Cloning and sequencing of a [NiFe] hydrogenase operon from *Desulfovibrio vulgaris* Miyazaki F

HARM M. DECKERS, FRANKIE R. WILSON and GERRIT VOORDOUW*

Division of Biochemistry, Department of Biological Sciences, The University of Calgary, Calgary, Alberta, Canada T2N 1N4

(Received 29 March 1990; revised 21 May 1990; accepted 12 June 1990)

A hydrogenase operon was cloned from chromosomal DNA isolated from *Desulfovibrio vulgaris* Miyazaki F with the use of probes derived from the genes encoding [NiFe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough. The nucleic acid sequence of the cloned DNA indicates this hydrogenase to be a two-subunit enzyme: the gene for the small subunit (267 residues; molecular mass = 28763 Da) precedes that for the large subunit (566 residues; molecular mass = 62495 Da), as in other [NiFe] and [NiFeSe] hydrogenase operons. The amino acid sequences of the small and large subunits of the Miyazaki hydrogenase share 80% homology with those of the [NiFe] hydrogenase from *Desulfovibrio gigas*. Fourteen cysteine residues, ten in the small and four in the large subunit, which are thought to co-ordinate the iron-sulphur clusters and the active-site nickel in [NiFe] hydrogenases, are found to be conserved in the Miyazaki hydrogenase. The subunit molecular masses and amino acid composition derived from the gene sequence are very similar to the data reported for the periplasmic, membrane-bound hydrogenase isolated by Yagi and coworkers, suggesting that this hydrogenase belongs to the general class of [NiFe] hydrogenases, despite its low nickel content and apparently anomalous spectral properties.

Introduction

Two classes of hydrogenases, the iron-only and nickel-containing hydrogenases have been found in sulphate-reducing bacteria. The cloning and sequencing of the structural genes for these hydrogenases has contributed considerably to their characterization and classification. Genes for the [Fe] hydrogenase have been isolated from two species, *D. vulgaris* Hildenborough and *D. vulgaris* subsp. *oxamicus* Monticello (Voordouw *et al.*, 1985, 1989b; Voordouw & Brenner, 1985). [Fe] hydrogenase is a periplasmic two-subunit enzyme (molecular mass = 46 kDa and molecular mass = 10 kDa for the large and small subunits, respectively). The small subunit has a 34 amino acid signal peptide for export of this hydrogenase to the periplasm (Prickril *et al.*, 1986). The [Fe] hydrogenase subunits do not show homology with those of the nickel-containing hydrogenases, which comprise two subclasses, the [NiFe] and [NiFeSe] hydrogenases. The nucleotide sequence of the structural genes of the [NiFe] hydrogenase from *D. gigas* (Li *et al.*,

1987; Voordouw *et al.*, 1989a) indicates molecular masses of 28 and 61 kDa for the processed small and the large subunit, respectively. The former has a 50 amino acid signal sequence for export of this hydrogenase. *D. gigas* hydrogenase is relatively easily extracted from the *Desulfovibrio* periplasm (Hatchikian *et al.*, 1978) and shares sequence homology with [NiFe] hydrogenases isolated and characterized from bacterial genera other than *Desulfovibrio*, i.e. *Bradyrhizobium japonicum* (Sayavedra-Soto *et al.*, 1988), *Azotobacter chroococcum* (Ford *et al.*, 1990), *Rhodobacter capsulatus* (Leclerc *et al.*, 1988), *Rhodocyclus gelatinosus* (Uffen *et al.*, 1990) and *Escherichia coli* (Menon *et al.*, 1990). The [NiFe] hydrogenases of these latter micro-organisms are generally found to be periplasmic and membrane-bound: they can be isolated only after trypsin and/or detergent treatment of cell membranes. The COOH terminus of these hydrogenases appears to have an extension of 40–50 amino acid residues, when compared with the *D. gigas* sequence. This extension has a central stretch of 20 hydrophobic amino acids (Menon *et al.*, 1990) and may function in the binding of these enzymes to the membrane.

Hydrogenases containing both selenium and nickel have been isolated from several species of *Desulfovibrio* and the cloning and sequencing of the genes from

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M33174.

Abbreviation: nt, nucleotide(s).

Fig. 1. (a) Restriction map for the 4.8 kb *ClaI*–*SmaI* insert of plasmid DV1 (Menon *et al.*, 1990), indicating the coding regions for the small (left) and large (right) subunits (hatched boxes) of the [NiFe] hydrogenase of *D. vulgaris* Hildenborough. Restriction sites for *ClaI* (C), *PstI* (P), *EcoRI* (E) and *SmaI* (S) are indicated, as well as the location of DNA fragments used as probes in the cloning of the Miyazaki hydrogenase genes (P_1 , P_2 , P_3). (b) Restriction map of a region of the *D. vulgaris* Miyazaki genome encoding the small and large subunits (hatched boxes) of Miyazaki hydrogenase. Plasmids pMZSS1-56 and pHCAL2 contained the indicated 3.1 and 3.8 kb *EcoRI* inserts cloned in pUC8. (c) Extent of DNA sequenced by the dideoxy-chain termination procedure (6190 nt). The sequence displayed in Fig. 3 is indicated (black box).

D. baculatus has been reported (Menon *et al.*, 1987; Voordouw *et al.*, 1989a). The sequences of the small (molecular mass = 31 kDa) and large (molecular mass = 57 kDa) subunits are homologous to those of the *D. gigas* [NiFe] hydrogenase, although the degree of sequence identity is less (38 and 34%, respectively, Voordouw *et al.*, 1989a) than that found within the [NiFe] hydrogenase family. The small subunit of [NiFeSe] hydrogenase has a 32 amino acid signal peptide, which shares sequence homology with those for the [NiFe] and [Fe] hydrogenase small subunits.

The hydrogenase from *D. vulgaris* Miyazaki F has been extensively studied by Yagi and co-workers (Yagi, 1970; Yagi *et al.*, 1976, 1985; Higuchi *et al.*, 1987). The subunit molecular masses (29 kDa and 60 kDa) and amino acid composition are similar to that of *D. gigas* hydrogenase (Yagi *et al.*, 1976; Hatchikian *et al.*, 1978). Nevertheless, the nickel content appears to be very low ($0.16 \text{ mol Ni mol}^{-1}$) and spectroscopic characteristics have been interpreted as an indication of the absence of bound nickel (Yagi *et al.*, 1985). In earlier studies the enzyme was shown to be inhibited by carbon monoxide, a characteristic of [Fe] hydrogenases (Yagi *et al.*, 1976), while lack of inhibition by carbon monoxide is diagnostic for the nickel-containing hydrogenases (Gow *et al.*, 1986; Lissolo *et al.*, 1986). The properties of the Miyazaki F hydrogenase described by Yagi and co-workers are thus unusual and a further investigation of hydrogenases in this strain with molecular biological methods is appropriate. In this study we investigate the possible presence of [Fe] and [NiFe] hydrogenase genes in this species with currently available probes and

describe the cloning and sequencing of a hydrogenase gene with high homology to the [NiFe] hydrogenase genes of *D. gigas*.

Methods

Biochemical reagents. Most of the enzymes used for cloning and sequencing work were obtained from Pharmacia. A sequencing kit containing T7 DNA polymerase ('Sequenase') was obtained from the United States Biochemical Corporation. Radioisotopes deoxyadenosine 5'- α -[^{35}S]thiotriphosphate [α - ^{35}S]dATP; (400 Ci mmol^{-1} , $14.8 \text{ TBq mmol}^{-1}$) and [α - ^{32}P]dATP ($3000 \text{ Ci mmol}^{-1}$, $111 \text{ TBq mmol}^{-1}$) were purchased from Amersham, and were used for dideoxynucleotide sequencing and nick-translation, respectively. Hybrid-N hybridization membranes were also obtained from Amersham.

Bacterial strains, plasmids and DNA isolation. DNA, isolated by the method of Marmur (1961), from *D. vulgaris* Miyazaki F (IAM 12604, Institute for Applied Microbiology, University of Tokyo, Japan) was kindly donated by Professor T. Yagi, Department of Chemistry, Shizuoka University, Oya, Shizuoka, Japan. DNA from *D. vulgaris* subsp. *vulgaris* Hildenborough (NCIB 8303) was similarly isolated in our own laboratory. Vectors pUC8 and M13mp8 or M13mp9 (Vieira & Messing, 1982; Messing & Vieira, 1982) were used for gene cloning and dideoxy sequencing, respectively. Plasmid DV1, containing the structural genes of the [NiFe] hydrogenase from *D. vulgaris* Hildenborough on a 4.8 kb *ClaI*–*SmaI* DNA insert in BLUESCRIPT was kindly donated by Dr A. E. Przybyla, Department of Biochemistry, School of Chemical Sciences, University of Georgia, Athens, GA, USA. A restriction map of this insert has been published (Menon *et al.*, 1990) and is presented in Fig. 1(a).

pHV150, which contains the *hydA*, *B* genes encoding [Fe] hydrogenase of *D. vulgaris* Hildenborough on a 1.9 kb *Bam*HI–*Bam*HI insert has been described elsewhere (Voordouw *et al.*, 1987). A 0.7 kb *hydC* probe, containing the 3' end of the *hydC* gene (Stokkermans *et al.*, 1989), was isolated from pHVC5 (Voordouw *et al.*, 1989b).

Fig. 2. Southern blotting of DNA from *D. vulgaris* Hildenborough and Miyazaki. Blots were incubated with: (a) a 1.9 kb probe containing the *hydA,B* genes for [Fe] hydrogenase from *D. vulgaris* Hildenborough or (b) probe P₁ (Fig. 1a). Hildenborough DNA was digested with *Hind*III (lane 1), *Eco*RI (lane 2) or *Pst*I (lane 3). Miyazaki DNA was digested with *Hind*III (lane 4), *Eco*RI (lane 5) or *Pst*I (lane 6). The sizes (kb) of selected fragments derived from the migration of molecular mass markers and the nucleic acid sequence (Fig. 3) are indicated.

Southern blotting, cloning and dideoxy sequencing. Southern blotting was done with the formamide procedure for Hybond-N membranes as described previously (Voordouw *et al.*, 1989b). Probes P₁ and P₂ (Fig. 1a) appeared to hybridize with 3.8 kb and 3.1 kb *Eco*RI fragments of *D. vulgaris* Miyazaki DNA, respectively (see Fig. 1b and Results). These hybridizing fragments were cloned in *Eco*RI-cleaved pUC8 and pHCAL2 and pMZSS1-56, containing the 3.8 and 3.1 kb *Eco*RI inserts, were isolated using techniques described previously (Voordouw *et al.*, 1989b). The inserts of these plasmids were sequenced with the dideoxy chain termination method of Sanger *et al.* (1977), using the procedures for generating M13 clones with random inserts obtained by sonication as outlined by Bankier & Barrell (1983). *E. coli* TG2 (from Toby Gibson, Laboratory of Molecular Biology, Cambridge, UK) was used as the host for these molecular biological manipulations. The sequence data obtained were compiled and analysed in a DEC-VAX computer using the programs of Staden (Staden, 1982, 1984a, b; Staden & McLachlan, 1982) and of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

Results

Using Southern blotting it can readily be shown that *D. vulgaris* Miyazaki F lacks the *hydA,B* genes, encoding a periplasmic [Fe] hydrogenase as found in the Hildenborough strain. Digests of chromosomal DNA from the two strains with restriction enzymes *Hind*III, *Eco*RI and *Pst*I were electrophoresed through agarose and Southern

blotted onto Hybond-N. Following hybridization of the blots with the *hydA,B* probe the results shown in Fig. 2(a) were obtained. In agreement with earlier data (Voordouw *et al.*, 1985), hybridizing fragments of 14 kb (*Hind*III), 4.8 kb (*Eco*RI) and 4.8 and 6.6 kb (*Pst*I) were observed for Hildenborough DNA (Fig. 2a, lanes 1–3), while Miyazaki DNA was entirely negative (Fig. 2a, lanes 4–6). A second gene in *D. vulgaris* Hildenborough, which could encode a [Fe] hydrogenase, was recently described by Stokkermans *et al.* (1989). This gene, *hydC*, is located immediately downstream from the Hildenborough *hydA,B* genes and has 30–50% sequence homology with these genes. Hybridization of Miyazaki DNA with a *D. vulgaris* Hildenborough *hydC* gene probe was tested but was also found to be negative (not shown).

Although *D. vulgaris* Miyazaki appears to lack genes for [Fe] hydrogenase with homology to those of the Hildenborough strain, its DNA does hybridize with probes derived from the [NiFe] hydrogenase genes of *D. vulgaris* Hildenborough. Probe P₁ hybridizes with a 3.8 kb *Eco*RI fragment and *Pst*I fragments of 1.3 and 0.58 kb (Fig. 2b, lanes 5 and 6) in agreement with the restriction map for this region of the Miyazaki chromosome, that was established by these experiments and nucleic acid sequencing (Fig. 1b). One can derive from the distribution of *Eco*RI sites in Fig. 1(b), that the 3.8 kb

pr1 pr2
 CGCGATGCGGAAATGTGTGCTCATTTCATTTCGAATAACGTTTCAATGTGTGTACCTTCCCGGCAACGTCGGGACAGGTACCCGCGGAAACGGATGCACGCAGAAGCGG
 10 20 30 40 50 60 70 80 90 100 110 120
 TCGCAGCCGGGGGCTTTCGACACAGTACGACCTTACCCCGCGCGCGCTGGCGGGGGGAGGCACGGTGATACGCGGTGCCGAAGGAGGCAGGGATGAAAATCTCGATCGGTCTCG
 130 140 150 160 170 180 190 200 210 220 230 240
 K E G V E E R L A E R G V S R R D F L K F C T A I A V T M G M G P A F A P E V A
 GCAAGGAGGCGTGGAGGAAAGCTTCCGGAACGCGCGTGTCTCGACGCGACTTCTCAAGTTCTGTACGGCCATCGCGTGACCATGGGCATGGGCCCCGCTTCGCGCCGGAAGTTG
 250 260 270 280 290 300 310 320 330 340 350 360
 R A L M G P R R P S V V Y L H N A E C T G C S E S V L R A F E P Y I D T L I L D
 CCCGCGCGCTCATGGGTCCCGCGCGCGCTCCGTGGTCTACCTGCACAACCGCAATGCACCGGTGTTCGGAATCGGTGTGCGCGGTTCGAACCTACATCGACACCTGATTCTGG
 370 380 390 400 410 420 430 440 450 460 470 480
 T L S L D Y H E T I M A A A G D A A E A A L E Q A V N S P H G F I A V V E G G I
 ACACGTTGCCCTCGATTACCATGAGACCATCATGGCGCGCGCGCGATGCGCGGAAGCGCGCTGGAGCAGGCGCTCAACAGCCCGCAGCGCTTCATCGCGGTGGGAAGCGGCA
 490 500 510 520 530 540 550 560 570 580 590 600
 P T A A N G I Y G K V A N H T M L D I C S R I L P K A Q A V I A Y G T C A T F G
 TTCCACGCGTCCCAACGGCATCTACGCAAGGTGGCAACACACCATGCTGGACATCTGCAGCCGATCCTGCCAAGGCCAGGCGCTCATCGGTACGGCACCTGCGCCACCTTCG
 610 620 630 640 650 660 670 680 690 700 710 720
 G V Q A A K P N P T G A K G V N D A L K H L G V K A I N I A G C P P N P Y N L V
 GCGCGTGCAGGCACCAAGCCCAACCCACCGGTGCCAAGGCGTCAACAGCGCGTGAAGCACCTTGGCGTCAAGGCCATCAACATCGCGGTTCGCCGCCAAGCCGTACAACCTGG
 730 740 750 760 770 780 790 800 810 820 830 840
 G T I V Y Y L K N K A A P E L D S L N R P T M F F G Q T V H E Q C P R L P H F D
 TCGGCACCATCGTGTACTACCTGAAGAACAAGGCCGCGCGAGCTGGACAGCTGAACGCGCCACCATGTTCTTCGGCCAGACCGTGCACGAACAGTGCCTCCGCTACCGCACTTCG
 850 860 870 880 890 900 910 920 930 940 950 960
 A G E F A P S F E S E E A R K G W C L Y E L G C K G P V T M N N C P K I K F N Q
 ACGCGGCGAATTCGCCCCGTGTTGCAATCGGAAGAAGCCCGCAAGGGTGGTGCCTTACGAGCTGGGTGCAAGGCCCGGTGACCATGAACACTGCCGAAGATCAAGTCAACC
 970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080
 T N W P V D A G H P C I G C S E P D F W D A M T P F Y Q N *
 AGACCAACTGGCCCGTGGACGCGGGGACCCCTGCATCGGGTGCAGCGAACCAGATTCTTGGGACGCCATGACCCGTTCTACAGAACTGATACGCGCACGCGTAGTCAACCAATA
 1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
 rbs M S G C R A Q N A P G G I P V T P K S S Y S G P I V V D P V
 CCCCCATAGGCAACGCCAAGGAGAATACCATGAGCGGTGCAGAGCCAGAAATGCTCCGGGCGCATCCCGTGACGCCCAAGAGCTCCTATAGCGGTCCCATCGTCTGACCCCGTT
 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
 T R I E G H L R I E V E V E N G K V K N A Y S S S T L F R G L E I I L K G R D P
 ACCCGCATCGAAGGCCACCTGCGCATCGAGGTGGAAGTGGAGAACGCAAGGTCAAGAACGCTACAGCAGTTCACGCTGTTCGGGGGCTTGAATATCTGAAGGGCGCGACCCC
 1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
 R D A Q H F T Q R T C G V C T Y T H A L A S T R C V D N A V G V H I P K N A T Y
 CGCGACGCCAGCACTTACCCAGCGCACTGCGCGGTGTGACCTATACCATGCGCTGGCTCCACCCGCTGCGTGACAAACGCGTGGCGGTGCACATTCCCAAGAACGCCACCTAC
 1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
 I R N L V L G A Q Y L H D H I V H F Y H L H A L D F V D V T A A L K A D P A K A
 ATCCGCAACCTGGTGTGGCGCGCAGTACCTGCACGACCATCTGCACTTCTACACCTGCACGCCCTGGACTTCGTGGAGTGACCGCGCGCTGAAGGCCGACCGGCCAAGGCC
 1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
 A K V A S S I S P R K T T A A D L K A V Q D K L K T F V E T G Q L G P F T N A Y
 GCCAAGGTGGCTCGTCCATCTGCCCCGCAAGACACGCGCGCGACCTGAAGGCGGTGCAGGACAAGCTGAAGACCTTCGTGGAACCGGGCAGCTCGGCCGTTACCAATGCCTAC
 1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
 F L G G H P A Y Y L D P E T N L I A T A H Y L E A L R L Q V K A A R A M A V F G
 TTCCTGGGCGGCCACCCGCTACTACCTGGATCCGGAACCAACCTCATCGCCACCGCCACTACCTGGAAGCCCTGCGCCTTCAGTGGAAGCGCGCGCCATGGCGCTTTTCGGC
 1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920
 A K N P H T Q F T V V G G V T C Y D A L T P Q R I A E F E A L W K E T K A F V D
 GCCAAGAAGCCGCACACCACTTACCCGTGGTGGCGCGGTGACCTGCTACGACGCCCTGACCCCGCAGCGCATCGCGCAATTCGAGGCGCTGTGAAGGAAACCAAGCGTTCTGTGAT
 1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040
 E V Y I P D L L V V A A A Y K D W T Q Y G G T D N F I T F G E F P K D E Y D L N
 GAAGTGTACATCCCGACCTGCTGGTGGTGGCGCGGCTACAAGGATTGGACGAGTACGGCGGCACCGACAACCTCATCACCTTCGGCGAATTCGGAAGGACGAGTACGACCTGAAC
 2050 2060 2070 2080 2090 2100 2110 2120 2130 2140 2150 2160

Fig. 3. Nucleotide sequence for the hydrogenase operon from *D. vulgaris* Miyazaki F. The coding regions for the small (nt 219–1169) and large (nt 1231–2931) subunits have been translated with the one letter amino acid code. Both genes are preceded by a purine-rich ribosome-binding site (rbs). Potential promoter sequences (pr1 and pr2), discussed in the text, are indicated, as well as a hairpin loop (—), that could function in transcription termination.

EcoRI fragment arises from partial digestion: a 1 kb fragment of Miyazaki DNA is expected to hybridize with P_1 upon complete digestion. This 1 kb fragment is present in *EcoRI* digests of Miyazaki DNA at low intensity (Fig. 2b, lane 5). Complete digestion of the *D. vulgaris* Miyazaki chromosome with *EcoRI* proved hard to achieve. In contrast complete *PstI* digests, showing only hybridization of P_1 with the 1.3 and 0.58 kb restriction fragments expected on the basis of the map (Fig. 1b), were achieved consistently, indicating that the more complex *EcoRI* band pattern is due to partial digestion rather than to the presence of multiple genes.

The 3.8 kb *EcoRI* fragment was next cloned by ligating a size-fractionated *EcoRI* digest (3–5 kb) of Miyazaki DNA to *EcoRI*-cut pUC8 and transforming the ligation mixtures into *E. coli* TG2. Recombinant colonies were probed with radiolabelled P_1 and a positive clone, containing pHCAL2 with the insert as outlined in Fig. 1(b), was isolated. Sequencing of this insert (see below) showed that it contains the entire large subunit gene and part of the 3' end of the small subunit gene (Fig. 1b). When Southern blots of *EcoRI*-digested *D. vulgaris* Miyazaki DNA were incubated with radiolabelled P_3 , two hybridizing fragments of 3.8 and 3.1 kb were observed, while just the 3.1 kb fragment was found to hybridize with probe P_2 (not shown). These results

indicated that the 3.1 kb fragment contains the 5' end of the small subunit gene. It was cloned by ligating 2–4 kb *EcoRI* fragments and probing the resulting recombinant clones with P_2 . pMZSS1-56 containing the 3.1 kb *EcoRI* insert as indicated in Fig. 1(b) was isolated.

Both the 3.1 and 3.8 kb *EcoRI* fragments were sequenced with the shotgun procedure outlined by Bankier & Barrell (1983). This led to complete determination of the sequence of the 3.8 kb fragment, while that of the 3.1 kb fragment was partially determined. Together the two sequences form a contig of 6190 nucleotides (nt), as indicated in Fig. 1(c). The sequence of 3100 nt of this contig is shown in Fig. 3, with the proposed reading frames for the small and large subunits of Miyazaki hydrogenase indicated by translation into protein. Every nt of the sequence shown in Fig. 3 was determined at least once on each strand. Several severe compressions could only be read by using T7 DNA polymerase ('Sequenase') rather than Klenow polymerase and by substituting dGTP by dITP in the sequencing reactions.

As indicated in Fig. 3, the gene for the small subunit (nt 219–1169) precedes that for the large subunit (nt 1231–2931) as in other operons encoding [NiFe] or [NiFeSe] hydrogenases (Li *et al.*, 1987; Menon *et al.*, 1987, 1990; Voordouw *et al.*, 1989; Leclerc *et al.*, 1988;

The coding regions for the small (nt 219–1169) and large (nt 1231–2931) subunit of Miyazaki hydrogenase are homologous to those for the small and large subunits of *D. gigas* [NiFe] hydrogenase. A comparison of the amino acid sequences, derived from the sequences of these genes, is shown in Fig. 4 and is discussed below. As expected from the Southern blotting results (Fig. 2a), there is no homology with the [Fe] hydrogenase genes of *D. vulgaris*.

The cloning and sequencing of [NiFe] hydrogenase genes from a variety of bacterial sources has allowed an extensive comparison of homologies in the small and large subunit polypeptide chains, which, in turn, has led to the definition of conserved amino acid, particularly cysteine, residues. Conserved cysteine side-chains are likely to co-ordinate iron-sulphur clusters and the active-site nickel in [NiFe] hydrogenases. There are three iron-sulphur clusters in the [NiFe] hydrogenase of *D. gigas*, which has probably been best characterized with spectroscopic methods of all enzymes in this class (Li *et al.*, 1987; Moura *et al.*, 1982). Two of these are of the Fe_4S_4 type, requiring four cysteine residues for co-ordination. The third is a Fe_3S_4 cluster, which is

presumably co-ordinated by three cysteines (Kissinger *et al.*, 1989). It thus appears that 11 cysteines are required for the co-ordination of iron-sulphur clusters in [NiFe] hydrogenases.

Comparison of primary structures of [NiFe] hydrogenase small and large subunits has shown that there are 14 strictly conserved cysteine residues. Perhaps surprisingly, it appears that 10 of these are present in the small, while only four are found in the large subunit. The small subunit appears, therefore, to be the main site for iron-sulphur cluster co-ordination in [NiFe] hydrogenases. Using the numbering of the *D. gigas* sequence in Fig. 4, the 10 strictly conserved cysteines of the small subunit are C-67, C-70, C-162, C-198, C-238, C-263, C-269, C-278, C-296 and C-299, while in the large subunit two groups of two residues are conserved at the NH₂-terminus, C-65 and C-68, and at the COOH terminus, C-530 and C-533. Cysteine residue C-530 serves a special function, since its TGC codon is replaced by a TGA codon, thought to encode selenocysteine, in the [NiFeSe] hydrogenase gene of *D. baculatus* (Voordouw *et al.*, 1989a). Since it has been shown by spectroscopic studies that this selenocysteine co-ordinates to the nickel (Eidsness *et al.*, 1989) it is now thought that C-530 is also a nickel ligand in [NiFe] hydrogenase.

Inspection of Fig. 4 reveals that all 14 cysteine residues listed above have been conserved in the sequence of the Miyazaki hydrogenase. In particular the conservation of C-530 (C-546 in the Miyazaki sequence, Fig. 4b) indicates this hydrogenase to belong to the [NiFe] hydrogenase family.

Apart from cysteine residues the two small subunits show 69% overall sequence identity and 81% sequence homology, as calculated with the GAP program (Devereux *et al.*, 1984). Amino acids 1–50 of the *D. gigas* small subunit (Fig. 4a) constitute a signal sequence for protein export to the periplasm and the NH₂ terminus of the mature small subunit starts with L-51 (Li *et al.*, 1987; Niviere *et al.*, 1988). The sequence of residues 1–50 of the Miyazaki small subunit is highly conserved. In particular a box of six amino acid residues (RRXFXX), which is present in the small subunit signal sequence of every hydrogenase determined to date (Voordouw *et al.*, 1989b) is also found in the Miyazaki sequence, while residues 50–51 comprise a signal peptidase processing site identical to that found in the *D. gigas* small subunit. Residues 1–50 of the Miyazaki small subunit are therefore also expected to function in the export of this hydrogenase to the periplasm. The amino acid sequence of the mature small subunit is 267 residues long and has a calculated molecular mass of 28 763 Da.

The *D. vulgaris* Miyazaki and *D. gigas* large subunits show 68% sequence identity and 80% sequence homology (Fig. 4b). The two sequences are highly homologous at the COOH terminus and end with the same sequence.

Table 1. Amino acid composition of *D. vulgaris* Miyazaki hydrogenase as determined from the sequence of the gene (n_G) and from the data (n_Y) of Yagi *et al.* (1976), recalculated for a total of 833 residues

The n_Y value for tryptophan, determined by the method of Bencze & Schmid (1957), may be inaccurate.

Amino acid	n_G	n_Y
K	48	45
H	30	25
R	34	27
D + N	77	78
T	52	50
S	31	38
E + Q	70	71
P	56	59
G	67	73
A	89	88
C	20	20
V	67	64
M	13	11
I	43	38
L	58	56
Y	32	28
F	35	34
W	11	28
Total	833	833

The Miyazaki large subunit thus also lacks the COOH terminal extension found in the membrane-bound [NiFe] hydrogenases of *E. coli*, *B. japonicum* and *R. capsulatus* (see Introduction). Apart from an NH₂-terminal extension of 16 residues in the Miyazaki large subunit, there are no major differences with the *D. gigas* sequence. The calculated large subunit molecular mass is 62 495 Da (566 residues, excluding the initiating methionine).

The Miyazaki hydrogenase genes, that were cloned and sequenced in the present study, are thus highly homologous to those encoding the periplasmic [NiFe] hydrogenase of *D. gigas*. We consider it very likely that these genes encode the periplasmic (Tamura *et al.*, 1988), membrane-bound enzyme purified and characterized by Yagi *et al.* (1976, 1985). The subunit molecular masses derived from the gene sequence are very similar to those determined by Yagi *et al.* (1985: 29 and 60 kDa) and the amino acid composition determined for purified hydrogenase (Yagi *et al.*, 1976) is in good agreement with that derived from the sequence of the gene (Table 1). It appears, therefore, that *D. vulgaris* Miyazaki harbours a [NiFe] hydrogenase and there are no obvious features in the amino acid sequence of the enzyme that can explain its unusual enzymic and spectroscopic properties (Yagi *et al.*, 1976, 1985).

This research was supported by an operating grant from the National Science and Engineering Research Council of Canada (NSERC) to G. V. The authors are indebted to Drs T. Yagi and A. E. Przybyla for gifts of chromosomal and plasmid DNA, to Drs P. M. Vignais and A. E. Przybyla for sending preprints of manuscripts and to J. K. Voordouw for technical support.

References

- BANKIER, A. T. & BARRELL, B. G. (1983). Shotgun DNA sequencing. In *Techniques in the Life Sciences*, pp. 1–34. Limerick, Ireland: Elsevier Scientific Publishers Ireland Ltd.
- BENCZE, W. L. & SCHMID, K. (1957). Determination of tyrosine and tryptophan in proteins. *Analytical Chemistry* **29**, 1193–1196.
- DEVEREUX, J., HAEBERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387–395.
- EIDSSNESS, M. K., SCOTT, R. A., PRICKRIL, B., DERVARTANIAN, D. V., LEGALL, J., MOURA, I., MOURA, J. J. G. & PECK, H. D., JR (1989). Evidence for selenocysteine coordination to the active site nickel in the [NiFeSe] hydrogenase from *Desulfovibrio baculatus*. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 147–151.
- FORD, C. M., GARG, N., GARG, R. P., TIBELIUS, K. H., YATES, M. G., ARP, D. J. & SEEFELDT, L. C. (1990). The identification, characterization, sequencing and mutagenesis of the genes (*hupSL*) encoding the small and large subunits of the H₂-uptake hydrogenase of *Azotobacter chroococcum*. *Molecular Microbiology* **4** (in the Press).
- GOW, L. A., PANKHANIA, I. P., BALLANTINE, S. P., BOXER, D. H. & HAMILTON, W. A. (1986). Identification of a membrane-bound hydrogenase of *Desulfovibrio vulgaris* (Hildenborough). *Biochimica et Biophysica Acta* **851**, 57–64.
- HATCHIKIAN, E. C., BRUSCHI, M. & LEGALL, J. (1978). Characterization of the periplasmic hydrogenase from *Desulfovibrio gigas*. *Biochemical and Biophysical Research Communications* **82**, 451–461.
- HIGUCHI, Y., YASUOKA, N., KAKUDO, M., KATSUBE, Y., YAGI, T. & INOKUCHI, H. (1987). Single crystals of hydrogenase from *Desulfovibrio vulgaris* Miyazaki F. *Journal of Biological Chemistry* **262**, 2823–2825.
- KISSINGER, C. R., ADMAN, E. T., SIEKER, L. C., JENSEN, L. H. & LEGALL, J. (1989). The crystal structure of the three-iron ferredoxin II from *D. gigas*. *FEBS Letters* **242**, 447–450.
- LECLERC, M., COLBEAU, A., CAUVIN, B. & VIGNAIS, P. M. (1988). Cloning and sequencing of the genes encoding the large and the small subunits of the H₂ uptake hydrogenase (*hup*) of *Rhodobacter capsulatus*. *Molecular and General Genetics* **214**, 97–108.
- LI, C., PECK, H. D., JR, LEGALL, J. & PRZYBYLA, A. E. (1987). Cloning, characterization and sequencing of the genes encoding the large and small subunits of the periplasmic [NiFe] hydrogenase of *Desulfovibrio gigas*. *DNA* **6**, 539–551.
- LISSOLO, T., CHOI, E. S., LEGALL, J. & PECK, H. D., JR (1986). The presence of multiple intrinsic membrane nickel containing hydrogenase in *Desulfovibrio vulgaris* (Hildenborough). *Biochemical and Biophysical Research Communications* **139**, 701–708.
- MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *Journal of Molecular Biology* **3**, 208–218.
- MENON, N. K., PECK, H. D., JR, LEGALL, J. & PRZYBYLA, A. E. (1987). Cloning and sequencing of the genes encoding the large and small subunits of the periplasmic (NiFeSe) hydrogenase of *Desulfovibrio baculatus*. *Journal of Bacteriology* **169**, 5401–5407.
- MENON, N. K., ROBBINS, J., PECK, H. D., JR, CHATELUS, C. Y., CHOI, E.-S. & PRZYBYLA, A. E. (1990). Cloning and sequencing of a putative *Escherichia coli* [NiFe] hydrogenase-1 operon containing six open reading frames. *Journal of Bacteriology* **172**, 1969–1977.
- MESSING, J. & VIEIRA, J. (1982). A new pair of of M13 vectors for selecting either DNA strand of double digest restriction fragments. *Gene* **19**, 269–276.
- MOURA, J. J. G., MOURA, I., HUYNH, B.-H., KRUGER, H.-J., TEXEIRA, M., DUVARNEY, R. C., DERVARTANIAN, D. V., XAVIER, A. V., PECK, H. D. JR & LEGALL, J. (1982). Unambiguous identification of the nickel EPR signal in ⁶¹Ni enriched *Desulfovibrio gigas* hydrogenase. *Biochemical and Biophysical Research Communications* **108**, 1388–1393.
- NIVIERE, V., FORGET, N., BOVIER-LAPIERRE, G., BONICEL, J. & HATCHIKIAN, C. (1988). Isolation, amino acid analysis and N-terminal sequence determination of the two subunits of the nickel-containing hydrogenase of *Desulfovibrio gigas*. *Biochimie* **70**, 267–271.
- PRICKRIL, B. C., CZECHOWSKI, M. H., PRZYBYLA, A. E., PECK, H. D. JR & LEGALL, J. (1986). Putative signal peptide on the small subunit of the periplasmic hydrogenase from *Desulfovibrio vulgaris*. *Journal of Bacteriology* **167**, 722–725.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- SAYAVEDRA-SOTO, L. A., POWELL, G. K., EVANS, H. J. & MORRIS, R. O. (1988). Nucleotide sequence of the genetic loci encoding subunits of *Bradyrhizobium japonicum* uptake hydrogenase. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 8395–8399.
- STADEN, R. (1982). Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. *Nucleic Acids Research* **10**, 4731–4751.
- STADEN, R. (1984a). A computer program to determine the function of nucleic acid sequences. *Nucleic Acids Research* **12**, 499–503.
- STADEN, R. (1984b). Graphic methods to determine the function of nucleic acid sequences. *Nucleic Acids Research* **12**, 521–538.
- STADEN, R. & MCLACHLAN, A. D. (1982). Codon preference and its use in identifying protein coding regions in long DNA sequences. *Nucleic Acids Research* **10**, 141–156.
- STOKKERMANS, J., VAN DONGEN, W., KAAAN, A., VAN DEN BERG, W. & VEEGER, C. (1989). *hydy*, a gene from *Desulfovibrio vulgaris* (Hildenborough) encodes a polypeptide homologous to the periplasmic hydrogenase. *FEMS Microbiology Letters* **58**, 217–222.
- TAMURA, A., KAWATE, T., OGATA, M. & YAGI, T. (1988). Interaction of cellular hydrogenase, cytochrome c3 and desulfovibrin in *Desulfovibrio vulgaris* Miyazaki with their antibodies. *Journal of Biochemistry* **104**, 722–726.
- UFFEN, R. L., COLBEAU, A., RICHAUD, P. & VIGNAIS, P. M. (1990). Cloning and sequencing the genes encoding uptake-hydrogenase subunits of *Rhodocyclus gelatinosus*. *Molecular and General Genetics* **221**, 49–58.
- VIEIRA, J. & MESSING, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
- VOORDOUW, G. & BRENNER, S. (1985). Nucleotide sequence of the gene encoding the hydrogenase from *Desulfovibrio vulgaris* (Hildenborough). *European Journal of Biochemistry* **148**, 515–520.
- VOORDOUW, G., WALKER, J. E. & BRENNER, S. (1985). Cloning of the gene encoding the hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) and determination of the NH₂-terminal sequence. *European Journal of Biochemistry* **148**, 509–514.
- VOORDOUW, G., HAGEN, W. R., KRUSE-WOLTERS, M., VAN BERKEL-ARTS, A. & VEEGER, C. (1987). Purification and characterization of *Desulfovibrio vulgaris* (Hildenborough) hydrogenase expressed in *Escherichia coli*. *European Journal of Biochemistry* **162**, 31–36.
- VOORDOUW, G., MENON, N. K., LEGALL, J., CHOI, E.-S., PECK, H. D. JR & PRZYBYLA, A. E. (1989a). Analysis and comparison of nucleotide sequences encoding the genes for [NiFe] and [NiFeSe] hydrogenase from *Desulfovibrio gigas* and *Desulfovibrio baculatus*. *Journal of Bacteriology* **171**, 2894–2899.
- VOORDOUW, G., STRANG, J. D. & WILSON, F. R. (1989b). Organization of the genes encoding [Fe] hydrogenase in *Desulfovibrio vulgaris* subsp. *oxamicus* Monticello. *Journal of Bacteriology* **171**, 3881–3889.
- YAGI, T. (1970). Solubilization, purification and properties of particulate hydrogenase from *Desulfovibrio vulgaris*. *Journal of Biochemistry* **68**, 649–657.
- YAGI, T., KIMURA, K., DAIDOJI, H., SAKAI, F., TAMURA, S. & INOKUCHI, H. (1976). Properties of purified hydrogenase from the particulate fraction of *Desulfovibrio vulgaris* Miyazaki. *Journal of Biochemistry* **79**, 661–671.
- YAGI, T., KIMURA, K. & INOKUCHI, H. (1985). Analysis of the active centre of hydrogenase from *Desulfovibrio vulgaris* Miyazaki by magnetic measurements. *Journal of Biochemistry* **97**, 181–187.