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Identification of a new class of nitrogen fixation genes in *Rhodobacter capsulatus*: a putative membrane complex involved in electron transport to nitrogenase

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Abstract. DNA sequence analysis of a 12236 bp fragment, which is located upstream of *nifE* in *Rhodobacter capsulatus nif* region A, revealed the presence of ten open reading frames. With the exception of *fdxC* and *fdxN*, which encode a plant-type and a bacterial-type ferredoxin, the deduced products of these coding regions exhibited no significant homology to known proteins. Analysis of defined insertion and deletion mutants demonstrated that six of these genes were required for nitrogen fixation. Therefore, we propose to call these genes *rnfA*, *rnfB*, *rnfC*, *rnfD*, *rnfE* and *rnfF* (for *R*hodobacter *n*itrogen *f*ixation). Secondary structure predictions suggested that the *rnf* genes encode four potential membrane proteins and two putative iron-sulphur proteins, which contain cysteine motifs ($C-X_2-C-X_2-C-X_3-C-P$) typical for [4Fe–4S] proteins. Comparison of the in vivo and in vitro nitrogenase activities of *fdxN* and *rnf* mutants suggested that the products encoded by these genes are involved in electron transport to nitrogenase. In addition, these mutants were shown to contain significantly reduced amounts of nitrogenase. The hypothesis that this new class of nitrogen fixation genes encodes components of an electron transfer system to nitrogenase was corroborated by analysing the effect of metronidazole. Both the *fdxN* and *rnf* mutants had higher growth yields in the presence of metronidazole than the wild type, suggesting that these mutants contained lower amounts of reduced ferredoxins.

Key words: *Rhodobacter capsulatus rnf* genes – In vivo and in vitro nitrogenase activities – Iron-sulphur proteins – Ferredoxins – Metronidazole

Introduction

During biological nitrogen fixation the nitrogenase enzyme complex catalyses the reduction of dinitrogen to ammonia. The conventional molybdenum-containing nitrogenase consists of two components: component 1, the molybdenum-iron protein or dinitrogenase, and component 2, the iron protein or dinitrogenase reductase (for review see Smith and Eady 1992). The Fe-protein is composed of two identical subunits bridged by a single [4Fe-4S] cluster, whereas the MoFe-protein is an $\alpha_2\beta_2$ tetramer harbouring two iron-molybdenum cofactors (FeMoco) and two P-clusters. The crystallographic structures of both nitrogenase components including all cofactors have been determined recently (Kim and Rees 1992; Georgiadis et al. 1992).

The complex structure of the nitrogenase enzyme system is also reflected by the organisation and regulation of genes required for its formation and activation. In *Klebsiella pneumoniae,* a facultatively anaerobic enterobacterium, 20 genes involved in the nitrogen fixation process are clustered on the chromosome (Arnold et al. 1988). Based on their functions the products encoded by these genes can be grouped into the structural components of the nitrogenase (NifH, NifD, NifK), proteins needed for full activation and catalytic stability of the nitrogenase (NifU, NifS, NifM, NifW, NifZ), gene products required for the synthesis and insertion of the FeMoco (NifH, NifQ, NifB, NifV, NifE, NifN), proteins involved in electron transport to nitrogenase (NifF, NifJ) and gene products responsible for the transcriptional regulation of all *nif* genes (NifA, NifL). For a review on the features of individual *nif* gene products, see Dean and Jacobson (1992).

In *Azotobacter vinelandii,* an obligately aerobic soil bacterium, homologues of 19 of these *nif* genes are present in the same sequential arrangement (Joerger and Bishop 1988; Jacobson et al. 1989; Bali et al. 1992). In terms of nitrogen fixation, the main difference between *A. vinelandii* and *K. pneumoniae* is the lack of a *nifJ* homologue and the existence of at least 15 open reading frames (ORFs), which are interspersed between the common *nif* genes in *A. vinelandii*. Although these ORFs are coregulated with genes required for nitrogen fixation, no clear function has been assigned to these genes.

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In *Rhodobacter capsulatus*, a non-sulphur, photosynthetic purple bacterium, homologues of *nif* genes and of some of the interspersed ORFs found in *A. vinelandii* are distributed in three *nif* regions, which are widely separated on the chromosome (Klipp et al. 1988; Fonstein et al. 1992). Four transcriptional units of *nif* region A (*nifENX*-ORF4-*fdxB-nifQ*, ORF6-*nifU*$_I$*SVW*, *nifA*$_I$, *nifB*$_I$-ORF1-*nifZ*), four operons of *nif* region B (*nifHDK*, *nifU*$_{II}$-*nifR4*, *nifA*$_{II}$, *nifB*$_{II}$) and the regulatory genes *nifR1* and *nifR2*, which are located in *nif* region C, have already been sequenced and genetically characterised in detail (for review see Klipp 1990). In addition, two genes encoding ferredoxins (*fdxC* and *fdxN*), which are expressed only under nitrogen-fixing conditions, were identified in *nif* region A (Schatt et al. 1989; Grabau et al. 1991; Saeki et al. 1991).

Although the biochemical features of nitrogenase and the organisation of genes necessary for the synthesis of this enzyme complex are very similar in different diazotrophs, the pathway for electron transport to nitrogenase seems to be species-specific. However, at present *K. pneumoniae* is the only organism for which the electron pathway to nitrogenase has been established unequivocally. A flavodoxin (NifF) and a pyruvate: flavodoxin-oxidoreductase (NifJ) couple the oxidation of pyruvate directly to the reduction of the iron protein (Shah et al. 1983; Nieva-Gomez et al. 1980). Although a *nifF* homologue is present in *A. vinelandii*, the corresponding flavodoxin is not essential for nitrogen fixation (Bennett et al. 1988). Another candidate for the reduction of nitrogenase reductase in *A. vinelandii* is ferredoxin I, encoded by the *fdxA* gene (Morgan et al. 1988). However, *A. vinelandii* mutants deleted for *fdxA*, or for both *fdxA* and *nifF*, are still able to fix nitrogen, suggesting that a third, as yet unidentified protein also serves as an electron donor to nitrogenase (Martin et al. 1989).

In this study we present the DNA sequence and mutational analysis of a new class of nitrogen fixation genes in *R. capsulatus*. These so-called *rnf* genes (for *R*hodobacter *n*itrogen *f*ixation), which are shown to be essential for nitrogen fixation, exhibit no homology to known sequences. The biochemical characterisation of *rnf* mutants demonstrated that the products of at least seven *R. capsulatus* genes are involved in electron transport to nitrogenase.

Materials and methods

Media and growth conditions. R. capsulatus wild type as well as the mutant strains were grown anaerobically in 50-ml bottles at 30° C and 3 klx of incandescent light. Growth experiments were performed with RCVB medium (Weaver et al. 1975) supplemented with 30 mM D,L-malate as a carbon source and either 2.5 mM $(NH_4)_2SO_4$ or 7 mM L-serine as nitrogen sources. Inocula were always grown in RCVB medium containing 15 mM $(NH_4)_2SO_4$. Cells were harvested by centrifugation and resuspended in growth medium to an optical density (OD_{660}) of 0.2 (Bausch and Lomb Spectronic 88). Growth with N_2 (Nif$^+$ phenotype) was tested in ammonium-free RCVB medium in microtiter plates incubated in an anaerobic jar (GasPak BBL Microbiology Systems). *Escherichia coli* strains were grown at 37° C in LB medium (Miller 1972). The antibiotic concentrations described by Masepohl et al. (1988) were used.

DNA sequencing. To determine an overlapping DNA sequence of both strands, appropriate restriction fragments were cloned into pSVB sequencing vectors (Arnold and Pühler 1988) and nested deletions were introduced either with the exonuclease III deletion kit (Pharmacia) or by partial digestion with *Sau*3A or *Hpa*II. Sequencing was performed by the chemical degradation method (Maxam and Gilbert 1980) or by the chain-termination method (Sanger et al. 1977). DNA sequences were analysed using the Staden software package (Staden 1986) and PC/GENE (IntelliGenetics). Homology searches were performed using the FASTA and TFASTA algorithms (Pearson and Lipman 1988).

Table 1. Plasmids

Designation	Relevant characteristics	Source or reference
pACYC 184	Cm^r, Tc^r	Chang and Cohen (1978)
pHP 45Ω	Ap^r, Sp^r	Prentki and Krisch (1984)
pML 5B$^+$	Tc^r, *lacZYA*	Labes et al. (1990)
pSUP 202	Ap^r, Cm^r, Tc^r, *mob*	Simon et al. (1983)
pSUP 401	Cm^r, Km^r, *mob*	Simon et al. (1983)
pSUP 2021	pSUP 202::Tn*5*	Simon et al. (1983)
pSVB 30	Ap^r, Lac$^+$	Arnold and Pühler (1988)
pTn*5*-B13	pBR 325::Tn*5*-B13 (Tc^r, *mob*)	Simon et al. (1989)
pTn*5*-B30	pBR 325::Tn*5*-B30 (Km, Tc^r)	Simon et al. (1989)
pWKR 56I	Km^r, Tc^r, *mob*	Klipp et al. (1988)
pWKR 102A	Cm^r, Gm^r, *mob*	Colonna-Romano et al. (1990)
pWKR 189	Ap^r, Gm^r	Moreno-Vivian et al. (1989a)
pWKR 339	Ap^r, Cm^r, Sp^r, *mob*	Schüddekopf et al. (1993)
pWKR 74	3.6 kb *Bam*HI fragment of pWKR 189 carrying Gm^r cloned into pSUP 401	This work

Cm, chloramphenicol; Tc, tetracycline; Ap, ampicillin; Sp, spectinomycin; Km, kanamycin; Gm, gentamicin

Table 2. Plasmids constructed for this work

Plasmid[a]	Mutation[b]	Cloned fragment (bp)[c]	Insertion site (bp)[c]	Vector
pWKR 219CI/II	*Bam*HIΔ::Gm	*Hin*dIII-*Xho*I (1–10 790)	Δ*Bam*HI (1307–1379)	pWKR 56I
pWKR 112AI/II	*rnfE*Δ::Tc_1	*Hin*dIII-*Hin*dIII (1–10 939)	Δ*Bgl*II (2567–4004)	pWKR 74
pWKR 219BI/II	*rnfE*Δ::Gm	*Hin*dIII-*Xho*I (1–10 790)	*Bam*HI (3708)	pWKR 56I
pKS 92I/II	*rnfD*::Gm	*Bam*HI-*Bam*HI (3708–6685)	*Eco*RI (5076)	pACYC 184, Tc-*mob*[d]
pWKR 155CI/II	*rnfC*::Gm	*Eco*RI-*Eco*RI (5076–8549)	*Bam*HI (6685)	pWKR 102A
pWKR 386I	*rnfB*::Tc_2	*Bam*HI-*Bcl*I (6685–7583)	*Sma*I (7091)	pWKR 339
pWKR 363I/II	*rnfA*Δ::Gm	*Bam*HI-*Eco*RI (6685–8549)	Δ*Bcl*I (7583–7766)	pSVB 30, Tc-*mob*[d]
pWKR 347I/II	ORF14Δ::Gm	*Bam*HI-*Xho*I (6685–10 790)	Δ*Pst*I (8803–9473)	pACYC 184, Tc-*mob*[d]
pWKR 346I/II	*fdxN*::Gm	*Pst*I-*Xho*I (9473–10 790)	*Bcl*I (10 082)	pSUP 202
pWKR 213BI/II	*rnfF*::Km	*Eco*RI-*Eco*RI (8549–12 236)	*Xho*I (10 790)	pSUP 202
pWKR 213AI/II	*rnfF*Δ::Km	*Eco*RI-*Eco*RI (8549–12 236)	Δ*Hin*dIII (10 939–11 105)	pSUP 202
pWKR 349AI/II	ORF10::Tc_2	*Hin*dIII-*Bcl*I (11 105–13 373)	*Bgl*II (12 138)	pWKR 339

[a] Roman numerals refer to the orientation of the interposon (I: non-polar mutations; II: polar mutations)

[b] The Gm interposon is derived from pWKR189, the Tc_1 interposon from pTn*5*-B30, the Tc_2 interposon from pML5B$^+$ and the Km interposon from pSUP2021

[c] The numbers correlate to the numbering of the DNA sequence data base entry and Fig. 2

[d] Tc-*mob* fragment derived from Tn*5*-B13

Construction of R. capsulatus *mutants.* The plasmids used for the construction of defined insertion or deletion mutants are listed in Tables 1 and 2. *R. capsulatus* wild type DNA fragments were cloned by standard methods (Sambrook et al. 1989) into mobilisable vector plasmids and the restriction sites shown in Figs. 1B and 2 were used to insert appropriate interposons (Table 2). Plasmids containing the desired mutations were mobilised from *E. coli* S17-1 (Simon et al. 1983) into *R. capsulatus* and homogenotisation of the corresponding insertions was selected for using the antibiotic resistances mediated by the interposons. A subsequent test for loss of the vector-encoded antibiotic resistance was used to identify strains carrying double cross-over events.

Nitrogenase assays in vivo and in vitro. H_2 production was measured as described by Klein et al. (1991). Acetylene reduction with 3 ml of the culture was carried out in 25-ml bottles under an argon atmosphere with 10% (v/v) acetylene after addition of 4 mM D,L-malate under saturating light intensity (30 klx). In vitro acetylene reduction assays were done after addition of CDAP (cetyltrimethyl-ammonium bromide), with dithionite as artificial electron donor, and an ATP regenerating system to 3 ml of the culture, as described by Haaker et al. (1982). Samples from the gas phase were analysed by gas chromatographic methods using a Shimadzu GC-8A equipped with an FID detector.

Immunodetection of nitrogenase proteins. Gel electrophoresis, Western blotting and immunodetection of nitrogenase proteins with an Amersham ECL system were carried out as described by Klein et al. (1993). After recording the results on Amersham Hyperfilm ECL, protein bands were scanned with an LKB Ultroscan densitometer. Protein concentrations were determined according to Lowry et al. (1951). Ammonium concentrations were measured with an ion-sensitive electrode (Ingold, Urdorf, Switzerland).

Results

DNA sequence analysis of R. capsulatus *genes located upstream of* nifE

Analysis of a contiguous DNA fragment of 13 946 bp from *R. capsulatus nif* region A, containing the *nifENX*-ORF4-*fdxB-nifQ*, ORF6-*nifU*$_r$-*SVW*, *nifA*$_I$ and *nifB*$_r$-ORF1-*nifZ* transcriptional units, has been described in detail (Masepohl et al. 1988, 1993; Moreno-Vivian et al. 1989a, b). As shown in Fig. 1A, this part of *nif* region A is preceded by a DNA fragment of about 10 kb which is characterised by Tn*5* insertions resulting in a Nif$^-$ phenotype (Klipp et al. 1988). A 12 236 bp fragment (Fig. 1B) located upstream of *nifE* was therefore subjected to DNA sequence analysis (Fig. 2). The complete nucleotide sequence data reported in this paper, which

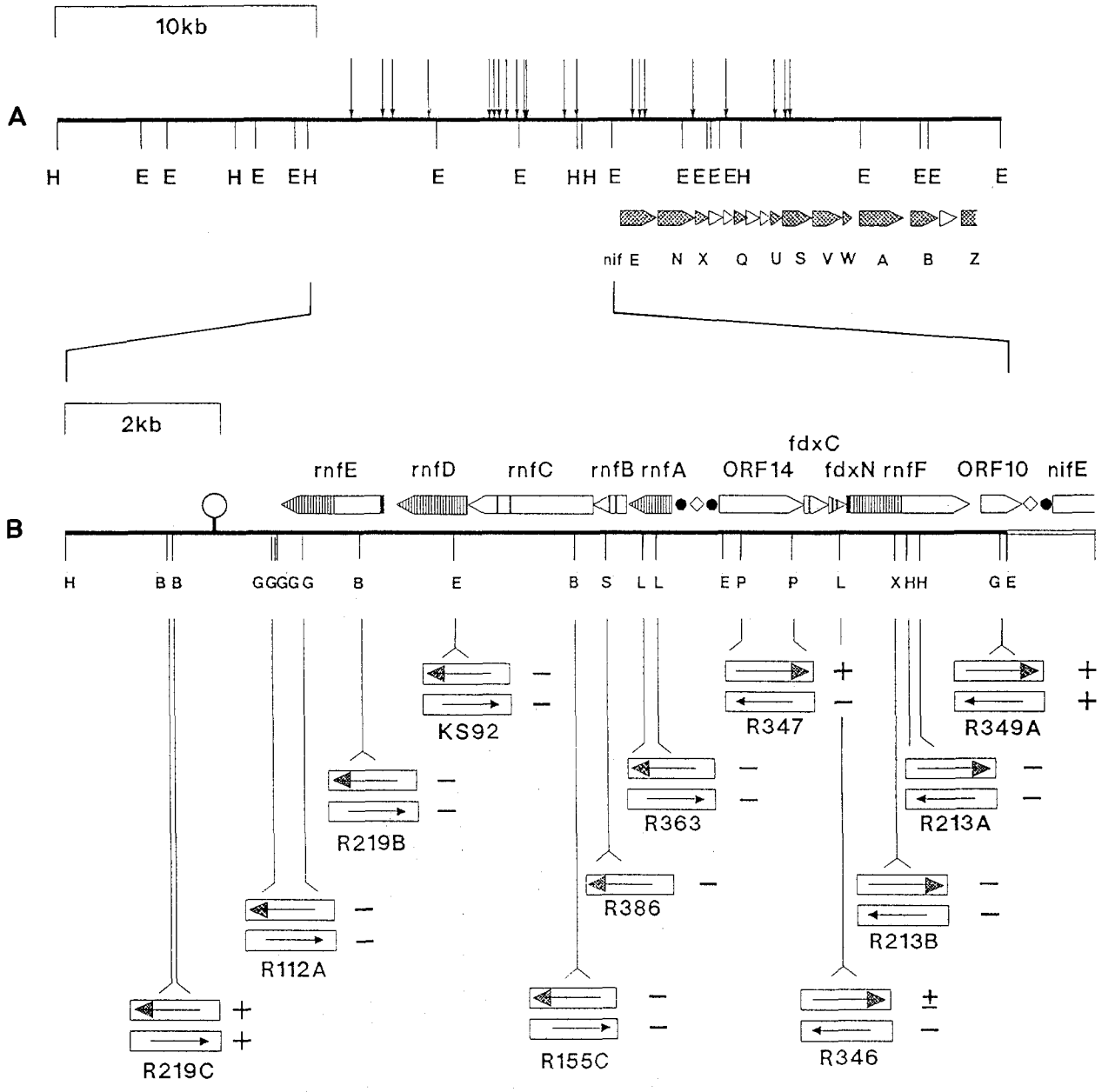

Fig. 1A, B. Genetic organisation and mutational analysis of *Rhodobacter capsulatus* nitrogen fixation genes. The physical and genetic map of *R. capsulatus nif* region A is given in **A**. *Vertical arrows* indicate the locations of Tn*5* insertions in different Nif$^-$ mutants. The location of coding regions as deduced from DNA sequence data is given below the restriction map. The DNA fragment subjected to sequence analysis in this study is given in more detail in **B** and is emphasised by a *black line*. The location and size of coding regions is given by *open arrows*. Potential membrane spanning parts are *striped*, putative leader peptides are marked by *black boxes* and cysteine motifs are indicated by *vertical lines*. Typical σ^{54}-dependent promoter sequences are indicated by *black circles* and NifA-binding sites by *open diamonds*. A potential stem-loop structure is indicated downstream of *rnfE*. The location of interposon insertions is shown below the restriction map. Details of the construction are given in Table 2. The direction of transcription of the antibiotic resistance genes located on the interposons (not drawn to scale) is symbolised by *arrows*. The phenotype of the corresponding mutants is given for each interposon mutant (+, diazotrophic growth like the wild type; +/−, slow diazotrophic growth, see Table 3; −, no growth at all with N_2 gas as nitrogen source). Only restriction sites relevant for cloning (Table 2) are shown in **B**. Abbreviations: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; L, *Bcl*I; P, *Pst*I; S, *Sma*I; X, *Xho*I

also includes the non-coding 2280 bp not presented in Fig. 2, will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Data Bases under the accession number X72888. Sequence analysis of an overlapping fragment demonstrated that the *Eco*RI site at position 12 236 directly abuts the sequence of the *nifENX* operon reported previously (Moreno-Vivian et al. 1989b).

Ten open reading frames (ORFs) were identified showing the codon usage frequency typical of *R. capsulatus nif* genes (Fig. 1B; Table 3). The designation of two of these ORFs as *R. capsulatus fdxC* and *fdxN* was based on their amino acid sequence homology to [2Fe-2S] plant-type ferredoxins and [4Fe-4S] bacterial-type ferredoxins (Schatt et al. 1989; Grabau et al. 1991;

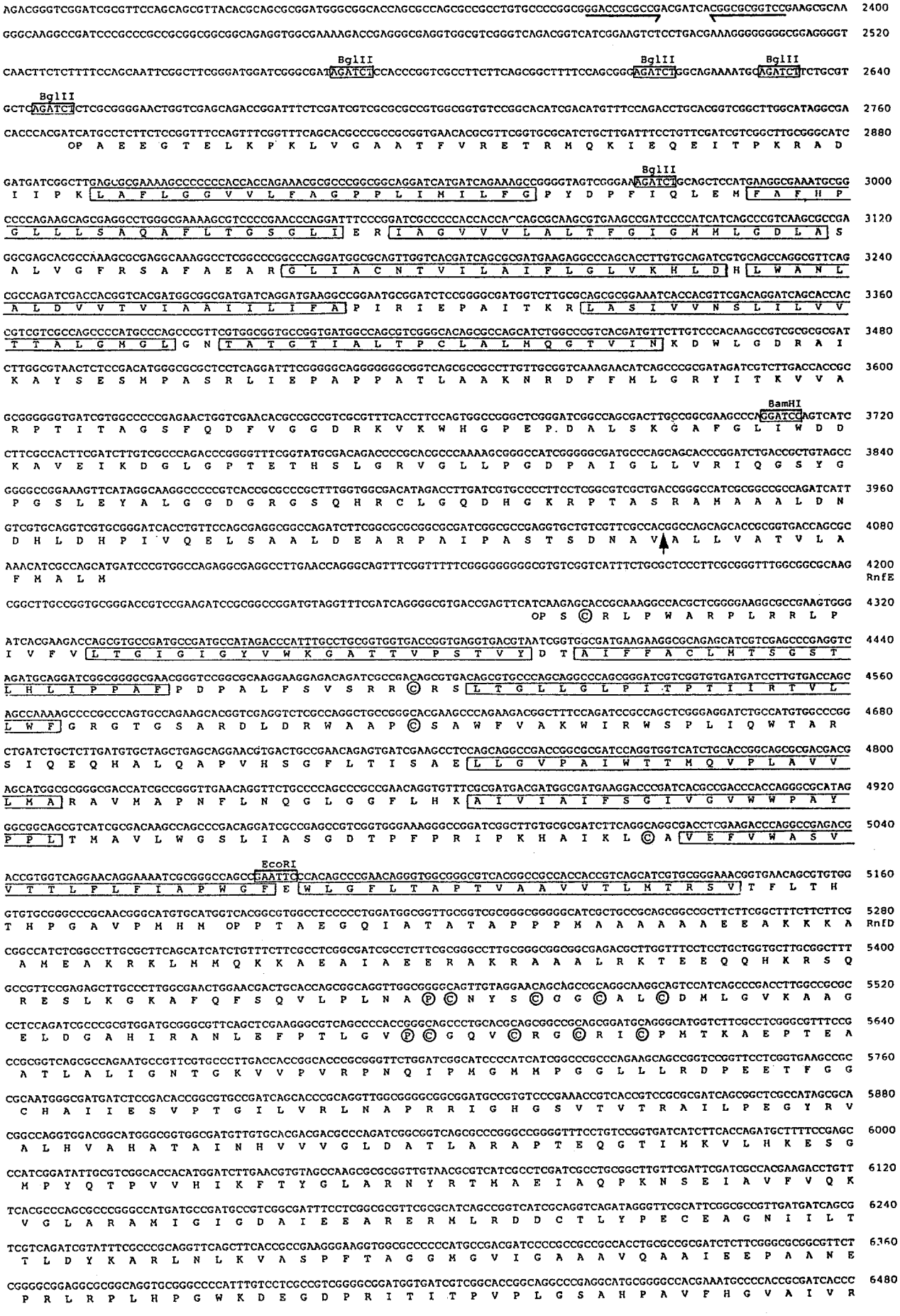

Fig. 2 (for legend see page 608)

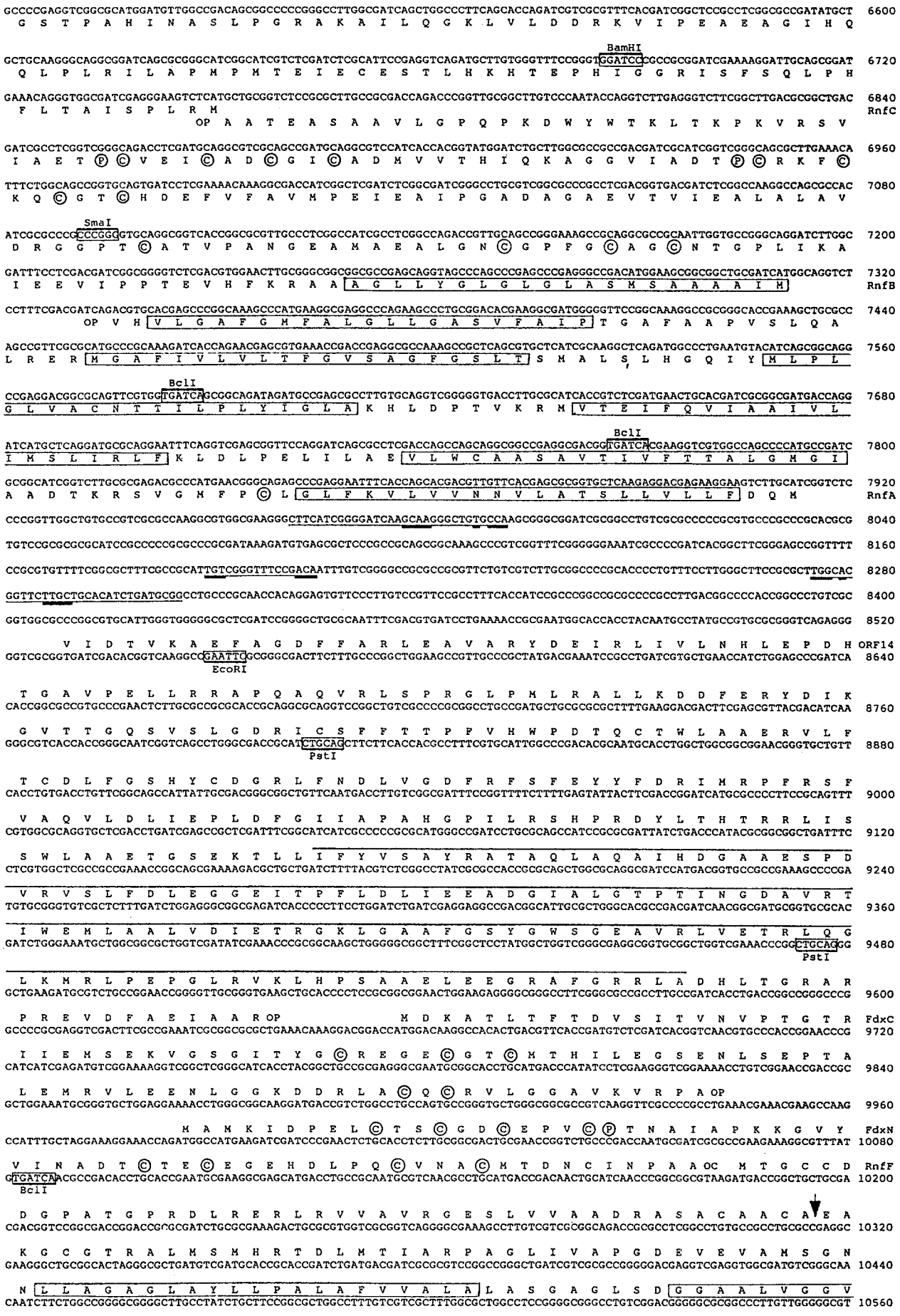

Fig. 2 (continued; for legend see page 608)

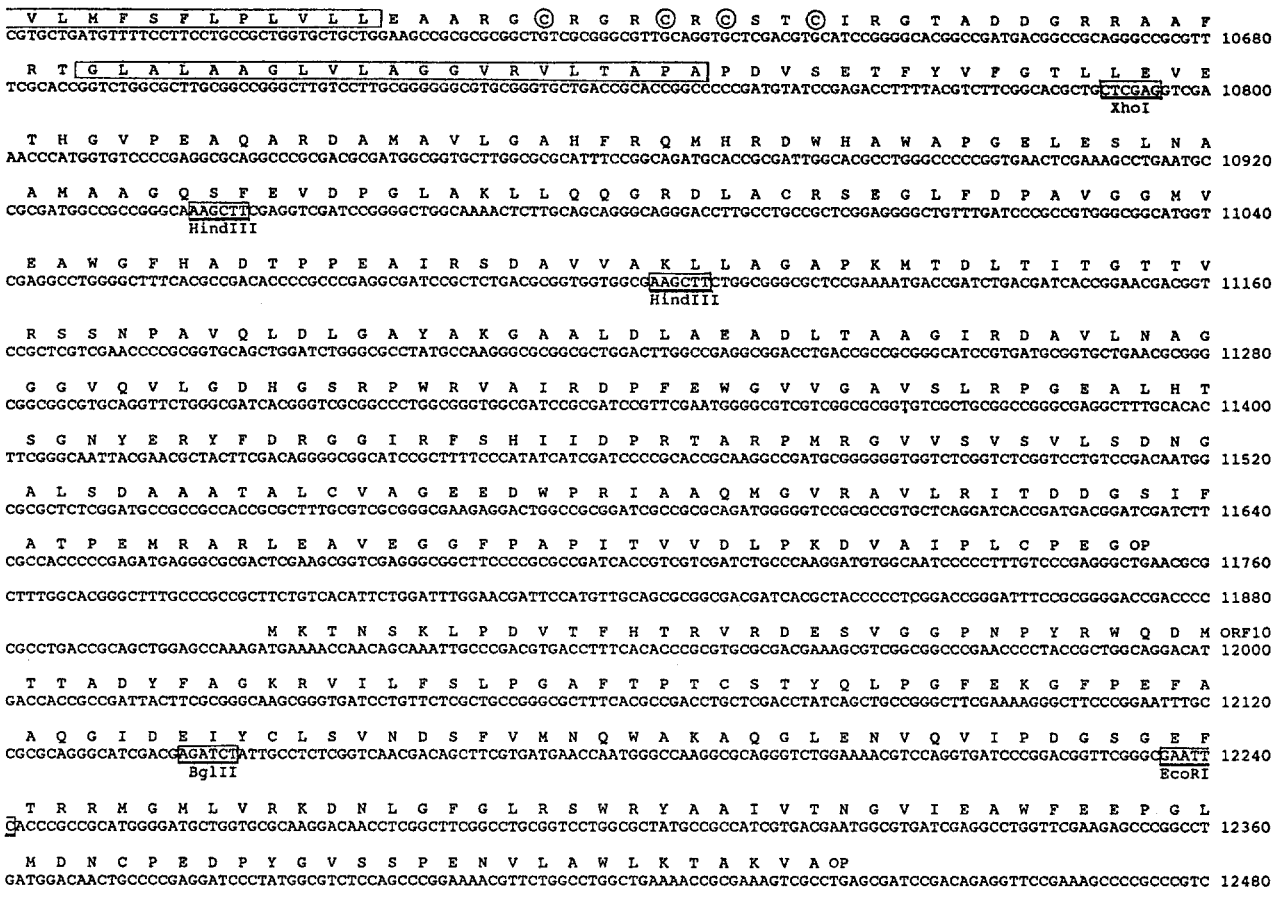

Fig. 2. Nucleotide sequence of a 12 480 bp DNA fragment located upstream of *nifE* in *R. capsulatus nif* region A. The DNA sequence is given in the 5′ to 3′ direction and numbering corresponds to the data base entry. The first non-coding 2280 bp are not shown. The predicted amino acid sequences of *rnfE, rnfD, rnfC, rnfB* and *rnfA* are given in single letter code below the nucleotide sequence and for ORF14, *fdxC, fdxN, rnfF* and ORF10 above the sequence. Transmembrane segments predicted by the method of Eisenberg et al. (1984) are *boxed* and putative cleavage sites of leader peptides are marked by *arrows*. Cysteine residues discussed in the text are *circled* and a motif in ORF14 which is homologous to *Clostridium* MP flavodoxin (Jouanneau et al. 1990) is emphasized by *overlining*. An inverted repeat located downstream of *rnfE* is marked by *arrows*. DNA motifs conforming to the consensus sequence for σ^{54}-dependent promoters are *underlined* and conserved nucleotides are marked by *filled bars*. The same symbols were used to indicate a DNA sequence which corresponds to NifA-binding sites. Restriction sites used for the construction of mutants are *boxed*

Table 3. Features of proposed products encoded by genes located upstream of *Rhodobacter capsulatus nifE*

Designation	Phenotype of mutants[a]	Total amino acids	Product molecular mass (daltons)	Predicted characteristics
rnfE	Nif$^-$	441	46 627	Secretory membrane protein
rnfD	Nif$^-$	304	33 277	Membrane protein
rnfC	Nif$^-$	519	55 587	Iron-sulphur protein
rnfB	Nif$^-$	187	19 090	Iron-sulphur protein
rnfA	Nif$^-$	193	20 424	Membrane protein
ORF14	Nif$^+$	370	41 433	Flavin-binding protein
fdxC	n.d.	95	10 163	[2Fe-2S] ferredoxin[b]
fdxN	Nif$^{+/-}$[c]	65	6 865	[4Fe-4S] ferredoxin[d]
rnfF	Nif$^-$	523	54 142	Secretory membrane protein
ORF10	Nif$^+$	179	19 988	–

[a] See Fig. 1
[b] Grabau et al. (1991); Saeki et al. (1991)
[c] Slow diazotrophic growth (no growth could be observed after 50 h as for wild type and Nif$^+$ strains, however, in contrast to Nif$^-$ strains, this mutant showed diazotrophic growth after 100 h)
[d] Schatt et al. (1989); Saeki et al. (1991)
n.d., not determined

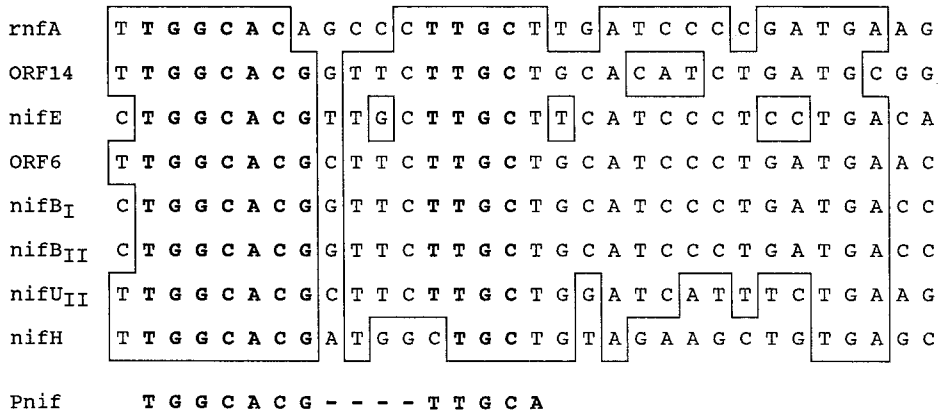

Fig. 3. Comparison of *R. capsulatus nif* promoters. The DNA sequence of σ^{54}-dependent promoters located in front of *rnfA* and ORF14 are compared to *R. capsulatus* promoters in front of *nifE* (Moreno-Vivian et al. 1989b), ORF6 (Masepohl et al. 1993), *nifB*$_I$ and *nifB*$_{II}$ (Masepohl et al. 1988), *nifU*$_{II}$ (Preker et al. 1992) and *nifH* (Pollock et al. 1988). Identical nucleotides are *boxed* and compared to the consensus sequence of σ^{54}-dependent promoters (P_{nif}) proposed by Morett and Buck (1989)

Saeki et al. 1991). All other ORFs in this part of the *R. capsulatus nif* region A exhibited no significant homology to known *nif* genes or to other sequences present in data bases. Therefore, these ORFs were called *rnf* genes (for *R*hodobacter *n*itrogen *f*ixation) if they were essential for nitrogen fixation or ORFs if mutations resulted in a Nif$^+$ phenotype. Five of these genes *(rnfABCDE)* are transcribed in the opposite direction to all other genes in *nif* region A (Fig. 1B; Fig. 2), whereas ORF14-*fdxC-fdxN-rnfF*-ORF10 are transcribed in the same direction. These two putative operons are preceded by DNA sequences corresponding to the consensus sequence of σ^{54}-dependent promoters (Morett and Buck 1989). As shown in Fig. 2, only one sequence agreed perfectly with the NifA-binding motif TGT-N_{10}-ACA (Cannon et al. 1991), which is located between these two putative σ^{54}-dependent promoters. This NifA-binding motif is located 197 bp upstream of the *rnfABCDE* promoter, whereas only 70 bp separate this element from the σ^{54}-dependent promoter of the ORF14-*fdxC-fdxN-rnfF*-ORF10 transcriptional unit.

Figure 3 shows a comparison of all σ^{54}-dependent promoters located in *R. capsulatus nif* region A (p_{rnfA}, p_{ORF14}, p_{nifE}, p_{ORF6}, p_{nifBI}) and *nif* region B (p_{nifH}, p_{nifUII}, p_{nifBII}), respectively. In contrast to σ^{54}-dependent promoters found in front of nitrogen fixation genes from a variety of diazotrophs, the *R. capsulatus nif* promoters contain not only the canonical $-12/-24$ motif but also additional invariant nucleotides between position -12 and the transcriptional start.

It is interesting to note that, based on the availability of start and stop codons and the codon usage frequencies, no putative coding regions could be localised within the 2769 bp sequence downstream of *rnfE*. However, a perfect stem-loop structure consisting of an 11 bp stem and a 6 bp loop (ΔG, -31.4 kcal), which might be the transcriptional stop signal of the *rnfABCDE* operon, was found 378 bp downstream of the *rnfE* stop codon (marked in Figs. 1B and 2).

Structure predictions bases on deduced amino acid sequences

Analyses based on three different methods (Eisenberg et al. 1984; Klein et al. 1985; Rao and Argos 1986) predicted RnfA, RnfD, RnfE and RnfF to be membrane proteins. As shown in Fig. 4, RnfA is predicted to contain six transmembrane segments, both RnfD and RnfE include seven of these elements and RnfF contains only three transmembrane segments. The exact localisation of these putative transmembrane segments is indicated in Fig. 2. The hydrophobic N-terminal parts of RnfE and RnfF are very likely to represent prokaryotic secretory signal sequences. According to the $-3/-1$ rule (for review see von Heijne 1988) RnfE is predicted to be a secretory membrane protein which is cleaved between amino acid residues 14 and 15 (marked in Fig. 2), whereas the putative cleavage site of RnfF is located between residues 44 and 45. The locations of connecting loops between transmembrane segments at the cytosolic or periplasmic side of the membrane could be predicted by the distribution of positively charged amino acid residues (von Heijne 1986). According to these rules, the loop separating the transmembrane segments I and II of RnfF, which contains no positively charged amino acid residues, should be exposed at the periplasmic side of the membrane. A cysteine motif C-X_3-C-X-C-X_2-C, which is located between transmembrane segments II and III of RnfF (marked in Fig. 2) would be located at the cytosolic side of the membrane, since this loop contains eight positively charged arginine residues. The large hydrophilic C-terminal part of this protein is predicted to be located at the periplasmic side of the membrane.

As shown in Fig. 2, the gene products of RnfB and RnfC are characterised by cysteine motifs typical for [4Fe-4S] proteins. Both proteins contain two C-X_2-C-X_2-C-X_3-C-P motifs, which are separated by 18 amino acid residues in RnfB and 27 residues in RnfC. In addition to these motifs, RnfB contains a further cysteine cluster C-X_2-C-X_4-C-X_{16}-C in the N-terminal region. This motif in RnfB is preceded by a putative transmembrane helix (Fig. 2), whereas RnfC is predicted to be a soluble protein.

A domain exhibiting significant similarities to FMN-binding sites was found in the C-terminal part of ORF14 (marked in Fig. 2). According to our DNA sequence data this domain is part of a large protein encoded by ORF14, which is predicted to consist of 370 amino acid residues. This is in contrast to data presented by Jouanneau et al. (1990) who proposed that the DNA region upstream of

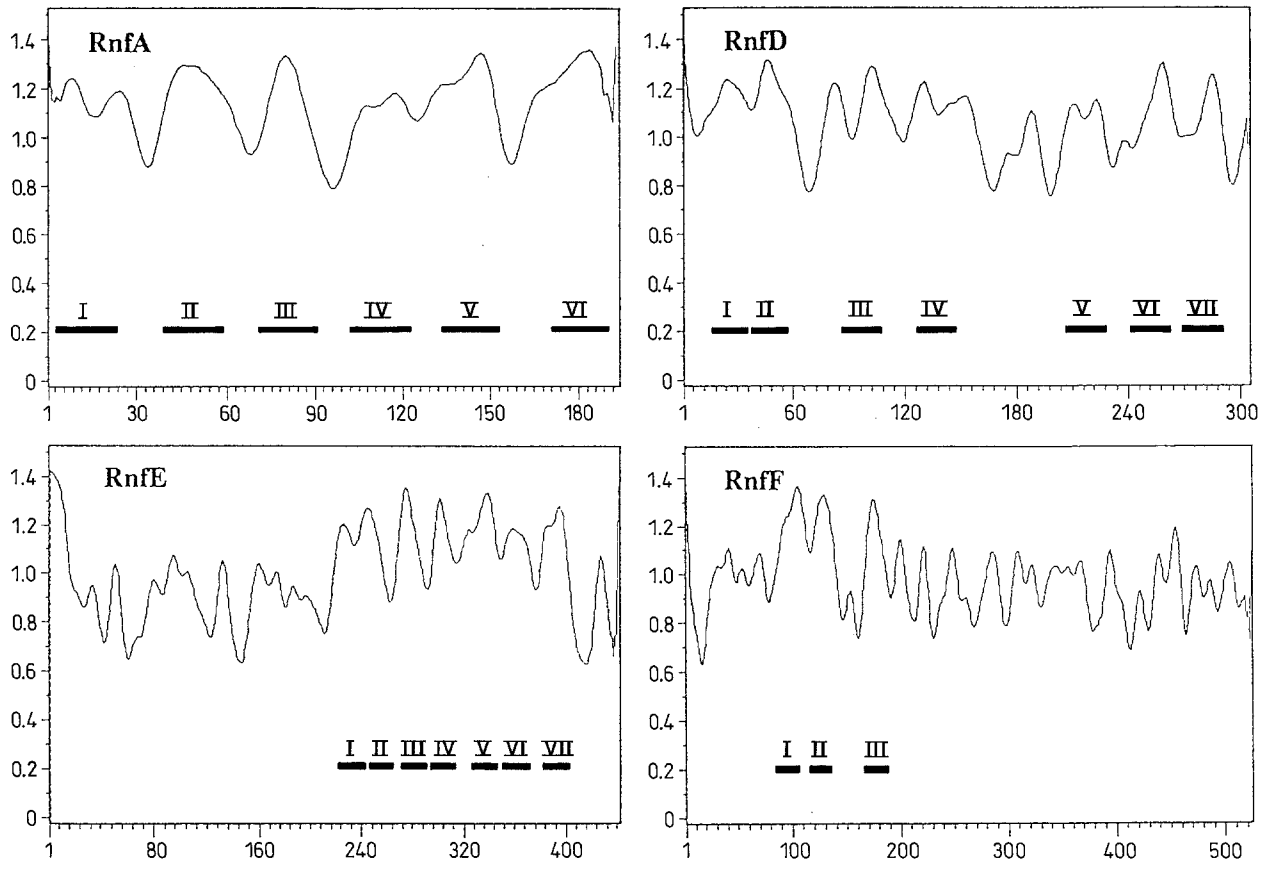

Fig. 4. Hydropathy plots of *R. capsulatus* RnfA, RnfD, RnfE and RnfF. Hydropathy indices were calculated by the method of Rao and Argos (1986) using a minimum window of 16 amino acid residues. The *black bars* indicate putative transmembrane segments also marked in Fig. 2

fdxC codes for a low molecular weight flavodoxin. Differences between the DNA sequence presented in this study and sequences published previously were also found downstream of *fdxN*. These changes cause the fusion of URF1 with the beginning of an ORF proposed by Saeki et al. (1991), resulting in the coding region of *rnfF*.

Mutational analysis

The DNA region sequenced in this study had initially been characterised by 13 transposon Tn*5*-induced mutations (Klipp et al. 1988). The Nif$^-$ phenotype of the corresponding mutants demonstrated that genes essential for nitrogen fixation are located in this part of *R. capsulatus nif* region A. To analyse the role of the sequenced genes in more detail, plasmids carrying defined insertions or deletions were constructed (Table 2, Fig. 1B). The interposons used for the construction of these mutations were previously shown to induce polar or non-polar mutations depending on their orientation (Moreno-Vivian et al. 1989a; Masepohl et al. 1993; Schüddekopf et al. 1993). The corresponding interposon-induced mutations were subsequently homogenotised into the *R. capsulatus* genome and the Nif phenotype of the resulting mutants was determined by analysing diazotrophic growth (Table 3; Fig. 1B). A clear Nif$^-$ phenotype was found for *rnfA, rnfB, rnfC, rnfD* or *rnfE* mutants, whereas deletion of a *Bam*HI fragment located 984 bp downstream of the stem-loop structure adjacent to *rnfE* resulted in a Nif$^+$ phenotype. Depending on the orientation of the interposon, ORF14 mutants exhibited a Nif$^+$ or a Nif$^-$ phenotype. This result demonstrated that the gene product of ORF14 itself is not essential for nitrogen fixation but the expression of genes absolutely required for this process, which are located downstream of ORF14, is dependent on the putative σ^{54}-dependent promoter located in front of ORF14. The analysis of insertion/deletion mutations located downstream of ORF14 proved that *rnfF* is the only gene of this transcriptional unit which is essential for diazotrophic growth. A very slow diazotrophic growth was found for a non-polar *fdxN* mutant. This is in contrast to data reported previously by Saeki et al. (1991) who described a Nif$^-$ phenotype for an *fdxN* mutant. Differences in the expression of *rnfF*, which was shown to be essential for nitrogen fixation (this study), may account for this discrepancy. Saeki et al. (1991) used a kanamycin cassette to inactivate *fdxN* and to drive expression of *rnfF*, whereas a gentamicin interposon was used in this study.

Determination of in vivo and in vitro nitrogenase activities of R. capsulatus *wild type and mutant strains*

During growth on D,L-malate and NH_4^+, no significant differences in growth rate or protein levels in the stationary phase of growth were observed between *R. capsulatus* wild type and the mutant strains described in this study (data not shown). As shown in Fig. 5, three different types of time courses for nitrogenase activity were found for *R. capsulatus* wild type and mutants carrying insertions/deletions in the sequenced part of *R. capsulatus nif* region A. The wild type (Fig. 5A) and the non-polar ORF14 mutant (R347I, data not shown) showed essentially the same behaviour. After exhaustion of NH_4^+, both strains produced considerable amounts of H_2 (data not shown) and exhibited specific activities of in vivo acetylene reduction of about 150 nmol per min per mg. The non-polar *fdxN* mutant (R346I) showed a low but significant nitrogenase activity of about 2 nmol per min per mg, which decreased slowly over 50 h (Fig. 5B). Almost identical values were found for the non-polar *rnfA* mutant (R363I, data not shown). In contrast, strains carrying mutations in *rnfE* (R112AI/II), *rnfC* (R155CI/II) and *rnfF* (R213AI/II, R213BI/II), as well as the polar *rnfA* and *fdxN* mutants (R363II and R346II), exhibited a short-lived increase in nitrogenase activity (0.5–2.5 nmol per min per mg), which was followed by a rapid decrease culminating in lack of activity after 30 h growth (Fig. 5C).

In addition to in vivo nitrogenase activities, in vitro nitrogenase activities of *R. capsulatus* wild type and representatives of both other types of time courses of the nitrogenase activity were analysed. The in vitro nitrogenase activities were tested after destruction of the cell envelope by CDAP, using an ATP-regenerating system and dithionite as an artificial electron donor. As shown in Table 4, after 20 h of growth the *rnfE* mutant (R112AII) and both *fdxN* mutants (R346I/II) exhibited significantly higher activities in vitro than in vivo (15.3–26.3% of wild type in vitro activity compared to 0.6–1.6% in vivo activity). Even after 50 h of growth, when *rnfE,* polar ORF14 and polar *fdxN* mutants

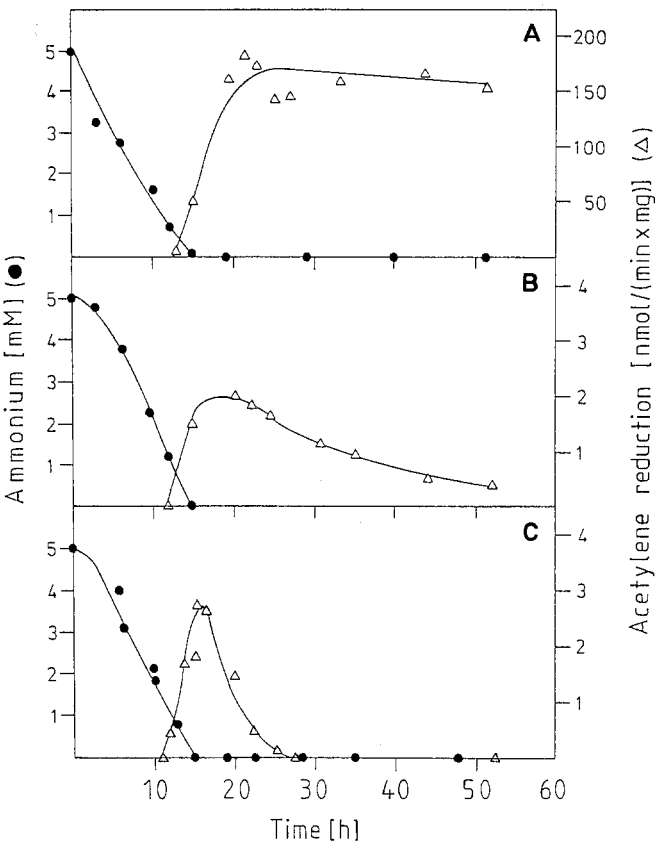

Fig. 5A–C. Time course of NH_4^+ consumption and nitrogenase activity. The NH_4^+ consumption *(filled circles)* and nitrogenase activity measured as acetylene reduction *(open triangles)* was followed for *R. capsulatus* wild type (**A**), for the non-polar *fdxN* insertion mutant R346I (**B**) and for the *rnfE* deletion mutant R112AI (**C**). Details of the construction of these mutants are given in Fig. 1B

(R112AII, R347II, R346II) showed no in vivo activity at all (Fig. 5C), considerable in vitro activity could still be detected (Table 4).

To analyse the correlation between nitrogenase activities and the relative amounts of nitrogenase proteins present in *R. capsulatus* wild type and mutant strains described in this study, Western blotting and immuno-

Table 4. Percentage of in vivo and in vitro acetylene reduction activity of *R. capsulatus* wild type and mutants

Strain	Genotype[a]	Type of time course[b]	Acetylene reduction[c]		
			% in vivo (20 h)	% in vitro (20 h)	% in vitro (50 h)
B10	Wild type	A	100	100	100
R112AII	*rnfE*Δ::Tc<	C	1.4	26.3	19.8
R347I	ORF14Δ::Gm>	A	86.5	n.d.	75.0
R347II	ORF14Δ::Gm<	C	1.6	n.d.	19.1
R346I	*fdxN*::Gm>	B	1.3	17.1	11.3
R346II	*fdxN*::Gm<	C	0.6	15.3	5.3

[a] Details of the construction of mutant strains are given in Table 2 and Fig. 1. Arrowheads indicate non-polar (>) and polar (<) insertions

[b] See Fig. 5

[c] Cells were grown on RCVB medium supplemented with 2.5 mM $(NH_4)_2SO_4$. In vivo activity of 100% was 150 nmol acetylene reduced per min per mg; 100% of in vitro activity was 6.3 nmol acetylene reduced per min per mg. The values shown are derived from at least two independent experiments with two determinations

n.d., not determined

Table 5. Relative levels of nitrogenase proteins in *R. capsulatus* mutants and wild type

Strain	Genotype[a]	20 h[b]			50 h[b]		
		Rc1α	Rc1β	Rc2	Rc1α	Rc1β	Rc2
B10	Wild type	100	100	100	100	100	100
R112AII	*rnfE*Δ::Tc<	39	38	46	18	38	22
R155CI	*rnfC*::Gm>	44	28	36	13	20	18
R155CII	*rnfC*::Gm<	60	30	33	14	17	23
R363I	*rnfA*Δ::Tc>	40	31	38	35	34	27
R363II	*rnfA*Δ::Tc<	52	35	12	39	31	39
R347I	ORF14Δ::Gm>	70	60	130	83	100	96
R347II	ORF14Δ::Gm<	20	36	25	14	35	31
R346I	*fdxN*::Gm>	18	43	25	30	59	50
R346II	*fdxN*::Gm<	20	43	26	11	27	27
R213AI	*rnfF*Δ::Km>	9	35	30	19	38	46
R213AII	*rnfF*Δ::Km<	7	21	31	13	37	41

[a] Details of the construction of mutant strains are given in Table 2 and Fig. 1

[b] Cells were grown on RCVB medium containing 2.5 mM $(NH_4)_2SO_4$. Samples were harvested after 20 h and 50 h, respectively, and subjected to gel electrophoresis followed by immunodetection of nitrogenase subunits Rc1α, Rc1β and Rc2 (see Fig. 6). Protein bands were scanned and relative amounts are given as percentage of levels in wild type

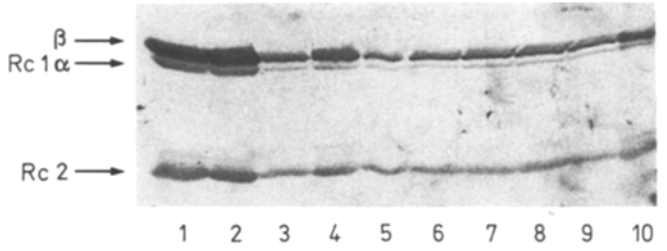

Fig. 6. Immunodetection of nitrogenase subunits. Cells were grown on RCVB medium containing 2.5 mM $(NH_4)_2SO_4$. Samples were harvested after 50 h. Equal amounts of total proteins were separated in SDS-polyacrylamide gels prior to Western blotting. Nitrogenase subunits were visualised using antisera against component 1 of *R. capsulatus* and component 2 of *Azotobacter vinelandii*. Lane 1, *R. capsulatus* B10 (wild type); lane 2, R347I (non-polar ORF14 mutant); lane 3, R347II (polar ORF14 mutant); lane 4, R346I (non-polar *fdxN* mutant); lane 5, R346II (polar *fdxN* mutant); lane 6, R213BI (non-polar *rnfF* mutant); lane 7, R213BII (polar *rnfF* mutant); lane 8, R213AI (non-polar *rnfF* deletion mutant); lane 9, R213AII (polar *rnfF* deletion mutant); lane 10, purified nitrogenase components of *R. capsulatus*

Table 6. Effect of 0.5 mM metronidazole on growth of *R. capsulatus* wild type and mutants

Strain	Genotype[a]	Percentage growth[b]
B10	Wild type	15
R112AII	*rnfE*Δ::Tc<	95
R155CI	*rnfC*::Gm>	88
R155CII	*rnfC*::Gm<	75
R363I	*rnfA*Δ::Tc>	70
R363II	*rnfA*Δ::Tc<	60
R347I	ORF14Δ::Gm>	18
R347II	ORF14Δ::Gm<	69
R346I	*fdxN*::Gm>	85
R346II	*fdxN*::Gm<	78
R213AI	*rnfF*Δ::Km>	75
R213AII	*rnfF*Δ::Km<	76

[a] Details of the construction of mutant strains are given in Table 2 and Fig. 1

[b] Cells were grown on RCVB medium containing 5 mM L-serine as nitrogen source and 0.5 mM metronidazole. The percentage growth is the ratio of protein values for strains grown with and without added metronidazole

detection were performed (Fig. 6). As shown in Table 5, all mutants that were unable to grow diazotrophically or showed reduced ability to grow with N_2 (R346I) contained significantly lower amounts of the α and β subunits of component 1, as well as lower amounts of component 2 of nitrogenase.

Inhibition of growth by metronidazole

Analyses of the in vitro nitrogenase activities of *R. capsulatus* mutants carrying insertions/deletions in *rnf* genes and the ferredoxin-encoding *fdxN* gene indicated that the products of these genes are involved in electron transfer to nitrogenase. The antimicrobial drug metronidazole can accept electrons from reduced ferredoxins and is reduced to lethal derivatives of the drug (Schmidt et al. 1977; Hallenbeck and Vignais 1981). Therefore, metronidazole can be used to select mutants defective in electron transport to nitrogenase (Willison and Vignais 1982; Wall et al. 1984). To analyse the effect of metronidazole on the mutants constructed in this study, growth under conditions of nitrogenase derepression (L-serine as nitrogen source) was compared in the absence and presence of 0.5 mM metronidazole. As shown in Table 6, the wild type and the non-polar ORF14 mutant (Nif$^+$ phenotype) reached only 15–18% of the biomass (protein) levels of cultures grown without metronidazole, whereas all other mutants reached 60–95% even in the presence of metronidazole.

Discussion

The DNA sequence data presented in this study complete the nucleotide sequence of *R. capsulatus nif* region A. A detailed genetic analysis of a contiguous DNA fragment of 26 182 bp containing 26 genes is now available. The Nif^+ phenotype of different insertion and deletion mutants upstream and downstream of *nif* region A proved that no further nitrogen fixation genes are located immediately adjacent to this gene cluster. In addition to genes which were identified in other nitrogen-fixing organisms, *R. capsulatus nif* region A harbours two transcriptional units that encode a new class of nitrogen fixation genes. Six of these so-called *rnf* genes, which exhibited no homology to known *nif* genes or other sequences present in data bases, were shown to be essential for nitrogen fixation, whereas the gene products of ORF14 and ORF10 were not required for this process. One of these newly identified transcriptional units also contains two genes, *fdxC* and *fdxN*, encoding a plant-type [2Fe-2S] and a bacterial-type [4Fe-4S] ferredoxin (Schatt et al. 1989; Grabau et al. 1991; Saeki et al. 1991), respectively. It was shown previously that purified ferredoxin I of *R. capsulatus*, which is encoded by *fdxN*, is capable of in vitro electron transfer to nitrogenase (Hallenbeck et al. 1982; Yakunin and Gogotov 1983). Therefore, it was assumed that the *fdxN* gene product is the actual physiological electron donor to nitrogenase. To test whether the gene products of *rnf* genes are also involved in electron transfer to nitrogenase, in vitro nitrogenase activities were determined using dithionite as an artificial electron donor. Extracts prepared from *fdxN* mutants as well as from *rnf* mutants could be complemented in vitro by dithionite (Table 4), indicating that the products of these genes are involved in electron transfer. However, the mutants could be complemented only to 5–26% of the activity found for wild type extracts. In *K. pneumoniae*, only two *nif* gene products, the pyruvate:flavodoxin-oxidoreductase NifJ and the flavodoxin NifF, are necessary for electron transfer to nitrogenase (Shah et al. 1983; Nieva-Gomez et al. 1980). In vitro nitrogenase activities of crude extracts of *nifJ* and *nifF* mutants could also be restored only partially by dithionite (Hill and Kavanagh 1980). To test if the reduced in vitro activities in *R. capsulatus fdxN* and *rnf* mutants correlate with the amount of nitrogenase proteins present in the cells, the nitrogenase components were quantified by immunological methods. All mutants defective in electron transfer to nitrogenase contained only about 30% components 1 and 2 of nitrogenase (Table 5; Fig. 6). Since this decrease in nitrogenase components was independent of the mutation site, a more general mechanism, influencing either the synthesis or stability of both nitrogenase components, must be responsible for the decrease of nitrogenase subunits and not a regulatory gene located in this part of *nif* region A. The hypothesis that *fdxN* and *rnf* gene products are involved in electron transport was corroborated by analysing the effect of metronidazole. These experiments (Table 6) demonstrated that all mutants analysed in this study had a higher growth yield in the presence of metronidazole compared to the wild type, indicating that the cells contained lesser amounts of reduced ferredoxins.

Analysis of in vivo nitrogenase activities demonstrated that all mutants in this part of *R. capsulatus nif* region A exhibited very low but significant acetylene reduction activities after ammonia depletion (Fig. 5). This activity disappeared completely in *rnfC*, *rnfE* and *rnfF* mutants after 30 h. In contrast, *fdxN* and *rnfA* mutants retained low nitrogenase activities for more than 50 h, indicating that the electron transport to nitrogenase can be maintained in these two mutants at a very low level by some other unidentified component. These results suggested that RnfC, RnfE and RnfF might be components of a ferredoxin-reducing system, which is necessary to reduce the not yet identified low efficiency electron donor under nitrogen-fixing conditions, whereas FdxN and RnfA are involved in supplying electrons to nitrogenase reductase directly.

Since the *rnf* gene products exhibited no homology to known proteins, possible functions can be deduced only from secondary structure analysis. These predictions suggested that the *rnf* genes encode four potential membrane proteins and two putative iron-sulphur proteins, each of which might contain at least two [4Fe-4S] clusters. Cysteine residues within the membrane proteins predicted to be exposed on the cytosolic side of the membrane, supported the hypothesis that these *rnf* gene products, and probably also the plant-type ferredoxin FdxC, might form a membrane complex involved in the reduction of the *fdxN*-encoded [4Fe-4S] ferredoxin. At the moment one can only speculate that this putative membrane complex might be an oxido-reductase with an unknown substrate or a system responsible for reverse electron flux, which uses the membrane potential generated by the photosystem to reduce ferredoxins, or that a direct, non-cyclic electron flux occurs to nitrogenase, which is catalysed by these gene products.

The two transcriptional units containing *fdx* and *rnf* genes are preceded by putative NifA- and σ^{54}-dependent promoters. This is in line with the finding that the expression of *fdxN* is negatively regulated by ammonium and oxygen (Schatt et al. 1989; Suetsugu et al. 1991). Only under nitrogenase-derepressing conditions were two mRNAs of approximately 330 and 750 nucleotides, which are homologous to *fdxN*, identified by Schatt et al. (1989). According to our sequence data and the analysis of polar and non-polar insertion mutants, *fdxN* is co-transcribed with ORF14-*fdxC*-*fdxN*-*rnfF* from a σ^{54}-dependent promoter in front of ORF14 (Fig. 2). Therefore, it is likely that cleavage of a long primary transcript results in one transcript species containing both *fdxC* and *fdxN*, and one transcript species containing only *fdxN*.

Comparison of all *R. capsulatus nif* promoters revealed a high degree of sequence conservation not only of the canonical $-12/-24$ promoter but also of additional nucleotides (Fig. 3). This extended motif might reflect differences in the regulation of *nif* genes in *R. capsulatus* compared to other nitrogen fixing organisms. In contrast to other diazotrophs, *nif* gene expression in *R. capsulatus* is not directly regulated by a general nitrogen regulation (*ntr*) system. Homologues to NtrA,

NtrB and NtrC, which are encoded by *nifR4, nifR2* and *nifR1* in *R. capsulatus* are specific for the expression of *nif* genes (for reviews see Hübner et al. 1991; Foster-Hartnett and Kranz 1992). The extended conserved motif in *R. capsulatus nif* promoters might therefore be necessary for the recognition by the NifR4-RNA polymerase complex or might be a target for additional regulatory proteins.

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