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Characterization of an Operon Encoding an NADP-Reducing Hydrogenase in *Desulfovibrio fructosovorans*

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A genomic DNA fragment from *Desulfovibrio fructosovorans*, which strongly hybridized with the *hydAB* genes from *Desulfovibrio vulgaris* Hildenborough, was cloned and sequenced. This fragment was found to contain four genes, named *hndA*, *hndB*, *hndC*, and *hndD*. Analysis of the sequence homologies indicated that HndA shows 29, 21, and 26% identity with the 24-kDa subunit from *Bos taurus* complex I, the 25-kDa subunit from *Paracoccus denitrificans* NADH dehydrogenase type I, and the N-terminal domain of HoxF subunit of the NAD-reducing hydrogenase from *Alcaligenes eutrophus*, respectively. HndB does not show any significant homology with any known protein. HndC shows 37 and 33% identity with the C-terminal domain of HoxF and the 51-kDa subunit from *B. taurus* complex I, respectively, and has the requisite structural features to be able to bind one flavin mononucleotide, one NAD, and three [4Fe-4S] clusters. HndD has 40, 42, and 48% identity with hydrogenase I from *Clostridium pasteurianum* and HydC and HydA from *D. vulgaris* Hildenborough, respectively. The 4.5-kb length of the transcripts expressed in *D. fructosovorans* and in *Escherichia coli* (pSS13) indicated that all four genes were present on the same transcription unit. The sizes of the four polypeptides were measured by performing heterologous expression of *hndABCD* in *E. coli*, using the T7 promoter/polymerase system. The products of *hndA*, *hndB*, *hndC*, and *hndD* were 18.8, 13.8, 52, and 63.4 kDa, respectively. One *hndC* deletion mutant, called SM3, was constructed by performing marker exchange mutagenesis. Immunoblotting studies carried out on cell extracts from *D. fructosovorans* wild-type and SM3 strains, using antibodies directed against HndC, indicated that the 52-kDa protein was recognized in extracts from the wild-type strain only. In soluble extracts from *D. fructosovorans* wild type, a 10-fold induction of NADP reduction was observed when H_2 was present, but no H_2-dependent NAD reduction ever occurred. This H_2-dependent NADP reductase activity disappeared completely in extracts from SM3. These results indicate that the *hnd* operon actually encodes an NADP-reducing hydrogenase in *D. fructosovorans*.

Hydrogenases (Hyds) are iron-sulfur enzymes which are responsible for the oxidation of molecular hydrogen, as well as for the reduction of protons during molecular hydrogen production. Hyds are key enzymes in the energy metabolism of the sulfate-reducing bacteria belonging to the genus *Desulfovibrio*, which use oxidized sulfur compounds as their terminal electron acceptors (31). Many *Desulfovibrio* species can use molecular hydrogen as their sole energy source (2); thus, the hydrogen oxidation which is thought to take place on the periplasmic side of the inner membrane would be involved in the electron transfer across the membrane and the creation of a proton motive force. Alternatively, *Desulfovibrio* species can grow on lactate or pyruvate, the oxidation of which in the cytoplasm may result in H_2 production (7, 15, 26). It has not yet been clearly established whether this H_2 production is attributable to regulation of the redox state of the electron transfer chain (8) or to H_2 cycling, which may drive ATP synthesis (13). The number, type, and cellular location of Hyds can vary from one *Desulfovibrio* species to another. It has emerged from a survey of 22 *Desulfovibrio* isolates that [Fe] Hyds have not always been detected, unlike the [NiFe] Hyds, which were found to be present in every strain tested (28). Four classes of bacteria have therefore been distinguished: those with [Fe], [NiFe], and [NiFeSe] Hyds (class 1), those with [NiFe] and [Fe] Hyds (class 2), those with [NiFe] and [NiFeSe] Hyds (class 3), and those with [NiFe] Hyds only (class 4). This diversity makes the role of these various Hyds difficult to determine. To study the role of Hyds in the sulfate-reducing bacteria, we decided some years ago to use *Desulfovibrio fructosovorans* as a model because of its ability to effect substrate-level phosphorylation by fermenting fructose. This property might make it possible to obtain Hyd-null mutations that are not lethal to the organism even if Hyds are involved in the mechanism underlying oxidative phosphorylation, as has been previously suggested (13). *D. fructosovorans* MR400, from which the [NiFe] Hyd structural genes (*hynAB*) had been deleted, was constructed in order to correlate the functional deficit with the genotype of the mutant (18). Significant levels of residual Hyd activity have been observed in MR400, which were attributable to additional enzymes. This residual activity was sufficiently strong for growth to occur on organic substrates as well as on H_2-sulfate medium.

With a view to identifying the Hyd responsible for this residual activity, the genomic DNA of this organism was probed by using the *hydAB* [Fe] Hyd genes from *D. vulgaris* Hildenborough (27). The results presented here led to the discovery of a new NADP-reducing hydrogenase, the existence of which was hitherto unsuspected in a sulfate-reducing bacterium.

MATERIALS AND METHODS

Bacterial strains and plasmids. *D. fructosovorans* DSM 3604 has been described by Olivier et al. (14). *D. fructosovorans* Δ*hndC* is a null mutant which was obtained by performing marker exchange mutagenesis as described by Rousset et

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al. (18). *Escherichia coli* DH5α [*endA1 hsdR17*(r_K^- m_K^+) *supE44 thi*$^{-1}$ λ$^-$ *recA1 gyrA96 relA1* Δ(*argF-lacZYA*)U169 ϕ 80*dlac*(Δ*lacZ*)*M15*] and *E. coli* K38 (HfrC λ, suppressor free) (24) were used as hosts for the recombinant plasmids.

Plasmid pHV150, containing the structural *hydAB* genes from *D. vulgaris* Hildenborough on a 1.9-kb inset, was kindly provided by G. Voordouw (27). Plasmids pT7-5, pT7-6, and pGP1-2 (24) were used in the heterologous expression experiments.

Medium. *D. fructosovorans* was grown anaerobically at 35°C on bicarbonate-buffered saline medium (31) supplemented with 20 mM fructose as the carbon and energy source and 30 mM sulfate as the terminal electron acceptor. Agar (1.6%) and thiamphenicol (40 μg/ml) were added to isolate the deletion mutant in plates.

Gene cloning. One 13-kb *Sac*I fragment from *D. fructosovorans* strongly hybridized with the *hydAB* genes from *D. vulgaris* Hildenborough in Southern blotting experiment (data not shown). Chromosomal DNA from *D. fructosovorans* was then digested by *Sac*I, and the 12- to 15-kb fractions were isolated by performing agarose gel electrophoresis and purified with prep A gene (Bio-Rad SA, Yvry sur Seine, France). The fractions were then ligated into dephosphorylated *Sac*I-digested pUC18 (Appligène, Illkirch, France), and the recombinant plasmids obtained were used to transform *E. coli* DH5α. Plasmid pSS13 was isolated after screening 800 transformants by performing in situ hybridization, using the nick translation labelled ^{32}P *hydAB* probe from *D. vulgaris* Hildenborough.

Subcloning and nucleotide sequence analysis. The locations of the genes in the 13-kb insert hybridizing with the *hydAB* probe were determined by performing restriction analysis and Southern blotting experiments. A 4.9-kb *Dra*I-*Sph*I fragment was subcloned into *Sma*I-*Sph*I-digested pUC18 and sequenced. The resulting recombinant plasmid was named pSD5 (see Fig. 8). Overlaps were generated by exonuclease III-exonuclease VII digestions (34). The sequencing reaction was routinely performed with a Sequenase kit (U.S. Biochemical, Cleveland, Ohio). The complementary strand was sequenced by using specific oligonucleotides as primers. The 354 nucleotides (nt) upstream from *Dra*I were sequenced by walking on the chromosome.

RNA extraction and Northern (RNA) blotting. *D. fructosovorans* cells grown in fructose-sulfate medium were harvested in the middle of the exponential phase and rapidly cooled to 0°C by adding crushed ice. The total RNAs were extracted using the hot phenol method described by Miller (11). The Northern blotting experiment was performed as previously described (20). The 1.3-kb *Pst*I-*Pvu*II and 1.7-kb *Eco*RI-*Bam*HI fragments radiolabeled by random priming were used for Northern blotting (see Fig. 6).

Expression of the *hndABCD* genes in a T7 promoter/polymerase system. The 4.9-kb *Dra*I-*Sph*I insert was subcloned in *Sma*I-*Sph*I-digested pUC18. The resulting plasmid was then digested at *Sac*I and *Hin*dIII restriction sites present in the pUC18 polylinker, and the fragment obtained was subcloned in pT7-5, placing the transcription of the genes under the control of the T7 ϕ10 promoter (24). The resulting plasmid, pTHnd, was used to transform *E. coli* K38(pGP1-2), and the expression of the cloned genes was monitored by incorporating L-[^{35}S]methionine into the gene products. The proteins were separated on a sodium dodecyl sulfate (SDS)–11% polyacrylamide gel and autoradiographed after overnight exposure.

Gene replacement. The *Bam*HI site was first removed from the polylinker by digesting plasmid pSD5 at the *Sac*I site located in the polylinker and at the *Eco*RV site located 400 bp after *Dra*I (see Fig. 8). The resulting plasmid was called pES5. The 1.2-kb *Bcl*I-*Hin*PI 1 fragment from pBR325 which contains the *cat* gene was then ligated into *Bam*HI-*Cla*I-digested pES5. The recombinant plasmid pES5C, which cannot replicate in sulfate-reducing bacteria, was introduced into *D. fructosovorans* by electropermeation (18). The thiamphenicol-resistant strains were isolated on solid medium. The gene replacement in *D. fructosovorans* Δ*hndC* was checked by performing Southern blotting experiments using *cat* and *hndC* genes as probes (see Fig. 8). One mutant, SM3, was selected.

Southern blotting. Chromosomal DNAs from *D. fructosovorans* wild-type and SM3 strains were isolated by Marmur's method (9). Genomic DNA was digested for 5 h with *Asu*II, subjected to electrophoresis on a 0.7% agarose gel, and transferred overnight onto a nylon membrane (Schleicher & Schuell, Ecquevilly, France). The ECL Random Prime Labelling kit and detection system were used as described by the manufacturer (Amersham, Les Ulis, France). Hybridization was performed at 65°C, and the 1.4-kb *Xho*I-*Bam*HI fragment containing the *hndC* gene and the 1.2-kb *Hin*PI-*Bcl*I fragment containing the chloramphenicol resistance gene (see Fig. 8) were used to probe chromosomal DNAs.

Construction and purification of the cytoplasmic MBP fusion proteins. A protein fusion and purification system was used as instructed by the manufacturer (Biolabs, Montigny le Bretonneux, France). pSD5 was digested by *Alu*I, and the 1.7-kb *Alu*I-*Alu*I fragment containing the *hndC* gene was ligated into *Stu*I-digested pMAL-c1, which contains the gene encoding a cytoplasmic maltose-binding protein (MBP) (see Fig. 9). After the transformation of *E. coli*, cell extracts from several clones were analyzed to check the expression of a 94-kDa protein, which corresponded to the size expected for an MBP-HndC fusion protein. The fusion protein was purified in one step by using a maltose affinity column and cleaved with the specific protease factor Xa.

Antibodies. The 52-kDa HndC protein was then injected into a rabbit at the rate of about 300 μg every 20 days for 4 months. The serum collected was then precipitated with 50% $(NH_4)_2SO_4$, and the pellet was resuspended in phosphate-buffered saline (PBS; 8‰ NaCl, 0.2‰ KCl, 1.6‰ Na_2HPO_4, 0.24‰ KH_2PO_4 [pH 7.2]). After an overnight dialysis at 4°C against PBS, the dialysate was fixed to DEAE-Trisacryl equilibrated with PBS. The resin was then pelleted by centrifugation for 30 min at 6,000 × *g*, and the supernatant containing immunoglobulins was used in immunoblotting studies.

Western blot (immunoblot) transfer and immunoblotting. Soluble proteins from wild-type *D. fructosovorans* and mutant SM3 were separated by electrophoresis in SDS–12% polyacrylamide gels and transferred onto BA83 nitrocellulose membranes (Schleicher & Schuell), using a Biometra Fastblot B33 electroblotting apparatus (Science-Tec, Les Ulis, France). Immunoreactive proteins were detected by using rabbit anti-HndC serum (diluted 1:1,000) and anti-rabbit peroxidase-labeled immunoglobulins (BM chemiluminescence Western blotting kit; Boehringer Mannheim, Meylan, France).

Enzyme assays. *D. fructosovorans* cells were broken with a French press and centrifuged for 30 min at 7,500 × *g*. Enzyme assays were performed on the soluble fraction obtained after additional centrifugation for 2 h at 150,000 × *g*. This fraction was carefully maintained under nitrogen atmosphere. The H_2-driven NAD(P) reduction was measured spectrometrically at 30°C at 340 nm in a tightly closed quartz cuvette (21).

Nucleotide sequence accession number. The nucleotide sequence of the *D. fructosovorans hnd* operon has been submitted to the GenBank Sequence Data Library under accession number U07229.

RESULTS AND DISCUSSION

Nucleotide sequence analysis. The nucleotide and derived amino acid sequences of *hndABCD* genes are given in Fig. 1. The nucleotide sequence showed the existence of four open reading frames (ORFs), designated *hndA*, *-B*, *-C*, and *-D*, all oriented in the same direction. Each of these was preceded by potential ribosome binding sites. These genes were 513, 378, 1,434, and 1,749 bp long and were separated by 38-, 24-, and 19-bp intergenic regions, respectively. The possibility that these ORFs encode polypeptides was examined with the MBS computer program, using the *D. fructosovorans* codon usage table (19). According to this analysis, all four ORFs exhibited a high probability of coding for proteins.

The gene *hndD* possesses four regions (nt 3561 to 3691, nt 3764 to 3954, nt 3933 to 4073, and nt 4121 to 4210) which share a high degree of homology (70, 62, 63, and 68%, respectively) with the central part of *hydA* (nt 501 to 630, nt 700 to 882, nt 857 to 980, and nt 1055 to 1140), encoding the large subunit of the [Fe] Hyd from *D. vulgaris* Hildenborough (data not shown) (27). These strong homologies are likely to be responsible for the nonambiguous response obtained in the Southern blotting experiments.

Amino acid sequence analysis. The 18.8-kDa protein encoded by *hndA* (Fig. 2) showed 29% identity with the 24-kDa subunit of bovine complex I (4), 21% identity with the 25-kDa subunit of the NADH dehydrogenase type I from *Paracoccus denitrificans* (33), and 26% identity with the N terminus (residues 1 to 149) of the HoxF subunit of the NAD-reducing Hyd from *Alcaligenes eutrophus* (25). On the basis of evidence from previous biochemical and electron paramagnetic resonance spectroscopic studies, one [2Fe-2S] cluster has been assigned to the 24- and 25-kDa subunits of the proteins of mammalian and bacterial origin, respectively (reference 30 and references therein). This cluster may be accommodated by the four conserved cysteine residues present at positions C-98, C-103, C-139, and C-143 in the amino acid sequence of HndA.

The product of the *hndB* gene is a 13.8-kDa protein, the amino acid sequence of which did not show any homology with the protein from either the Swissprot or GenEMBL data bank.

HndC (Fig. 3) is a 51.7-kDa protein. Using the Swissprot data bank, we found that the region extending from residues 1 to 441 of HndC shows 37% identity with the C-terminal region (residues 161 to 612) of the HoxF subunit of the NAD-reducing Hyd from *A. eutrophus* (25) and 33% identity with the C-terminal region (residues 67 to 444) of the 51-kDa subunit from *Bos taurus* complex I (4). These proteins are known to be

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AATTCCATTATTTTTTGAAATCGGTCCGGAGCGCTCCACGCCCCGGGACCGGTGCCGGACGTATCCGGGGGAACCGCTTTCCGGGTGACTCTCCCCTGGG- 100
                                                                                        -35
TGTTTTTGTTAATAGCCTGTGTATACAGGCTTTTTTTTGTTGGTCCGGTTATTGCTGTTTCCGGGGCAGGCGTGAGGAAGAAGGGCCTGCTTTGGCGTGG- 200
         -10
AAGACGGCAGACATGGCTCAAGGGAATAAGCCCCCTTAGATTGTGAAAATAATCACAATCTAAGGGGGCAGGAATGTAACGGGGATAACGGATCAAGGCC- 300

TTCCGGGCAGGAGAAAGGACGGTGGGCGTAGCGCCTCGTTTTTCCAAGAACTTTAAAATGCCACAAGGAGAGGTGTTTTTGTATGCAAAACTCAACTTGC- 400
                                                                    rbs     HndA  M  Q  N  S  T  -   6

CAAGCGGTCGGCGAATGCAGGGTGCCGGAACATGCGGTTCTGCCGCAGCCGCTGTACCGGGAAGTCGTTCAGTTTATTGAATCCCTCCCTCAGAAAGAGG- 500
  Q  A  V  G  E  © R  V  P  E  H  A  V  L  P  Q  P  L  Y  R  E  V  V  Q  F  I  E  S  L  P  Q  K  E-  39

GGCATCTCGTCACCGTGCTCCATAAGGCGCAAAGTGTTTTTGGCTATCTGCCCATCGAAGTGCAGCAGTTTGTGGCCGATCACATGGAAGTTCCCCTGGC- 600
 G  H  L  V  T  V  L  H  K  A  Q  S  V  F  G  Y  L  P  I  E  V  Q  Q  F  V  A  D  H  M  E  V  P  L -  72

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A  Q  V  Y  G  V  V  S  F  Y  T  F  F  T  M  V  P  K  G  K  Y  P  I  S  V  © M  G  T  A  © F  V  K- 106

GGCGCGGACAAAGTGGTCCATGCCTTCAAAGAGCAGCTCAAGATCGATATCGGCGACGTGACCCCGGACGGCAGGTTCTCCATCGACACCCTGCGTTGCG- 800
  G  A  D  K  V  V  H  A  F  K  E  Q  L  K  I  D  I  G  D  V  T  P  D  G  R  F  S  I  D  T  L  R © -139

TCGGCGGCTGCGCCCTGGCTCCCATCGTCATGGTCGGCGAGAAGGTCTACGGCAACGTGACGCCGGGGCAGGTCAAGAAAATCCTGGCTGAGTACTAGCG- 900
 V  G  G  © A  L  A  P  I  V  M  V  G  E  K  V  Y  G  N  V  T  P  G  Q  V  K  K  I  L  A  E  Y  *  - 171

CAACCGCGTGCGATTTACAGATAAGGAGCGCGTTAAATGAGCACTATCCGATCCTTTGAAGATCTCAAGGCCAAGCGCCAGGAGATTCTTGACCGCAAGG-1000
                        rbs   HndB   M  S  T  I  R  S  F  E  D  L  K  A  K  R  Q  E  I  L  D  R  K-  21

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 A  A  R  N  G  K  T  I  I  N  V  S  L  A  T  © S  I  A  A  G  G  K  V  A  M  E  A  M  Q  D  E  V  -  54

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A  K  N  G  L  T  G  V  E  F  M  Q  S  S © M  T  Y  © Y  A  E  P  T  V  E  I  T  L  P  G  K  D  P-  88

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 E  R  V  V  L  *  *           rbs  HndC  M  A  A  T  T  T  E  K  K  Q  L  R  I  A  T  R  N  © G  -  19

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GCAACGCCGACGAAGGCGACCCGGAGTTCATGGACCGCGCCGTGCTCGAGGGCGACCCCCACTCGGTGGTGGAGGCCATGGCCATCGGCGGCTACGCCAT-1700
©  N  A  D  E  G  D  P  E  F  M  D  R  A  V  L  E  G  D  P  H  S  V  V  E  A  M  A  I  G  G  Y  A - 119

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TGCCGCATCGGCTCCAAGCGCCTCTACGAGATCCTGGACCGGATCACCAAGGGCAAGGGCACCCGGGCCGACCTCGATCGGCTCAAGTCCCTGTCCGAGA-2500
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 F  G  I  R  E  S  P  Y  D  G  G  E  M  S  H  Y  R  K  D  I  S  A  S  I  I  R  D  M  D  K Ⓒ I  M  - 155

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Ⓒ R  R Ⓒ  E  T  M Ⓒ  N  T  V  Q  T Ⓒ  G  V  L  S  G  V  N  R  G  F  T  A  V  V  A  P  A  F  E  M- 189

AACCTGGCCGACACCGTGTGCACCAACTGCGGCCAGTGCGTGGCCGTGTGCCCCACCGGCGCCCTGGTCGAGCACGAGTACATCTGGGAAGTGGTCGAGG-3500
 N  L  A  D  T  V Ⓒ  T  N Ⓒ  G  Q Ⓒ  V  A  V Ⓒ  P  T  G  A  L  V  E  H  E  Y  I  W  E  V  V  E - 222

CCCTGGCCAACCCGGACAAGGTCGTCATCGTCCAGACCGCCCCGGCCGTGCGCGCGGCCCTCGGCGAGGACCTCGGCGTCGCCCCCGGCACCTCGGTCAC-3600
 A  L  A  N  P  D  K  V  V  I  V  Q  T  A  P  A  V  R  A  A  L  G  E  D  L  G  V  A  P  G  T  S  V  - 255

CGGCAAGATGGCCGCCGCCCTGCGTCGCCTGGGCTTCGACCACGTCTTCGACACCGACTTCGCCGCCGACCTGACCATCATGGAGGAAGGCTCGGAATTC-3700
T  G  K  M  A  A  A  L  R  R  L  G  F  D  H  V  F  D  T  D  F  A  A  D  L  T  I  M  E  E  G  S  E  F- 289

CTCGACCGCCTGGGCAAGCACCTGGCCGGCGACACGAATGTCAAGCTGCCCATCCTCACCTCCTGCTGCCCGGGCTGGGTCAAGTTCTTCGAACACCAGT-3800
 L  D  R  L  G  K  H  L  A  G  D  T  N  V  K  L  P  I  L  T  S Ⓒ Ⓒ  P  G  W  V  K  F  F  E  H  Q - 322

TCCCGGACATGCTCGACGTGCCCTCCACGGCCAAGTCGCCGCAGCAGATGTTCGGCGCCATCGCCAAGACCTACTACGCCGACCTGCTCGGCATCCCGCG-3900
 F  P  D  M  L  D  V  P  S  T  A  K  S  P  Q  Q  M  F  G  A  I  A  K  T  Y  Y  A  D  L  L  G  I  P  - 355

CGAAAAGCTCGTGGTCGTCTCGGTCATGCCCTGCCTGGCCAAGAAGTACGAGTGCGCCCGTCCGGAATTCTCCGTGAACGGCAACCCGGACGTCGACATC-4000
R  E  K  L  V  V  V  S  V  M  P Ⓒ  L  A  K  K  Y  E Ⓒ  A  R  P  E  F  S  V  N  G  N  P  D  V  D  I- 389

GTCATCACCACCCGTGAGCTGGCCAAGCTCGTCAAGCGCATGAACATCGACTTCGCCGGGCTGCCCGACGAGGACTTCGATGCGCCGCTCGGCGCGTCCA-4100
 V  I  T  T  R  E  L  A  K  L  V  K  R  M  N  I  D  F  A  G  L  P  D  E  D  F  D  A  P  L  G  A  S - 422

CGGGCGCGGCCCCGATCTTCGGCGTCACCGGCGGCGTCATCGAGGCGGCGCTGCGCACCGCCTACGAGCTGGCCACCGGCGAAACCCTTAAGAAGGTCGA-4200
 T  G  A  A  P  I  F  G  V  T  G  G  V  I  E  A  A  L  R  T  A  Y  E  L  A  T  G  E  T  L  K  K  V  - 455

CTTCGAGGACGTGCGCGGCATGGACGGCGTCAAAAAGGCCAAGGTCAAGGTCGGCGACAACGAGCTTGTCATCGGCGTGGCCCACGGCCTTGGCAACGCG-4300
D  F  E  D  V  R  G  M  D  G  V  K  K  A  K  V  K  V  G  D  N  E  L  V  I  G  V  A  H  G  L  G  N  A- 489

CGGGAACTCTTGAAGCCGTGCGGCGCGGGCGAGACCTTCCACGCCATCGAGGTCATGGCCTGTCCGGGCGGCTGCATCGGCGGCGGCGGACAGCCCTACC-4400
 R  E  L  L  K  P Ⓒ  G  A  G  E  T  F  H  A  I  E  V  M  A Ⓒ  P  G  G Ⓒ  I  G  G  G  G  Q  P  Y - 522

ACCACGGCGATGTGGAACTGCTCAAAAAGCGCACCCAGGTCCTCTACGCCGAGGACGCGGGCAAGCCGCTTCGCAAGTCCCACGAGAACCCGTACATCAT-4500
 H  H  G  D  V  E  L  L  K  K  R  T  Q  V  L  Y  A  E  D  A  G  K  P  L  R  K  S  H  E  N  P  Y  I  - 555

CGAACTCTACGAGAAGTTCCTGGGCAAGCCGCTCTCCGAACGCTCGCACCAGCTGCTGCACACCCACTACTTCAAGCGGCAGCGCTTATAACAGAGGAAG-4600
I  E  L  Y  E  K  F  L  G  K  P  L  S  E  R  S  H  Q  L  L  H  T  H  Y  F  K  R  Q  R  L  *           - 585

AGTCTGGGAGGGGCGTCGCCCCTCCCAGACCCTCCTCACCAGAAGGGGCGCCGCCCCTTCTGGACTTCCCCGCCGGGGGAATAATTCCCCCCCGGCCCCC-4700
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TTTAACGGGGCAAGGTTCTTTGACAGTGTTCCTCCTTAACTCCTCCCGTGGCAGGCTGGTGACCTGTCCGTGGCGCCGGAATCGACGCGGCGTCGGCTTA-4800
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FIG. 1. Nucleotide sequence of the *hnd* region. The amino acid sequence deduced from the nucleotide sequence is given in the single-letter code. Cysteine residues are in circles. Stop codons are indicated by asterisks. The Shine-Dalgarno sequences (rbs) are underlined. The convergent arrows indicate inverted repeats.

involved in the NAD^+ oxidoreductase activity, where flavin mononucleotide (FMN) acts as a cofactor (reference 4 and references therein). The HndC region extending from residues 54 to 91, which is likely to be involved in the formation of the ADP pocket in the NAD^+ binding site, was identified by the method of Wierenga et al. (32). The consensus sequence possibly involved in the binding of FMN was not established. However, the multiple alignment of HndC and related proteins, shown in Fig. 3, shows that the region involved in the binding of FMN is probably located in the glycine-rich conserved region (16) encompassing amino acids 170 to 218 in HndC. HndC harbors 21 cysteine residues with five conserved positions. The sequence motif CysXXCysXXCys corresponding to residues 348 to 354 along with cysteine 392 in HndC may coordinate one [4Fe-4S] cluster. A cluster of this kind has been identified in electron paramagnetic resonance spectroscopic studies carried out on both HoxF from *A. eutrophus* and the bovine 51-kDa subunit of *B. taurus* complex I (reference 30 and references therein). The other conserved cysteine residue, at position 177 in HndC, falls within the region thought to be involved in the binding of FMN. The C-terminal domain of HndC is characterized by the presence of eight cysteine residues arranged into two canonical sequence motifs, CysXXCysXXCysXXXCys(Pro/Lys), and may coordinate two [4Fe-

FIG. 2. Multiple alignment of the derived amino acid sequences of the *hndA* gene product and related proteins. A, HndA from *D. fructosovorans*; B, 24-kDa subunit from *B. taurus* complex I; C, 25-kDa subunit from *P. denitrificans* NADH dehydrogenase type I; D, HoxF subunit (residues 1 to 200) from *A. eutrophus* NAD-reducing hydrogenase. Identical amino acids are shaded, and cysteine residues present in conserved positions are boxed.

4S] ferredoxin-like clusters (3). HndC therefore probably houses one NAD^+ and one FMN binding site and coordinates three [4Fe-4S] clusters, arranged in that linear order in the sequence from N to C terminal. Since HndA and HndC together are potentially able to harbor one NAD^+ cluster, one FMN cluster, three [4Fe-4S] clusters, and one [2Fe-2S] cluster, it can be postulated that this dimer might form a functional unit having NAD^+ reductase activity, as does the HoxF-HoxU dimer of *A. eutrophus* (25).

The polypeptide encoded by *hndD* has a predicted molecular mass of 63.6 kDa. The amino acid sequence of HndD was compared with those of the Hyd I from *Clostridium pasteurianum* (10), the large subunit (HydA) of the dimeric [Fe] Hyd from *D. vulgaris* Hildenborough (27), and the putative monomeric [Fe] Hyd (HydC) from *D. vulgaris* Hildenborough (23). As shown in Fig. 4, identities of 48, 42, and 40% were found to exist between HndD and HydA, HydC, and Hyd I, respectively. A longer N-terminal domain consisting of 117 amino acids was present in Hyd I, HydC, and HndD but was lacking in HydA. A recent Raman spectroscopic investigation (5) indicated that in addition to the H cluster, three [4Fe-4S] clusters and one [2Fe-2S] cluster are present in Hyd I from *C. pasteurianum* and only two [4Fe-4S] clusters are present in HydAB from *D. vulgaris* Hildenborough, which suggests that the N-terminal domain of Hyd I may harbor one of the three [4Fe-4S] clusters and the [2Fe-2S] cluster. In the case of HndD (Fig. 4), the N-terminal domain harbors seven conserved cysteine residues and lacks the eighth cysteine residue, which is to be found in a nonconserved position in Hyd I and HydC. Since eight residues are required to accommodate two iron-sulfur clusters, histidine 101 might be the fourth actual ligand of one cluster (9a). It seems quite likely that the N-terminal region of HndD may bind one [2Fe-2S] and one [4Fe-4S] center. The subsequent sequence (residues 118 to 239 in HndD) contains eight conserved cysteine residues showing the characteristic motif responsible for coordinating two [4Fe-4S] ferredoxin-like clusters (3). The highest similarity between the four sequences aligned in Fig. 4 lies in the central part of the proteins (from residues 240 to 520 in HndD), which possesses five matching

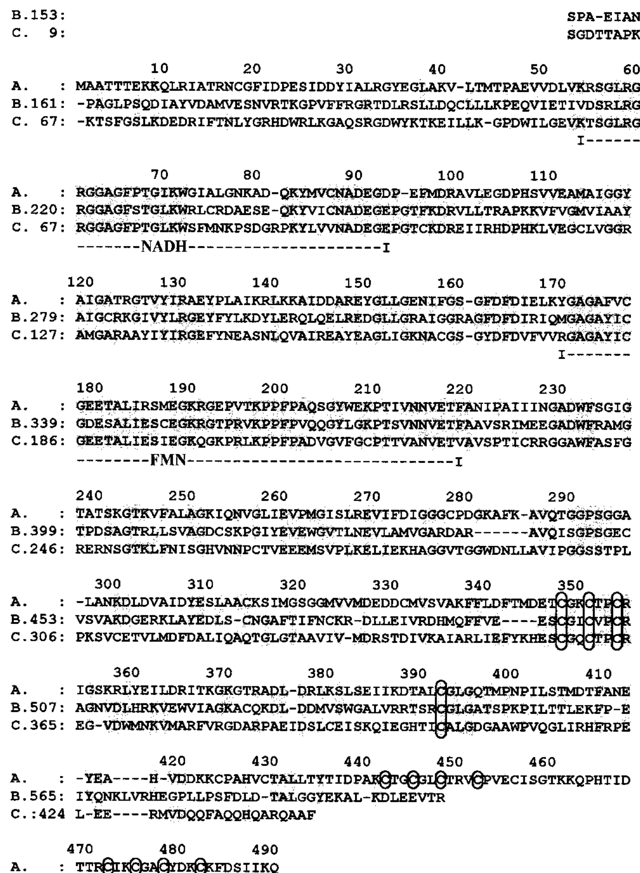

FIG. 3. Multiple alignment of the derived amino acid sequences of the *D. fructosovorans hndC* gene product and related proteins. A, HndC from *D. fructosovorans*; B, HoxF (residues 153 to 602) from *A. eutrophus*; C, 51-kDa subunit from *B. taurus* complex I. Identical amino acids are shaded, and cysteine residues present in conserved positions are boxed.

cysteine residues. This highly similar region is thought to harbor the hydrogen-activating H cluster (10, 29). HndD therefore has all of the features necessary to chelate one [2Fe-2S] cluster, three [4Fe-4S] ferredoxin-like clusters, and one activating H cluster and might therefore constitute a monomeric [Fe] Hyd, as does Hyd I from *C. pasteurianum*.

On the basis of these sequence homology data, it can be hypothesized that HndA, -B, -C, and -D assembled in a multimeric entity might bring together all of the elements necessary to obtain a NAD^+-reducing hydrogenase. This assumption is summarized in Fig. 5, where the salient structural features of HndA, -B, -C, and -D from *D. fructosovorans* are compared with those from the tetrameric NAD^+-reducing hydrogenase from *A. eutrophus*. On the other hand, the absence of the signal peptide consensus box (12) in the amino acid sequences of the four proteins indicates that they may have a cytoplasmic location, as does *A. eutrophus* NAD^+-reducing hydrogenase (17).

Northern blotting analysis. A 1.3-kb *Pst*I-*Pvu*II fragment containing the major part of *hndD* was used in Northern experiments. When the total RNAs from *D. fructosovorans* were probed, a major 4.5-kb transcript was observed (Fig. 6, lane 3). Minor 2.9-kb transcripts were also observed, which may have been either some of the degradation products of the 4.5-kb transcripts or the 4.5-kb transcripts swept along by the rRNAs. The same 4.5-kb transcripts were obtained when RNAs from

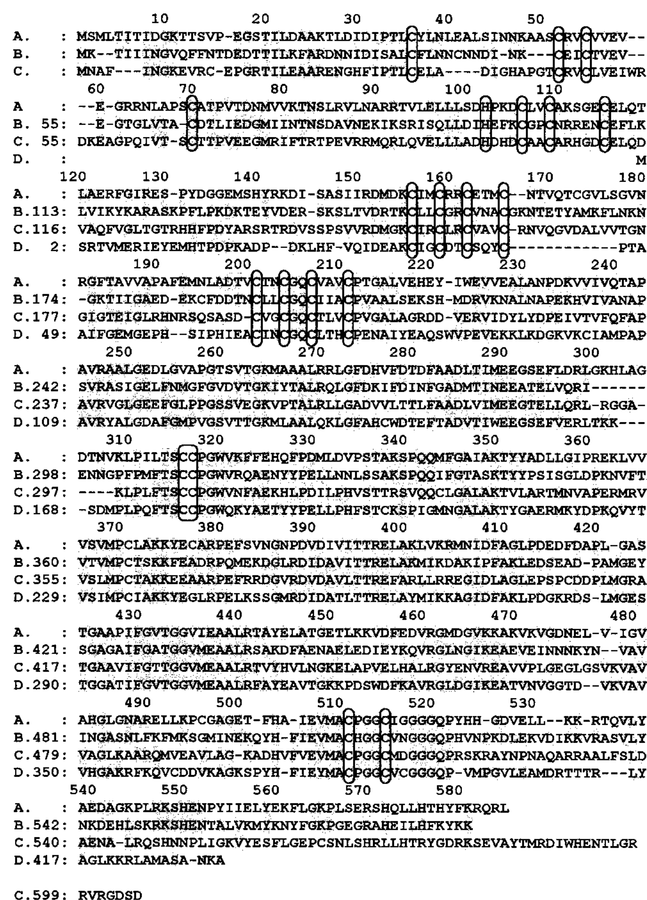

FIG. 4. Multiple alignment of the derived amino acid sequences from HndD from *D. fructosovorans* (A), Hyd I from *C. pasteurianum* (B), HydC from *D. vulgaris* Hildenborough (C), and HydA from *D. vulgaris* Hildenborough (D). Identical amino acids are shaded, and cysteine residues present in conserved positions are boxed.

E. coli DH5α(pSS13) were probed (Fig. 6, lane 2). When the 1.7-kb *Eco*RI-*Bam*HI probe, encompassing *hndC* and a large part of *hndB*, was used, the same transcripts were observed (Fig. 6, lane 4). These results indicated that *hndA*, *hndB*, *hndC*, and *hndD* are transcribed in *D. fructosovorans* and that these

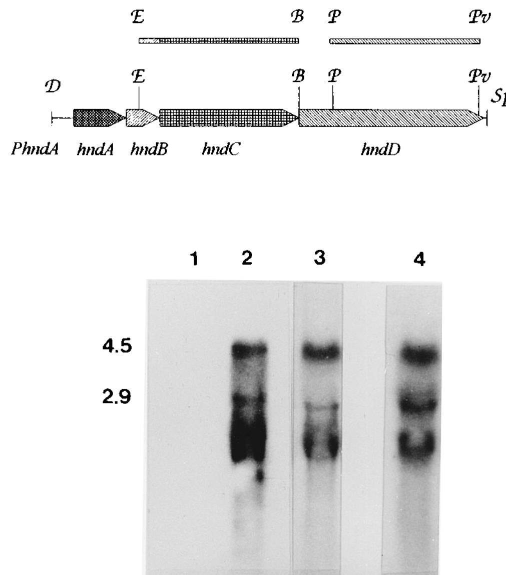

FIG. 6. Northern blotting experiments. The *Pst*I-*Pvu*II intragenic fragment was used to probe RNAs from *E. coli* DH5α(pUC18) (lane 1), *E. coli* DH5α(pSS13) (lane 2), and *D. fructosovorans* (lane 3). Lane 4, total RNAs from *D. fructosovorans* probed with the *Eco*RI-*Bam*HI intergenic probe. Restriction enzyme abbreviations: B, *Bam*HI; E, *Eco*RI; D, *Dra*I; P, *Pst*I; Pv, *Pvu*II; Sp, *Sph*I. Sizes are indicated in kilobases.

genes belong to the same transcription unit. The coregulation of the expression of these genes can be interpreted as the need for the four related proteins to be present at the same time in the cell, either because they might work together in a multimeric structure or because they might act sequentially in a series of reactions.

Heterologous expression studies. To determine whether this operon had any real biochemical existence, the four genes were cloned in pT7-5 and pT7-6 and overexpressed in *E. coli*. Four polypeptides with apparent molecular masses of 13.6, 16, 52, and 65 kDa were synthesized (Fig. 7). The molecular masses observed were in good agreement with the theoretical values of 13.8, 18.8, 51.7, and 63.6 kDa predicted for the products of the genes *hndB*, *hndA*, *hndC*, and *hndD*, respectively. No translation products were observed in the transformants carrying the *hnd* region cloned in the opposite orientation in pT7-6 (data

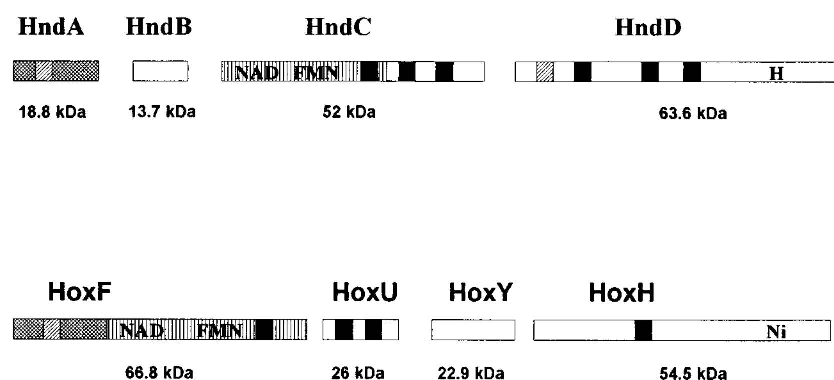

FIG. 5. Summary of related motifs in the proteins involved in the NADP-reducing Hyd from *D. fructosovorans* (top) and in the NAD-reducing Hyd from *A. eutrophus* (bottom). H, the H cluster present at the active center of [Fe] Hyds; Ni, the nickel atom present at the active center of [NiFe] Hyds. The possible sites for prosthetic group coordination are as follows: ▨, [2Fe-2S] center; ▩, HndA homologous region; □, region showing no homology; ■, [4Fe-4S] center; and ▥, HndC homologous region.

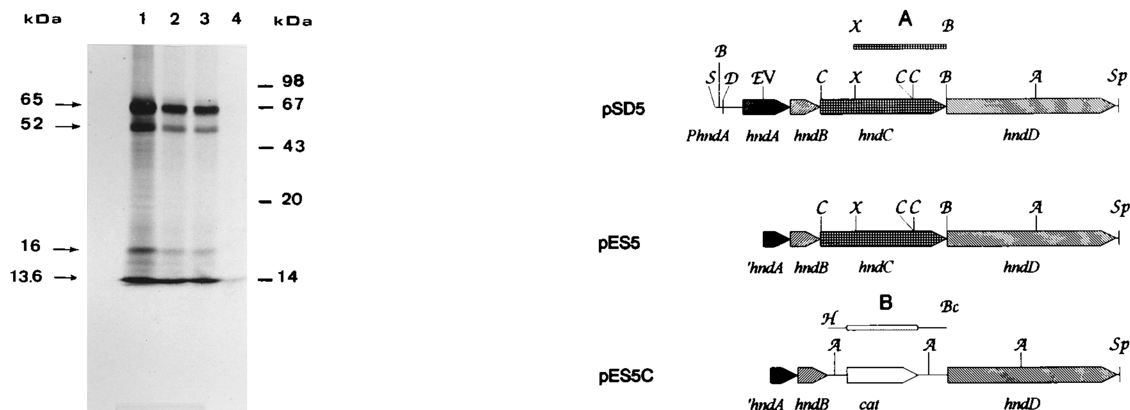

FIG. 7. Heterologous expression of the *hnd* genes in the T7 promoter/polymerase system. In the autoradiogram of ^{35}S-labeled proteins, 40, 20, and 10 μl of cell extract from *E. coli* K38(pGP1-2) harboring the expression plasmid pTHnd were loaded in lanes 1, 2, and 3, respectively. Lane 4, control experiment, performed with *E. coli* K38 harboring plasmid pT7-5 alone. The positions of molecular mass standards are indicated.

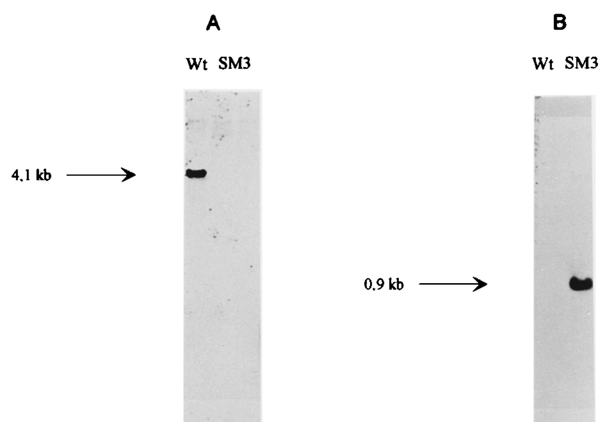

FIG. 8. Construction of strain SM3 and Southern blotting experiment. Restriction enzyme abbreviations: A, *Asu*II; B, *Bam*HI; Bc, *Bcl*I; C, *Cla*I; D, *Dra*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*PI; S, *Sac*I; Sp, *Sph*I; X, *Xho*I. The *Xho*I-*Bam*HI fragment was probe A, which was used to probe blot A, and the *Hin*PI/*Bcl*I fragment was probe B, which was used to probe blot B. Wt and SM3, wild-type and SM3 strains of *D. fructosovorans*, respectively.

not shown). These results indicated that the identified operon may be translated into four proteins exhibiting the predicted sizes.

Gene replacement. For the purpose of checking the biochemical characteristics of this newly identified enzyme, a Δ*hndC* mutant was constructed by marker exchange mutagenesis (see Materials and Methods). The chromosomal DNAs of the wild type and mutant SM3 were digested with *Asu*II to obtain a differential response to probing. In the chromosome of the wild-type strain, there are two *Asu*II sites of interest, one located in the middle of the *hndD* gene (Fig. 8, pSD5) and the other located 700 bp upstream of the *Dra*I site (not shown). The locations of these two sites were expected to produce one 4.1-kb fragment, which was what actually occurred when the chromosomal DNA of the wild-type strain was tested with probe A (Fig. 8A). As expected, no hybridization occurred when the chromosomal DNA of SM3 was tested with probe A, indicating that the *hndC* gene had been deleted (Fig. 8A). Conversely, the presence of two additional *Asu*II sites in the chloramphenicol cassette inserted into SM3 (Fig. 8, pES5C) released one 0.9-kb fragment which hybridized with probe B (Fig. 8B). No hybridization occurred when the wild-type strain chromosomal DNA was tested with probe B (Fig. 8B). These results indicated that the genotype of SM3 was Δ*hndC* Cm^r.

Immunoblot analysis. With a view to identifying the subunits of this enzyme in *D. fructosovorans*, antibodies were produced by using HndD and HndC expressed in *E. coli* in the pMAL-c1 system. Only HndC induced the production of an antibody exhibiting a measurable immunological response. Anti-HndC antibodies were then used to test cell extracts from *D. fructosovorans* wild-type and SM3 strains to determine whether HndC was present (Fig. 9). One band corresponding to a protein of 52 kDa, which is the size to be expected for HndC, was observed in the wild-type extract but was lacking in the SM3 extract (Fig. 9). It can be deduced from these results that HndC was actually expressed in *D. fructosovorans* and that the SM3 phenotype was $HndC^-$.

Enzymatic activity. The last step in the preliminary characterization of this new enzyme consisted of investigating the activity of this enzyme in *D. fructosovorans*. The sequence comparisons suggested that the enzyme might be able to reduce NAD^+ in the presence of molecular H_2, with HndA and HndC acting as the possible NAD-reducing subunits and HndD acting as the hydrogenase subunit. No H_2-induced NAD^+ reduction ever occurred, however, in soluble extracts from *D. fructosovorans* (Fig. 10). When $NADP^+$ was tested as the electron acceptor, the characteristic increase in the reduction speed was observed immediately after addition of H_2 (Fig. 10). As expected, SM3 failed to effect any H_2-dependent NADP reduction (Fig. 10). This observation clearly indicated that the enzyme discovered was a NADP-reducing hydrogenase, showing complete specificity toward NADP under our experimental conditions.

It is not yet clear whether this enzyme is a multimeric complex. The purification of the enzyme is under way at our laboratory. The specificity of this hydrogenase toward $NADP^+$ makes it the first of its kind to be described. Since $NADPH_2$ is almost exclusively used in anabolism, it can be hypothesized that *D. fructosovorans* is able to use the reducing power of H_2 directly to synthesize biomolecules. This is probably not the only means whereby *D. fructosovorans* produces $NADPH_2$, however. For example, the ability of this bacterium to oxidize fructose and malate suggests that $NADPH_2$ might also be produced via the pentose pathway and by the malic enzyme, respectively. This intriguing enzyme might therefore play a crucial role under conditions in which alternative pathways do

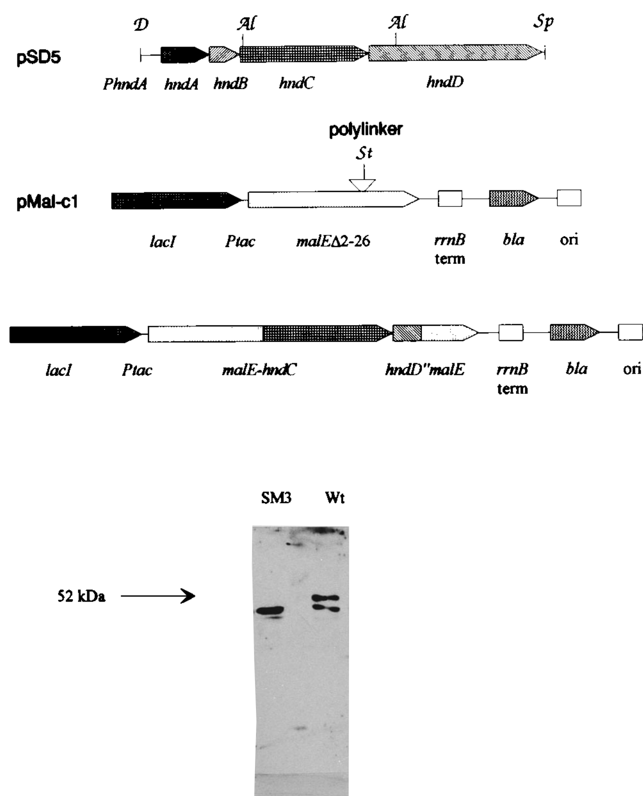

FIG. 9. Construction of the protein fusion MalE-HndC and Western immunoblotting. Restriction enzyme abbreviations: Al, *Alu*I; D, *Dra*I; Sp, *Sph*I; St, *Stu*I. Wt and SM3, wild-type and SM3 strains of *D. fructosovorans*, respectively.

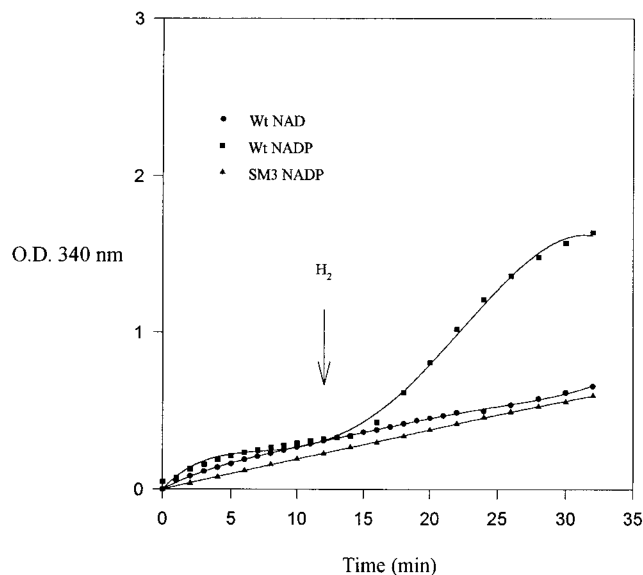

FIG. 10. Enzymatic activity. Soluble extract (1.8 mg of total protein) from the *D. fructosovorans* wild-type (Wt) strain was incubated with NAD^+ (●) or $NADP^+$ (■), and soluble extract (1.8 mg of total protein) from *D. fructosovorans* SM3 was incubated with $NADP^+$ (◆), both under nitrogen atmosphere. Hydrogen was injected into the cuvette as indicated by the arrow. O.D. optical density.

not work, as might be the case when *D. fructosovorans* is growing on hydrogen with only very little available organic carbon source. Under these growth conditions, which might occur quite commonly in anaerobic biotopes, CO_2 fixation presumably becomes essential (1, 6, 22) and the bacterium therefore cannot afford to use organic molecules to synthesize $NADPH_2$. Further studies of mutant SM3 will surely yield new insights into *Desulfovibrio* metabolism.

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