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Clostridium pasteurianum W5 synthesizes two NifH-related polypeptides under nitrogen-fixing conditions

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Previous studies identified five *nifH*-like genes (*nifH2* through *nifH6*) in *Clostridium pasteurianum* (strain W5), where the *nifH1* gene encodes the nitrogenase iron protein. Transcripts of these *nifH* genes, with the exception of *nifH3*, were detected in molybdenum-sufficient nitrogen-fixing cells. However, the size of the transcripts, the level of transcription and the presence of polypeptides encoded by the *nifH*-like genes were not reported. The *nifH2* and *nifH6* genes were extremely similar, as they seemed to differ by only two bases in a span of 2481 bp, one in the coding region and another in the upstream region. Re-examination of the DNA sequences revealed that the coding region of *nifH2* and *nifH6* was identical, whereas the difference in the upstream region was confirmed. Results from the authors' ongoing study of the *nif* genes of single-colony isolates of *C. pasteurianum* suggest that the *nifH6* designation should be eliminated. Here the size of mRNA from *nifH2* and the detection of the NifH2 polypeptide in nitrogen-fixing cells of *C. pasteurianum* are reported. Northern blot analysis of periodically collected nitrogen-fixing cells showed that the *nifH1* and *nifH2* mRNAs were present throughout growth. Addition of ammonium acetate repressed the transcription of both these genes similarly. Using an antiserum raised against NifH of *Azotobacter vinelandii*, two NifH-related bands were detected by Western blot analysis after electrophoretic separation of proteins in extracts of nitrogen-fixing *C. pasteurianum* cells. After separation of proteins by preparative SDS-PAGE, the NifH polypeptides were characterized by MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) and by ES-MS/MS (electrospray tandem mass spectrometry) analyses. The results confirmed the presence of NifH2, in addition to NifH1, in nitrogen-fixing *C. pasteurianum* cells.

INTRODUCTION

Clostridium pasteurianum was the first free-living nitrogen-fixing organism that was isolated (Winogradsky, 1895), and with which the first consistently active cell-free nitrogen fixation was demonstrated (Carnahan *et al.*, 1960; Burris, 1988). Early biochemical studies with *C. pasteurianum* revealed several salient features of biological nitrogen fixation, including the discovery of ferredoxin, a low redox-potential electron donor for nitrogenase (Mortenson, 1964). The nitrogenase component proteins are structurally conserved to such an extent that heterologous pairs are usually active (Emerich *et al.*, 1981). However, the Fe and MoFe proteins of *C. pasteurianum* have unique structures (Chen *et al.*, 1986; Wang *et al.*, 1988; Kim & Rees, 1993; Schlessman *et al.*, 1998; Chen, 2004), which could explain the lack of activity in heterologous pairings involving the nitrogenase of *C. pasteurianum*. Whether or not the distinctive nitrogen-fixing system of *C. pasteurianum* contributes to the robustness of this organism is not known. Further characterization of the nitrogen-fixing system of this organism could identify targets suitable for experimentation to address this question.

The nitrogen-fixation (*nif*) gene cluster of *C. pasteurianum* consists of *nifH2*, *nifH1*, *nifD*, *nifK*, *nifE*, *nifN-B*, *modA*, *modB*, *nifVω* and *nifVα* genes in the same orientation (Chen & Johnson, 1993; Chen, 2004). Four additional *nifH*-like genes were reported: Chen *et al.* (1986) identified *nifH3*, whereas Wang *et al.* (1988) identified *nifH4* to *nifH6*. The *nifH1*gene encodes the characterized Fe protein (273 amino acids) of nitrogenase (Chen *et al.*, 1986; Chen & Johnson, 1993). The *nifH2* gene encodes a polypeptide of 272 amino acids that differs from NifH1 by 22 amino acids. The

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Abbreviations: ES-MS/MS, electrospray tandem mass spectrometry; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

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deduced NifH-related polypeptides of *C. pasteurianum* fall into four phylogenetic groups represented by NifH1 through NifH4 (Wang *et al.*, 1988; Ueda *et al.*, 1995). NifH3 is the most distinct among them as it differs from the others by 87 to 97 amino acids (31·9–35·5 %). NifH3 of *C. pasteurianum* is, however, closely related to AnfH of *Azotobacter vinelandii* (Chen *et al.*, 1990).

The assignment of the *nifH6* gene was based on its differences from *nifH2*: Gly-13 encoded by GGA in *nifH6* versus Ala-13 encoded by GCA in *nifH2*, and the absence of a *Hin*dIII site in its upstream region (AAGACTT preceding *nifH6* versus AAGCTT preceding *nifH2*). Because of the similarity between these two genes and their flanking regions, our laboratory recently resequenced the inserts in pCP114 (harbouring *nifH2*; Chen *et al.*, 1986) and pCP600 (harbouring *nifH6*; Wang *et al.*, 1988). It turned out that the coding region of *nifH2* and *nifH6* was identical, with Gly-13 being the conserved amino acid. The single nucleotide difference in the upstream region was, however, confirmed. Our laboratory is now characterizing the *nif* and *nif*-like genes of single-colony isolates of *C. pasteurianum* strain W5 (wild-type). So far, all of the isolates have been found to contain the sequence previously cloned in pCP600 (C. Tollin, A. Tran, J. Toth & J.-S. Chen, unpublished results). The new results suggest that the laboratory stock of *C. pasteurianum* W5 contained two populations that differed by the presence or absence of a *Hin*dIII site preceding the *nifH2* gene, and the vast majority appears to lack the *Hin*dIII site. Although the characterization of the single-colony isolates is continuing, it is now appropriate to amend the *nifH2* sequence and eliminate the *nifH6* designation.

Transcripts of the *nifH*-like genes, with the exception of *nifH3*, were detected in nitrogen-fixing *C. pasteurianum* (Wang *et al.*, 1988). Except for NifH1, it was not known if other NifH-related polypeptides were synthesized. In this paper, we report the presence of NifH2 in nitrogen-fixing cells of *C. pasteurianum*.

METHODS

Growth conditions. *C. pasteurianum* strain W5 was grown at 35 °C in a medium containing sucrose, 30 g l^{-1}; K_2HPO_4, 0·69 g l^{-1}; $Na_2SO_4.10\ H_2O$, 0·19 g l^{-1}; mineral solution (George & Chen, 1983), 1 ml l^{-1}; $CaCO_3$, 5 g l^{-1}; and resazurin, 1 mg l^{-1}. The headspace of the culture was flushed with nitrogen throughout incubation.

Whole-cell acetylene-reduction assay. The acetylene-reduction assay was used to measure nitrogenase activity as described by Chen *et al.* (2001), except that the vial contained 2 ml bacterial culture and was incubated at 35 °C.

Preparation of cell-free extracts and protein determination. Cell paste was thawed under argon in anaerobic 50 mM Tris/HCl (pH 8·0) buffer (3 ml per g cells), containing 20 % (v/v) glycerol, 1 mM dithiothreitol, 0·1 mg DNase I ml^{-1} and 2 mg lysozyme ml^{-1}. Other steps were as described by Yan & Chen (1990). A cell-free extract of nitrogen-fixing *A. vinelandii*, prepared as described by Vichitphan (2001), was a gift from the laboratory of W. E. Newton (Deptartment of Biochemistry, Virginia Tech, Blacksburg, VA, USA). Protein concentration was determined by the dye-binding assay (Bradford, 1976) with bovine gamma globulin as a standard.

Western blot analysis. Proteins in cell-free extracts were separated by SDS-PAGE or non-denaturing PAGE on 12 % acrylamide gel. The SDS-PAGE was performed according to the method of Laemmli (1970). The non-denaturing PAGE was also performed according to Laemmli (1970), but in the absence of SDS and at a constant voltage of 100 V at 4 °C. Proteins were electrophoretically transferred onto positively charged nitrocellulose membranes in a semi-dry electrophoretic transfer cell (Bio-Rad). Western blots were probed with a NifH antiserum using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). The NifH antiserum was raised in rabbit against the nitrogenase Fe protein of *A. vinelandii*, and was a gift from the laboratory of W. E. Newton (Department of Biochemistry, Virginia Tech, Blacksburg, VA, USA).

Preparation of the *nifH* probe. The probe for the *nifH*-like genes was generated by PCR of a conserved region of the *C. pasteurianum* *nifH* gene as described previously (Chen *et al.*, 2001).

Northern analysis of *nif* mRNA. For RNA isolation, the guanidine isothiocyanate procedure described by Johnson (1994) was used, except that at the final step, the RNA pellet was dissolved in 250 µl water containing diethyl pyrocarbonate (0·1 %, w/v) and SDS (0·05 %, w/v). The size of RNA fragments was estimated by using two different RNA ladders, one from New England BioLabs and the other from Gibco-BRL Life Technology. The RNA species were routinely transferred to positively charged nylon membranes (Hybond-N^+) for 16 to 18 h by capillary elution with 20× SSC. The ECL signal was generated and detected according to the manufacturer's instructions (Amersham Biosciences).

Direct RT-PCR amplification of the 0·9 kb mRNA bound on a membrane. cDNA strands of the 0·9 kb mRNA were synthesized in 0·5 ml microcentrifuge tubes using a 2 × 3 mm piece of nitrocellulose membrane containing the 0·9 kb mRNA as recommended in the Omniscript reverse transcriptase handbook (Qiagen). Ten microlitres of the reverse transcriptase reaction mixture containing the cDNA strand was then used for PCR amplification of *nifH*-like gene fragment as described previously (Chen *et al.*, 2001)

Separation of the NifH-related polypeptides by preparative SDS-PAGE. Preparative SDS-PAGE was run for 8 h using a 12 % acrylamide gel in a model 491 Prep Cell (Bio-Rad) with the discontinuous buffer system of Laemmli (1970). The sample for each run contained 25 mg protein. The elution position of NifH-related polypeptides was determined by Western blotting. Selected fractions containing NifH-related polypeptides were subjected to analyses by MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) and ES-MS/MS (electrospray tandem mass spectrometry).

Analysis of NifH-related polypeptides by MALDI-TOF MS. For in-gel digestion of proteins with trypsin, a modification of the procedure of Shevchenko *et al.* (1996) was used, and trypsin (20 ng μl^{-1}) was prepared in 50 mM ammonium bicarbonate buffer. Disulfide bonds, if present, were reduced with 10 mM DTT. Proteins were alkylated with 50 mM iodoacetamide. When necessary, the gel pieces were dehydrated in acetonitrile and rehydrated in 100 mM ammonium bicarbonate buffer. The peptides were recovered with Zip-Tips as described by the manufacturer (Millipore). At the final step, the peptides were eluted with 3 µl of a saturated solution of 4-hydroxy-α-cyanocinnamic acid in 1 : 1 (v/v) acetonitrile/acidified water.

Mass spectra of the peptides were obtained on a Kratos Kompact SEQ (Kratos Analytical) time-of-flight mass spectrometer. Pulses of

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radiation at 337·1 nm and 3 ns duration were directed at the sample/matrix mixture. The resulting ions were accelerated through a 1·8 m flight tube by a potential difference of 20 kV. The laser fluence and spot position were varied manually during data acquisition. Spectra were recorded and processed using the Kratos LAUNCHPAD MALDI software, version 1.2.0.

To match proteins in the SWISS-PROT database, the determined peptide masses were compared with values computed from the database entries according to the cleavage specificity of trypsin. The computed peptide masses include those resulting from modifications of cysteine (alkylation by iodoacetamide or acrylamide) or oxidation of methionine, and allow up to one missed cleavage. All necessary computation was implemented using PEPTIDENT, available at the ExPASy (Expert Protein Analysis System) molecular biology server (http://us.expasy.org).

Analysis of NifH-related polypeptides by ES-MS/MS. ES-MS/MS analysis of NifH-related polypeptides was performed by the W. M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia (Charlottesville, VA, USA). The LC-MS system consisted of a Finnigan LCQ ion trap mass spectrometer system with a Protana nanospray ion source interfaced to a Phenomenex Jupiter 10 μm C18 reversed-phase capillary column. The peptides were eluted from the column by an acetonitrile/0·1 M acetic acid gradient. The nanospray ion source was operated at 2·8 kV. The digest was analysed using the double-play capability of the instrument, acquiring full-scan mass spectra to determine peptide molecular masses, and product-ion spectra to determine amino acid sequence in sequential scans. The data were analysed by database searching using the SEQUEST search algorithm. Peptides that were not matched by this algorithm were interpreted manually and searched versus the EST databases using the SEQUEST algorithm.

RESULTS

Detection of a second *nifH*-related mRNA in *C. pasteurianum*

Northern blot analysis of RNA from nitrogen-fixing cells of *C. pasteurianum* detected two major mRNA populations (4 and 0·9 kb) that hybridized to the *nifH* probe (Fig. 1a). The 4 kb mRNA is the transcript of the structural genes (*nifH1DK*) for the molybdenum-containing nitrogenase; the other *nifH*-containing regions are not expected to produce a transcript of this size under molybdenum-sufficient growth conditions (Wang *et al.*, 1988; Zinoni *et al.*, 1993). The 0·9 kb transcript was not reported previously, and it could have arisen from either degradation or processing of the 4·0 kb mRNA, or it might be a *bona fide* transcript from one of the *nifH*-like genes. After performing Northern blot analysis, the 0·9 kb region (the dotted square in Fig. 1a) of the RNA-containing membrane was cut out and used in an RT-PCR experiment. A 350 bp fragment was amplified (Fig. 1b) and sequenced (by Davis Sequencing). The sequenced fragment encompassed nucleotides 198 to 548 of the coding region, which contains characteristic nucleotides of each *nifH*-like gene (Wang *et al.*, 1988). The sequence revealed that the 0·9 kb message was from the *nifH2* gene of *C. pasteurianum*.

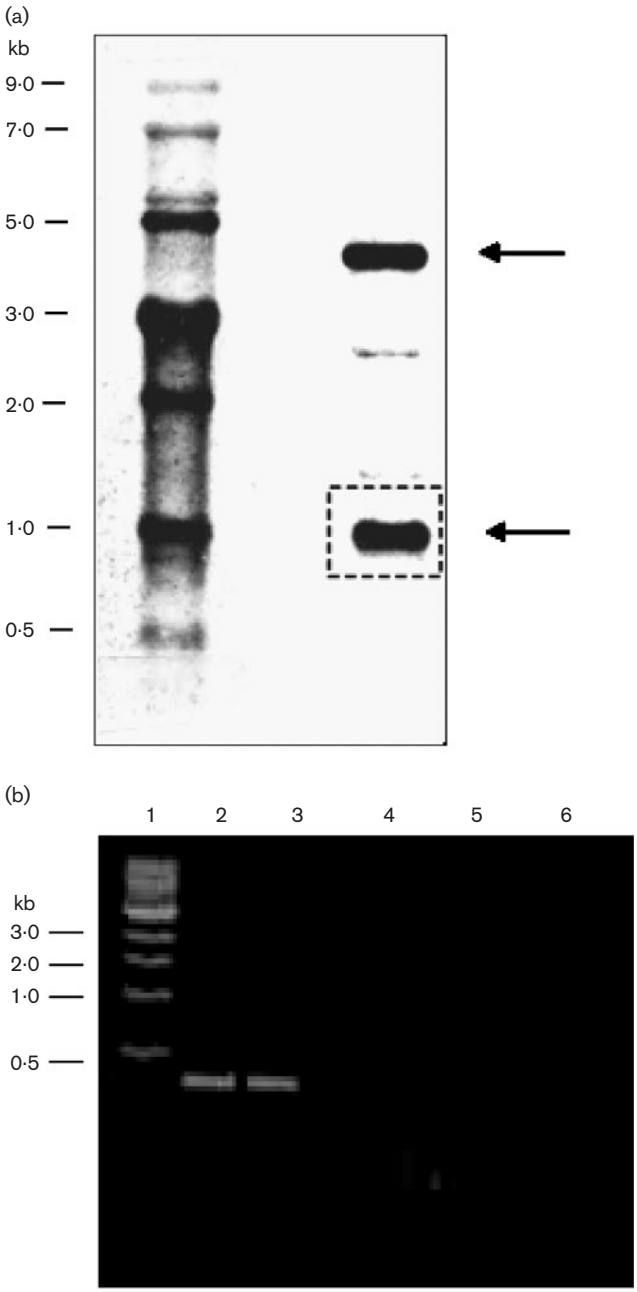


Fig. 1. Detection and RT-PCR amplification of the 0·9 kb *nifH*-like mRNA of *C. pasteurianum*. (a) Northern blot of mRNA from nitrogen-fixing cells of *C. pasteurianum*. Total RNA was hybridized with an HRP-labelled, *nifH1*-derived probe. Two mRNA bands at the sizes of 4·0 and 0·9 kb are clearly visible (arrows). With the RNA markers and the X-ray films as references, the 0·9 kb message was localized and a portion (shown as a dotted square) of the membrane was cut out from the running track of the sample and used in a RT-PCR experiment. (b) Separation of RT-PCR products in a 1 % agarose gel. Lane 1, DNA ladder; lane 2, positive control with genomic DNA as the template; lane 3, RT-PCR products; lane 4, negative control with a membrane piece cut from the 2·0 kb region as the template; lane 5, RT-PCR with the RNA isolated from a non-nitrogen fixing culture of *C. pasteurianum*; lane 6, negative control (without reverse transcriptase).

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Expression of *nifH2* versus *nifH1*

To compare the expression of *nifH1* and *nifH2*, *C. pasteurianum* was grown in a nitrogen-fixing medium, and cells were collected periodically for the isolation of RNA. The RNA was analysed by Northern blotting with a *nifH* probe. As expected, two major signals, at 4 and 0·9 kb, were observed. A visual examination of the signal intensity of the 0·9 kb and the 4 kb bands in the periodical RNA samples showed that the *nifH2* gene was expressed in parallel to the *nifH1DK* genes under the growth conditions used in this study, and the level of mRNA did not change significantly throughout the entire measuring period, although the level of the *nifH2* mRNA was somewhat higher in early samples (3, 6 and 11 h) than in late samples (25 and 33 h) relative to the *nifH1DK* mRNA (Fig. 2a).

In a similar experiment, a nitrogen-fixing culture of *C. pasteurianum* was supplemented with ammonium acetate to a concentration of 4 mM when the culture was 5 h old, and RNA was isolated before and after ammonium acetate addition to study the effect of ammonium ions on the synthesis and stability of the *nifH2* mRNA. As shown in Fig. 2(b), the addition of ammonium acetate caused almost complete disappearance of the *nifH2* signal as well as the *nifH1DK* signal (the 8 h time point). Synthesis of the *nifH2* mRNA resumed later (the 11 h timepoint in Fig. 2b). The concentration of ammonium ion in the medium was not measured in this study. However, earlier studies established that under similar conditions ammonium ions would be exhausted about 4 h after the start of growth (Daesch & Mortenson, 1972), and synthesis of new *nif* mRNA would ensue (Seto & Mortenson, 1974). No significant differences were observed in the expression patterns of the *nifH1DK* and *nifH2* genes until the culture was 27 h old. At that point, the level of the *nifH1DK* mRNA was significantly reduced, but the level of the *nifH2* mRNA was still relatively high. This pattern differed from that shown in Fig. 2(a). Whether or not this difference between the expression of the *nifH2* gene and the *nifH1DK* genes is physiologically important is yet to be determined.

Immunological detection of NifH-related polypeptides in nitrogen-fixing cells of *C. pasteurianum*

Proteins in crude extracts were separated by SDS-PAGE, and analysed by Western blotting using an antiserum raised against the Fe protein of *A. vinelandii*. The signal given by *C. pasteurianum* was compared with that given by *Clostridium beijerinckii* and *A. vinelandii*. When an equal amount of cellular proteins was analysed, the signal given by *C. pasteurianum* was much broader than that given by *C. beijerinckii* or *A. vinelandii* (data not shown). A closer examination of the Western blots suggested that more than one NifH-related polypeptide, having a molecular mass similar to that of the NifH1 polypeptide, were present in nitrogen-fixing cells of *C. pasteurianum* (Fig. 3). To further resolve the polypeptides by SDS-PAGE, different acrylamide concentrations (10, 12 and 15 % and 4–20 % gradient gels) were tested. SDS-PAGE on 15 % gels provided the best resolution, and the band patterns suggested the presence of a NifH-related polypeptide in addition to the NifH1 polypeptide in the crude extracts of nitrogen-fixing *C. pasteurianum* (Fig. 3a,b).

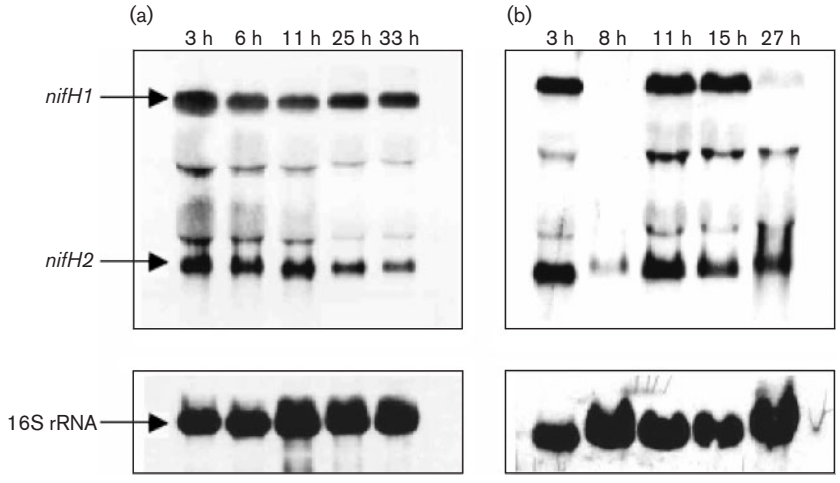


Fig. 2. Northern blot analysis of the *nifH1* and *nifH2* mRNAs of *C. pasteurianum*. Cells were grown in a defined nitrogen-fixing medium under a stream of nitrogen gas and collected periodically throughout incubation; total RNA was isolated from these fresh cells. Total RNA (6 μg per well) was resolved on a 0·7 % formaldehyde-agarose gel. The RNA species were then transferred to positively charged nylon membranes by capillary elution with 20× SSC, and the resulting membranes were incubated with a 350 bp HRP-labelled, *nifH1*-derived probe at 42 °C in a hybridization buffer containing 0·5 M NaCl. For comparison, the membranes were stripped in 0·1 % SDS solution and incubated with a HRP-labelled 16S rRNA probe. (a) Expression of *nifH1* and *nifH2* in a nitrogen-fixing culture; (b) expression of *nifH1* and *nifH2* in an ammonia-supplemented nitrogen-fixing culture. For the ammonia-supplemented culture, ammonium acetate was added to a nitrogen-fixing culture when the culture was 5 h old.

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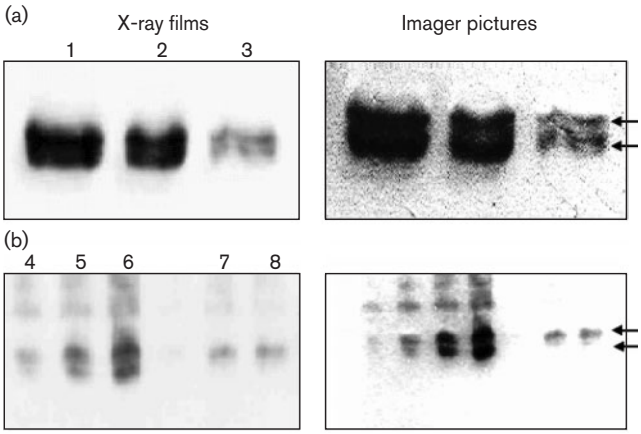


Fig. 3. Western blot analysis of the NifH proteins of *C. pasteurianum*. A cell-free extract prepared from nitrogen-fixing cells was analysed by SDS-PAGE with 15 % total acrylamide (a) and non-denaturing PAGE with 12 % total acrylamide (b). Electrophoretic transfer of proteins onto a positively charged nitrocellulose membrane was done in a semi-dry electrophoretic transfer cell. Western blots were probed with an anti-NifH serum and analysed using chemiluminescent detection. Lanes 1 to 6 contain different quantities of the same cell-free extract; lanes 7 and 8 contain a cell-free extract prepared from nitrogen-fixing cells of *C. beijerinckii* NRRL B593, which served as a control. The pictures shown were generated from the X-ray films with the use of an imager to enlarge the region containing the NifH bands. The arrows indicate relative positions of the NifH proteins.

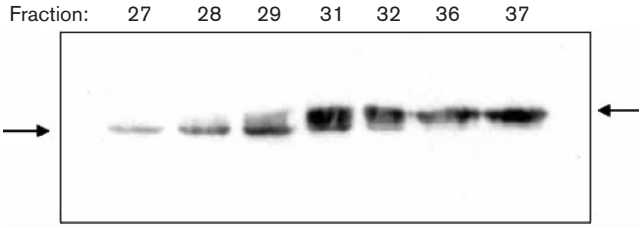


Fig. 4. Western blot analysis of NifH polypeptides of *C. pasteurianum* after separation by preparative gel electrophoresis. A cell-free extract (approximately 25 mg protein) prepared from nitrogen-fixing cells of *C. pasteurianum* was resolved with a Bio-Rad Prep Cell (model 491). Fractions 27, 28, 29, 31, 32, 36 and 37 were analysed by SDS-PAGE with 15 % total acrylamide. Electrophoretic transfer of proteins onto a positively charged nitrocellulose membrane was done in a semi-dry electrophoretic transfer cell. The membranes were probed with an anti-NifH serum using chemiluminescent detection. Fractions 27 and 28 contained one of the NifH polypeptides, fractions 29, 31 and 32 contained both of the NifH polypeptides, and fractions 36 and 37 contained the other NifH polypeptide. The arrows indicate the relative positions of NifH polypeptides.

Separation of NifH-related polypeptides from *C. pasteurianum* by preparative SDS-PAGE

The NifH-related polypeptides of *C. pasteurianum* were separated by preparative SDS-PAGE. Selected fractions were subjected to analytical SDS-PAGE, and the Coomassie blue-stained gels were examined for the presence of polypeptides with a size similar to that of NifH1 (M_r 29 666). Fractions 10 to 37, which spanned this size range, were analysed by Western blotting for the presence of NifH-related polypeptides, and fractions 27 to 37 were found to be NifH-positive. A closer examination of the Western-blot membrane (Fig. 4) suggested that fractions 27, 28, 36 and 37 each contained one NifH-related band. Consistent with the order of collection during SDS-PAGE, the polypeptides present in fractions 27 and 28 moved slightly faster than that present in fractions 36 and 37. Fractions 29 through 35, on the other hand, each gave two NifH-related bands, corresponding to the NifH-related band found in fractions 28 and 36, respectively.

Identification of NifH-related polypeptides of *C. pasteurianum* by MALDI-TOF-MS analysis

An analysis of the tryptic peptides from the proteins in fractions 28, 31 and 37 (see Fig. 4 for a description of these fractions) provided the first clue that distinct NifH-related polypeptides were present in *C. pasteurianum*, rather than the presence of NifH1 and its modified products. For fraction 28, peptide-mass fingerprinting (PEPTIDENT) gave the following number (in parentheses) of matching peptides with each NifH-related polypeptide of *C. pasteurianum*: NifH1 (7), NifH2 (5), NifH3 (5), NifH4 (6) and NifH5 (8). It should be noted that because of a high degree of similarity between the NifH sequences (Wang *et al.*, 1988), some of the matching peptides are common to two or more NifH-like sequences, hence limiting the number of peptide masses that can be specifically assigned to a NifH-like polypeptide. At present, there is no compelling evidence for the presence of NifH2, NifH3 or NifH4 in fraction 28. The number of matched peptides indicated the presence of NifH1 or NifH5 or both in fraction 28. Although eight peptides could be matched with the NifH5 sequence, only one (for positions 261–273) of them is specific for NifH5. To make this peptide mass compatible with the NifH5 sequence, it had to have an oxidized methionine residue and a mass tolerance of −1·1326 Da. It is most probable that NifH5 was not present in fraction 28. For NifH1, two of the seven matching peptides (positions 75–81 and 211–216) can be considered specific for this polypeptide, if NifH5 is excluded, whereas the other five matching peptides are conserved among NifH1 through NifH5. Because *nifH1* encodes the purified Fe protein and there is no strong evidence for the presence of NifH2 through NifH5 in fraction 28, we concluded this fraction contained NifH1.

For fraction 31, peptide-mass fingerprinting gave the following number (in parentheses) of matching peptides with each NifH-related polypeptide of *C. pasteurianum*: NifH1 (7), NifH2 (6), NifH3 (5), NifH4 (4) and NifH5 (6). None of the four matching peptides with NifH4 was specific for

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this NifH isoform. Among the five matching peptides with NifH3, three were specific; however, in order for the peptide masses to fit, Cys-39 in the peptide for positions 34–47 must be modified by iodoacetamide or acrylamide. It was concluded (Wang *et al.*, 1988) that *nifH3* mRNA was absent in nitrogen-fixing and non-nitrogen-fixing cells grown under molybdenum-sufficient conditions (conditions used in this study). Therefore, the presence of NifH3 in the fraction was unlikely. For the seven matching peptides with NifH1, one was specific (positions 222–242), whereas two were common with NifH5 (positions 75–81 and 244–260). For the six matching peptides with NifH5, none was specific for this isoform, while two were shared only with NifH1. The six matching peptides for NifH2 contained two (positions 223–238) that are specific for this isoform, but the peptide masses required modification of Cys-231 by iodoacetamide or acrylamide. It may be tentatively concluded that fraction 31 contained NifH1 and NifH2.

For fraction 37, there was no specific matching peptide for NifH4, although there were two peptides (positions 82–97; one with both Cys residues alkylated by iodoacetamide, and the other with both Cys residues forming acrylamide adducts) that could match NifH1 through NifH5. There were five peptides matching NifH1/NifH5, three of which were specific for NifH1/NifH5. There were also five peptides matching NifH2, three of which were specific for NifH2. Thus, the MALDI-TOF-MS results suggest that NifH1 was present in fractions 28, 31 and 37. On the other hand, NifH2 was only found in fractions 31 and 37, indicating a slower mobility of this isoform(s) than NifH1 on SDS-PAGE.

In a separate experiment, we subjected a crude extract of nitrogen-fixing *C. pasteurianum* to analytical SDS-PAGE and analysed a gel slice that contained polypeptides with a nominal M_r of 30 000. The peptide masses obtained from two runs of MALDI-TOF-MS were used in a database search, and the highest scores and numbers of matching peptides (in parentheses) were with NifH5 (18), NifH1 (16) and NifH2 (11). In addition, eight peptides matched NifH3, of which four were specific. For NifH5, four of the five NifH5-specific peptides (different modifications of positions 261–273, 217–238 and 222–238) were assignable only when a relatively large mass tolerance (deviations of −1·132, −1·133, −1·157, and 1·263 Da) was allowed. Therefore, the results again suggested the presence of NifH1 and NifH2 in *C. pasteurianum*.

To confirm the resolving power of MALDI-TOF-MS, we

Table 1. Tryptic peptides of *C. pasteurianum* NifH isoforms identified by ES-MS/MS

NifH isoform	Mass*	Position (start and end residue)
NifH2	2274·2	55–74
	2001·8 (C^A)	223–238
	1426·7 (M^O)	261–272
	1410·7	261–272
	1095·6 (M^O)	1–9
	792·46	3–9
	737·39	75–81
	618·35	211–216
NifH1 + NifH2†	2519·2 (M^O, M^O)	141–163
	2503·2 (M^O)	141–163
	2487·2	141–163
	1616·6 (C^A, C^A)	82–97
	1602·6 (C^A, C^I)	82–97
	1588·6 (C^I, C^I)	82–97
	1610·8	185–198
	1482·7	186–198
	1395·8	199–210
	1131·6 (C^A)	176–185
	1117·6 (C^I)	176–185
	1003·5 (C^A)	176–184
	989·5 (C^I)	176–184
	912·59	46–54
	574·32	217–221
NifH1†	2992·5	55–81
	2700·2 (C^A)	217–238
	2260·2	55–74
	2131·1 (M^O)	243–260
	2115·1	243–260
	1662·8 (M^O)	15–30
	1646·8	15–30
	1599·8 (M^O, M^O)	261–273
	1372·8	243–254
	1233·7 (M^O)	211–221
	1149·5 (C^A, M^O)	31–40
	1135·5 (C^I, M^O)	31–40
	1133·5 (C^A)	31–40
	1119·5 (C^I)	31–40
	1081·6 (M^O)	1–9
	1065·6	1–9
	778·45	3–9
	678·35 (M^O)	211–216
	662·35	211–216

Table 1. cont.

*The mass reported here was from a peptide containing a modified cysteine or methionine residue, when it is denoted by C^I (cysteine alkylated by iodoacetamide), C^A (cysteine alkylated by acrylamide) or M^O (methionine oxidized to methionine sulfoxide). †The deduced amino acid sequences of NifH1, H2, H4 and H5 are identical in the regions (start and end residue numbers) 56–74 and 119–212. Some of the peptides listed under NifH1 or under (NifH1 + NifH2) here fall into these regions and could have arisen from *nifH4* or *nifH5*, but there is no other evidence to suggest the presence of the NifH4 or NifH5 protein in the nitrogen-fixing cells of *C. pasteurianum*. See Fig. 5 for the locations of the tryptic peptides in the NifH isoforms.

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analysed several other samples: (i) the purified Fe protein of *A. vinelandii*, (ii) bovine serum albumin, (iii) polypeptides with a nominal M_r of 70 000 from nitrogen-fixing *C. pasteurianum*, and (iv) a gel piece from SDS-PAGE that did not contain proteins. The *A. vinelandii* Fe protein and bovine serum albumin were correctly identified along with several other proteins with similar sequences (data not shown). The gel piece without proteins did not yield any relevant peaks. *C. pasteurianum* polypeptides with a M_r of 70 000 did not yield any peptide masses assignable to any NifH-related proteins. The results further supported the conclusion that, in addition to NifH1, one or more NifH-related polypeptides were present in *C. pasteurianum*. To further identify the NifH isoforms in *C. pasteurianum*, we subjected fraction 37 to analysis by electrospray tandem mass spectrometry (ES-MS/MS).

Identification of NifH-related polypeptides of *C. pasteurianum* by ES-MS/MS analysis

The ES-MS/MS analysis determines the amino acid sequence, in addition to the mass, of the tryptic peptides and hence allows more conclusive identification of the NifH isoforms in a sample. Table 1 lists the NifH-related peptides that were detected in fraction 37 by ES-MS/MS. Eight of the detected tryptic fragments were NifH2-specific (the eight discrete fragments represented seven sequences, as one sequence was represented by two fragments that differed in the oxidation state of the methionine residue). These tryptic fragments are conclusive evidence for the presence of the NifH2 polypeptide in *C. pasteurianum* (Fig. 5).

Fifteen of the detected tryptic fragments, which represented nine sequences, could be from NifH1/NifH5 as well as NifH2, as these fragments were from regions that are conserved in NifH1/H2/H5. It is conceivable that at least some of these fragments were derived from NifH2 but not from NifH5, as explained in the preceding section. These 15 fragments are therefore considered to be from NifH1 and NifH2.

The NifH4 sequence is highly related to the NifH1/NifH5 and NifH2 sequences (Wang *et al.*, 1988). For example, the NifH1/H2/H4/H5 sequences are identical at positions 32–54, 79–117 and 119–212. Therefore, some of the peptides (such as those covering positions 82–97, 141–163 and 185–198) that we assigned to NifH1 and NifH2 could be from NifH4. However, because we did not detect any NifH4-specific peptide by either MALDI-TOF-MS or ES-MS/MS analysis, there is no reason to suggest the presence of the NifH4 polypeptide in nitrogen-fixing cells of *C. pasteurianum*.

Nineteen of the detected tryptic fragments, which represented 12 discrete sequences, have been assigned to NifH1, although 17 of them could also be from NifH5. NifH1 and NifH5 differ by two amino acid residues, Tyr-227 and Gln-267 in NifH1 versus Phe-227 and Glu-267 in NifH5. The two fragments with masses 2700·2 (positions 217–238) and 1599·8 (positions 261–273), respectively, contained Tyr-227 and Gln-267 characteristic of NifH1. Furthermore, among the possible tryptic peptides from the five NifH isoforms of *C. pasteurianum*, the peptide covering positions 217–238 (AEINKQTVIEYDPTCEQAEEYR) is unique to NifH1 (Fig. 5). Because a NifH5-specific peptide has not been detected, we assign these 19 peptides to NifH1 (Table 1).

DISCUSSION

Multiple *nifH* or *nifH*-related sequences are present in several diazotrophs. Besides the *vnfH* and *anfH* genes for the alternative nitrogen-fixation systems (Bishop &

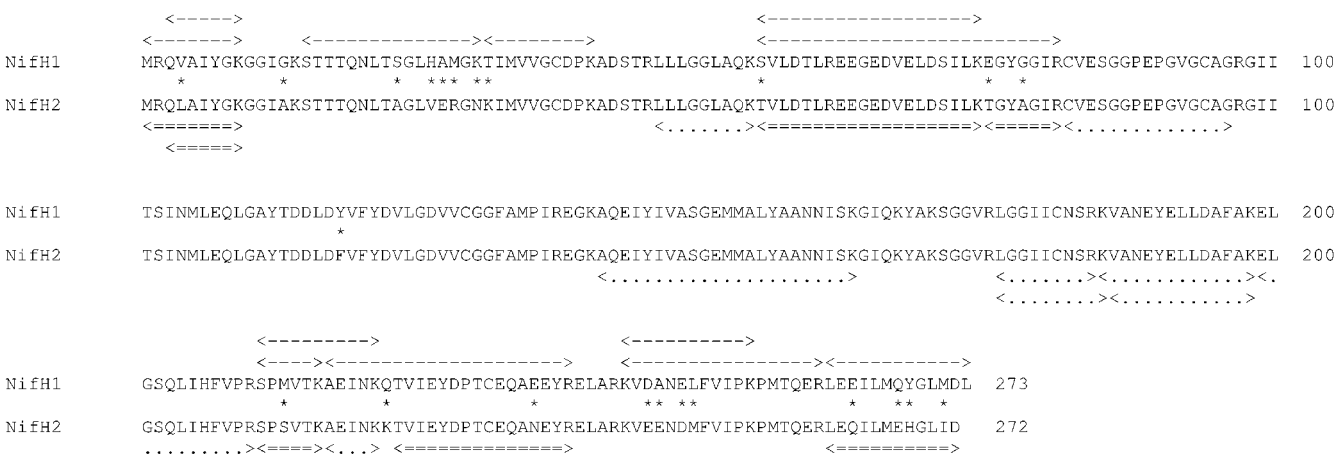


Fig. 5. Alignment of NifH1 and NifH2 amino acid sequences and positions of tryptic peptides detected by ES-MS/MS. *, Residues different between NifH1 and NifH2; <-->, NifH1-specific peptides detected by ES-MS/MS analysis; <===>, NifH2-specific peptides detected by ES-MS/MS analysis; <.>, peptides common in NifH1 and NifH2 detected by ES-MS/MS analysis.

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Premakumar, 1992), there are reiterated *nifH* genes in *Rhizobium* (Martinez *et al.*, 1985) and *Paenibacillus* (Choo *et al.*, 2003). In *Rhizobium* ORS571, two *nifH* genes were characterized (Norel & Elmerich, 1987), and both genes appear functional. However, activity measurements (*ex planta*) showed that *nifH1* accounts for about 70 % of the nitrogen-fixing activity, whereas *nifH2* accounts for only 30 %. In *Rhizobium etli* (formerly *Rhizobium phaseoli*), three *nifH* genes were identified; none is indispensable for nitrogen fixation and at least two are functionally expressed (Morett *et al.*, 1988).

The presence of five *nifH*-related genes in an organism is uncommon. Whether or not the *nifH*-like genes (*nifH2* through *nifH5*) of *C. pasteurianum* are functional and what physiological roles they play are important questions to be answered. The purified Fe protein (NifH1 dimer) of *C. pasteurianum* was sequenced (Tanaka *et al.*, 1977). If certain amino acids were detected at specific sequencing steps, it could indicate the contamination of the purified Fe protein with one or more of NifH-related polypeptides. An examination of the protein sequencing data showed that the purified Fe protein could not contain more than trace amounts of proteins from the *nifH*-like genes (Wang *et al.*, 1988). However, this conclusion does not rule out the possibility that other NifH-related proteins are present in the cell but are separated from the *nifH1*-encoded Fe protein during the purification of an active Fe protein. This study provided conclusive evidence for the synthesis of NifH2 in addition to NifH1. If NifH2 forms a homodimer or a NifH2-NifH1 heterodimer, such a dimer is probably not an active Fe protein. This conjecture is based on the results from several laboratories. During the purification of the Fe protein from *C. pasteurianum*, the NifH1 dimer was the only active component that complemented the MoFe protein in a nitrogenase assay (Tso *et al.*, 1972; Zumft & Mortenson, 1973).

To deduce a possible function for NifH2, it should be useful to compare the structures of NifH2 and NifH1. The predicted main-chain fold of the NifH2 polypeptide was obtained by modelling NifH2 against the crystallographically determined structure of the Fe protein of *C. pasteurianum*, and the two structures were similar (data not shown). Between NifH1 and NifH2, 10 of the 22 different amino acids occur near residues that are either involved in

Table 2. Location and properties of the 22 different amino acid residues between NifH1 and NifH2 of *C. pasteurianum*

Residue no.	NifH1	NifH2	Location in the protein*	No. of residues involved in the proposed function*
4	Val	Leu†	β1 (2–8)	
23	Ser	Ala	α1 (14–27)	
26	His	Val†		
27	Ala	Glu†		
28	Met	Arg†	Loop (28–31)	
30	Lys	Asn†		
31	Thr	Lys†		
55	Ser	Thr	Switch I (38–55)	4, dimer interface; 4, nucleotide binding
75	Glu	Thr†	β3 (73–75)	
78	Gly	Ala	3_{10} (76–80)	
118	Tyr	Phe	β5 (118–124)	
213	Met	Ser†	α7 (212–220)	6, dimer interface; 3, nucleotide binding
222	Gln	Lys†	Loop (221–231)	1, dimer interface
235	Glu	Asn†	α8 (232–246)	2, nucleotide binding
245	Asp	Glu		
246	Ala	Glu†		
248	Glu	Asp	Loop (247–257)	
249	Leu	Met		
262	Glu	Gln†	α9 (258–268)	4, dimer interface
267	Gln	Glu†		
268	Tyr	His†		
271	Met	Ile	C-terminal	1, dimer interface

*Nomenclature of the secondary structure (with the start and end residues in parentheses), and number of residues involved in the proposed functions are according to Schlessman *et al.* (1998) for NifH1. See Georgiadis *et al.* (1992) for a schematic representation of the secondary structure.

†The side-chains of these residues, when compared with those in NifH1, are either larger in volume, different in charge properties or different in tendency to form helices.

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dimer interactions or implicated in nucleotide binding (Table 2). When compared with NifH1, the side-chain of 14 of the 22 different residues in NifH2 is either larger in volume, different in charge properties, or different in tendency to form α-helices. The changes of Met-213 to Ser (a drastic decrease in tendency to form α-helix) and Gln-222 to Lys (appearance of a positive charge) could be significant. They occur in or near α7 (nomenclature of Schlessman *et al.*, 1998; see Georgiadis *et al.*, 1992, for a schematic representation of the secondary structure), which encompasses eight amino acids (residues 210, 212, 215, 216, 218–221) that are involved in dimer interactions and three amino acids (residues 214, 215, 218) that are involved in nucleotide binding. The presence of Ser-213 and Lys-222 in NifH2 may cause the NifH2 monomer (or dimer, if one is formed) to interact differently with the MoFe protein, when compared with NifH1.

The change of Ala-246 to Glu increases negative charges (from 2 to 3 among residues 245–248) in a region connecting α8 and α9 (α9 is next to the C-terminus). The C-terminal region of *C. pasteurianum* NifH1 is shorter than that of NifH of other organisms (Chen *et al.*, 1986), which limits its involvement in intersubunit interaction and contribution to dimer stabilization (Schlessman *et al.*, 1998). The cluster of negative charges near α9 may alter the topography of this region in NifH2 and allow a positively charged metabolite to bind to NifH2 and cause the NifH2 monomer or dimer to assume an altered conformation. If a putative NifH2 dimer cannot donate electrons to the MoFe protein or if the altered conformation of the NifH2 dimer or monomer has a higher affinity than the NifH1 dimer for the MoFe protein, the tight binding between NifH2 and the MoFe protein could be a mechanism, albeit archaic, for switching off nitrogenase activity. This is similar to the proposal of Wang *et al.* (1988) that, under certain physiological conditions, products from some of the *nifH*-like genes might serve to modulate (e.g. down-regulate) nitrogenase activity by acting as an inhibitor of the MoFe protein. Studies on heterologous pairs showed that an incompatible Fe protein could act as an inhibitor of nitrogenase activity (Emerich & Burris, 1978; Clarke *et al.*, 2000).

NifH2 is expressed throughout growth in parallel to NifH1, and addition of ammonium acetate to the culture affected the expression of both genes similarly. A proposed regulatory role for NifH2 may involve the protein as a signal-transducing protein to relay the nitrogen status to the MoFe protein. In this regard, it may be noted that in *C. acetobutylicum* and *C. beijerinckii*, the $nifI_1$ (*glnB1*) and $nifI_2$ (*glnB2*) genes occur between the *nifH* and *nifD* genes, but *C. pasteurianum* does not have similar genes according to results of Southern hybridization and PCR (J. Toth & J.-S. Chen, unpublished results).

Because *C. pasteurianum* is at present not amenable to genetic manipulations, further biochemical and functional characterization of the NifH2 protein cannot be readily performed. On the other hand, the nitrogen-fixing *C. acetobutylicum* and *C. beijerinckii* can now be genetically manipulated (Chen, 2004), so it might be possible to express NifH2 in these species. Because the Fe and MoFe proteins are highly conserved in these three nitrogen-fixing clostridia, the presence of NifH2 in *C. acetobutylicum* or *C. beijerinckii* may allow a physiological study of NifH2. Using appropriate vectors, NifH2 may be expressed in *C. acetobutylicum* or *C. beijerinckii* under non-nitrogen-fixing conditions so that NifH2 can be purified from cells without NifH1.

The nitrogen-fixing species within the genus *Clostridium* are traditionally considered representatives of anaerobic, free-living nitrogen-fixers. These nitrogen fixers, because of their free-living lifestyle, were not considered active contributors of fixed nitrogen for supporting plant growth. A recent study, however, could change this view because clostridia were found as nitrogen-fixing endophytes in consortia with nondiazotrophic bacteria in tissues of gramineous plants from a wide region of Asia (Minamisawa *et al.*, 2004). The newly isolated nitrogen-fixing clostridia from gramineous plants are phylogenetically close to known nitrogen-fixing species, including *C. beijerinckii* and *C. pasteurianum*, as well as to species such as *Clostridium intestinale* (formerly *Clostridium intestinalis*) and *Clostridium saccharoperbutylacetonicum*, which were not previously reported as nitrogen fixers. The newly discovered niches for nitrogen-fixing clostridia suggest a more significant role for the obligate anaerobes in supporting plant growth via nitrogen fixation. It will be interesting to examine these newly isolated nitrogen-fixing clostridia for their composition of *nif* genes.

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