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The presence of five *nifH*-like sequences in *Clostridium pasteurianum*: sequence divergence and transcription properties

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ABSTRACT

The nifH gene encodes the iron protein (component II) of the nitrogenase complex. We have previously shown the presence in Clostridium pasteurianum of two nifH-like sequences in addition to the nifH1 gene which codes for a protein identical to the isolated iron protein. In the present study, we report that there are at least five nifH-like sequences in C. pasteurianum. DNA sequencing data indicate that the six nifH (nifH1) and nifH-like (nifH2, nifH3, nifH4, nifH5 and nifH6) sequences are not identical and vary from each other to different extents with sequence identity ranging between 68 to 99.9% within the nifH coding regions. Under normal N_2-fixing growth conditions (molybdenum-containing medium), transcripts of nifH1 and most of the nifH-like sequences accumulate. The above results suggest the functioning of more than one "nifH" gene under N_2-fixing growth conditions for C. pasteurianum. A common sequence was found around the -100 regions of all nif or nif-like transcription units. Sequences identical to or very similar to the consensus Escherichia coli promoter were found in the -35 and -10 regions.

INTRODUCTION

Biological nitrogen fixation is catalyzed by an enzyme complex called nitrogenase. Nitrogenase consists of two separately purified protein components: the Fe protein and the MoFe protein. In the N_2 fixation process, Fe protein is first reduced by a reductant (ferredoxin in C. pasteurianum); the reduced Fe protein then transfers electrons to MoFe protein with the consumption of ATP. MoFe protein contains the active site for N_2 reduction to NH_3. The Fe protein has two identical subunits which are encoded by a gene designated nifH. The MoFe protein is a tetramer with an $\alpha_2\beta_2$ structure. The two genes which encode the α and β subunits are nifD and nifK, respectively. The three structural genes in C. pasteurianum (designated nifH1, nifD and nifK) have been previously isolated and sequenced in our laboratory (1-3). The deduced amino acid sequence of nifH1 is identical to that of the isolated protein (1,4) suggesting that nifH1 is the functional nifH gene. The coding sequence of nifD begins 41 nucleotides after the stop codon of nifH1. The start codon for nifK overlaps with the stop codon for nifD.

We previously reported the presence of two nifH-like sequences (designated nifH2 and nifH3) in C. pasteurianum (1). The presence of extra nifH or nifH-like sequences has also been reported in other diazotrophs. In Rhizobium phaseoli, three nifH genes with identical coding regions were found and none of the three genes is indispensable for N_2 fixation (5). More recently, Norel and Elmerich (6) reported the presence of two functional but not identical nifH genes in Rhizobium ORS571. Two additional nifH homologous sequences have been identified in Azotobacter vinelandii (7) and one in Azotobacter chroococcum (8). Transcripts from these nifH homologous sequences of Azotobacter were identified only under molybdenum- and NH_3-starvation conditions. Consequently, they were presumed to be involved in the alternative N_2-fixation pathway (8, 9).

In this paper we report that there are five nifH-like sequences in addition to nifH1 in C. pasteurianum. The nucleotide sequence similarities among these nifH and nifH-like sequences range from 68 to nearly 100%, and most of them are transcribed under N_2-fixing conditions.

MATERIALS AND METHODS

Materials

Restriction and modification enzymes used in this study were obtained either from Bethesda Research Laboratories or International Biotechnologies, Inc. ^{125}I was obtained from Amersham and the ^{35}S-labeled dATP was from New England Nuclear Corporation.

DNA cloning and sequencing

nifH1 DNA was digested from the insert of pCP114 (1) and labelled with ^{125}I for use as a hybridization probe. High molecular weight DNA from C. pasteurianum W5 was digested with different restriction endonucleases: AhaIII, EcoRI, HindIII, KpnI and PstI. The digested DNA was subjected to gel electrophoresis, Southern transfer (10), and hybridization analysis using ^{125}I-labeled nifH1 DNA as a probe. Regions showing homology to nifH1 were cut out from an agarose gel, and DNA eluted from the gel slices was ligated into pUC plasmids, and transformed into E. coli JM109.

Inserts from positive clones were subcloned into M13 phage. Exonucleases III and VII were used for sequential deletions of the inserts in M13 replicating form to generate templates for sequencing (11). The dideoxy method (12) and ^{35}S-labeled dATP were used for DNA sequencing (13).

RNA preparation

For the isolation of total RNA from N_2-fixing and ammonia-grown cells, C.

pasteurianum W5 was grown under the following conditions: Spores maintained in soil were used to inoculate a potato medium supplemented with sucrose and $CaCO_3$ (14). Actively growing cultures in the potato medium were used to inoculate 500 ml of either N_2-fixing or ammonia medium (see below). Cultures at the mid-exponential phase of growth ($A_{550\ nm}$ at 2.5 to 3.5; Bausch and Lomb Spectronic 20) in 500 ml of medium were used to inoculate 8 l of the corresponding medium. The N_2-fixing medium contained (per liter): a mineral stock (14), 1 ml; K_2HPO_4, 0.688 g; $Na_2SO_4.10\ H_2O$, 0.19 g; $CaCO_3$, 3.75 g; biotin, trace; sucrose 30 g. Molybdate was present at 10 μM in the medium. The ammonia medium contained $(NH_4)_2SO_4$, (2 g per liter) and p-aminobenzoic acid (trace) in addition to the above ingredients. The cultures were sparged with N_2. The N_2-fixing culture was harvested at an A_{550} of 6.2 (late exponential phase). The ammonia-grown (non-N_2-fixing) culture was harvested at an A_{550} of 3.5 (mid-exponential phase), and the culture had been growing in the ammonia medium for over eight generations. Fresh cells were used in the isolation of total RNA (15).

S_1 nuclease mapping

M13 clones containing the noncoding strand (mRNA strand) of nifH1 and each of the five nifH-like sequences plus 5'-flanking sequences of at least 290 nucleotides were used as templates for the synthesis of ^{35}S-labeled DNA probes using the Klenow fragment (large fragment of DNA polymerase I). Synthesis was initiated from nifH-specific primers (21-mer), which hybridized at around 80 nucleotides downstream from ATG (the translation start codon), and extended upstream from there. Typically, to 1 μg of template DNA in 7.5 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), the followings were added: 1 μl of primer (2 ng) and 1.5 μl of 10X priming buffer (100 mM Tris-HCl, pH 8.5, 100 mM $MgCl_2$). The priming step was carried out at 55-60°C for 1.5 hr. After priming, 1 μl of ^{35}S-dATP (10 μCi), 1 μl of Klenow fragment (1 unit/μl), and 8 μl of 2 mM each of dCTP, dGTP and dTTP were added and the mixture was incubated at 30°C for 20 min. At the end of the incubation, 8 μl of chasing solution containing 2 mM each of dATP, dCTP, dGTP and dTTP was added to the solution and incubated for another 15 min. The reaction was stopped by adding 1 μl of 0.5 M EDTA. Of the synthesized probe, 2 μl was mixed with 100 μg of total RNA either from N_2-fixing cells or non-N_2-fixing cells and precipitated. The pellet was dissolved into 50 μl of freshly prepared hybridization solution containing 80% formamide, 2X SSC, 0.4 mM HEPES, pH 7.0 and 0.1% SDS. The solution was heated at 55°C for 3 min, then transferred to a 39°C water bath and incubated overnight. After hybridization, the solution was diluted with

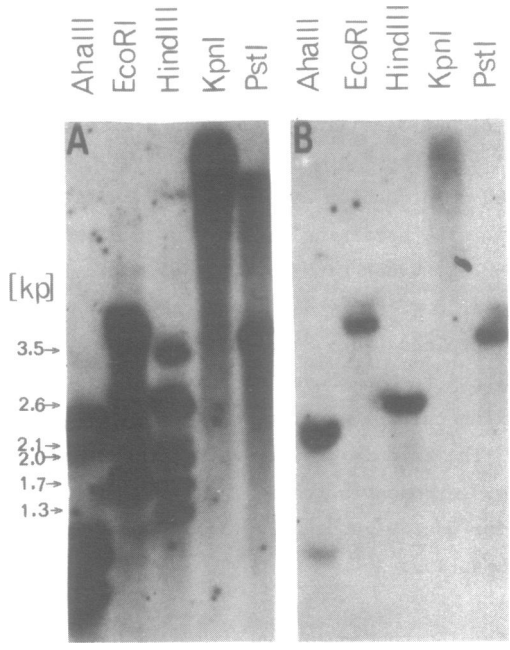

Fig. 1. Southern blot analysis of restriction endonuclease digested C. pasteurianum DNA with nifH1 (A) and nifD (B) DNA as probes. The nifD probe began within the region between nifH1 and nifD and extended to the EcoRI site within nifD. It hence detected an AhaIII fragment (weak band) containing nifH1 (ref. 1).

250 µl of S_1 buffer (50 mM Na acetate, pH 4.8, 0.5 mM $ZnCl_2$, 50 mM NaCl). S_1 nuclease (55 units) was added and the reaction mixture was incubated at 25°C for 30 min. After precipitation, the DNA/RNA hybrids were dissolved in 15 µl TE and analyzed by denaturing gel electrophoresis and autoradiography.

Primer extension

The primer extension assays were performed as described by Alam et al. (16).

RESULTS

Cloning and sequence analysis of multiple nifH-like sequences

Using ^{125}I-labeled nifH1 DNA as a probe, multiple bands were detected in Southern analysis of DNA fragments from C. pasteurianum. As shown in Fig. 1A, five AhaIII bands, four EcoRI bands, six HindIII bands, and two PstI bands were observed. Only one band, however, was observed for the same DNA samples when ^{125}I-labeled nifD DNA was used as the probe (Fig. 1B). This result

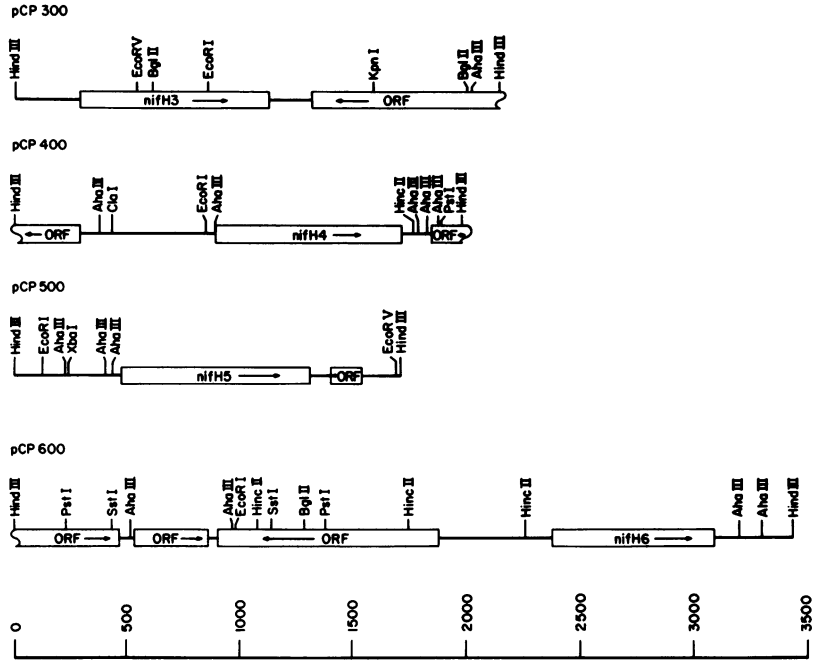

Fig. 2. Restriction maps and locations of nifH-like sequences on newly cloned HindIII DNA fragments of C. pasteurianum. Arrows indicate the direction of transcription. ORF: Open Reading Frame. Numbers at the bottom are in base pairs.

indicated the presence of multiple nifH-like but not nifD-like sequences in C. pasteurianum. Two of the six bands of HindIII-digested DNA contained nifH1 (the second band from the top) and nifH2 (the bottom band) (Fig. 1A) which are carried on the clone pCP114 as previously described (1). The remaining four bands became candidates for cloning and sequencing.

The newly cloned DNA fragments were sequenced, and each contained a nifH-like sequence (Fig. 2). Thus, these sequences resided on different restriction fragments. One of the four nifH-like sequences is nifH3, part of which has been previously sequenced (1). The others were designated as nifH4, nifH5, and nifH6. The nucleotide sequences of all six nifH and nifH-like DNA fragments are shown in Fig. 3. When the nucleotide sequences of the coding regions of five nifH-like sequences were compared with that of nifH1, it was found that nifH5 had a sequence identity of 96%, nifH4 of 91%, nifH2 and nifH6 of 90%, and nifH3 of 68%. High levels of sequence identity extend to the putative ribosome-binding sites in the sequences. Beyond that point little

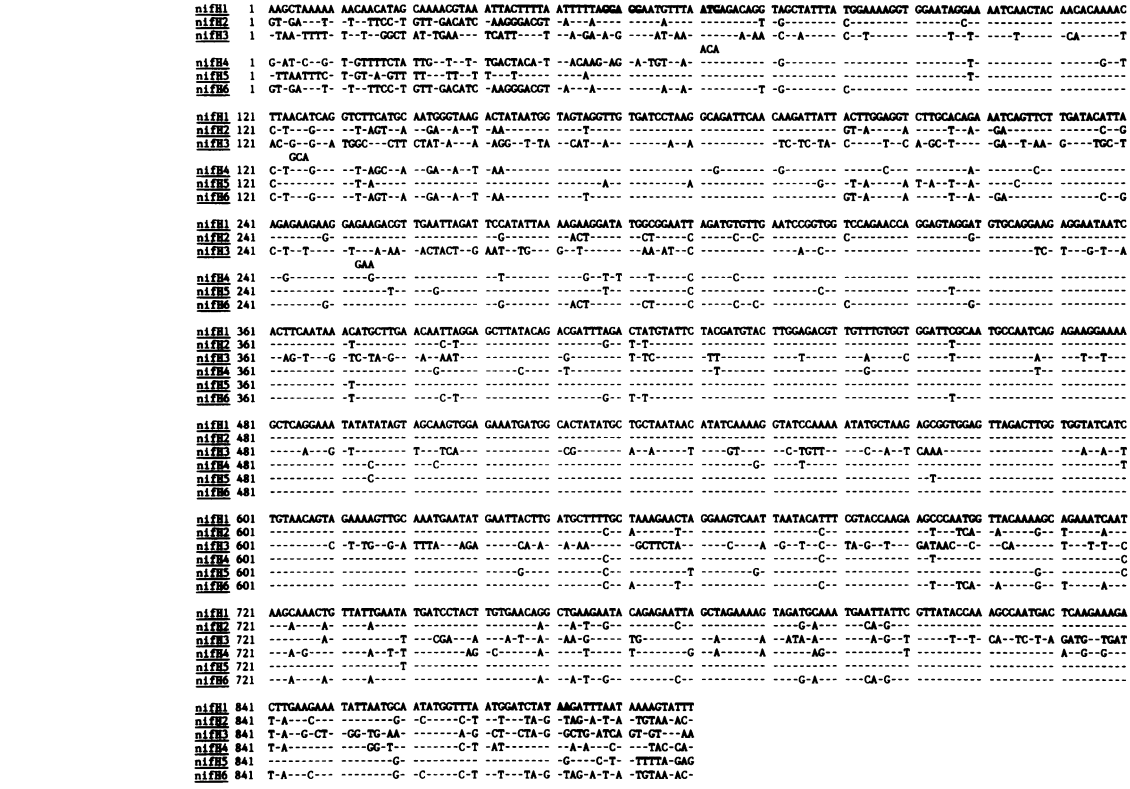

Fig. 3. Nucleotide sequences of nifH1 and five nifH-like sequences from C. pasteurianum. For the five nifH-like sequences, letters are given only for the nucleotides that are different from nifH1, and the ones that are the same are indicated by "-". The bold letters are putative ribosome binding sites, start codons (nucleotides 61-63) and termination codons, respectively. ACA, GCA and GAA underneath nifH3 in first three panels are extra codons of nifH3, which follow the codon above.

identity in the 5' flanking regions is apparent, even between nifH1 and nifH5 which have a very high sequence identity. DNA fragments of nifH2 (clone pCP114) and nifH6 (clone pCP600) require special attention. Within the corresponding region of 2482 bp, there are only two nucleotide changes. The first occurs in the HindIII site of clone pCP114, which results in the loss of that restriction site in clone pCP600. The second is in the coding region of nifH-like sequences and results in an amino acid change. The high sequence identity between nifH2 and nifH6 (99.9%) results in difficulties in distinguishing their transcripts.

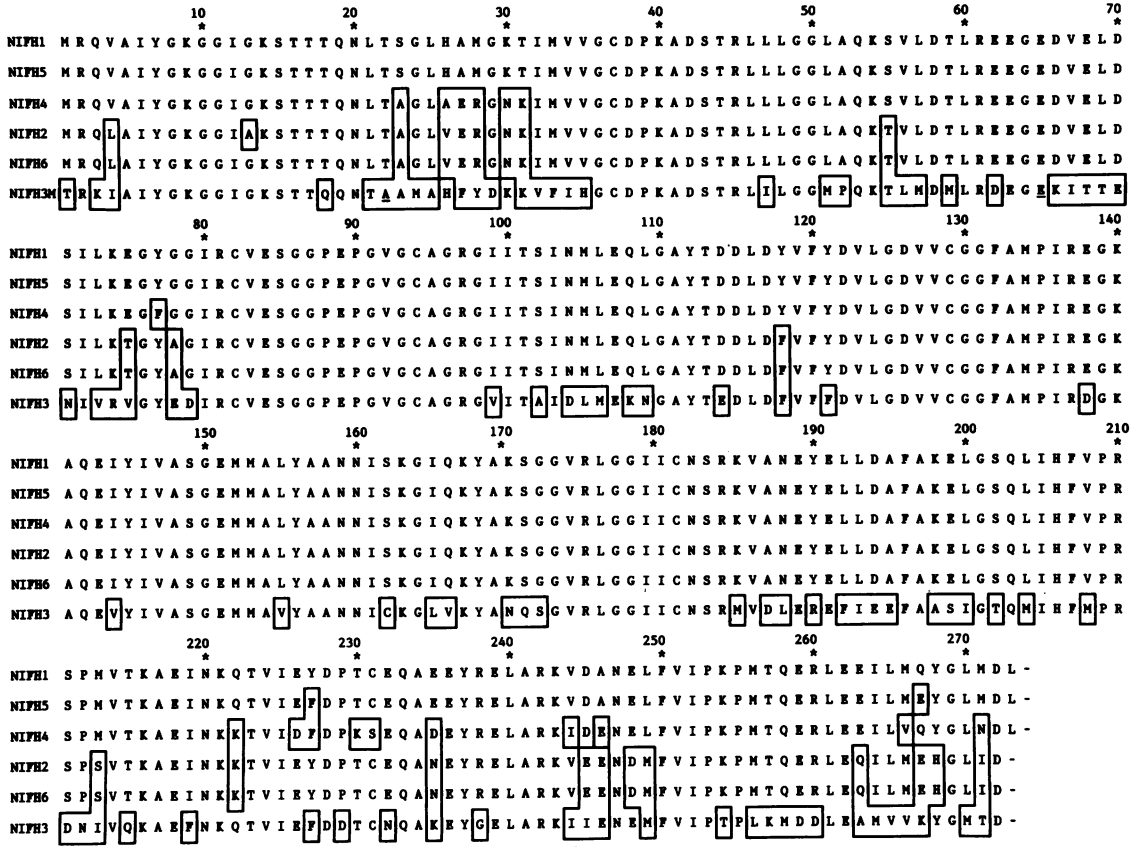

Fig. 4. The deduced proteins of nifH1 and five nifH-like sequences. Amino acids that are different from nifH1 are boxed. Underlined A and E in the NIFH3 sequence indicate that there was an extra A or E at that position.

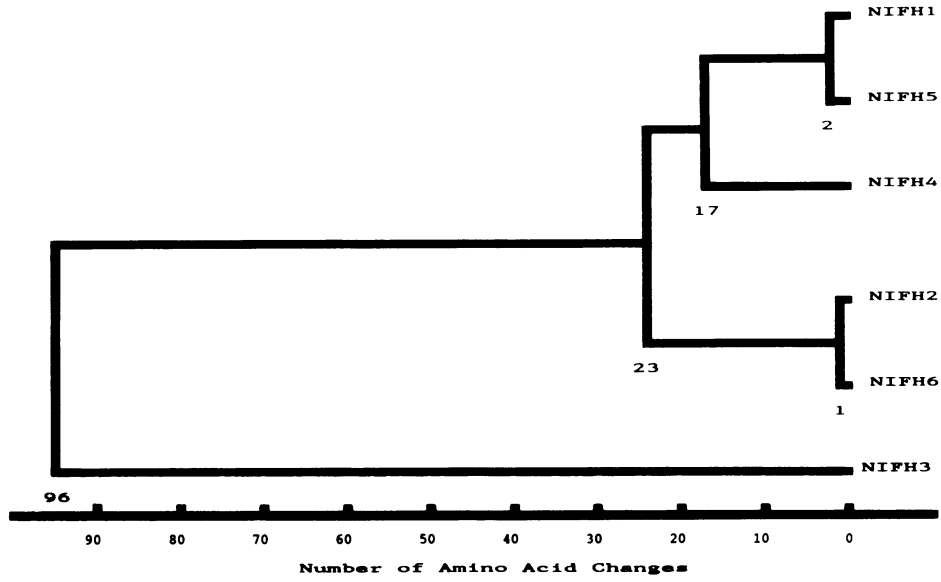

Fig. 5. Summary of amino acid changes in the deduced NIFH1 and NIFH-like proteins.

The deduced amino acid sequences are shown in Fig. 4. They are more divergent in the terminal regions than in the middle region, which contained a little over one hundred highly conserved residues. The five cysteine residues (37, 82, 94, 129, 181) are conserved among all six sequences. Based on the deduced amino acid sequences, they can be divided into four groups. Group 1 consists of nifH1 and nifH5 which differ from each other by two amino acid residues in the C-terminal region. These changes involved Tyr ⇌ Phe and Gln ⇌ Glu. Group 2 includes only nifH4 which has 17 amino acid differences from group 1 and 22-23 from group 3. nifH2 and nifH6 constitute group 3, with only a single amino acid difference of Ala ⇌ Gly between them. This difference is a result of a single nucleotide change as mentioned earlier. nifH3 (Group 4) is the most distinct, having 95-97 amino acid differences from the rest, plus having three extra amino acids in the N-terminal region. The overall interrelationship of the deduced proteins is shown in Fig. 5.

Transcription of nifH sequences

S_1 nuclease mapping of the 5' end of nifH1 mRNA indicated that transcription initiates 62 base pairs (bp) preceding the translation start site (Fig. 6A, lane 2). The primer extension experiment, however, indicated transcription initiates 60 bp preceding the translation start site (lane 7).

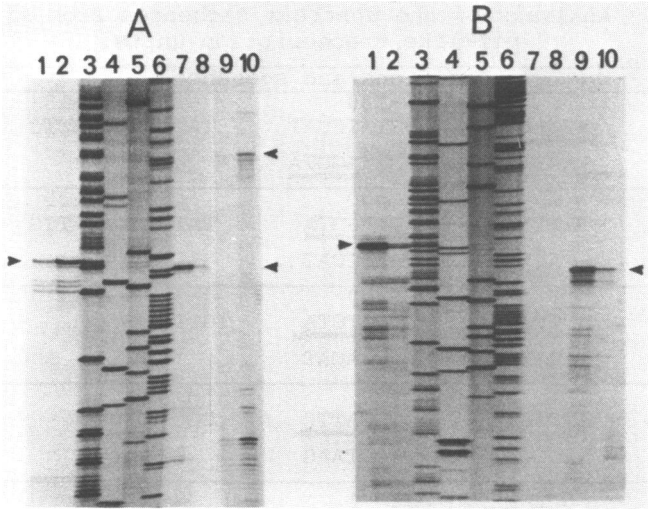

Fig. 6. S_1 nuclease mapping and primer extension assays detecting mRNAs of nifH1 and five nifH-like sequences and identifying their transcription start sites. Arrows indicate the major bands that were used for determining the transcription start sites. A: S_1 nuclease mapping for nifH1 (lanes 1 & 2) and for nifH5 (lane 9 and 10) and primer extension for nifH1 (lanes 7 and 8). In lanes 1, 8, and 9 total RNA from non-N_2-fixing cells was used. In lanes 2, 7, and 10 total RNA from N_2-fixing cells was used. Lanes 3, 4, 5, & 6 are sequencing ladders (A, C, G and T, respectively). B: Primer extension assays for the transcripts of nifH2/nifH6 (lanes 1 and 2), nifH3 (lanes 7 and 8) and nifH4 (lanes 9 and 10). Total RNA from N_2-fixing cells was used in lanes 1, 7, and 9. Total RNA from non-N_2-fixing cells was used in lanes 2, 8, and 10. Lanes 3, 4, 5, and 6 are A, C, G, and T sequencing ladders.

To determine which of the two results was correct, M13 single-stranded DNA cleaved at the distal end of the multiple-cloning region was used in a primer extension experiment. Both Klenow fragment and reverse transcriptase replicated to the end of the template (data not shown). This result suggests that the original primer extension performed was correct and that S_1 nuclease digestion might not have been complete in the other experiment. Therefore, it was concluded that the transcription of nifH1 started at -60. There was much more nifH1 mRNA in N_2-fixing cells (lanes 2 and 7) than in non-N_2-fixing cells (lanes 1 and 8), confirming that nifH1 is a functional nifH gene in C. pasteurianum.

When probe DNA, synthesized off the nifH5 template using the nifH1 primer, was used in the S_1 mapping experiment, a band was observed which was 25 nucleotides longer than that observed for nifH1. This result indicate (1) the presence of nifH5 mRNA and (2) a transcription start site at -85 (Fig. 6A,

Table 1. Analysis of the upstream sequences from six nif and nif-like transcription units.

Gene	Positions and sequences			
nifH1	-130	-35	-15	-10
	ATCAATAT- -GTATCCAT	TATTG	TATTG	TATAT
	TAGTTATA-N_{10}-CATAGGTA			
nifH5	-100	-40	-25	-15
	TATTTGAT- -ATAAGGTA	TATTG	TATTG	TATACT
	ATAAACTA-N_6-TATTCCAT			
nifH2 & nifH	-130	-39		-14
	ATCTATAA- -ATGGTGTA	TGTTGACA		TATAAT
	TAGATATT-N_7-TACCACAT			
nifH4	-90	-30		-10
	ATCAATAT- -ATGGATTC	GGGTA		TAAATTAA
	TAGTTATA-N_8-TACCTAAG			
nifE	-90	-35		-20
	ATATTGAT- -ATGGATTC	GGGTA		TAAATTAA
	TATAACTA-N_9-TACCTAAG			
Consensus	ATCAATAT-N_{6-10}-ATGGATTC			
Genes of Gram+ (19)		-35 TTG	-15 TG	-10 TATAAT
E. coli		-35 TTGACA		-10 TATAAT
Kp^1 $(Av)^2$ nif	-100 TGT-N_{10}-ACA		-25 CTGG	-14 TTGCA

1, Klebsiella pneumoniae; 2, Azotobacter vinelandii.

lane 10). There was a difference in the amount of nifH5 mRNA between N_2-fixing (lane 10) and non-N_2-fixing cells (lane 9). This suggests that nifH5, which has the greatest sequence identity with nifH1 (96% at the DNA level for nifH coding sequences), may also be involved in the N_2-fixation process. The less intense band for nifH5 may reflect less DNA probe, due to using a heterologous primer for the probe synthesis (the 21-mer oligonucleotide had template mismatches at primer positions 19 and 21).

The presence of mRNAs for other nifH-like sequences was also investigated by primer extension experiments. Due to the high sequence identity between nifH2 and nifH6 (one change out of 816 nucleotides in nifH coding regions), a single primer had to be used for both. Specific primers were used for nifH3 and nifH4. Using the nifH2/nifH6 primer and RNA from N_2-fixing cells resulted in two bands, at positions -76 and -77 (Fig. 6B lane 1). The -76 band may be the result of some of the primer being one nucleotide shorter, since doublets occurred when using this primer for sequencing (data not included). Only

trace amounts of primer extension occurred when RNA from non-N_2-fixing cells was used (lane 2). No primer extension was observed for nifH3 using RNA from either N_2-fixing (lane 7) or non-N_2-fixing (lane 8) cells, indicating the absence of nifH3 mRNA. Using the nifH4 primer resulted in a strong band with RNA from N_2-fixing cells (lane 9) and only a light band with RNA from non-N_2-fixing cells (lane 10), suggesting the presence of its transcript in N_2-fixing cells. The transcription start site for nifH4 is at -80 (the priming position was different for nifH4).

Promoter sequence analysis

A total of eight nif genes or nif-like sequences from C. pasteurianum have been isolated and sequenced in our laboratory. In addition, part of nifE has also been isolated and sequenced (unpublished results). These genes/sequences belong to six transcription units, excluding nifH3 which did not appear to have any transcripts. With the information of the transcription start sites available, the 5' upstream sequences for each of the six transcription units were compared and regions with apparent similarities are summarized in Table 1.

DISCUSSION

Deduced protein sequences

It is interesting to note that in the deduced amino acid sequences of nifH2, nifH4 or nifH6, Ala-23, Val-26, Glu-27, Lys-31, Thr-55, Thr-75, Phe-118, Asn-235 or Asp-235, Ile-244, Glu-267, His-268, and Ile-271 differed from corresponding residues in the C. pasteurianum Fe protein. However, these residues are either present or highly conserved in Fe proteins of other organisms (1). It is also interesting to note that among the nifH-like sequences, the nifH3-encoded amino acid sequence differed most from the sequence of C. pasteurianum Fe protein and the other nifH-like sequences (Fig. 4). However, twenty nine of these distinct residues in the nifH3-encoded sequence are present or conserved in Fe proteins of eight other organisms (1). These residues are (numbering refers to the nifH3 sequence which is 1 to 2 residues longer than the nifH1 sequence before residue 65 of nifH1 and 3 residues longer afterwards) 5, 34, 36, 49, 59, 61, 73, 82, 102, 105, 112, 169, 173, 190, 196, 197, 201, 205, 207, 214-216, 218, 235, 257, 261-262, 266-267. The sequence Asp-Asn-Ile-Val-Gln (residues 214-218) in nifH3 is distinctively conserved in Fe protein from eight other organisms (1). Ile-216 is also present in the deduced Fe protein sequences of Methanococcus voltae (17) and Thiobacillus ferroxidans (18). This raises a very interesting question

concerning the evolutionary relationship between the nifH-like sequences of C. pasteurianum and nifH genes of the other organisms.

Transcription of nifH-like sequences

nifH1 is considered the functional nifH gene in C. pasteurianum based on the deduced amino acid sequence and the protein sequence. But our S_1 nuclease mapping experiments and primer extension assays indicated the presence of transcripts of nifH2/nifH6, nifH4 and nifH5, in addition to nifH1, in N_2-fixing cells. Whether or not any of these mRNAs from the nifH-like sequences is translated is not known. If they are translated, the physical properties of the proteins probably differ significantly from those of the Fe protein. Otherwise, one would expect some co-purification with the Fe protein, which was not the case as indicated by protein sequencing data (see the last section).

Promoter sequences of nif transcription units in C. pasteurianum

The promoter regions of the C. pasteurianum nif transcription units have sequences common to those of other gram-positive organisms (19) and of E. coli. Both nifH1DK and nifH5 have sequences of TATTG at the -35 and -15 regions and TATAT or TATACT at the -10 region which is homologous to that of other Gram-positive bacteria. nifH2 and nifH6 match perfectly the E. coli promoter sequences of TTGACA at -35 region and TATAAT at -10 region. nifH4 and nifE, which have several homologous blocks in the upstream sequences (data not included) share GGGTA at -35 region and TAAATTAA at -10 region. No homologies were observed with the consensus nif promoter sequence proposed for K. pneumoniae (20, 21). In the -100 regions, however, the sequence is homologous among all six transcription units. The consensus sequence is ATCAATAT-N_{6-10}-ATGGATTC (Table 1), which has no homology to the TGT-N_{10}-ACA sequence of K. pneumoniae (22). This sequence consists of three segments, with N_{6-10} appearing to serve as a bridge which separates the first segment, ATCAATAT, from the third segment, ATGGATTC. For some of the transcription units, the first and last sequences can only be noticed if the reverse complements (nifH1, nifH5 and nifE) and reverse sequence (nifH5) are used. This suggests that both the first and last segments may be orientation-independent for its function. It has been observed that the activator sequence of TGT-N_{10}-ACA at -100 is independent of its orientation for function in K. pneumoniae (22). However, this sequence differs from the C. pasteurianum -100 region in that the key sequences TGT and ACA are palindromic in nature and do not change regardless of the orientation; thus, they may not be directly comparable. The enhancer and Sp1 of eukaryotes and viruses have

also been found to be bidirectionally functional (23). Thus it is possible that the C. pasteurianum -100 region is a binding site for some regulatory molecules, as is TGT-N_{10}-ACA for the nifA protein in K. pneumoniea (24, 25).

Possible functions of multiple nifH-like sequences

Except for nifH3, the deduced amino acid sequences from the other four nifH-like sequences showed a very high sequence identity with the amino acid sequence of the Fe protein (nifH1 product); nifH5 differed from nifH1 in only two amino acid residues: Tyr-227 (H1) vs. Phe-227 (H5) and Gln-267 (H1) vs. Glu-267 (H5). Since transcripts from these nifH-like sequences were present in the cell and proteins (if synthesized) encoded by these sequences may have similar chromatographic properties, we wonder if one or several of them might be co-purified with the nifH1 product. A careful examination of the published sequencing result of the Fe protein (4, 26, and 27) indicated that the purified Fe protein sample could not have more than trace amounts, if any, of proteins from the nifH-like sequences. Specifically, nifH2, nifH3 and nifH6 would give Asp, instead of Leu, as the C-terminus, but hydrazinolysis of the carboxymethyl Fe protein gave leucine in 100% yield (4). The tryptic peptides (T-n; ref. 26) and CNBr peptides (B-n; ref. 27) were then examined for other critical differences. T-4 and T-5 were generated by cleavage of a Lys-Thr linkage (residues 30 to 31). For nifH2, nifH4 and nifH6, the corresponding regions had the sequence of Asn-Lys-Ile (residues 30 to 32). Thus, trypsin would cleave the Lys-Ile linkage to give a "T-5" with Ile as the N-terminus. The N-terminus of T-5 was found to be Thr with a 92% yield (26). The corresponding "T-4" may not co-purify with T-4 because of significant sequence differences, and data of the C-terminal sequence of T-4 do not suggest the presence of a different sequence. A similar conclusion concerning nifH2 and nifH6 can also be made by examining the N-termini (Ser-55, 96%; Glu-75, 100%) of T-8 and T-9. The nifH5 product was not in the purified Fe protein because amino acid analysis of T-22 (containing tyr-227) gave two Tyr but no Phe (Table I in ref. 26), which is also supported by analysis of B-8 (27). A similar situation is also found by an analysis (data not shown) of peptides containing Tyr-118, Gln-222, Glu-235, and Gln-267.

That the purified Fe protein does not contain any measurable amount of products from the nifH-like sequences does not exclude the possibility that some of the products are present in the cell. Two conflicting reports (28, 29) were published concerning whether another species of Fe protein-like molecules was present in ammonia-grown as well as in N_2-fixing cells of C. pasteurianum W5. The result of Jeng et al. (29) was further supported by the

finding that no precipiting band resulted when crude extracts of ammonia-grown cells were tested with antiserum raised against the Fe protein (30). However, in light of the presence of transcripts from these nifH-like sequences, it may be worthwhile to re-investigate this problem using cells grown under different conditions. It would be important to the understanding of nitrogenase function if different ratios of acetylene-reducing versus N_2-reducing activities are observed when MoFe protein is tested with proteins encoded by the nifH-like sequences. Earlier studies using heterologous nitrogenase components demonstrated that the two nitrogenase components together play a role in determining the ratio of acetylene-reducing to N_2-reducing activities (31) and an incompatible Fe protein can act as an inhibitor of nitrogenase activities (32). It thus seems possible that some products from the nifH-like sequences might serve to modulate (such as to down-regulate) the nitrogenase activity under certain physiological conditions.

It was found that nifH is required for the molybdenum-iron cofactor (FeMoco) synthesis or activity in K. pneumoniae (33) and A. vinelandii (34), suggesting multiple functions of nifH in these organisms. It was also noticed that, in K. pneumoniae, the C-terminal end of nifH was not required for FeMoco synthesis although it was for Fe protein activity (33), which might relate to the divergence in the C-terminal region of nifH-like sequences in C. pasteurianum. It is possible that the multiple functions of nifH in K. pneumoniae and A. vinelandii are carried out by different genes in C. pasteurianum. The promoter sequences of nifH4 is homologous to that of nifE which is required for FeMoco synthesis (35), suggesting that they may be co-regulated and involved in the same process. In other words, nifH4, but not nifH1, might be functioning in FeMoco biosynthesis in C. pasteurianum. Also, some of the multiple nifH-like sequences might be involved in the alternative N_2 fixation pathway which has been implicated to exist in C. pasteurianum (36) and could be deduced from results of Mo-depleted growth experiments (37; also our unpublished data). The functions of the multiple nifH-like sequences need to be further investigated and defined. In this respect, the presence of a multigene family for the molybdenum-pterin binding protein in C. pasteurianum (38) is also an intriguing phenomenon.

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