al.*, 1995) and (ii) subunits of bacterial H^+-NDH complexes from *Paracoccus denitrificans* (PdNqo1) (Xu *et al.*, 1991), *E. coli* (EcNuoF) (Weidner *et al.*, 1993), *Salmonella typhimurium* (StNuoF) (Archer *et al.*, 1993), and *Thermus thermophilus* (TtNqo1) (NCBI sequence ID 1279866), subunits of mitochondrial Complex I from *Bos taurus* (BtFP51) (Patel *et al.*, 1991; Pilkington *et al.*, 1991), *Caenorhabditis elegans* (CeFP51) (Wilson *et al.*, 1994), *Neurospora crassa* (NcFP51) (Prais *et al.*, 1991), and *Salanum tuberosum* (PoFP51) (NCBI sequence ID 1076668), and subunits of NAD(P)-reducing hydrogenase from *Alcaligenes eutrophus* (AeHase) (Tran-Betcke *et al.*, 1990), *Anaebaena variabilis* (AvHase) (Schmitz *et al.*, 1995), and *Desulfovibrio fructosovorans* (DfHase) (Malki *et al.*, 1995).

the oxygen sensor FixL is a membrane protein in *R. meliloti* (Lois *et al.*, 1993). Mutants of *rnf* genes were previously shown to have higher growth yields in the presence of metronidazole compared to that of wild type (Schmehl *et al.*, 1993). The observations led Masepohl and Klipp (1996) to propose that the Rnf proteins form a membrane complex which is an oxidoreductase to utilize membrane potential for ferredoxin reduction. Our immunochemical results which show that loss of one of the three Rnf products destabilized other Rnf product(s), RnfB and/or RnfC, support the assumption that the Rnf products form a membrane complex. The subunits that fail to form the complex may be digested by endogenous proteases. Although we cannot completely exclude the possibility that the RnfA protein has a regulatory function for expression of other Rnf proteins, this possibility is unlikely because *rnfA* and its downstream genes belong to a single transcriptional unit.

On the basis of the experimental results and comparisons of the homologs, a possible construction of the Rnf complex can be predicted. The complex seems to have a chimeric construction between H^+-NDH and Na^+-NDH (or Complex I). The peripheral proteins, RnfB and RnfC, may form a subcomplex analogous to the flavoprotein fraction (FP) of bacterial H^+-NDH and bovine Complex I (Hatefi, 1985; Yano *et al.*, 1996), because (1) RnfC has Cys motifs to chelate [4Fe-4S] clusters and potential binding sites for NADH and FMN as do the Nqo1 protein of *P. denitrificans* and the 51 kDa subunit of the Complex I and (2) RnfB has a capacity to chelate Fe-S clusters as do the bacterial Nqo2 protein and the bovine 24 kDa subunit. The number and the type of Fe-S clusters in RnfB may be different from those in Nqo2 and the 24 kDa subunit, each of which has a single [2Fe-2S] cluster (Ohnishi *et al.*, 1985; Ragan *et al.*, 1982; Yano *et al.*, 1994); in these respects, RnfB can be more similar to Nqo9 and the bovine 23 kDa subunit (TYKY) (Fearnley & Walker, 1992; Walker, 1992; Yagi, 1993; Yagi *et al.*, 1993). However, this difference may be explained as a difference of the substrate other than NADH. Since *R. capsulatus* is known to have a H^+-NDH as a separate complex (Dupuis, 1992; Dupuis *et al.*, 1995), ferredoxin I rather than quinone seems to be the other substrate for the Rnf complex as previously proposed (Masepohl & Klipp, 1996; Saeki *et al.*, 1991; Schmehl *et al.*, 1993). On the other hand, the polytopic membrane proteins, RnfA, RnfD, and RnfE, may form another subcomplex analogous to the predicted ion channel that is formed in *V. alginolyticus* by NqrB, NqrD, and NqrE proteins (Rich *et al.*, 1995). It might be noted that the NqrC protein which has similarity to the N-terminal portion of RnfE protein is the γ-subunit of the purified Na^+-NDH complex (Hayashi *et al.*, 1995; Pfenninger-Li *et al.*, 1996) and that the NqrB protein is supposed to be the fourth subunit of the Na^+-NDH complex of which Pfenninger-Li *et al.* (1996) recently reported a new purification. The RnfE protein can possibly serve to link ion or proton translocation through the channel and redox reaction at the peripheral subcomplex with its N-terminal moiety. The proton-motive force or membrane potential in chromatophores might be thus coupled by the Rnf protein complex to achieve the energetically uphill reaction to reduce ferredoxin by NADH. This predicted function might account for the previously observed correlation between membrane potential and whole cell nitrogenase activity in *Rhodobacter sphaeroides* (Haaker *et al.*, 1982).

The occurrence of operons similar to the *rnfABCDE* operon may indicate that *H. influenzae* and *E. coli* each have a membrane complex analogous to the Rnf complex. Notably, the two bacteria each possess a putative ferredoxin that has a structural feature similar to that of *R. capsulatus* FdxN; *H. influenzae* Hi0527 protein (Fleischmann *et al.*, 1995; NCBI sequence ID 1074078) and *E. coli* ORF_o86 protein (NCBI sequence ID 1723603) have extra residues between the second and third Cys residues of the second cluster-binding Cys motifs [comparisons are in Saeki *et al.* (1996) and Moulis (1996)]. It is possible that the putative membrane complexes in these nondiazotrophic bacteria also couple the ion translocation with ferredoxin reduction. In conclusion, we predict a new family of energy-coupling NADH oxidoreductases that are distributed in a wide range of bacteria and function at redox potentials as low as those of FdxN proteins. The Rnf protein complex might have evolved to serve specifically in nitrogen fixation; however, other members of the family can serve in rather general metabolic pathways. Since the RnfB-$(His)_6$ is functional to some extent in the strain KF11 (Figures 3 and 6), the tagged protein can be a clue for biochemical study on the Rnf complex.

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