Cloning and Nucleotide Sequences of the Genes for the Subunits of NAD-Reducing Hydrogenase of *Alcaligenes eutrophus* H16

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The genes *hoxF*, -*U*, -*Y*, and -*H* which encode the four subunit polypeptides α, γ, δ, and β of the NAD-reducing hydrogenase (HoxS) of *Alcaligenes eutrophus* H16, were cloned, expressed in *Pseudomonas facilis*, and sequenced. On the basis of the nucleotide sequence, the predicted amino acid sequences, and the N-terminal amino acid sequences, it was concluded that the structural genes are tightly linked and presumably organized as an operon, denoted *hoxS*. Two pairs of -24 and -12 consensus sequences resembling RpoN-activatable promoters lie upstream of *hoxF*, the first of the four genes. Primer extension experiments indicate that the second promoter is responsible for *hoxS* transcription. *hoxF* and *hoxU* code for the flavin-containing dimer (α and γ subunits) of HoxS which exhibits NADH:oxidoreductase activity. A putative flavin-binding region is discussed. The 26.0-kilodalton (kDa) γ subunit contains two cysteine clusters which may participate in the coordination of two [4Fe-4S] centers. The genes *hoxY* and *hoxH* code for the small 22.9-kDa β subunit and the nickel-containing 54.8-kDa β subunit, respectively, of the hydrogenase dimer of HoxS. The latter dimer exhibits several conserved regions found in all nickel-containing hydrogenases. The roles of these regions in coordinating iron and nickel are discussed. Although the deduced amino acid sequences of the δ and β subunits share some conserved regions with the corresponding polypeptides of other [NiFe] hydrogenases, the overall amino acid homology is marginal. Nevertheless, significant sequence homology (35%) to the corresponding polypeptides of the soluble methylviologen-reducing hydrogenase of *Methanobacterium thermoautotrophicum* was found. Unlike the small subunits of the membrane-bound and soluble periplasmic hydrogenases, the HoxS protein does not appear to be synthesized with an N-terminal leader peptide.

*Alcaligenes eutrophus* H16 is a gram-negative facultatively lithoautotrophic bacterium which can assimilate CO₂ and utilize H₂ as an energy source (reviewed in reference 5). Hydrogen oxidation is catalyzed by hydrogenases. These enzymes are found in phylogenetically diverse microorganisms, where they are responsible for both consumption and production of H₂ (reviewed in reference 13). Two distinct hydrogenases have been purified and characterized from extracts of *A. eutrophus* (39, 44). These two enzymes differ in cellular localization, cofactor content, polypeptide composition, and apparent molecular weight. The membrane-bound hydrogenase (HoxP) is a representative of the more common hydrogenase type. It consists of two heterologous polypeptides and is coupled to the respiratory chain via an unknown electron acceptor (43). The second hydrogenase (HoxS) of *A. eutrophus* resides in the cytoplasm (35). It is composed of four heterologous polypeptides, reduces NAD as the physiological electron acceptor, and contains flavin mononucleotide as the prosthetic group. Hydrogenases of this type have been found only in *Alcaligenes* species and in the gram-positive lithoautotrophic bacterium *Nocardia opaca* (reviewed in reference 5). A multimeric hydrogenase which utilizes deazaflavin as electron acceptor has been identified in methanogenic bacteria (23).

Three classes of hydrogenases can be distinguished on the basis of their metal content. The class of iron [Fe] hydrogenases is characterized by two ferredoxinlike [4Fe-4S] clusters and a third atypical iron-sulfur center supposedly involved in the reaction with H₂. Both *A. eutrophus* hydrogenases belong to the nickel-iron [NiFe] type. These enzymes typically have two [4Fe-4S] centers and one [3Fe-3S] cluster in addition to nickel. A third class of hydrogenases contains iron, nickel, and selenium [NiFeSe]. An enzyme of this type has been described for *Desulfovibrio* and *Methanococcus* strains (reviewed in reference 13).

The genes coding for the two hydrogenases of *A. eutrophus* H16 lie in a cluster of genes on a 450-kilobase-pair (kb) conjugative megaplasmid (15, 24). Molecular cloning of megaplasmid DNA in *Escherichia coli* and screening of the resultant hybrid plasmids for complementation of hydrogenase-deficient mutants led to the identification of hydrogenase (*hox*) genes. Subsequent studies revealed that the two structural gene loci *hoxS* and *hoxP* coding for the soluble NAD-linked hydrogenase and the membrane-bound hydrogenase, respectively, mark the left and right borders of the *hox* gene complex (12).

In this communication, we report the cloning of the *hoxS* locus, consisting of the four structural genes, *hoxF*, *hoxU*, *hoxY*, and *hoxH*, and the heterologous expression of this locus in *Pseudomonas facilis*. The complete nucleotide sequence of the *hoxS* region was determined, and the primary amino acid sequence was compared with that of other hydrogenases. Putative cofactor-binding sites are discussed.

**MATERIALS AND METHODS**

**Organisms and plasmids.** The bacterial strains, vectors, and recombinant plasmids used in this study are shown in Table 1.

**Media and growth conditions.** Strains of *A. eutrophus* and *P. facilis* were grown in mineral salts medium (40). Autotrophic cultures were grown under an atmosphere of...
TABLE 1. Bacterial strains and plasmids used in this study

| Strain          | Plasmid | Relevant markers | Reference or source          |
|-----------------|---------|-----------------|-----------------------------|
| *Alcaligenes eutrophus* |         |                 |                             |
| H16             | pHG1    | HoxP⁺ HoxS⁺     | DSM 428, ATCC 17699         |
| HF14            | pHG1-16 | HoxP⁺ HoxS⁻     | 41                          |
| HF15            | pHG1-15 | HoxP⁺ HoxS⁻     | 18                          |
| HF89            | pHG1-13 | HoxP⁺ HoxS⁻     | 18                          |
| *Pseudomonas facilis* |         |                 |                             |
| K               | pHG22-a | HoxP⁺ HoxS⁻     | DSM 620                     |
| J               | pHG22-b |                 |                             |
|                 | pHG20   | HoxP⁺ HoxS⁻     | This study                  |
|                 |         |                 |                             |
| *Escherichia coli* |         |                 |                             |
| JM101           |         | Δ(lac-proAB) F' [proAB lacP lacZ ΔM15] | 31 |
| NM522           |         | hsdR Δ(lac-proAB) F' [proAB lacP lacZ ΔM15] | 16 |
| NM522           | pTZ18R  | Ap⁺ lacZ’ fl ori | 33                          |
| NM527           | pTZ19R  | Ap⁺ lacZ’ fl ori | 33                          |
| HB101           |         |                 |                             |
| HB101           | pVK101  | Km⁺ Te⁺ pHG1 insert (11.6 kb) | 12 |
| HB101           | pVK102  | Km⁺ Te⁺ pHG1 insert (32 kb) | 22 |
| HB101           | pRK2013 | Km⁺                | 11                          |
| HB101           | pGE5    | Te⁺ pHG1 insert (32 kb) | This study                  |
| HB101           | pGE16   | Te⁺ pHG1 insert (15 kb) | This study                  |
| HB101           | pGE15   |                 |                             |

* Hox, Ability to oxidize hydrogen; HoxP, membrane-bound hydrogenase activity; HoxS, NAD-reducing hydrogenase activity.

hydrogen, oxygen, and carbon dioxide in a ratio of 8:1:1 (vol/vol/vol). Organic carbon sources were routinely added at a concentration of 0.4% (wt/vol). The concentration of the nitrogen source was 0.2% (wt/vol). Strains of *E. coli* were propagated in M9 medium (29) or in Luria broth (LB). LB medium with 0.2% (wt/vol) sodium chloride was used as the complex medium for the cultivation of hydrogen-oxidizing bacteria. Selective media contained antibiotics at the following concentrations: 15 µg of tetracycline per ml and 350 µg of kanamycin per ml for *A. eutrophus*; 3 µg of tetracycline per ml for *P. facilis*; 15 µg of tetracycline per ml, 30 µg of kanamycin per ml, and 50 µg of ampicillin per ml for *E. coli*.

Cloning of hoxS DNA. A cosmid library of megaplasmid pHG1 was constructed as described previously (12). pHG1 DNA was partially digested with the restrictionendonuclease HindIII and ligated with HindIII-digested cosmid vector pVK102. The ligation products were packaged in vitro into bacteriophage lambda with a DNA packaging kit (Boehringer & Soehne GmbH, Mannheim, Federal Republic of Germany) according to the instructions of the manufacturer. After infection of *E. coli* HB101, tetracycline-resistant colonies were selected and screened for the presence of hydrogenase genes.

To identify hoxS clones, we transferred the cosmids to HoxS⁻ mutants of *A. eutrophus* (Table 1) via a triparental cross with pRK2013 as the mobilizing vector (11). Transconjugants were selected on mineral medium with 15 µg of tetracycline per ml and tested for autotrophic growth on hydrogen.

**Standard DNA techniques.** Standard DNA techniques were essentially as described by Maniatis et al. (29). Rapid, small-scale DNA isolation for cloning analysis was done by the method of Birnboim and Doly (4). For large-scale preparation of vector and recombinant plasmid DNA from *E. coli*, the DNA was further purified by ethidium bromide gradient centrifugation. Single-stranded template DNA was prepared after precipitation of the phage with polyethylene glycol. *E. coli* strains were transformed with the ligated DNA by the method of Mandel and Higa (28). DNA-DNA hybridization was conducted by the method of Southern (46). For labeling and detection of DNA, nick translation kits and Blue Gene kits (GIBCO-Bethesda Research Laboratories, Eggenstein, Federal Republic of Germany) were used as recommended by the manufacturer.

**DNA sequence determination and analysis.** Overlapping restriction fragments were cloned into phage M13mp18 and M13mp19 (32) or into plasmids pTZ18R and pTZ19R (33). Nested deletions were constructed by unidirectional exonuclease III-S1 nuclease digestion (Erase-a-base-system; Promega Corp., Madison, Wis.). The dideoxy-chain termination method was applied (37) with 32P-labeled α-dATP from Dupont, NEN Research Products (Dreieich, Federal Republic of Germany). Sequencing reactions were done with Klenow-polymerase, using 7-deaza-dGTP to avoid compression (PUC sequencing kit; Boehringer) or alternatively with T7 DNA polymerase (T7 sequencing kit; Pharmacia, Freiburg, Federal Republic of Germany) or Taq polymerase (Taqence DNA sequencing kit; United States Biochemical Corp., Cleveland, Ohio). The complete sequence was compiled from overlapping partial sequences determined throughout for both strands. Nucleotide and amino acid sequences (open reading frames, translation into amino acids, codon usage, sequence comparison, hydrophobicity) were analyzed with the programs of the MacMolly sequence analysis package (Softgene GmbH, Berlin, Federal Republic of Germany).

**Transcript mapping.** Total RNA of *A. eutrophus* H16 was a gift from U. Oelmüller (Institut für Mikrobiologie, Universität Göttingen). The transcription start point was determined by primer extension reaction as described by Ausubel et al. (2) with single-stranded template DNA. Synthetic oligonucleotides 24 nucleotides long were supplied by the Institut für Genbiologische Forschung, Berlin.

**Enzyme assay and immunochemical analysis.** For assays of hydrogenase activity, cultures were grown autotrophically on hydrogen or heterotrophically on a mixture of fructose and glycerol (0.2% [wt/vol] each). HoxS activity (hydrogen: NAD⁺ oxidoreductase, EC 1.12.1.2) was measured by mon-
itoring hydrogen-dependent NAD reduction with detergent-treated cells (14). Ouchterlony immunodiffusion analyses were performed as described previously (15). Protein was determined with bovine serum albumin as the standard (27).

Chemicals. Restriction endonucleases, T4 DNA ligase, and the lambda DNA packaging kit were obtained from Boehringer & Soehne GmbH. All other chemicals were from E. Merck AG, (Darmstadt, Federal Republic of Germany).

RESULTS

Cloning of structural genes for NAD-reducing hydrogenase (HoxS) from A. eutrophus H16. Previous studies showed that genes coding for HoxS map in a 100-kb long region of megaplasmid pHG1 (24). An 11.6-kb EcoRI fragment from this region restored HoxS activity in some but not all mutants bearing structural gene mutations (12), indicating that only a part of the hoxS locus was present. To obtain a clone with the complete locus, we constructed a cosmid library of pHG1 by partial HindIII digestion. Resultant fragments were cloned into the broad-host-range vector pVK102 (11). Six recombinant plasmids were identified, which efficiently complemented all HoxS− mutants. These recombinants shared a common 15-kb HindIII fragment, which was subcloned into pVK102 yielding the cosmid pGE15 (Table 1). The 15-kb HindIII fragment and the 11.6-kb EcoRI fragment overlapped for a distance of 2.3 kb (Fig. 1).

Indirect evidence for the assumption that the 15-kb HindIII fragment encodes the entire set of structural genes for the NAD-reducing hydrogenase was obtained by heterologous expression of the hoxS DNA in P. facilis, which contains only a membrane-bound hydrogenase. Transfer of the recombinant cosmid pGE15 into two wild-type strains of P. facilis yielded transconjugants which grew faster on hydrogen than the parent strains. Thus, the acquisition of HoxS activity apparently enhanced the lithoautotrophic growth of P. facilis (Table 2). Enzymatic assays showed that the HoxS-containing transconjugants of P. facilis obtained NAD-reducing hydrogenase activity (Table 2). The HoxS protein of the P. facilis transconjugants was immunologically identical to that of A. eutrophus (Fig. 2A). In this context a prominent protein of A. eutrophus, designated B protein, is of interest. Its formation is coordinately regulated with HoxS activity, and it occurs only in those hydrogen bacteria which contain NAD-reducing hydrogenase. The physiological function of the B protein is still unknown (21). It was absent in wild-type strains of P. facilis but immunologically detectable in transconjugants containing the hoxS cosmid pGE15 (Fig. 2B). Thus, the gene of the B protein must also be located on the 15-kb HindIII fragment.

Attempts to express the hoxS genes of A. eutrophus in E.
coli were unsuccessful. This may be attributable to the complex regulation of hox gene expression, to the inactivity of the hoxS promoter in E. coli, and/or to the requirement for specific processing of the HoxS gene products.

Nucleotide and derived amino acid sequences of NAD-reducing hydrogenase. The region presumed to encode the structural genes was sequenced. The nucleotide sequence revealed four adjacent open reading frames, each of which is preceded by a tentative ribosomal binding site (underlined in Fig. 3). Codon usage (data not shown) was in good agreement with that of previously sequenced genes of A. eutrophus (1, 20). The open reading frames were designated as hoxF, -U, -Y, and -H. On the basis of comparison of the deduced amino acid sequences with the previously published N-terminal amino acid sequences (54), it was possible to assign these four genes to the subunits α, γ, δ, and β of HoxS. Some discrepancies between the experimentally determined amino acid sequences and the deduced sequences were found. Our data predict Trp at position 24 of the α subunit and Val, Ala, and Val at positions 29, 31, 36, respectively, of the β subunit. Interestingly, the same amino acids were found at the corresponding positions in the NAD-reducing hydrogenase of N. opaca (54). Subunits β and γ lack the initial methionine, which is apparently removed posttranslationally. The deduced molecular weights of the subunits were in good agreement with the biochemical values (Table 3).

Stop and start codons of the genes hoxF, -U, and -Y were found to overlap. The genes hoxY and hoxH are separated by 20 bases (Fig. 3) This arrangement predicts an organization as an operon. Two possible RpoN-specific promoter sequences with typical −24 and −12 consensus elements (17) lie upstream of the hoxF start codon, whereas typical prokaryotic −35 and −10 consensus sequences were absent in this region. Primer extension experiments (data not shown) revealed a signal at nucleotide 654 (Fig. 3) indicating that this is the transcription start point of the hoxS operon. This is compatible with the assumption that promoter 2 does in fact direct transcription of the hoxS operon. A large inverted repeat (indicated by arrows in Fig. 3) immediately following the hoxH stop codon could form a hairpinlike 10mer and may be involved in transcription termination. The free energy of this structure is 35.8 kcal (approximately 149.6 kJ/mol) according to the base-pairing rules of Tinoco et al. (47).

### Amino acid sequence comparison of hydrogenase dimer

Sequence data are now available for six hydrogen-consuming hydrogenases (25, 26, 30, 34, 38, 50, 51). Similarities exist between [NiFe] and [NiFeSe] enzymes. They are predominantly heterodimers of comparable molecular weight. The respective structural genes are apparently organized as operons in which the genes for the small subunits precede those for the large ones (reviewed in reference 13). The genes for the NAD-dependent hydrogenase of A. eutrophus show a similar organization: the genes hoxY and hoxH encoding the hydrogenase dimer lie downstream of the genes hoxF and hoxU coding for the flavin-containing moiety of HoxS.

While there is little homology between the predicted primary amino acid sequences of the δ and β subunits of HoxS and the corresponding membrane-bound and soluble periplasmic polypeptides, a considerable overall homology to the soluble hydrogenase subunits γ (34.0%) and δ (36.6%) of *Methanobacterium thermoautotrophicum* ∆H (34) was found (Fig. 4).

The deduced amino acid sequences reveal that the δ subunit of HoxS contains nine cysteine residues. A total of 9 to 13 cysteines are commonly found in the small hydrogenase subunits of various organisms (Fig. 5A). A number of these are located at homologous positions within conserved sequence motifs (34). A conserved domain at the C terminus is not found in the small polypeptide of *A. eutrophus*; it seems to be truncated (Fig. 5A). On the other hand, an additional stretch of amino acids occurs at the N terminus which is missing in the corresponding polypeptides. Since this N-terminal moiety is present in the mature δ subunit (54), a leader peptide, which has been predicted for the small subunits of membrane-bound and soluble periplasmic hydrogenases, can be excluded.

Our data indicate the presence of six cysteine residues in the β subunit, four of which are highly conserved. Two of these are located near the N terminus, and the other two are at the C terminus. The primary amino acid sequence in their immediate neighborhood is also highly conserved. The cysteine clusters are located in hydrophobic regions, which is most explicit in the *A. eutrophus* enzyme (Fig. 5B).

**Comparison of flavin-containing HoxS dimer and other flavoproteins.** Subunits α and γ of HoxS constitute an NADH:oxidoreductase and contain one molecule of enzyme-bound flavin mononucleotide (42, 45). Comparison of the amino acid sequences of the α subunit and flavin-containing succinate dehydrogenase (53), fumarate reductase (9), and glyceraldehyde-3-phosphate dehydrogenase (10) from *E. coli* revealed no extensive homology (data not shown). In the N-terminal region, we found a pair of glycines (residues 42 and 44, Fig. 3) within a βββ structure reminiscent of flavin-binding sites of other oxidoreductases (52). Two cysteine clusters occur in the γ polypeptide of HoxS; cysteine

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**TABLE 2. Heterologous expression of HoxS activity in *P. facilis***

| Strain   | Hybrid plasmid | Doubling time (h) | Hydrogenase activity (U/mg of protein) |
|----------|----------------|------------------|---------------------------------------|
| DSM 620  | pGE15          | 11.4a            | 0.01a                                  |
| DSM 620  | pGE15          | 8.8a             | 0.27a                                  |
| DSM 550  | pGE15          | 13.5a            | 0.05a,* 0.03b                         |
| DSM 550  | pGE15          | 9.2a             | 0.10a,* 0.35b                         |

* Cells were grown in mineral salts medium in an atmosphere of H₂-CO₂-O₂ (8:1:1, vol/vol/vol).

* Cells were grown in fructose-glycerol medium (14).
FIG. 3. Complete nucleotide sequence of the *hoxS* locus. Both strands were sequenced, and all clones fully overlapped. Potential ribosome-binding sites are underlined, and conserved nucleotides of putative σ^54^ promoters are boxed. A dot indicates the start site of transcription. Stop codons are marked by asterisks, and possible hairpin structures are emphasized by inverted arrows. Derived amino acid sequences are given in one-letter code. Cysteine residues are circled. The initiator methionine of *hoxU* and *hoxS* is in parentheses, since this amino acid is absent in the protein sequence (54).
TABLE 3. Comparison of biochemical and sequence data for the hoxS locus

| Gene  | No. of base pairs | No. of amino acids | No. of cysteine residues | M₁ (sodium dodecyl sulfate gel) | M₁ (DNA sequence) |
|-------|-------------------|--------------------|--------------------------|-------------------------------|-------------------|
| hoxF  | 1,806             | 602                | 17                       | 63,000                        | 66,792            |
| hoxU  | 702               | 233                | 12                       | 30,000                        | 26,027            |
| hoxY  | 627               | 209                | 9                        | 26,000                        | 22,884            |
| hoxH  | 1,464             | 487                | 6                        | 56,000                        | 54,744            |

* Data according to Schneider and Schlegel (44).

DISCUSSION

The four structural genes of the NAD-reducing hydrogenase of *A. eutrophus* H16 are tightly linked. Genes hoxF and hoxU and in turn hoxU and hoxY overlap at their respective stop and start codons. Genes hoxY and hoxH are separated by an intergenic region of 20 bases. This suggests that the four genes belong to an operon. Immunochemical analyses of HoxS' insertion mutants with antibodies raised against the individual subunits revealed a polar effect of mutations in hoxF and hoxU on the expression of the downstream genes hoxY and hoxH (U. Warnecke and B. Friedrich, unpublished data), indicating that all four genes are transcribed from a common promoter upstream of hoxF.

It has been shown previously that an rpoN-like gene of *A. eutrophus*, designated hno, controls the expression of diverse metabolic pathways including hydrogen oxidation (36). Various evidence suggests that this gene encodes an alteration of the native sigma factor of the RNA polymerase. Indeed, sequence analysis of the hno gene revealed extensive homology to the *rpoN* genes of enteric bacteria (J. Warrelmann and B. Friedrich, unpublished data). Not surprisingly, therefore, −24 and −12 consensus elements were found upstream of hoxF. These sequences are typical of RpoN-activatable promoters (3). The large inverted repeat immediately downstream of the hoxH stop codon may have a role in transcription termination. Preliminary DNA-RNA hybridization experiments indicate that the hoxS region directs the formation of an approximately 5-kb large transcript of hoxS (U. Oelmüller and C. G. Friedrich, personal communication). This length is in good agreement with the value postulated from the sequence data (4.8 kb).

A model for the spacial and functional organization of the NAD-reducing hydrogenase of *A. eutrophus* is presented in Fig. 6. This model is based on the results of sequence analyses, ultrastructural electron microscopic investigations (48; W. Johannsen and F. Mayer, personal communication), biochemical data, and electron spin resonance spectroscopy (45). The essential features of this model can be summarized as follows: The so-called hydrogenase dimer, consisting of subunits β and δ, contains the catalytic center involved in the generation of protons and reducing equivalents. Alignment of the amino acid sequences of the β subunit and the corresponding polypeptides of [NiFe] and [NiFeSe] hydrogenases suggests that highly conserved amino acids, in particular cysteine residues at the C- and N-terminal regions, play an important role in the catalytic function of the enzyme.

The nine cysteine residues of the small δ subunit are not arranged in ferredoxin-like clusters as is the case in the [Fe] hydrogenase of *Desulfovibrio vulgaris* (19, 50). However, at least four of them lie in conserved domains common to the small subunits of [NiFe] hydrogenases. It has been suggested that [NiFe] hydrogenases contain one [3Fe-3S] and
two [4Fe-4S] non-heme iron centers (reviewed in reference 13). Since the cysteine content of the δ subunit of HoxS is lower and its size is about 30% smaller than that reported for corresponding hydrogenase polypeptides (Fig. 5A), we propose a hydrogenase dimer of HoxS containing only one [4Fe-4S] center and one [3Fe-3S] center. These clusters have been detected previously by electron spin resonance spectroscopy (45). The [3Fe-3S] center is presumably located in the small δ subunit. The function of this subunit in catalysis is at present unclear. It may be involved in redox regulation of hydrogenase activity (8). Alternatively, it may represent an intermediate site in electron transport to the acceptor site. The acceptor site is tentatively assigned to the moiety of HoxS (45) composed of subunits α and γ (Fig. 6).

The γ subunit contains sufficient cysteiny1 residues to enable coordination of two [4Fe-4S] centers. Similar [4Fe-4S] centers have been identified in the NADH:oxidoreductase dimer of N. opaca (42). The predicted amino acid sequence reveals similarities to bacterial ferredoxins (7). Unlike the ferredoxins, however, the cysteine clusters are split into two parts, with the two parts separated by 47 and 46 amino acids (aa) respectively: (i) Cys-Leu-2aa-Cys-2aa-Cys-47aa-Cys-Pro and (ii) Cys-Ile-2aa-Cys-2aa-Cys-46aa-Cys-Pro.

The [2Fe-2S] center (Fig. 6) is spectroscopically distinguishable from the [4Fe-4S] cluster by its slow electron spin relaxation rate (45). Our data suggest that this cluster is coordinated by a series of cysteine residues of the α subunit. Three of these cysteines lie closely clustered: Cys-2aa-Cys-2aa-Cys at position 499. It is unclear which of the other cysteines belongs to this cluster. Sequence comparisons revealed that the α subunit contains some structural features of putative flavin-binding sites. This may be the site where reduction of the electron acceptor NAD⁺ takes place. Since NAD⁺ accepts two reducing equivalents at a time, flavin

![Diagram of hydrogenase and diaphorase](https://jb.asm.org/)
mononucleotide has been postulated as the terminal electron donor. The [Fe-S] clusters are one-electron redox groups (49). Therefore, reduction of flavin mononucleotide must involve two such clusters acting cooperatively. The pair of [4Fe-4S] clusters of the γ subunit may mediate this step.

Sequence data indicate that archaeabacterial and eubacterial hydrogenases have evolved from a common ancestor. Remarkably, the soluble methylviologen-reducing hydrogenase of *M. thermoautotrophicum* (34) and the hydrogenase dimer of *H. eutrophus* share only slight homology with the corresponding membrane-bound and soluble periplasmic enzymes found in aerobic and anaerobic bacteria including HoxP of *A. eutrophus* (K. Horstmann, C. Kortlücke, and B. Friedrich, unpublished data). In this context, it is worth noting that the plasmid-encoded *hox* S genes of *A. eutrophus* are flanked by two copies of an insertion element (E. Schwartz and B. Friedrich, unpublished data). This transposonlike structure suggests that mobile elements play a role in the evolution of the lithoautotrophy gene cluster of megaplasmid pHG1.

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**LITERATURE CITED**

1. Andersen, K., and J. Caton. 1987. Sequence analysis of the *Alcaligenes eutrophus* chromosomally encoded ribulose bisphosphate carboxylase large and small subunit genes and their gene products. J. Bacteriol. 169:4547-4558.
2. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, Inc., New York.
3. Reynor, J. M., Cannon, V. Buchanan-Wollaston, and F. C. Cannon. 1983. The nfi promoters of *Klebsiella pneumoniae* have a characteristic primary structure. Cell 34:655-671.
4. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.
5. Bowien, B., and H. G. Schlegel. 1981. Physiology and biochemistry of aerobic hydrogen-oxidizing bacteria. Annu. Rev. Microbiol. 35:405-452.
6. Boyer, H. W., and D. Roulland-Dussoux. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41:459-472.
7. Bruschi, M., and F. Guerlesquin. 1988. Structure, function and evolution of bacterial ferredoxins. FEMS Microbiol. Rev. 54:155-176.
8. Cammack, R., V. M. Fernandez, and K. Schneider. 1986. Activation and active sites of nickel containing hydrogenases. Biochimie 68:85-91.
9. Cole, S. T. 1982. Nucleotide sequence coding for the flavoprotein subunit of the fumarate reductase of *Escherichia coli*. Eur. J. Biochem. 122:479-484.
10. Cole, S. T., K. Eglmeier, S. Ahmed, N. Honore, L. Elmes, W. F. Anderson, and J. H. Weiner. 1988. Nucleotide sequence and gene-polyepitope relationships of the *gpi*ABC operon encoding the anaerobic sn-glycerol-3-phosphate dehydrogenase of *Escherichia coli* K-12. J. Bacteriol. 170:2448-2456.
11. Ditta, G., S. Stanfield, C. Corbin, and D. R. Helinski. 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.
12. Eberz, G., C. Hogrefe, C. Kortlücke, A. Kaminski, and B. Friedrich. 1986. Molecular cloning of structural and regulatory hydrogenase (hox) genes of *Alcaligenes eutrophus* H16. J. Bacteriol. 168:636-641.
13. Fauque, G., H. D. Peck, Jr., J. J. G. Moura, B. H. Huynh, Y. Berlier, D. V. DerVartanian, M. Teixeira, A. E. Przybyla, P. A. Lepinat, I. Moura, and J. LeGall. 1988. The three classes of hydrogenases from sulfate-reducing bacteria of the genus *Desulfovibrio*. FEMS Microbiol. Rev. 54:299-344.
14. Friedrich, B., E. Heine, A. Fink, and C. G. Friedrich. 1981. Nickel requirement for native hydrogenase formation in *Alcaligenes eutrophus*. J. Bacteriol. 145:1144-1149.
15. Friedrich, B., C. Hogrefe, and H. G. Schlegel. 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. 147:198-205.
16. Gough, G. A., and N. E. Murray. 1983. Sequence diversity among related genes for recognition of specific targets in DNA molecules. J. Mol. Biol. 166:1-19.
17. Gussin, G. N., C. W. Ronson, and F. M. Ausubel. 1986. Regulation of nitrogen fixation genes. Annu. Rev. Genet. 20:567-591.
18. Hogrefe, C., D. Römermann, and B. Friedrich. 1984. *Alcaligenes eutrophus* hydrogenase genes (Hox). J. Bacteriol. 158:43-48.
19. Huynh, B. H., D. S. Patil, I. Moura, M. Teixeira, J. G. Moura, D. V. DerVartanian, M. H. Czechowski, B. C. Prickril, H. D. Peck, Jr., and J. LeGall. 1987. On the active sites of the [NiFe] hydrogenase from *Desulfovibrio gigas*. J. Biol. Chem. 262:795-800.
20. Jendrossek, D., A. Steinbüchel, and H. G. Schlegel. 1986. Alcohol dehydrogenase gene from *Alcaligenes eutrophus*: subcloning, heterologous expression in *Escherichia coli*, sequencing, and location of the Tn5 insertions. J. Bacteriol. 170:5248-5256.
21. Kärst, U., S. Suetin, and C. C. Friedrich. 1987. Purification and properties of a protein linked to the soluble hydrogen-oxidizing bacteria. J. Bacteriol. 169:2079-2082.
22. Knauf, V. C., and E. W. Nester. 1982. Wide host range cloning vectors: a cosm id clone bank of an *Agrobacterium* Ti plasmid. Plasmid 8:45-54.
23. Kojima, N., J. A. Fox, R. P. Hauslinger, L. Daniels, W. H. Orme-Johnson, and C. Walsh. 1983. Paramagnetic centers in the nickel-containing, deazaflavin-reducing hydrogenase from *Methanobacterium thermautotrophicum*. Proc. Natl. Acad. Sci. USA 80:378-382.
24. Kortlücke, C., C. Hogrefe, G. Eberz, A. Pühler, and B. Friedrich. 1987. Genes of lithoautotrophic metabolism are clustered on the megaplasmid pHG1 in *Alcaligenes eutrophus*. Mol. Gen. Genet. 212:122-128.
25. Leclerc, M., A. Colbeau, B. Caufin, and P. M. Vignais. 1988. Cloning and sequencing of the genes encoding the large and the small subunits of the *H2* uptake hydrogenase (hup) of *Rhodobacter capsulatus*. Mol. Gen. Genet. 214:97-108. (Erratum, 215:368.)
26. Li, C., H. D. Peck, Jr., J. LeGall, and A. E. Przybyla. 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.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
28. Mandel, M., and A. Higa. 1970. Calcium dependent bacterio- phage DNA infection. J. Mol. Biol. 53:159-162.
29. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. Menon, N. K., H. D. Peck, Jr., J. LeGall, and A. E. Przybyla. 1987. Cloning and sequencing of the genes encoding the large and small subunits of the periplasmic [NiFeSe] hydrogenase of *Desulfovibrio bacinulans*. J. Bacteriol. 169:5401-5407. (Erratum, 170:4429.)
31. Messing, J., R. Crea, and P. H. Seeburg. 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. 9:309-321.
32. Norrander, J., T. Kempe, and J. Messing. 1983. Construction of
improved M13 vectors using oligonucleotide-directed mutagenesis. Gene 26:101–106.
33. Perbal, B. 1988. A practical guide to molecular cloning, 2nd ed., p. 278–296. John Wiley & Sons, Inc., New York.
34. Reeve, J. N., G. S. Beckler, D. S. Cram, P. T. Hamilton, J. W. Brown, J. A. Krzycki, A. F. Kołodziej, L. Alex, W. H. Orme-Johnson, and C. T. Walsh. 1989. A hydrogenase linked gene in Methanobacterium thermoautotrophicum DH encodes a polyfrerodoxin. Proc. Natl. Acad. Sci. USA 86:3031–3035.
35. Rhode, M., W. Johannsen, and F. Mayer. 1986. Immunocytochemical localization of the soluble NAD-dependent hydrogenase in cells of Alcaligenes eutrophus. FEMS Microbiol. Lett. 36:83–86.
36. Römermann, D., J. Warrelmann, R. A. Bender, and B. Friedrich. 1989. An rpoN-like gene of Alcaligenes eutrophus and Pseudomonas facilis controls expression of diverse metabolic pathways, including hydrogen oxidation. J. Bacteriol. 171:1093–1099.
37. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
38. Sayavedra-Soto, L. A., G. K. Powell, H. J. Evans, and R. O. Morris. 1988. Nucleotide sequence of the genetic locus encoding subunits of Bradyrhizobium japonicum uptake hydrogenase. Proc. Natl. Acad. Sci. USA 85:8395–8399.
39. Schink, B., and H. G. Schlegel. 1979. The membrane-bound hydrogenase of Alcaligenes eutrophus I. Solubilization, purification, and biochemical properties. Biochim. Biophys. Acta 567:315–324.
40. Schlegel, H. G., H. Kaltwasser, and G. Gottschalk. 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierrnder Bakterien: Wachstumspysilogische Untersuchungen. Arch. Microbiol. 38:209–222.
41. Schlesier, M., and B. Friedrich. 1982. Effect of molecular hydrogen on histidine utilization by Alcaligenes eutrophus. Arch. Microbiol. 132:260–265.
42. Schneider, K., R. Cammack, and H. G. Schlegel. 1984. Content and localization of FMN, Fe-S clusters and nickel in the NAD-linked hydrogenase of Nocardioid opaca 1b. Eur. J. Biochem. 142:75–84.
43. Schneider, K., D. S. Pattil, and R. Cammack. 1983. ESR properties of membrane bound hydrogenases from aerobic hydrogen bacteria. Biochim. Biophys. Acta 748:353–361.
44. Schneider, K., and H. G. Schlegel. 1976. Purification and properties of soluble hydrogenase from Alcaligenes eutrophus H16. Biochim. Biophys. Acta 452:66–80.
45. Schneider, K., H. G. Schlegel, R. Cammack, and D. O. Hall. 1979. The iron sulfur centers of soluble hydrogenase from Alcaligenes eutrophus. Biochim. Biophys. Acta 578:445–461.
46. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
47. Tinoco, I., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla. 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. 246:40–41.
48. Tsuprin, V. L., I. B. Utkin, V. O. Popov, A. M. Egorov, I. V. Berezin, and N. A. Kiselev. 1986. Electron microscopy of the hydrogenase from the hydrogen-oxidizing bacterium Alcaligenes eutrophus Z1. FEBS Lett. 197:225–228.
49. van Belzen, R., and S. P. J. Albracht. 1989. The pathway of electron transfer in NADH:Q oxidoreductase. Biochim. Biophys. Acta 974:311–320.
50. Voordouw, G., and S. Brenner. 1985. Nucleotide sequence of the gene encoding the hydrogenase from Desulfovibrio vulgaris (Hildenborough). Eur. J. Biochem. 148:515–520.
51. Voordouw, G., N. K. Menon, J. LeGall, E.-S. Choi, H. D. Peck, Jr., and A. E. Przybyla. 1989. Analysis and comparison of nucleotide sequences encoding the genes for [NiFe] and [NiFeSe] hydrogenases from Desulfovibrio gigas and Desulfovibrio baculatus. J. Bacteriol. 171:2894–2899.
52. Wierenga, R. K., P. Terpstra, and W. G. J. Hol. 1986. Prediction of the occurrence of the ADP-binding ββ-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol. 187:101–107.
53. Wood, D., M. G. Darrison, R. J. Wilde, and J. R. Guest. 1984. Nucleotide sequence encoding the flavoprotein and hydrophobic subunits of the succinate dehydrogenase of Escherichia coli. Biochem. J. 222:519–534.
54. Zaborosch, C., K. Schneider, H. G. Schlegel, and H. Kratzin. 1989. Comparison of the NH2-terminal amino acid sequences of the four non-identical subunits of the NAD-linked hydrogenase from Nocardioid opaca 1b and Alcaligenes eutrophus H16. Eur. J. Biochem. 181:175–180.