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Isolation and Characterization of the *nifUSVW-rpoN* Gene Cluster from *Rhodobacter sphaeroides*

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The *rpoN* gene from *Rhodobacter sphaeroides* was isolated from a genomic library via complementation of a *Rhodobacter capsulatus rpoN* mutant. The *rpoN* gene was located on a 7.5-kb *Hin*dIII-*Eco*RI fragment. A Tn*5* insertion analysis of this DNA fragment showed that a minimal DNA fragment of 5.3 kb was required for complementation. Nucleotide sequencing of the complementing region revealed the presence of *nifUSVW* genes upstream from *rpoN*. The *rpoN* gene was mutagenized via insertion of a gene encoding kanamycin resistance. The resulting *rpoN* mutant was not impaired in diazotrophic growth and was in all respects indistinguishable from the wild-type strain. Southern hybridizations using the cloned *rpoN* gene as a probe indicated the presence of a second *rpoN* gene. Deletion of the *nifUS* genes resulted in strongly reduced diazotrophic growth. Two conserved regions were identified in a NifV LeuA amino acid sequence alignment. Similar regions were found in pyruvate carboxylase and oxaloacetate decarboxylase. It is proposed that these conserved regions represent keto acid-binding sites.

The sigma factor of RNA polymerase confers promoter specificity to the holoenzyme. After initiation of transcription, the sigma factor is released, and the core RNA polymerase proceeds to transcribe the DNA. In addition to the major sigma factor, other sigma factors are frequently employed by bacteria (20). One of these alternative sigma factors is RpoN (23). RNA polymerase containing RpoN initiates transcription at promoters containing the consensus sequence CTGG..8 bp..TTGCA 26 bp upstream from the transcription initiation site (9). Although RpoN was initially recognized for its role in nitrogen metabolism, it now is firmly established that RpoN is required for a wide range of metabolic activities, including hydrogen oxidation, use of C_4 dicarboxylic acids, assimilation and dissimilation of nitrate, flagellation, and formation of pili (37). The metabolic role of RpoN is species dependent. The *rpoN* gene has been cloned from a number of organisms and was found to be flanked by two conserved open reading frames (ORFs) of unknown function (6, 12, 27, 28, 35, 36, 41–43, 49a, 50, 58). In *Rhodobacter capsulatus*, the *rpoN* gene is not flanked by these conserved ORFs but is located between *nifHDK*, which encodes the Fe and FeMo proteins of nitrogenase, and *nifA*, which encodes a transcriptional activator of the nitrogen fixation (*nif*) genes (31). The *nifA* gene is duplicated in *R. capsulatus*. Upstream from the duplicated *nifA* gene, the *nifUSVW* genes are present instead of *rpoN* (34). NifV catalyzes the synthesis of homocitrate, an integral part of the FeMo cofactor of nitrogenase (24–26). The *nifU*, *nifS*, and *nifW* genes are involved in maturation or stability of nitrogenase (30, 46).

Rhodobacter sphaeroides, a metabolically versatile organism, can grow in five different growth modes. During autotrophic or photoheterotrophic growth, it employs two forms of ribulose bisphosphate carboxylase (RuBisCO). These forms of RuBisCO are encoded in two similar but not identical operons in which genes encoding other enzymes required for CO_2 fixation are also found (16–19). In the upstream region of the form I operon, there exists a sequence very similar to the RpoN consensus promoter sequence (17), suggesting that transcription of this operon may be dependent on RpoN (57). To probe the role of RpoN in the expression of the *cfx* operons and metabolism of *R. sphaeroides* in general, we set out to clone and inactivate the *rpoN* gene of *R. sphaeroides*. In this article, we report the cloning and sequence analysis of *rpoN* from *R. sphaeroides* and its organization in an operon with the *nifUSVW* genes. Evidence for the presence of a second copy of the *rpoN* gene is also presented.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *R. capsulatus* and *R. sphaeroides* strains were grown photoheterotrophically in Ormerod's medium (45) supplemented with malate (MSM) at 30°C in screw-cap tubes or in GasPak anaerobic jars (American Scientific Products, McGaw Park, Ill.) as previously described (61). Nitrogen fixation capacity was tested by growth under photoheterotrophic conditions with MSM without NH_4Cl. *Rhodobacter* strains were also grown heterotrophically on peptone-yeast extract or minimal medium supplemented with fructose in Erlenmeyer flasks or on agar plates (61). *Escherichia coli* strains were grown on Luria-Bertani (LB) or M9 medium at 37°C (51). When appropriate, the following supplements were added (concentrations given in micrograms per milliliter except as otherwise noted): ampicillin, 50; 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 20; isopropyl-β-D-thiogalactoside, 0.1 mM; kanamycin, 25; rifampin, 50; spectinomycin, 100; streptomycin, 50; and tetracycline, 12.5 (*E. coli*) or 1 (*R. capsulatus* and *R. sphaeroides*). Agar (1.5%) was added for solid media.

DNA manipulations. Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly (10). Chromosomal DNA was isolated via the sarcosyl-lysate procedure

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TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Source or reference
E. coli		
JM107	*endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15	59
S17-1	*thi pro* r$^-$ m$^+$ Sm^r Tp^r *recA* RP4-2 (Tc::Mu, Km::Tn*7*)	54
SM10	*supE44 hsdR thi thr leuB6 lacY1 tonA21 recA* (Tc::Mu, Km::Tn*7*)	54
RM1628	*pro met* Km^r (Tn*5*)	R. J. Meyer
R. sphaeroides		
HR	Sm^r	61
HRI	Sm^r Rif^r	This study
EM1	Sm^r *rpoN*::Km^r	This study
EM11	Sm^r Rif^r Δ*nifUS*::Sp^r	This study
R. capsulatus		
LJ1	*rpoN*	60
Plasmids		
pRK404	Tc^r, broad-host-range vector	13
pSUP202	Ap^r Tc^r Cm^r *mob*$^+$	54
pUC1318	Ap^r, pUC vector with hybrid cloning site	32
pTZ18U	Cloning vector	Bio-Rad
pTZ19U	Cloning vector	Bio-Rad
pHP45-Ω	Ap^r Sp^r/Sm^r	48
pUC1318K	Km^r Ap^r, pUC1318K with a 1.5-kb *Hin*dIII-*Sal*I fragment from Tn*5* encoding Km^r	This study
pNIT1	Tc^r, pVK102 with a 18-kb fragment of *R. sphaeroides* chromosomal DNA encoding *rpoN*	This study
pNIT2	Tc^r, pVK102 with a 20-kb fragment of *R. sphaeroides* chromosomal DNA encoding *rpoN*	This study
pNIT11	Tc^r, pRK404 with a 9-kb *Hin*dIII fragment from pNIT1 encoding *rpoN*	This study
pNIT12	Tc^r, pRK404 with a 9-kb *Hin*dIII fragment from pNIT2 encoding *rpoN*	This study
pNIT122	Tc^r, pRK404 with a 7.5-kb *Hin*dIII-*Eco*RI fragment from pNIT12	This study
pSNT108	Ap^r, pTZ18U with a 2.7-kb *Hin*dIII-*Sal*I fragment from pNIT122	This study
pSNT4	Ap^r, pTZ18U with a 1.2-kb *Bam*HI fragment from pNIT122	This study
pKTN1	Ap^r Tc^r, pSUP202 with a 1.2-kb *Bam*HI fragment encoding *rpoN*	This study
pKTN2	Ap^r Tc^r Km^r, pKTN4 with a 1.5-kb *Sac*I fragment from pUC1318K encoding Km^r	This study
pKTN3	Ap^r Sp^r, pSNT108 with a 1.2-kb *Stu*I deletion and a 2.0-kb *Sma*I fragment from pHP45-Ω encoding Sp^r	This study
pKTN4	Ap^r Tc^r Sp^r, pSUP202 with a 3.5-kb *Pvu*II fragment from pKTN3	This study

(15). DNA-modifying enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, Md.) and were used according to the manufacturer's instructions. Analysis of restriction digests by gel electrophoresis and transformation of *E. coli* were performed as described by Sambrook et al. (51). DNA fragments were isolated from agarose gels by adsorption to glass (Geneclean kit; Bio 101, La Jolla, Calif.).

Southern hybridizations. DNA was transferred to nylon membranes (GeneScreen Plus; Du Pont) by electroblotting as specified by the manufacturer. Prehybridization, hybridization, and washing procedures were performed at 63°C according to protocols suggested by the manufacturer, unless specified otherwise. DNA fragments used as probes were labelled with [^{32}P]dCTP by using a random primed labelling kit supplied by Boehringer (Mannheim, Germany).

Mobilization of plasmids. Mobilization of plasmids using *E. coli* S17-1 or SM10 containing the appropriate plasmids as a donor was performed essentially as described by Simon et al. (54). Triparental mating procedures were described previously (62).

Tn*5* mutagenesis. *E. coli* RM1628 containing pNIT122 was grown on LB medium containing tetracycline and kanamycin until the late exponential growth phase; 0.1 ml of the culture was used to inoculate 50 ml of LB medium, while the kanamycin concentration was increased to 300 μg/ml. When the culture reached the late exponential phase, 0.1 ml was used to inoculate the same medium and allowed to grow overnight. Plasmid DNA was subsequently isolated and used to transform *E. coli* S17-1 to kanamycin resistance (Km^r) and tetracycline resistance (Tc^r).

Construction of *R. sphaeroides rpoN* and *nifUS* mutants. A 1.4-kb *Hin*dIII-*Sal*I fragment from Tn*5*, encoding kanamycin resistance, was treated with Klenow enzyme and ligated into *Hin*dIII-digested, Klenow enzyme-treated pUC1318, generating a Km^r cartridge flanked by five unique restriction sites (pUC1318K). The 1.2-kb *Bam*HI fragment internal to *rpoN* was treated with Klenow enzyme and ligated into *Eco*RI-digested, Klenow enzyme-treated pSUP202, generating pKTN1. A 1.4-kb *Sac*I fragment containing the Km^r cartridge derived from pUC1318K was cloned into the unique *Sac*I site within the *rpoN* gene, generating pKTN2.

A 1.4-kb *Stu*I fragment from pSNT108 was replaced with the 2.0-kb *Sma*I fragment (Ω element) from pHP45-Ω, generating pKTN3. pKTN3 was digested with *Pvu*II, and the fragment containing the Ω element was cloned into *Sca*I-digested pSUP202, generating pKTN4.

E. coli SM10 and S17-1 were transformed with pKTN2 and pKTN4, respectively. The pKTN2 plasmid was subsequently mobilized to *R. sphaeroides* HR, and pKTN4 was mobilized to *R. sphaeroides* HRI. Exconjugants were selected by screening for streptomycin resistance (Sm^r) and Km^r (pKTN2) or rifampin resistance (Rif^r) and spectinomy-

cin resistance (Sp^r) (pKTN4). Since pSUP202 cannot replicate in *R. sphaeroides*, Km^r or Sp^r can be obtained only via integration of the plasmid into the chromosome. Selection for replacement of the wild-type gene with the mutated gene via a double recombination event was achieved by screening exconjugants for tetracycline susceptibility (Tc^s). Southern hybridizations confirmed that a double recombination had taken place.

Nucleotide sequencing. The 2.6-kb *Hin*dIII-*Sal*I fragment of pNIT122 was subcloned in both orientations in pTZ19U. A nested set of unidirectional deletions was subsequently created by digestion with exonuclease III and mung bean nuclease, essentially as described by Henikoff (21). Other subclones were obtained by digestion of pNIT122 with the appropriate restriction enzymes and subsequent ligation with pTZ18U and pTZ19U. Infection of *E. coli* JM107 containing the derivatives of pTZ18U and pTZ19U with the helper phage M13KO7 (59) and purification of single-stranded DNA were performed as previously described (51). Dideoxy sequencing reactions were performed with modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio) and [^{35}S]dATP as described by the manufacturer. In addition to the sequencing reactions using dGTP, at least one strand was also sequenced with dITP to eliminate compressions. The nucleotide sequence data were compiled and analyzed by using the programs supplied in the GCG sequence analysis software package (Genetics Computer Group, Madison, Wis.).

Nucleotide sequence accession number. The sequences reported in this article have been entered into GenBank under accession number M86823.

RESULTS

Cloning of the *R. sphaeroides rpoN* gene. *R. capsulatus* LJ1 carries a mutation in *rpoN* (31) and is unable to grow under nitrogen-fixing conditions (60). A genomic library of *R. sphaeroides* DNA previously used to isolate carbon dioxide fixation genes (62) was mobilized into *R. capsulatus* LJ1 via triparental mating as described in Materials and Methods. The mating mixture was plated on MSM plates lacking a nitrogen source and incubated under nitrogen-fixing growth conditions to select for complementation of *R. capsulatus* LJ1. Nitrogen-fixing colonies were purified on fructose-tetracycline agar. Plasmid DNA was subsequently isolated and used to transform *E. coli* to Tc^r. Two different plasmids which had retained the capability to complement *R. capsulatus* LJ1 could be distinguished (pNIT1 and pNIT2). Restriction mapping showed that these plasmids share a 9-kb *Hin*dIII fragment.

Localization of *rpoN*. The 9-kb *Hin*dIII fragment common to pNIT1 and pNIT2 was subcloned in pRK404, generating pNIT11 and pNIT12, respectively. The 9-kb *Hin*dIII fragment was found to complement the *rpoN* mutation of *R. capsulatus* LJ1. Subsequent subcloning of the 9-kb *Hin*dIII fragment reduced the complementing region to a 7.5-kb *Hin*dIII-*Eco*RI fragment, generating pNIT122. The ability to complement was not dependent on the orientation of the insert with respect to the *lac* promoter of pRK404. Plasmid pNIT122 was introduced into *E. coli* RM1628, and transposition of Tn*5* from the chromosome to pNIT122 was allowed to occur. The resulting pNIT122:Tn*5* plasmids were mapped by restriction analysis, and those plasmids containing Tn*5* within the 7.5-kb *Hin*dIII-*Eco*RI insert were mobilized to *R. capsulatus* LJ1 via conjugation. Tc^r exconjugants were tested for their ability to grow under nitrogen-fixing conditions (Fig. 1). The Tn*5* insertions delineated a 5.3- to 5.7-kb complementing region on pNIT122.

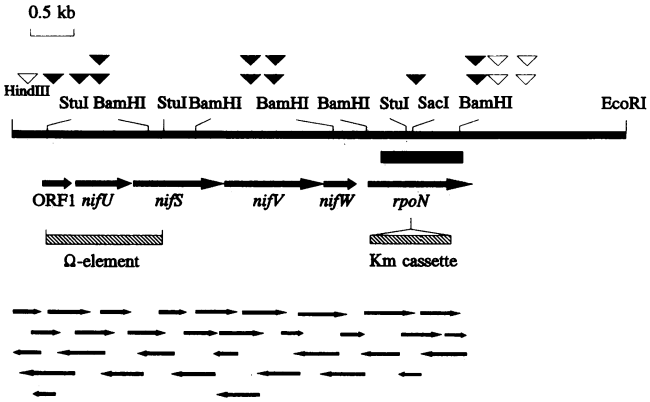

FIG. 1. Restriction map of the *nifUSVW-rpoN* cluster from *R. sphaeroides*. Closed triangles represent Tn*5* insertions preventing the complementation of *R. capsulatus* LJ1. Open triangles represent Tn*5* insertions that do not affect complementation. The positions and directions of transcription of the genes and ORF1 are indicated by arrows below the restriction map. The black bar shows where the heterologous *rpoN* probe hybridized. The replacement of the *Stu*I fragment with the Ω element and the insertion of the kanamycin resistance (Km) cassette in *rpoN* are indicated with striped bars. The sequencing strategy is shown beneath the restriction map.

The position of *rpoN* was determined by Southern hybridization of restriction digests of pNIT122 using the *rpoN* gene from *R. capsulatus* as a heterologous probe. The probe hybridized to an *Eco*RI-*Pst*I fragment (data not shown and Fig. 1). Combining the results from the Tn*5* insertional analysis and the Southern hybridizations, we concluded that *rpoN* is located at the right end of the complementing region and is transcribed from left to right (Fig. 1). Since RpoN has a molecular weight of about 54,000 (37), the coding capacity of the region delineated by the Tn*5* insertions is in excess of what is needed to accommodate *rpoN*. This suggested that transcription of *rpoN* was dependent upon transcription through upstream genes.

Nucleotide sequence of the *rpoN* gene and upstream DNA. In order to characterize *rpoN* and the upstream sequences, the nucleotide sequence of the complementing region was determined according to the sequencing strategy depicted in Fig. 1. Six ORFs, transcribed in the same direction and preceded by plausible ribosome binding sites, could be distinguished. An analysis of the codon usage showed that all six ORFs could have a coding function (data not shown). The ORFs may encode proteins with molecular weights of 10,780 (ORF1), 25,407 (ORF2), 41,225 (ORF3), 40,664 (ORF4), 11,905 (ORF5), and 47,870 (ORF6). The intergenic region between the first five ORFs is small, varying from 0 bp (ORF2-ORF3-ORF4) to 9 bp (ORF1-ORF2) and 10 bp (ORF4-ORF5). The intergenic region between ORF5 and ORF6 is larger (100 bp). A 33-bp stretch of only C and G nucleotides containing a 24-bp inverted repeat is present in the last intergenic region. A stretch of T's following the inverted repeat, characteristic of a *rho*-independent terminator, is not present (47). The nucleotide sequence and the predicted amino acid sequences are shown in Fig. 2.

Southern hybridization experiments strongly suggested that ORF6 might be an *rpoN* gene of *R. sphaeroides*. A search of the Swiss-Protein data base, using the deduced amino acid sequence of ORF6 as the query sequence,

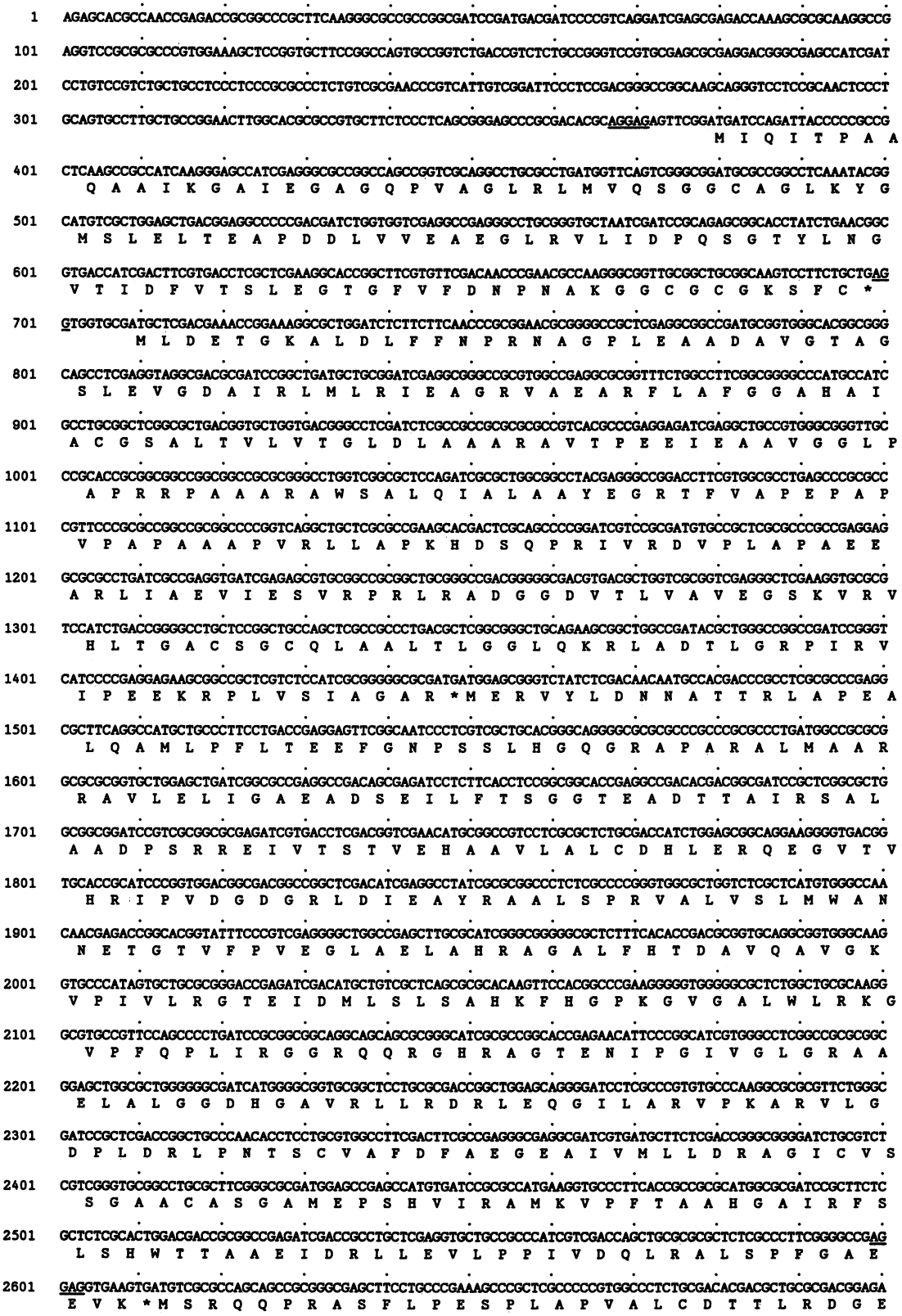

FIG. 2. Nucleotide sequence of the DNA fragment encoding ORF1, *nifUSVW-rpoN*. Amino acids are represented below the nucleotide sequence by the single-letter code. Putative ribosome binding sites are underlined. Asterisks represent stop codons.

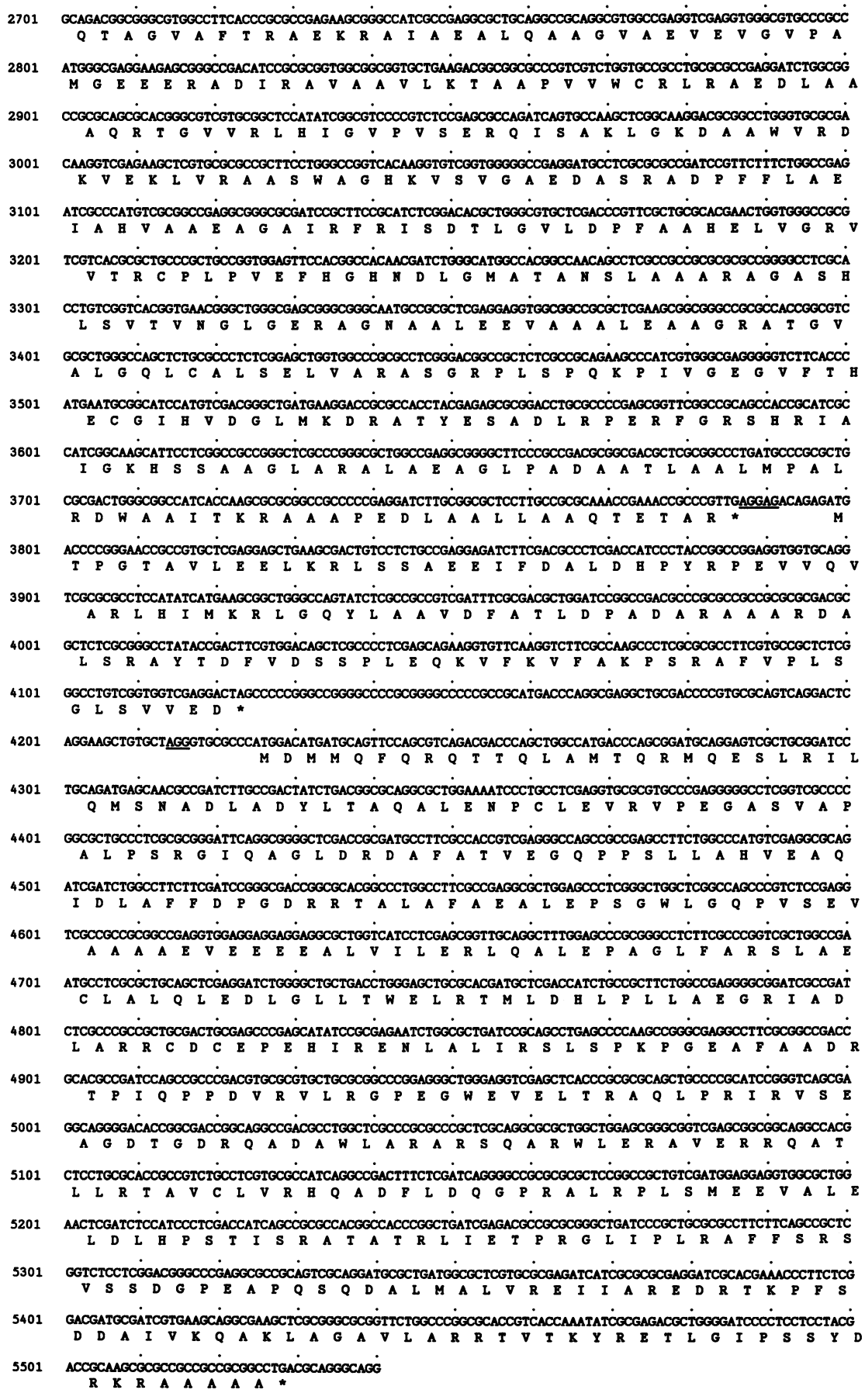

FIG. 2—*Continued*

revealed extensive similarities with other RpoN proteins. We therefore identified ORF6 as *rpoN*.

RpoN. The *rpoN* gene has three possible initiation codons. Only the first initiation codon is preceded by a possible ribosome binding site, and it was chosen as the start of the *rpoN* coding sequence (Fig. 3). The RpoN proteins from the two purple nonsulfur bacteria are more closely related to each other than to any other RpoN protein (51% identity). The similarity with the other RpoN proteins in Fig. 3 did not exceed 35% identity. With the exception of the *R. capsulatus* protein, which has a pI of 6.8, the RpoN proteins all exhibit a pI of <5. This is also true for *R. sphaeroides* (pI 4.9). Merrick et al. (41) identified three regions of similarity in the RpoN sequences from several sources. Region I (residues 1 to 68) is characterized by a high Gln content; region III (residues 69 to 434) contains a helix-turn-helix motif (residues 315 to 340) and a stretch of highly conserved residues, the so called RpoN box (residues 408 to 417). The poorly conserved spacer region between regions I and III is virtually absent in the *R. sphaeroides* and *R. capsulatus* sequences.

ORF1, *nifU*, *nifS*, and *nifW*. Searches of the Swiss-Protein data base using the ORFs upstream from *rpoN* as query sequences led to the tentative identification of *nifU* (ORF2), *nifS* (ORF3), and *nifW* (ORF5). The alignment of the ORF1, NifU, NifS, and NifW proteins from *R. sphaeroides* with those from other sources is shown in Fig. 4. ORF1 displayed a high degree of similarity to ORF6 of the *Azotobacter vinelandii* nitrogen fixation cluster (7, 29). NifU proteins from other diazotrophic organisms have seven conserved Cys residues, which are present as Cys-X-X-Cys and Cys-X-Cys motifs (3, 7, 8, 11, 29, 44). The *R. sphaeroides* NifU protein was poorly conserved compared with the other NifU proteins (Fig. 4B). In fact, only three Cys residues, two of which are also conserved in the other NifU proteins, can be found in the *R. sphaeroides* NifU protein. In the NifU proteins from *A. vinelandii* and *Klebsiella pneumoniae*, a consensus sequence (Cys-X-X-Cys-His) typical of a heme-binding site of cytochrome *c* proteins is present (40). The heme-binding site is present in a region completely absent from the NifU protein of *R. sphaeroides*.

In contrast to the NifU proteins, the NifS proteins displayed a high degree of similarity throughout the amino acid sequence (Fig. 4C).

ORF1 upstream from *nifU* displayed a high degree of similarity with an ORF in the nitrogen fixation clusters from an *Anabaena* sp. (11, 42) and *A. vinelandii* (7, 29), especially at the carboxyl terminus (Fig. 4A). This ORF is found at the same position in *A. vinelandii*, upstream from *nifU*. Starting at residue 97 in the *R. sphaeroides* sequence, a motif (G-X-X-G-X-G-K-S) characteristic of a nucleotide-binding site (22) was present in a highly conserved region of the protein.

Analysis of NifV. The data base searches were used to identify ORF4 as *nifV*. Besides being similar to other NifV proteins, NifV is similar to isopropylmalate synthase (LeuA), as previously noted (14). Inspection of an amino acid sequence alignment between the NifV and LeuA proteins reveals two regions of high similarity (Fig. 5, regions 1 and 2). To determine whether these conserved regions are also present in other proteins, region 2 in the sequence alignment was used to construct a profile as described by Gribskov et al. (19a). The Swiss-Protein data base was searched in order to find proteins matching the region 2 profile. By using this technique, a data base can be searched with a sequence alignment, a procedure which is more sensitive than conventional searches with a single amino acid sequence.

Pyruvate carboxylase and oxaloacetate decarboxylase were found to contain a sequence similar to region 2 of NifV. In addition, a sequence similar to region 1 was present at the proper spacing relative to region 2, compared with the sequence alignment in Fig. 5. The sequence between regions 1 and 2 was not highly conserved.

***R. sphaeroides rpoN* mutant.** To determine the role of *rpoN* in the metabolism of *R. sphaeroides*, an *rpoN* mutant strain, EM1, was constructed. The *rpoN* mutation did not prevent diazotrophic growth of *R. sphaeroides* EM1. *R. sphaeroides* EM1 was not a glutamine auxotroph, nor was growth on malate impeded. These phenotypes are generally associated with a *rpoN* mutation. In the Southern hybridization used to verify the construction of the *rpoN* mutation (Fig. 1), a 4-kb band could be seen in addition to the 1.2-kb band representing *rpoN* (data not shown). The Southern hybridization was repeated at a lower stringency (60°C), using a 0.5-kb *Sac*I-*Bam*HI fragment internal to *rpoN* and lacking the variable 3′ end of the *rpoN* gene as a probe. Again, 1.2- and 4.0-kb *Bam*HI fragments hybridized to the *rpoN* probe (Fig. 6).

***R. sphaeroides nifUS* mutant.** The functionality of the *nifUSVW-rpoN* operon was determined by deleting part of the *nifUS* genes and replacing them with an Ω element (Fig. 1). Since the Ω element is flanked by transcriptional terminators, it has a polar effect on the transcription of the genes downstream (48), *nifVW-rpoN*. Therefore, any phenotype resulting from this insertion may be caused by either the deletion of *nifUS* or the lack of transcription of downstream genes. *R. sphaeroides* EM11 and three other independently isolated mutants displayed strongly reduced diazotrophic growth. The *R. sphaeroides* EM11 colonies appearing after 1 month of incubation were very small and only slightly pigmented compared with the wild-type colonies. The growth of the mutant strain was indistinguishable from that of the parental strain on media containing NH_4^+.

DISCUSSION

In contrast to the situation in other bacteria, where the *rpoN* gene is flanked by two conserved ORFs of unknown function, the *rpoN* gene of *R. capsulatus* is located between *nif* genes. The present study shows that this is also true for another purple nonsulfur bacterium, *R. sphaeroides*. In *R. capsulatus*, the *nifAB* genes are duplicated. The genetic organization of region A is *nifUSVW-nifAB*, and the organization of region B is *nifHDK-nifU-rpoN-nifAB* (1, 33, 34, 39). A duplication of a *nifUSVW-rpoN-nifAB* cluster and subsequent deletion of *nifSVW-rpoN* in region B and deletion of *rpoN* in region A could explain this organization in *R. capsulatus*. Furthermore, it would explain the unusual organization in region B, where a *nifU* gene is present in the absence of *nifSV*. Our finding that a *nifUSVW-rpoN* cluster actually exists in a bacterium belonging to the same genus lends credence to this hypothesis.

Insertions of Tn*5* in the *R. sphaeroides nifUSVW* genes located on pNIT122 prevented complementation of the *rpoN* mutation of *R. capsulatus* (31, 60) by this plasmid. This strongly suggests that transcription of *rpoN* of *R. sphaeroides* is dependent on a promoter upstream from *nifUSVW*. The absence of a recognizable terminator structure between *nifW* and *rpoN* further supports this conclusion. The cotranscription of *rpoN* with upstream genes has not been found in other bacteria. The promoter of the *R. capsulatus rpoN* gene was mapped downstream from *nifHDK* via

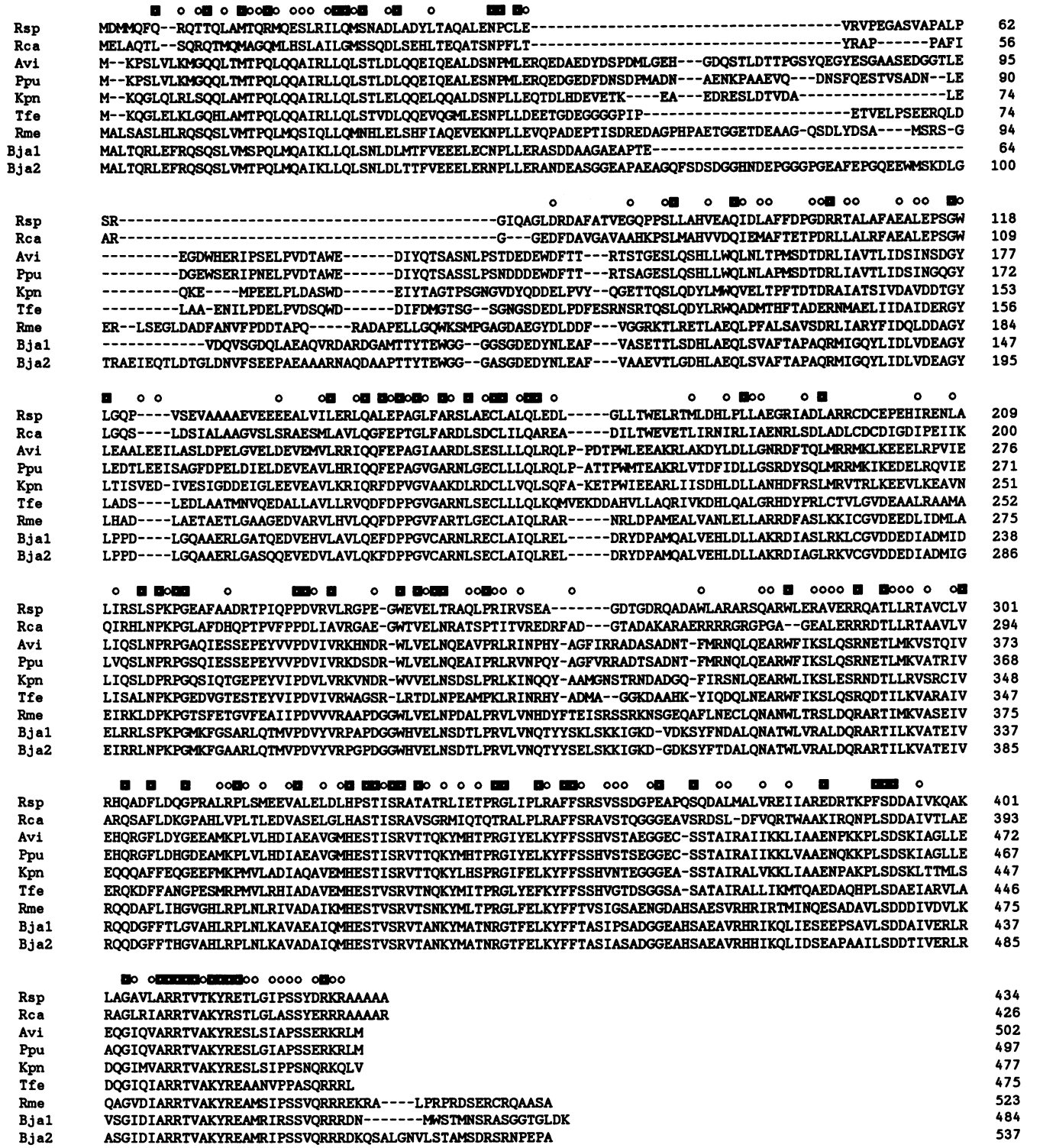

FIG. 3. Alignment of the *R. sphaeroides* (Rsp) RpoN amino acid sequence with those of other bacteria. Abbreviations: Rca, *R. capsulatus* (31); Avi, *A. vinelandii* (41); Ppu, *Pseudomonas putida* (27); Kpn, *K. pneumoniae* (42); Tfe, *Thiobacillus ferrooxidans*; Rme, *Rhizobium meliloti*; Bja1, *B. japonicum* RpoN1 (36); Bja2, *B. japonicum* RpoN2 (36). Symbols: ■, identical residue in at least eight sequences; ○, conservative substitution in at least eight sequences according to the scheme PAGST, QNED, ILVM, HKR, YFW, C. Numbers at the right refer to amino acid residues.

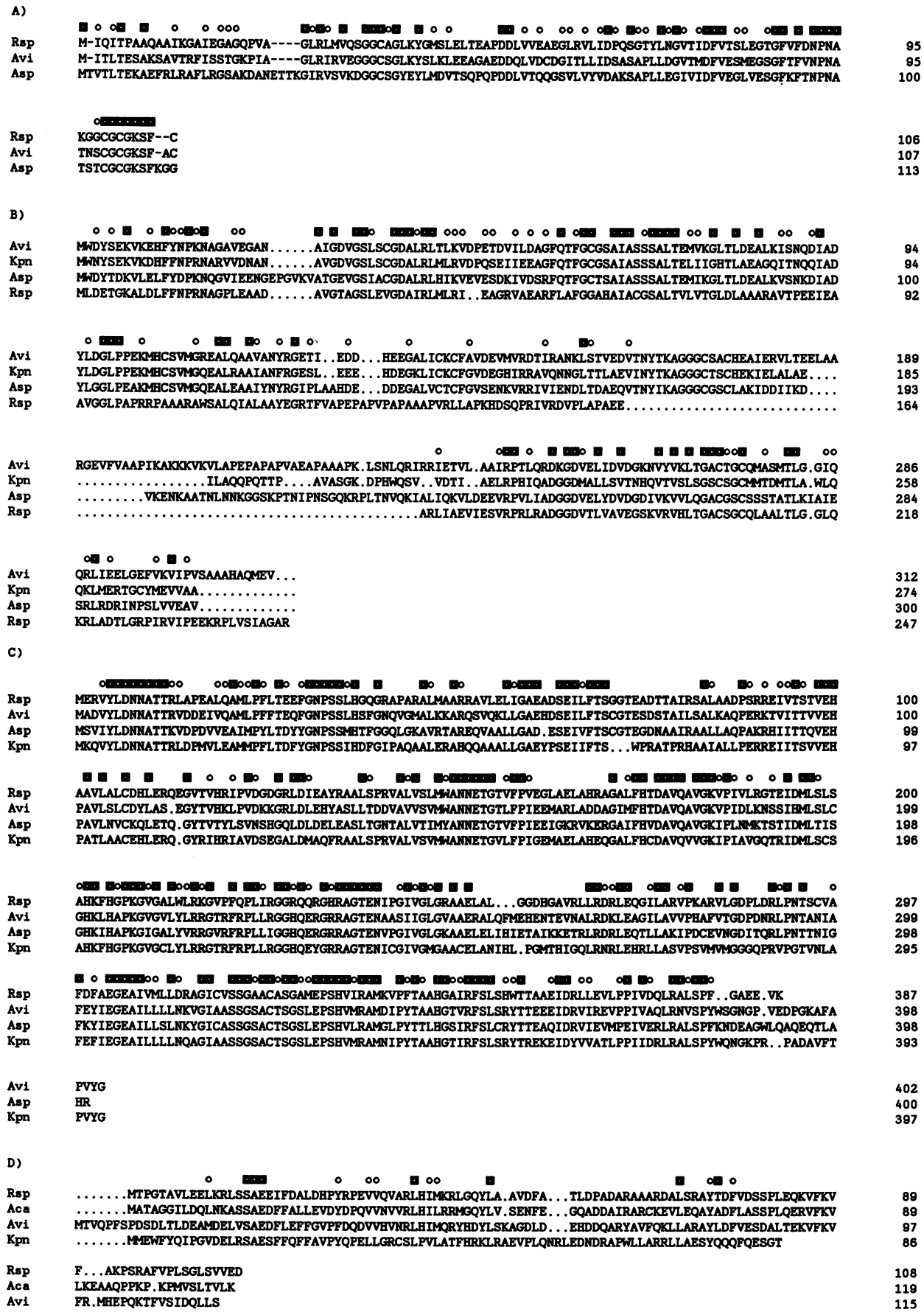
A)
Rsp M-IQITPAAQAAIKGAIEGAGQPVA----GLRLMVQSGGCAGLKYGMSLELTEAPDDLVVEAEGLRVLIDPQSGTYLNGVTIDFVTSLEGTGFVFDNPNA 95
Avi M-ITLTESAKSAVTRFISSTGKPIA----GLRIRVEGGGCSGLKYSLKLEEAGAEDDQLVDCDGITLLIDSASAPLLDGVTMDFVESMEGSGFTFVNPNA 95
Asp MTVTLTEKAEFRLRAFLRGSAKDANETTKGIRVSVKDGGCSGYEYLMDVTSQPQPDDLVTQQGSVLVYVDAKSAPLLEGIVIDFVEGLVESGFKFTNPNA 100
Rsp KGGCGCGKSF--C 106
Avi TNSCGCGKSF-AC 107
Asp TSTCGCGKSFKGG 113
B)
Avi MWDYSEKVKEHFYNPKNAGAVEGAN.....AIGDVGSLSCGDALRLTLKVDPETDVILDAGFQTFGCGSAIASSSALTEMVKGLTLDEALKISNQDIAD 94
Kpn MWNYSEKVKDHFFNPRNARVVDNAN.....AVGDVGSLSCGDALRLMLRVDPQSEIIEEAGFQTFGCGSAIASSSALTELIIGHTLAEAGQITNQQIAD 94
Asp MWDYTDKVLELFYDPKNQGVIEENGEPGVKVATGEVGSIACGDALRLHIKVEVESDKIVDSRFQTFGCTSAIASSSALTEMIKGLTLDEALKVSNKDIAD 100
Rsp MLDETGKALDLFFNPRNAGPLEAAD.....AVGTAGSLEVGDAIRLMLRI..EAGRVAEARFLAFGGAHAIACGSALTVLVTGLDLAAARAVTPEEIEA 92
Avi YLDGLPPEKMHCSVMGREALQAAVANYRGETI..EDD...HEEGALICKCFAVDEVMVRDTIRANKLSTVEDVTNYTKAGGGCSACHEAIERVLTEELAA 189
Kpn YLDGLPPEKMHCSVMGQEALRAAIANFRGESL..EEE...HDEGKLICKCFGVDEGHIRRAVQNNGLTTLAEVINYTKAGGGCTSCHEKIELALAE.... 185
Asp YLGGLPEAKMHCSVMGQEALEAAIYNYRGIPLAAHDE...DDEGALVCTCFGVSENKVRRIVIENDLTDAEQVTNYIKAGGGCGSCLAKIDDIIKD.... 193
Rsp AVGGLPAPRRPAAARAWSALQIALAAYEGRTFVAPEPAPVPAPAAAPVRLLAPKHDSQPRIVRDVPLAPAEE..... 164
Avi RGEVFVAAPIKAKKKVKVLAPEPAPAPVAEAPAAAPK.LSNLQRIRRIETVL.AAIRPTLQRDKGDVELIDVDGKNVYVKLTGACTGCQMASMTLG.GIQ 286
Kpn .....ILAQQPQTTP....AVASGK.DPHWQSV..VDTI..AELRPHIQADGGDMALLSVTNHQVTVSLSGSCSGCMMTDMTLA.WLQ 258
Asp .....VKENKAATNLNNKGGSKPTNIPNSGQKRPLTNVQKIALIQKVLDEEVRPVLIADGGDVELYDVDGDIVKVVLQGACGSCSSSTATLKIAIE 284
Rsp .....ARLIAEVIESVRPRLRADGGDVTLVAVEGSKVRVHLTGACSGCQLAALTLG.GLQ 218
Avi QRLIEELGEFVKVIPVSAAAHAQMEV... 312
Kpn QKLMERTGCYMEVVAA..... 274
Asp SRLRDRINPSLVVEAV..... 300
Rsp KRLADTLGRPIRVIPEEKRPLVSIAGAR 247
C)
Rsp MERVYLDNNATTRLAPEALQAMLPFLTEEFGNPSSLHGQGRAPARALMAARRAVLELIGAEADSEILFTSGGTEADTTAIRSALAADPSRREIVTSTVEH 100
Avi MADVYLDNNATTRVDDEIVQAMLPFFTEQFGNPSSLHSFGNQVGMALKKARQSVQKLLGAEHDSEILFTSCGTESDSTAILSALKAQPERKTVITTVVEH 100
Asp MSVIYLDNNATTKVDPDVVEAIMPYLTDYYGNPSSMHTFGGQLGKAVRTAREQVAALLGAD.ESEIVFTSCGTEGDNAAIRAALLAQPAKRHIITTQVEH 99
Kpn MKQVYLDNNATTRLDPMVLEAMMPFLTDFYGNPSSIHDFGIPAQAALERAHQQAAALLGAEYPSEIIFTS...WFRATPRHAAIALLPERREIITSVVEH 97
Rsp AAVLALCDHLERQEGVTVHRIPVDGDGRLDIEAYRAALSPRVALVSLMWANNETGTVFPVEGLAELAHRAGALFHTDAVQAVGKVPIVLRGTEIDMLSLS 200
Avi PAVLSLCDYLAS.EGYTVHKLPVDKKGRLDLEHYASLLTDDVAVVSVMWANNETGTLFPIEEMARLADDAGIMFHTDAVQAVGKVPIDLKNSSIHMLSLC 199
Asp PAVLNVCKQLETQ.GYTVTYLSVNSHGQLDLDELEASLTGNTALVTIMYANNETGTVFPIEEIGKRVKERGAIFHVDAVQAVGKIPLNMKTSTIDMLTIS 198
Kpn PATLAACEHLERQ.GYRIHRIAVDSEGALDMAQFRAALSPRVALVSVMWANNETGVLFPIGEMAELAHEQGALFHCDAVQVVGKIPIAVGQTRIDMLSCS 196
Rsp AHKFHGPKGVGALWLRKGVPFQPLIRGGRQQRGHRAGTENIPGIVGLGRAAELAL...GGDHGAVRLLRDRLEQGILARVPKARVLGDPLDRLPNTSCVA 297
Avi GHKLHAPKGVGVLYLRRGTRFRPLLRGGHQERGRRAGTENAASIIGLGVAAERALQFMEHENTEVNALRDKLEAGILAVVPHAFVTGDPDNRLPNTANIA 299
Asp GHKIHAPKGIGALYVRRGVRFRPLLIGGHQERGRRAGTENVPGIVGLGKAAELELIHIETAIKKETRLRDRLEQTLLAKIPDCEVNGDITQRLPNTTNIG 298
Kpn AHKFHGPKGVGCLYLRRGTRFRPLLRGGHQEYGRRAGTENICGIVGMGAACELANIHL.PGMTHIGQLRNRLEHRLLASVPSVMVMGGGQPRVPGTVNLA 295
Rsp FDFAEGEAIVMLLDRAGICVSSGAACASGAMEPSHVIRAMKVPFTAAHGAIRFSLSHWTTAAEIDRLLEVLPPIVDQLRALSPF..GAEE.VK 387
Avi FEYIEGEAILLLLNKVGIAASSGSACTSGSLEPSHVMRAMDIPYTAAHGTVRFSLSRYTTEEEIDRVIREVPPIVAQLRNVSPYWSGNGP.VEDPGKAFA 398
Asp FKYIEGEAILLSLNKYGICASSGSACTSGSLEPSHVLRAMGLPYTTLHGSIRFSLCRYTTEAQIDRVIEVMPEIVERLRALSPFKNDEAGWLQAQEQTLA 398
Kpn FEFIEGEAILLLLNQAGIAASSGSACTSGSLEPSHVMRAMNIPYTAAHGTIRFSLSRYTREKEIDYVVATLPPIIDRLRALSPYWQNGKPR..PADAVFT 393
Avi PVYG 402
Asp HR 400
Kpn PVYG 397
D)
Rsp .....MTPGTAVLEELKRLSSAEEIFDALDHPYRPEVVQVARLHIMKRLGQYLA.AVDFA...TLDPADARAAARDALSRAYTDFVDSSPLEQKVFKV 89
Aca .....MATAGGILDQLNKASSAEDFFALLEVDYDPQVVNVVRLHILRRMGQYLV.SENFE...GQADDAIRARCKEVLEQAYADFLASSPLQERVFKV 89
Avi MTVQPFSPDSDLTLDEAMDELVSAEDFLEFFGVPFDQDVVHVNRLHIMQRYHDYLSKAGDLD...EHDDQARYAVFQKLLARAYLDFVESDALTEKVFKV 97
Kpn .....MMEWFYQIPGVDELRSAESFFQFFAVPYQPELLGRCSLPVLATFHRKLRAEVPLQNRLEDNDRAPWLLARRLLAESYQQQFQESGT 86
Rsp F...AKPSRAFVPLSGLSVVED 108
Aca LKEAAQPPKP.KPMVSLTVLK 119
Avi FR.MHEPQKTFVSIDQLLS 115

FIG. 4. Alignment of ORF1 (A), NifU (B), NifS (C), and NifW (D) deduced amino acid sequences of *R. sphaeroides* (Rsp) with those of other bacteria. Abbreviations: *Kpn*, *K. pneumoniae* (3, 7, 8); Avi, *A. vinelandii* (7, 29); Aca, *Azotobacter caulinodans* (2); Asp, *Anabaena* sp. (11, 44). Symbols: ■, identical residue; ○, conservative substitution according to the scheme PAGST, QNED, ILVM, HKR, YFW, C. Numbers at the right refer to amino acid residues.

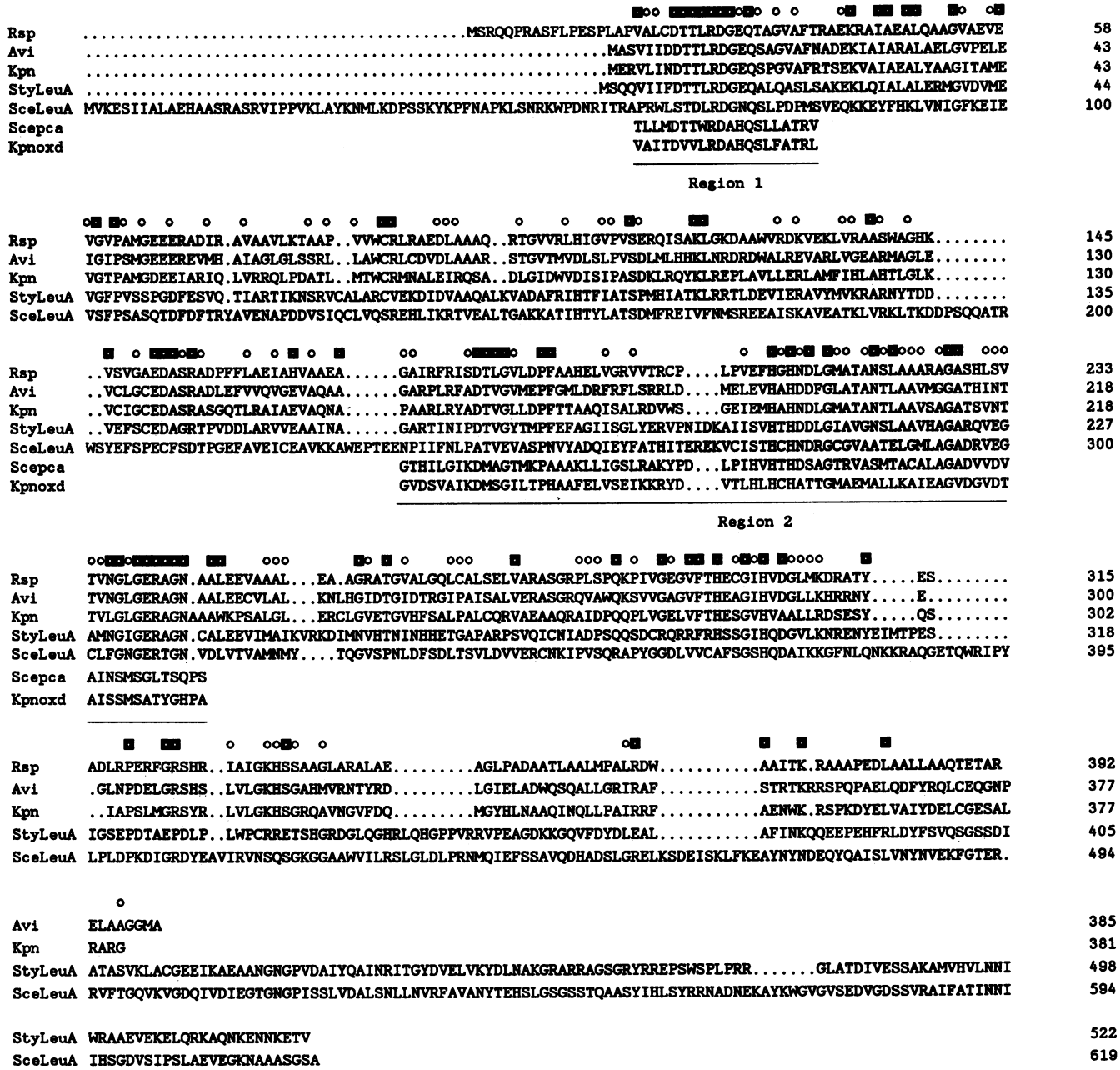

FIG. 5. Alignment of the *R. sphaeroides* (Rsp) NifV amino acid sequence with NifV from *K. pneumoniae* (Kpn) (3, 7) and *A. vinelandii* (Avi) (7) and with LeuA from *Salmonella typhimurium* (StyLeuA) (49) and *Saccharomyces cerevisiae* (SceLeuA) (5). For oxaloacetate decarboxylase from *K. pneumoniae* (Kpnoxd) (residues 2 to 21 and 165 to 239) and pyruvate carboxylase from *S. cerevisiae* (Scepca) (residues 557 to 576 and 727 to 802), only the parts similar to regions 1 and 2 are shown (38, 53). Symbols: ◘, identical residue in at least four sequences; ○, conservative substitution in at least four sequences according to the scheme PAGST, QNED, ILVM, HKR, YFW, C. Numbers at the right refer to amino acid residues.

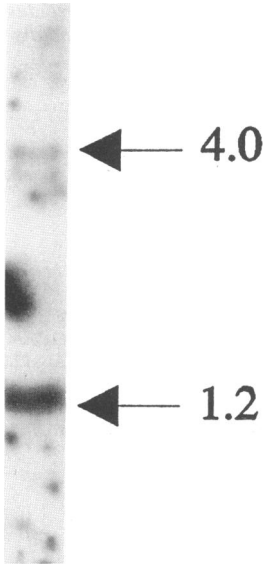

FIG. 6. Hybridization of a 0.5-kb *Sac*I-*Bam*HI fragment internal to *rpoN* to *Bam*HI-digested chromosomal DNA from *R. sphaeroides*. Sizes are indicated in kilobases.

mini-Mu insertions (4). However, the exact transcription initiation site is unknown, and therefore it may be possible that the *rpoN* promoter is situated upstream from the recently discovered *nifU* gene (33), as is the case in *R. sphaeroides*. The *nifUSVWZM* gene cluster from *A. vinelandii* is also transcribed from a promoter upstream from *nifU* (30).

The organization of the *nifUSVW* genes in *R. sphaeroides* is similar to those in *R. capsulatus* and *K. pneumoniae* (3, 33); however, *A. vinelandii* has two ORFs between *nifV* and *nifW* (29). The cotranscription of the *nifUSVW* genes of *R. sphaeroides*, as indicated by the Tn*5* insertional analysis, is underscored by the overlapping stop and start codons of the *nifU*, *nifS*, and *nifV* genes, which are indicative of translational coupling.

The complementation of the *R. capsulatus rpoN* mutant with the genomic library of *R. sphaeroides* resulted in the isolation of only one *rpoN* gene. Much to our surprise, the insertion of a Km^r gene in *rpoN* had no phenotypic effect. Since *rpoN* is essential for diazotrophic growth in all organisms examined thus far, the possibility of the existence of a second *rpoN* gene was examined. Using the *R. sphaeroides rpoN* gene as a probe in Southern blots revealed a second hybridizing fragment. Duplication of nitrogen fixation genes is not without precedent; as previously described, *R. capsulatus* contains a duplicated set of *nif* genes in regions A and B (34). In addition, *Bradyrhizobium japonicum* was recently found to possess two functional copies of *rpoN*; one gene is expressed constitutively, while the expression of the second *rpoN* gene is controlled by oxygen (36). In *R. sphaeroides*, there are two operons encoding duplicate copies of many of the Calvin cycle CO_2 fixation structural genes (16–19, 56). Interestingly, these operons appear to be located on different chromosomes (55). A comparable situation could exist for the *rpoN* genes.

Detailed comparisons of deduced amino acid sequences indicated that the RpoN proteins from *R. sphaeroides* and *R. capsulatus* belong to a different group from other RpoN proteins. The most striking difference between the two RpoN families is the virtual absence of region II in the *R. sphaeroides* and *R. capsulatus* RpoN proteins. Since it has been proposed that region II is involved in melting of the promoter (52), the absence of this domain in the *R. capsulatus* and *R. sphaeroides* RpoN proteins suggests that, at least in these proteins, region II is not critical. Despite the low overall similarity of the RpoN proteins of the purple nonsulfur bacteria and those of other organisms, most residues thought to be functionally important are conserved.

In the NifU amino acid sequences of *A. vinelandii* and *K. pneumoniae*, a possible heme-binding site was identified (40). The His residue in the consensus sequence is the ligand of the heme group and is not conserved in the *Anabaena* sp. sequence (11), where it is replaced by a Leu residue. In *R. sphaeroides*, the heme-binding site is completely absent. Since the heme-binding site is conserved in only two NifU proteins, we conclude that NifU is not a heme-binding protein. The ORF upstream from *nifU* contains a consensus ATP phosphate-binding site (22), suggesting that the gene product of this ORF may bind ATP or other nucleotides. Although no function could be assigned to this ORF, the fact that it is highly conserved among unrelated organisms suggests that it is a functionally important protein.

The product of the reaction catalyzed by NifV has been identified as homocitrate (25). Substrates of the reaction catalyzed by NifV leading to the formation of homocitrate have not been identified. However, as has been noted by Evans et al. (14), the NifV primary structure is similar to that of isopropylmalate synthase, an enzyme which catalyzes the formation of isopropylmalate from acetyl coenzyme A (acetyl-CoA) and α-ketovalerate. Homocitrate synthase present in the lysine biosynthetic pathway catalyzes the formation of homocitrate from acetyl-CoA and α-ketoglutarate, a similar reaction. It is therefore reasonable to assume that the substrates for NifV are acetyl-CoA and α-ketoglutarate.

By using a profile search, a sequence similar to region 2 of the NifV-LeuA sequence alignment was identified in pyruvate carboxylase and oxaloacetate decarboxylase. Upon inspection of the amino acid sequence of these two proteins, a sequence similar to region 1 was also identified at the proper spacing relative to region 2. The conservation of these regions suggests that they play an important role in catalysis, substrate binding, or both. It has been suggested that the fragment of pyruvate carboxylase containing regions 1 and 2 (residues 550 to 900) is involved in the binding of the substrate, pyruvate (38). Pyruvate has a keto acid group in common with oxaloacetate, α-ketoglutarate, and α-ketovalerate, the substrates of the other enzymes used in the sequence alignment. Therefore, on the basis of the common chemical nature of the substrates of these enzymes, we propose that region 1 and region 2 are involved in the binding of the keto acid group of the respective substrates. Obviously, subsequent enzymological investigations are required to confirm these hypotheses.

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