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Nucleotide Sequences and Genetic Analysis of Hydrogen Oxidation (*hox*) Genes in *Azotobacter vinelandii*

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Azotobacter vinelandii **contains a heterodimeric, membrane-bound [NiFe]hydrogenase capable of catalyzing the reversible oxidation of H_2. The β and α subunits of the enzyme are encoded by the structural genes *hoxK* and *hoxG*, respectively, which appear to form part of an operon that contains at least one further potential gene (open reading frame 3 [ORF3]). In this study, determination of the nucleotide sequence of a region of 2,344 bp downstream of ORF3 revealed four additional closely spaced or overlapping ORFs. These ORFs, ORF4 through ORF7, potentially encode polypeptides with predicted masses of 22.8, 11.4, 16.3, and 31 kDa, respectively. Mutagenesis of the chromosome of *A. vinelandii* in the area sequenced was carried out by introduction of antibiotic resistance gene cassettes. Disruption of *hoxK* and *hoxG* by a kanamycin resistance gene abolished whole-cell hydrogenase activity coupled to O_2 and led to loss of the hydrogenase α subunit. Insertional mutagenesis of ORF3 through ORF7 with a promoterless *lacZ*-Km^r cassette established that the region is transcriptionally active and involved in H_2 oxidation. We propose to call ORF3 through ORF7 *hoxZ*, *hoxM*, *hoxL*, *hoxO*, and *hoxQ*, respectively. The predicted *hox* gene products resemble those encoded by genes from hydrogenase-related operons in other bacteria, including *Escherichia coli* and *Alcaligenes eutrophus*.**

The obligately aerobic dinitrogen (N_2)-fixing organism *Azotobacter vinelandii* is capable of oxidizing dihydrogen (H_2) either supplied exogenously or produced endogenously as a byproduct of N_2 fixation. Energy derived from H_2 oxidation can be coupled to ATP synthesis (22) or can drive mannose uptake (33). The oxidation of H_2 formed during nitrogenase turnover has been postulated to increase the efficiency of N_2 fixation (15).

H_2 oxidation in *A. vinelandii* is catalyzed by a membrane-bound [NiFe]hydrogenase (22). Electrons produced by the enzyme flow to O_2 as the terminal electron acceptor through a respiratory chain involving ubiquinone (46) and type *b*, *c*, and *d* cytochromes (66). The enzyme was first purified from membranes, and many of its catalytic properties were described by Kow and Burris (26). The hydrogenase was tentatively thought to be a monomer of approximately 60 kDa but has since been shown to have a native molecular mass of 98 kDa and to be composed of two subunit types (57). The larger subunit (α) and the smaller subunit (β) were estimated to be 67 and 31 kDa, respectively. The enzyme was found to contain nickel and iron in the ratio of 0.68 and 6.6 g atoms per mol of protein (57), respectively, suggesting a ratio of 1 nickel to 10 iron atoms.

The genetic determinants for hydrogenase in *A. vinelandii* are poorly understood. Previously, we described the isolation and nucleotide sequence of a segment of the chromosome of this organism spanning the adjacent structural genes *hoxK* and *hoxG* for the β and α subunits of the [NiFe]hydrogenase (40). The deduced NH_2-terminal amino acid sequence of the *hoxK* gene product was found to encode a 45-residue extension that is absent in the purified β subunit. This sequence was postulated to be a signal sequence involved in localization of hydrogenase in the membrane.

hoxK and *hoxG* appear to be cotranscribed with a third potential gene, open reading frame 3 (ORF3), which apparently codes for an integral membrane protein. Here we present evidence, based on the nucleotide sequence of an additional 2,344 bp 3′ to ORF3 and mutagenesis of the entire region sequenced so far, which suggests that a minimum of seven genes are involved in H_2 oxidation in *A. vinelandii*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *Escherichia coli* and *A. vinelandii* and the plasmids used in this study are listed in Table 1.

Media and growth conditions. *E. coli* strains were routinely grown aerobically at 37°C in Luria-Bertani (LB) medium (34). Antibiotics were added, as required, at the following final concentrations (in micrograms per milliliter): ampicillin, 100; kanamycin, 20. *A. vinelandii* strains were grown aerobically in batch cultures at 30°C on rich medium (RM) (50) or Burks medium with 2% glucose (44). Ammonium acetate (15 mM) was added as a nitrogen source when necessary. When needed, kanamycin was added at 1 μg/ml and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was added at 0.1 μg/ml.

Genetic and recombinant DNA techniques. *E. coli* strains were transformed by the $CaCl_2$ method of Dagert and Erlich (13). *A. vinelandii* was transformed with linearized plasmid DNA by the method of Page and von Tigerstrom (47). Plasmid preparations were made by the alkaline lysis method of Birnboim and Doly (5). *A. vinelandii* genomic DNA was extracted as described by Robson et al. (50). Southern hybridization analyses were conducted as described previously (40), and probes were labeled by nick translation (49), using 5′-[α-^{32}P]dCTP (3,000 Ci/mmol; Amersham International). Restriction enzymes were purchased from Promega, Boehringer Mannheim, and New England Biolabs, and restriction digests were carried out in TAS buffer (17). Plasmid DNA to be digested with *Stu*I was isolated from a *dam dcm* mutant strain, *E. coli* GM1674. DNA ligations were performed with T4 DNA ligase from New England Biolabs, as

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
71/18	Δ(*lac-pro*) [F′ *lacI*^{q} *lacZ*ΔM15 *proAB*] *supE*	41
JM109	*recA1 endA1 gyrA thi hsdR17 supE44 relA1* λ$^-$ Δ(*lac-proAB*)	69
GM1674	[F′ *proAB lacI*^{q} *lacZ*ΔM15] Δ(*lac-pro*) *dam-3 dcm-6 thi-1 tsx-63*	F. Zinoni
A. vinelandii CA	Wild type	9
Plasmids		
pTZ19R	T7 promoter, *lacZ′*, f1 *ori*, Ap^r	36
pTZ19RΔEB	pTZ19R from which *Eco*RI through *Bam*HI polylinker sites were deleted	This work
pBluescriptKS(−)	T3 and T7 promotors, *lacZ′*, f1 *ori*, Ap^r	Stratagene
pKOK6	*lacZ*::Km^r cassette, Ap^r Tc^r Cm^r	23
pUC4-K	Km^r cassette, Ap^r	63
pALM21	40-kb *Sau*3A genomic fragment of *A. vinelandii* in cosmid pTBE	40
pALMZ′1	8.9-kb pALM21 *Xba*I-*Bam*HI fragment in pTZ19R	40
pALM26	2.1-kb pALM21 *Sal*I-*Bgl*II fragment in pBluescriptKS(−)	This work
pALM27	3.15-kb pALM21 *Sph*I-*Sph*I fragment in pTZ19RΔEB	This work
pALM28	2.6-kb pALM21 *Sph*I-*Sph*I fragment in pTZ19RΔEB	This work

recommended by the manufacturer. DNA was electrophoresed in 0.8% agarose gels in TAE buffer (34) and, when required, extracted from low-melting-point agarose (Bethesda Research Laboratories) by the freeze-squeeze method (59).

DNA sequencing and analysis. Overlapping subclones for sequencing of pALMZ′1 were generated as described previously (40). Bidirectional deletions of pALM26 were generated by the exonuclease III procedure of Henikoff (21) with the Promega Erase-a-Base system. Double-stranded template DNA was prepared from subclones transformed into *E. coli* 71/18 or *E. coli* JM109 by modified alkaline lysis procedures, which included polyethylene glycol, RNase A, and proteinase K treatments (40). Sequencing was performed by the dideoxy chain termination method (51) with 5′-[α-^{35}S] dATP (600 Ci/mmol; Amersham International) and Sequenase (U.S. Biochemical Corp.) as per the manufacturers' instructions. To eliminate compressions observed with G+C-rich DNA, 7-deaza-dGTP was used in place of dGTP (3, 43). Universal and reverse sequencing primers were obtained from Boehringer Mannheim Biochemicals. A number of additional site-specific primers were synthesized to sequence regions for which no suitable deletions were available. Sequencing reactions were run out on 5 or 6% polyacrylamide wedge gels (0.25 to 0.75 mm) containing 7 M urea in a Tris-borate-EDTA buffer system (34). Autoradiography was done at room temperature with Kodak X-OMAT AR film. Sequence analyses and alignments were performed on a VAX computer, with the University of Wisconsin Genetics Computer Group programs (14).

Mutagenesis. Insertion mutations were first constructed in vitro in appropriate plasmids and maintained in *E. coli*. The mutations were confirmed by mapping the recombinant plasmids, after which they were recombined into the *A. vinelandii* chromosome.

Two methods were used to mutagenize the *hox* gene cluster in *A. vinelandii*. (i) A Km^r cassette, isolated as a *Bam*HI fragment from pUC4-K (63), was ligated into *Sau*3A-linearized pALMZ′1 DNA. (ii) The *lacZ*::Km^r cassette from pKOK6 (23) was inserted in both orientations (except in the case of ORF7) into unique restriction sites by using appropriate subclones of the *hox* region. All recombinant clones were confirmed by restriction mapping, and *lacZ*::Km^r clones were tested for β-galactosidase expression to determine the position and orientation of the cassette with respect to the insert.

The mutations were transferred into the *A. vinelandii* chromosome by transformation with linearized plasmid DNA. In most cases, Km^r transformants resulted from the transfer of the resistance marker into the chromosome by homologous recombination. Putative mutants were analyzed by Southern blots of genomic DNA to confirm the mutant genotype.

Western immunoblot analysis. Polypeptides from denatured whole-cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27) and electroblotted onto nitrocellulose paper (0.45 μm; Bio-Rad) for analysis by enzyme-linked immunosorbent assays (4, 62). Rabbit antibody raised against the purified *Bradyrhizobium japonicum* [NiFe]hydrogenase α subunit (a gift from D. Arp) and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibodies (Promega) were used at dilutions of 1:1,000 and 1:7,500, respectively, to detect the hydrogenase α subunit. The *B. japonicum* hydrogenase has been shown previously to be immunologically related to the hydrogenase from *A. vinelandii* (2). The immunocross-reactive bands were detected colorimetrically in 0.1 M Tris (pH 9.5) containing 0.1 M NaCl and 5 mM $MgCl_2$ by using 0.35 mM 5-bromo-4-chloro-3-indolyl phosphate (Sigma) and 0.37 mM Nitro Blue Tetrazolium (Sigma) as substrates for the alkaline phosphatase reaction.

Enzyme assays. Whole-cell H_2 oxidation activity coupled to O_2 reduction was determined amperometrically (64). β-Galactosidase activity in whole cells of *A. vinelandii lacZ*-Km^r mutants was estimated by the method of Miller (42). For both assays, *A. vinelandii* wild-type and mutant strains were grown under nitrogen-fixing conditions in RM.

Nucleotide sequence accession number. The sequence described in this work has been assigned GenBank accession number M80522.

RESULTS

Subcloning and sequence analysis of *hox*-related ORFs. The structural genes *hoxK* and *hoxG*, which encode the β and α subunits, respectively, of the *A. vinelandii* heterodimeric

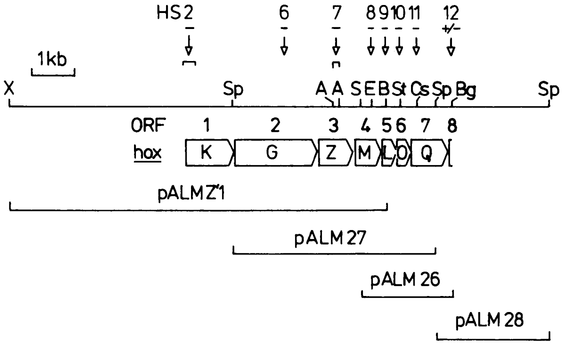

FIG. 1. Physical and genetic map of the *A. vinelandii hox* region. The figure shows a restriction map of a 10.5-kb subfragment of the 40-kb chromosomal fragment cloned into the cosmid clone pALM21 (40). Restriction sites used for cloning and mutagenesis are indicated for the following enzymes: A, *Asp*718; B, *BamH*I; Bg, *Bgl*II; Cs, *Csp*451; E, *EcoR*I; S, *Sal*I; Sp, *Sph*I; St, *Stu*I; X, *Xba*I. Overlapping subfragments of this region used to construct plasmids for sequencing and mutagenesis are indicated as bars in the lower part of the figure. The relative positions of seven ORFs and a partial eighth ORF (ORF8′), reading from left to right, are indicated by boxes with their genetic assignment. The sites and numbering of kanamycin resistance cassette insertions (from pUC4-K and pKOK6) into the *A. vinelandii* chromosome are indicated with arrows in the upper part of the figure, along with the phenotype obtained with respect to the hydrogen-oxidizing activity of whole cells grown in RM under nitrogen-fixing conditions: −, no detectable H_2 oxidation in whole cells; +/−, approx. 30% of wild-type H_2 oxidation activity.

[NiFe]hydrogenase (40), were characterized previously. A third ORF (ORF3), which potentially encodes a 28-kDa hydrophobic polypeptide, is located immediately downstream from *hoxG*. To determine the nucleotide sequence of the region downstream of ORF3, two overlapping subclones, pALMZ′1 and pALM26, were generated from the *A. vinelandii* cosmid clone pALM21 (Fig. 1). Analysis of the newly obtained sequence suggests the presence of four additional, complete ORFs (ORF4 through ORF7). These ORFs are probably genes, since they exhibit a codon usage typical of *A. vinelandii* and each is preceded by a purine-rich region typical of potential ribosome-binding sites (58). The nucleotide and derived amino acid sequences are shown in Fig. 2. ORF3 and ORF4 are separated by a 61-bp intergenic region. The remaining genes are closely linked, i.e., they have overlapping stop and start codons or are separated by less than 17 bp, and it is likely that the translation of adjacent genes is tightly coupled (45). All the ORFs identified here, together with ORF1 through ORF3 identified earlier, apparently use ATG and TGA as the initiation and termination codons, respectively. Codon usage analysis suggests the presence of an additional ORF (ORF8′), 3′ to and overlapping ORF7 by 4 bp, which may be a gene, though in this case the initiator codon appears to be GTG, which would be unusual for this species.

Mutagenesis of the *A. vinelandii hox* gene cluster. The involvement in H_2 oxidation of the entire sequenced region, including *hoxK*, *hoxG*, and ORF3, which were sequenced earlier, was examined by gene disruption (Fig. 1). Km^r or Km^r::*lacZ* gene cassettes were introduced into each ORF in the chromosome of *A. vinelandii*. Precise interpretation of the phenotypic effects of such gene disruptions is complicated by the likely polarity induced by introduction of the antibiotic resistance cassettes. However, we used this approach to establish the functionality and transcriptional activity of the region as a whole rather than to ascribe a specific function to each ORF.

Strains HS2 and HS6 contain mutations in *hoxK* and *hoxG*, respectively. As expected, both HS2 and HS6 exhibited no detectable H_2 oxidation activity, and the mutations resulted in a complete loss of immunologically cross-reactive [NiFe]hydrogenase α subunit (Table 2, Fig. 3). These results therefore confirm that these genes are required for hydrogen oxidation in *A. vinelandii*.

Mutations in the region downstream of the structural genes were constructed in vitro by the insertion of a *lacZ*::Km^r cassette into unique restriction sites in pALM27 (ORF3 to ORF5), pALM26 (ORF6 and ORF7), and pALM28 (ORF 8′) (Fig. 1). These mutations were recombined into and stably maintained in *A. vinelandii*. Mutations in ORF3 through ORF7 abolished H_2 oxidation activity (Table 2). All these mutants contained apparently reduced levels of immunologically cross-reactive α subunit (HoxG). Strains bearing mutations in ORF3 (Fig. 4), ORF6, or ORF7 (data not shown) apparently synthesize not only the wild-type form of the α subunit but also a form which migrates more slowly in SDS-PAGE. Moreover, only the slower-migrating form was detected in strains with mutations in ORF4 and ORF5 (Fig. 4). An ORF8′ mutant (HS12a) exhibited intermediate levels of hydrogenase activity when *lacZ* was inserted in the same orientation as the ORF. However, HS12b, a mutant in which the cassette was present in the reverse orientation, was Hox^-.

Mutations throughout this region had no obvious effect on the growth of the organism in a simple salts medium or RM, with glucose or sucrose as the carbon source, with or without added nitrogen (NH_4^+). This indicates that, at least under the conditions tested, this enzyme is not essential to *A. vinelandii*, though there may be conditions, e.g., carbon or energy limitations, under which [NiFe]hydrogenase expression is beneficial, as has been shown for *Azotobacter chroococcum* (1).

Use of the promoterless *lacZ*::Km^r cassette enabled us to establish that this region is transcriptionally active. Only mutants containing the *lacZ* gene oriented in the same direction as the potential *hox* genes gave significant levels of β-galactosidase activity. *hoxK* currently defines the 5′ end of the *hox* cluster. We propose to name ORF3 and ORF4 *hoxZ* and *hoxM*, respectively, based on their homology to the *Alcaligenes eutrophus hoxZ* and *hoxM* genes (20), and to call the potential genes encoding ORF5 through ORF7 *hoxL*, hoxO, and *hoxQ*, respectively.

Properties of *A. vinelandii* potential *hox* gene products and comparison with other known gene products. The properties of the predicted *hox* gene products are summarized in Table 3. The *hoxK* and *hoxG* products were discussed in an earlier article (40). Previous analysis suggested that HoxZ (ORF3) could be an integral membrane protein, potentially containing four hydrophobic, membrane-associated domains (40). Homologous gene products are encoded at equivalent positions in hydrogenase operons from other bacteria, including *Escherichia coli* (38), *Rhodobacter capsulatus* (48), *A. eutrophus* (20), and probably *B. japonicum* (56) and *A. chroococcum* (18). Alignment of several of these gene products highlights the conservation of six histidine residues associated with the potential membrane-spanning regions predicted for the *A. vinelandii* gene product (Fig. 5). A more recent data base search indicated a similarity between HoxZ and cytochrome *b* from *Neurospora crassa* (8). *b*-type cytochromes are also encoded at a similar relative position within operons for other membrane-bound enzyme com-

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3801 CCGCCGGCTCCGGGCGACGGTGAGGCAACGATGACAGCCCCCAACATCCTCATTCTCGGT
                              M  T  A  P  N  I  L  I  L  G
                              ORF4 / hoxM>
3861 ATCGGCAACCTGCTCTGGGCGGACGAAGGCTTCGGCGTGCGTTGCGTGGAGCTGCTCAAC
     I  G  N  L  L  W  A  D  E  G  F  G  V  R  C  V  E  L  L  N

3921 GAGCGCTATCGCTTCCCCGACGGGGTGCGACTGATGGACGGCGGCACCCAGGGCATCTAC
     E  R  Y  R  F  P  D  G  V  R  L  M  D  G  G  T  Q  G  I  Y

3981 CTGGTGCAGCACGTCCAGCAGGCCGACTGCCTGATCGTCTTCGACGCCGTCGACTACGGC
     L  V  Q  H  V  Q  Q  A  D  C  L  I  V  F  D  A  V  D  Y  G

4041 CTGGCGCCCGGCACCCTGAAGATCGTGCGCGACGACGAGGTGCCCCGCTTCATGGGCGCC
     L  A  P  G  T  L  K  I  V  R  D  D  E  V  P  R  F  M  G  A

4101 AAGCGCATGAGCCTGCACCAGACCGGCTTCCAGGACGTGCTGGCGCTGGCCGCGTTCAGC
     K  R  M  S  L  H  Q  T  G  F  Q  D  V  L  A  L  A  A  F  S

4161 GGCGCCTACCCGCGCGAGCTGCTGCTGATCGGCGTGCAGCCCGAGGAACTGGAAGACTTC
     G  A  Y  P  R  E  L  L  L  I  G  V  Q  P  E  E  L  E  D  F

4221 GGCGGCAGCCTGCGCGAGCCGGTGCGCGCCCAGCTGGAGCCGGCGCTGCGGGTCGCCCTG
     G  G  S  L  R  E  P  V  R  A  Q  L  E  P  A  L  R  V  A  L

4281 GAATTCCTCGCCGAGCGCGGGGTGGTCGCCGCAGCGCGCGACGGCGACGCCGAGCGCCTG
     E  F  L  A  E  R  G  V  V  A  A  A  R  D  G  D  A  E  R  L

4341 GCTCCGGCGCCACTGGCCCTGGGCCGCTACGAGGCCGGCCGCCCGGCCGAGGAACTGGCC
     A  P  A  P  L  A  L  G  R  Y  E  A  G  R  P  A  E  E  L  A

4401 TACCGCCACGGCGATATCCGCTTCATCCCGCAGCAGCCTCTGGAGGACGACTGAGCCATG
     Y  R  H  G  D  I  R  F  I  P  Q  Q  P  L  E  D  D  *     M
                                                          ORF5/
4461 TGCATCGGTATTCCCCTGCGGGTGCTGGAATGCGCGCCGGGCCGCGCCCTGTGCGGCGAC
     C  I  G  I  P  L  R  V  L  E  C  A  P  G  R  A  L  C  G  D
     hoxL>
4521 GAGAACGGCGTGCGGTGGATCGACACGCGGCTGGTCGAGCCGCCGGCGCCCGGCGACTGG
     E  N  G  V  R  W  I  D  T  R  L  V  E  P  P  A  P  G  D  W

4581 CTGCTGGTGTTCCTCGACGCCGCCCGCGAAATCCTCGACGCCGGGCGCGCCGCGCGGATC
     L  L  V  F  L  D  A  A  R  E  I  L  D  A  G  R  A  A  R  I

4641 CGCGAGGCCCTGCGCGCGCTGCAGGCCGTACAGGCGGGCGATCCGGCCGCGCTCGCCGGC
     R  E  A  L  R  A  L  Q  A  V  Q  A  G  D  P  A  A  L  A  G

4701 CTGTTCGCCGACCTCGACCGCGAGCCGCAACTCCCGCCCCACCTGCAGGCGCAACTGCCG
     L  F  A  D  L  D  R  E  P  Q  L  P  P  H  L  Q  A  Q  L  P

4761 CCCAAGGAACCGACATGATCCATCCCCTGATCCAGCGTCTGACCACGACGCTCGGCTACC
     P  K  E  P  T  *
                  M  I  H  P  L  I  Q  R  L  T  T  T  L  G  Y  P
                  ORF6 / hox O>
4821 CCCTGCTGGATGCCGACGGCCTCGACCGCCAGGTGCGGACGCAGCCGTTCTCCGTGCTGT
       L  L  D  A  D  G  L  D  R  Q  V  R  T  Q  P  F  S  V  L  F

4881 TCTTCGCCGGCGATCCGCAGCGCTTTCCCGAGGCGCTCGACGTCGCGGTGATCCTGCCGG
       F  A  G  D  P  Q  R  F  P  E  A  L  D  V  A  V  I  L  P  E

4941 AACTGGTCAAGGCCTTCCCGCAGCTCGCTCCCGCGTTGATCGCCGGCGCGGACGAGGCCC
       L  V  K  A  F  P  Q  L  A  P  A  L  I  A  G  A  D  E  A  R

5001 GTCTGCAGGGGCGCTACGGTTTCTCCGTCTGGCCGAGCCTGGTGTTCCTCGCGGGGGAGC
       L  Q  G  R  Y  G  F  S  V  W  P  S  L  V  F  L  A  G  E  R

5061 GCTACCTGGGCTGCCTGTCGCGGGTACTGGACTGGGGCGAATACCTGGAGCGCATCCCGG
       Y  L  G  C  L  S  R  V  L  D  W  G  E  Y  L  E  R  I  P  A

5121 CGATCCTCGCCGGCGAGAACGAAGACCTGCCACGCATTCCCGTGCTGTCGCCGGAGAGCG
       I  L  A  G  E  N  E  D  L  P  R  I  P  V  L  S  P  E  S  G

5181 GCACACCTTCCTGCAGCCCGATGGAGACCAGTGAATCATGAACGACGACCTGCCCATTCT
                                           M  N  D  D  L  P  I  L
       T  P  S  C  S  P  M  E  T  S  E  S  *
                                          ORF7 / hox Q>
5241 CCCGCCCGGCTTCGGCCCCGGCTCGCATGGCGAAGAGGAGCGGCCCGACTGCCCGTCGAT
      P  P  G  F  G  P  G  S  H  G  E  E  E  R  P  D  C  P  S  M

5301 GCCGCGCGCCATGCGCACCTTCGAACGTCCCGCGCTGCCGGAGCCCGGACAACTGAGCGG
      P  R  A  M  R  T  F  E  R  P  A  L  P  E  P  G  Q  L  S  G

5361 ACACCCCATCGCCCTGGCGCTGCTGGAGCGGCTGCAGGAGGCGCTGGGCGCCTACCGGAT
      H  P  I  A  L  A  L  L  E  R  L  Q  E  A  L  G  A  Y  R  I

5421 CGGCGAGCAGAGCCGGGTGATCGGCCTCGACCGCCAGCCCAAGGCCGACCTGAAGCTGCT
      G  E  Q  S  R  V  I  G  L  D  R  Q  P  K  A  D  L  K  L  L

5481 CCAGCAGATCCTCGGCGAAGGCGAGGTGGCGATCCAGGTCGGCGGCCAGCGCCCCGCGCG
      Q  Q  I  L  G  E  G  E  V  A  I  Q  V  G  G  Q  R  P  A  R

5541 CATCCAGGAAACCGTGCTGGCCGGGGTCTGGTGGGTGCAACTGCAGATCGGCCGGGGCGA
      I  Q  E  T  V  L  A  G  V  W  W  V  Q  L  Q  I  G  R  G  E

5601 GGTCGTCGGCCAGTGGCTGGAGGTGGCCGACGTTCCCGCTCTGGTGCGCCGCCGGGCCTT
      V  V  G  Q  W  L  E  V  A  D  V  P  A  L  V  R  R  R  A  F

5661 CGCCGAAACCCGCTGGCCGCAGCTCGGTGAGTTGCCGGACGGCCTGCTCAACGCCGGCCC
      A  E  T  R  W  P  Q  L  G  E  L  P  D  G  L  L  N  A  G  P

5721 GGTGCTGGTGGAGTTGCTCGACGCGGCGAAGCGGCATGCCGAGCGCGCGCTCGCCACGCC
      V  L  V  E  L  L  D  A  A  K  R  H  A  E  R  A  L  A  T  P

5781 CCACGCGGTCAACCTGTCGTTGCTGCCGTTCTCCCCGGAGGACCGGCGCTTTCTCGCCGA
      H  A  V  N  L  S  L  L  P  F  S  P  E  D  R  R  F  L  A  E

5841 AAGGCTCGGCGAGGGGTCGGTGACCCTGCTCTCGCGCGGCTACGGCAACTGCCGCATCGC
      R  L  G  E  G  S  V  T  L  L  S  R  G  Y  G  N  C  R  I  A

5901 CAGCACTGCCACCCCCGGCATCTGGTGCGTGCAGTACTTCAACAGCAGCGACCGGCTGAT
      S  T  A  T  P  G  I  W  C  V  Q  Y  F  N  S  S  D  R  L  I

5961 CCTCGACACCCTGGAGGTGACGGGCATCCCGCAGGTGGCCTGCGCCGCCCAGGAGGACAT
      L  D  T  L  E  V  T  G  I  P  Q  V  A  C  A  A  Q  E  D  I

6021 CGACGACTCCGCCGAGAGGCTGCGCGAGATCCGCGAGGCTCTGGAGTGAGCGCGCGCTTC
      D  D  S  A  E  R  L  R  E  I  R  E  A  L  E  *
                                                  V  S  A  R  F
                                                  ORF8'>
6081 GAGGGCAGCTACCTGGGCGACGCCACGCGCCTGGCGGACGACGCCGTGCTCGAATGCAAG
     E  G  S  Y  L  G  D  A  T  R  L  A  D  D  A  V  L  E  C  K

6141 ATCT
     I  C
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FIG. 2. Nucleotide and deduced amino acid sequence of the *hoxM* to *hoxQ* region of *A. vinelandii*. The nucleotide sequence for the region spanning *hoxM* to ORF8′ is shown. The amino acid sequences for the predicted ORFs are indicated below in single-letter code. Potential ribosome-binding sites are underlined. Gene assignments and direction of transcription are indicated by boxed arrows below the potential initiation codon of each ORF.

plexes involved in substrate-linked electron transfer. These include FrdC, encoded within the *Wolinella succinogenes* fumarate reductase operon (25), and the *sdhA* gene product from the *Bacillus subtilis* succinate dehydrogenase operon (32). Alignment of FrdC to SdhA with respect to histidine residues gives the maximum degree of identity (20%) and shows the conservation of five potential membrane-spanning hydrophobic stretches with four associated histidine resi-

TABLE 2. Cassette mutagenesis of *A. vinelandii hox* region

Strain[a]	Cassette	ORF/gene	Restriction site (position)	Relative β-galactosidase activity[b]	H_2 oxidation activity[c]
CA				–	+
HS2	Km^r	ORF1/*hoxK*	*Sau*3A (partial)	NT	–
HS6	Km^r	ORF2/*hoxG*	*Sau*3A (partial)	NT	–
HS7a	*lacZ*::Km^r	ORF3/*hoxZ*	*Asp*718 (3540–3771)	+	–
HS7b	*lacZ*::Km^r	ORF3/*hoxZ*	*Asp*718 (3540–3771)	–	–
HS8a	*lacZ*::Km^r	ORF4/*hoxM*	*Eco*RI (4410)	+	–
HS8b	*lacZ*::Km^r	ORF4/*hoxM*	*Eco*RI (4410)	–	–
HS9a	*lacZ*::Km^r	ORF5/*hoxL*	*Bam*HI (4765)	+	–
HS9b	*lacZ*::Km^r	ORF5/*hoxL*	*Bam*HI (4765)	–	–
HS10a	*lacZ*::Km^r	ORF6/*hoxO*	*Stu*I (5081)	+	–
HS10b	*lacZ*::Km^r	ORF6/*hoxO*	*Stu*I (5081)	–	–
HS11a	*lacZ*::Km^r	ORF7/*hoxQ*	*Csp*45I (5450)	+	–
HS12a	*lacZ*::Km^r	ORF8′	*Bgl*II (6268)	+	±
HS12b	*lacZ*::Km^r	ORF8′	*Bgl*II (6268)	–	–

[a] a and b refer to the orientation of the *lacZ* gene with respect to the orientation of the ORF into which it was inserted. a, *lacZ* and ORF read in the same orientation; b, *lacZ* and ORF read in opposite directions.
[b] +, 100% of the β-galactosidase activity for the particular ORF; –, less than 2% of that activity; NT, not tested.
[c] Activity measured amperometrically.

dues (32). It is likely that HoxZ is a membrane-bound heme protein which could serve as a component of an electron transfer chain from hydrogenase.

hoxM (ORF4) encodes an acidic polypeptide of 23 kDa with 32% hydrophobic residues. HoxM was 43% identical to HyaD, encoded within the *E. coli* hydrogenase-1 (Hyd1) gene cluster (Fig. 6A) (38).

hoxL (ORF5) codes for an 11.5-kDa acidic polypeptide with only 1% serine and threonine, compared with 13% in an average protein, but unusually high proportions of alanine (16%; average protein, 8.7%) and arginine (9.5%; average protein, 4.5%), which are most abundant between residues 47 and 85. This region is predicted to form an α-helix with low surface probability, extending for 25 residues. The *hoxL* polypeptide showed significant identity to three other gene products, two of which are hydrogenase related. HoxL was 30 and 31% identical to the *E. coli* hydrogenase-related HypC and HybG polypeptides, respectively (Fig. 6B). HypC is encoded within the *hyp* operon, required for the activity of all three *E. coli* hydrogenases (31). HybG is the product of the last gene in the hydrogenase-2 (HYD2) structural gene operon (37). HoxL also showed 31% identity to OrfC, encoded within an operon of unknown function which is transcribed divergently from the fumarate reductase operon in *Proteus vulgaris* (12). *E. coli* HypC and HybG and *P. vulgaris* OrfC have a very high degree of identity (>50%), whereas HoxL is more distantly related. All four proteins have the N-terminal sequence Met-Cys-Ile/Leu-Gly, a motif common to a large variety of metalloproteins, including ferredoxins, cytochromes, oxidoreductases, and hydrogena-

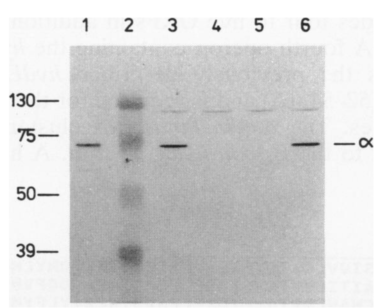

FIG. 3. Western immunoblot analysis of *A. vinelandii hoxK* and *hoxG* structural gene mutants. Purified *A. vinelandii* hydrogenase (lane 1) or *A. vinelandii* wild-type (lanes 3 and 6), HS2 (lane 4), and HS6 (lane 5) mutant whole-cell extracts were fractionated on 9% acrylamide SDS-PAGE gels, electroblotted onto nitrocellulose, and analyzed by enzyme-linked immunosorbent assays with antibody to the hydrogenase α subunit as described in Materials and Methods. Bio-Rad prestained SDS-PAGE size markers (lane 2) include phosphorylase b (130,000 Da), bovine serum albumin (75,000 Da), ovalbumin (50,000 Da), and carbonic anhydrase (39,000 Da). The molecular masses (in kilodaltons) are indicated to the left. The position of the cross-reactive *A. vinelandii* hydrogenase α subunit is noted to the right.

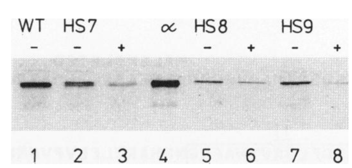

FIG. 4. Influence of disruptions in *hoxZ*, *hoxM*, and *hoxL* on the hydrogenase α subunit. The experiment was performed essentially as described in the legend to Fig. 3. Extracts were prepared from cultures grown with (+) or without (–) ammonia. Samples were subjected to SDS-PAGE. Lane 1, wild type (WT) grown under N_2-fixing conditions (–); lanes 2 and 3, strain HS7 (ORF3, *hoxZ*); lane 4, purified hydrogenase α subunit; lanes 5 and 6, strain HS8 (ORF4, *hoxM*); lanes 7 and 8, strain HS9 (ORF5, *hoxL*). Western blots prepared from SDS-PAGE gels were developed with antibody to the hydrogenase α subunit.

TABLE 3. Properties of predicted *A. vinelandii hox* gene products

ORF	Gene	Sequence[a]	No. of aa[b]	Mol wt	pI
1[c]	*hoxK*	149–1225	358	39,259	8.05
2[c]	*hoxG*	1222–3030	602	66,736	6.62
3[c]	*hoxZ*	3047–3769	240	27,737	9.12
4	*hoxM*	3831–4454	207	22,769	4.52
5	*hoxL*	4458–4778	106	11,436	4.70
6	*hoxO*	4775–5221	148	16,252	4.36
7	*hoxQ*	5218–6069	283	31,015	4.82

[a] Numbers refer to nucleotide positions in the sequence shown in Fig. 2 and reference 40.
[b] Number of amino acid residues, including the N-terminal methionine.
[c] Based on sequence data from reference 40.

ses. In HoxL, this sequence is a subset of the sequence Met-Cys-Ile-Gly-Ile-Pro-Leu-Arg-Val-Leu-Glu-Cys. A similar sequence was also found in *E. coli* HycF, a product of the *E. coli* hydrogenase-3 (HYD3) *hyc* operon (6), though there is no overall similarity between these putative polypeptides. This sequence is similar, in part, to cysteine motifs that ligand the 4Fe4S centers of some ferredoxins (7). A consensus sequence, Cys-Ile-X-X-X-X-X-Val-X-X-Cys, identified only 4 of 12,476 protein data base entries with a similar sequence, which included three members of the ferredoxin superfamily and a thiolase. These observations imply that HoxL may be a metalloprotein or contribute part of a metal-binding domain in a protein complex.

HoxO (ORF6) encodes an acidic, hydrophobic, 16-kDa polypeptide, with 26% identity to the *E. coli* HYD1 operon-encoded HyaE (38) (Fig. 6C). Secondary-structure predictions for HoxO indicate the presence of a 21-amino-acid hydrophobic α-helix with low surface probability.

HoxQ (ORF7) is a 31-kDa, acidic polypeptide which is 32% identical to *E. coli* HyaF (38) (Fig. 6D). The C termini of these two proteins have a 25-amino-acid stretch with over 50% identity. This region in HoxQ is predicted to form an amphiphilic α-helix. There are many examples of proteins with amphiphilic helices that are capable of liganding metals, e.g., *E. coli* cytochrome b_{562} ligands heme via four amphiphilic helices (35), forming transmembrane channels (16) or having structural functions (e.g., myosin) (10).

The *A. vinelandii hoxM*, *hoxL*, *hoxO*, and *hoxQ* gene products are homologous to and colinear with the predicted products of *A. eutrophus hoxM* and three adjacent potential ORFs in the *hoxP* region of *A. eutrophus* (20). The predicted amino acid sequence of ORF8′ is 80% identical to the N terminus of an ORF immediately downstream of the putative *hoxQ* gene equivalent in *A. eutrophus* (20).

DISCUSSION

The nucleotide sequence data and mutagenesis work have established that a locus of at least 6.1 kb is involved in H_2 oxidation in *A. vinelandii*. This region contains seven tightly clustered putative genes which are transcribed in the same direction. The close spacing of the genes, five of which overlap, suggests that they form a single operon. Alternatively, if the 61-bp *hoxZ* to *hoxM* intergenic region contains a promoter, then the genes may be arranged into at least two transcriptional units. The possibility of overlapping operons, as reported for the *E. coli hyp* gene cluster (31), cannot be excluded.

A high degree of conservation is apparent between the *A. vinelandii hox* gene cluster and membrane-bound [NiFe]hydrogenase gene clusters from other bacteria. Those which resemble *A. vinelandii* in apparently possessing a single hydrogenase have genes clustered in a single locus extending for more than 15 kb. These organisms include *A. chroococcum* (60, 61), *B. japonicum* (28), *Rhizobium leguminosarum* (30), and *R. capsulatus* (11, 67, 68). In all these organisms, the arrangement of the genes for the α and β subunits and a third ORF corresponding to *hoxZ* is conserved (18, 29, 48, 56).

A. eutrophus contains both soluble and membrane-bound Ni-containing hydrogenases (19). Genes for the membrane-bound enzyme show high identity to and are colinear with those in *A. vinelandii* over the region described (20). The high degree of similarity between these gene clusters is surprising considering that the *hox* genes are plasmid-borne in *A. eutrophus* (19, 24) but chromosomally located in *A. vinelandii* and the other organisms discussed above. This suggests that all the genes identified so far are essential for hydrogenase function or that the *hox* gene clusters may have been acquired by relatively recent lateral gene transfer events.

In *E. coli*, the situation is yet more complex because at least three hydrogenases, HYD1, HYD2, and HYD3 (55), can be expressed. These enzymes are encoded by the *hya* (38), *hyb* (37), and *hyc* (6) operons, respectively. Each operon encodes four to five ORFs in addition to the structural genes. A fourth operon containing the *hyp* genes (31) encompasses the previously described *hydE*, *hydF*, and *hydB* genes (52–54, 65) and is required for the activity of all three enzymes. The *A. vinelandii hox* cluster corresponds most closely to the *E. coli hyaA* operon. A high degree of

```
        1                                                                        I                                100
AVHOXZ                                MALEKSLETGD....GQEKVRKQTAVYVVYEAPLRLWHWVTALSIVVLGVTGYFIGAPLPTHP.GEAMDNYLMGYIRFAHFAAG
RCORFX  MKGVSDERINAPVRGPDEIFEASRLTGDATREDLESIRRRTSVYVYEAPVRVWHWVNALAITILVVTGYFIASPLPSMQIGEATDQFVMGYIRFAHFAAG
ECHYAC                                                    MQQKSDNVVSHYVFEAPVRIWHWLTVLCMAVLMVTGYFIGKPLPSVS.GEATYLFYMGYIRLIHFSAG

        101     II                                                                 III                            200
AVHOXZ  YVLAIGFLGRVYWAFVGNHHARELFLVPVHRKAWWKELWHEVRWYLFLEKTPKKYIGHNPLGQLAMFCFFVVGAVFMSVTGFALYAEGLGRDSWADRLFG
RCORFX  VVMSVAFFGRIYWAFVGNRHAWQMFYIPIFNKRYWKEFVFELRWYFFLEEEPKKYIGHNPLAHAAMFTFITLGITFMMITGWALYAEGAGQGGVTDSLFG
ECHYAC  MVFTVVLLMRIYWAFVGNRYSRELFIVPVWRKSWWQGVWYEIRWYLFLAKRPSADIGHNPIAQAAMFGYFLMS.VFMIITGFALYSEHSQYAIFAP..FR

        201                  IV                                 271
AVHOXZ  WVIPLF....GQSQDVHTWHHLGMWYLVVFVMVHVYLAVREDIVSRQSLISTMVGGWRMFKDDRPD
RCORFX  WVLGYV....QNSQRLHTLHHLGMWAIVIFAIIHIYAAVREDVMSRQSMVSTMISGHRTFKDDRIE
ECHYAC  YVVEFFYWTGGNSMDIHSWHRLGMWLIGAFVIGHVYMALREDIMSDDTVISTMVNGYRSHKFGKISNKERS
```

FIG. 5. Conserved histidines and hydrophobic domains in *A. vinelandii* HoxZ (AVHOXZ) (ORF3) (40) and comparable potential gene products from [NiFe]hydrogenase structural gene operons in *R. capsulatus* ORFX (RCORFX) (48) and *E. coli* HyaC (ECHYAC) (38). Potential membrane-associated hydrophobic domains (I, II, III, and VI) and conserved histidine residues are boxed. Numbers serve as reference points and are not indicative of absolute amino acid residue positions.

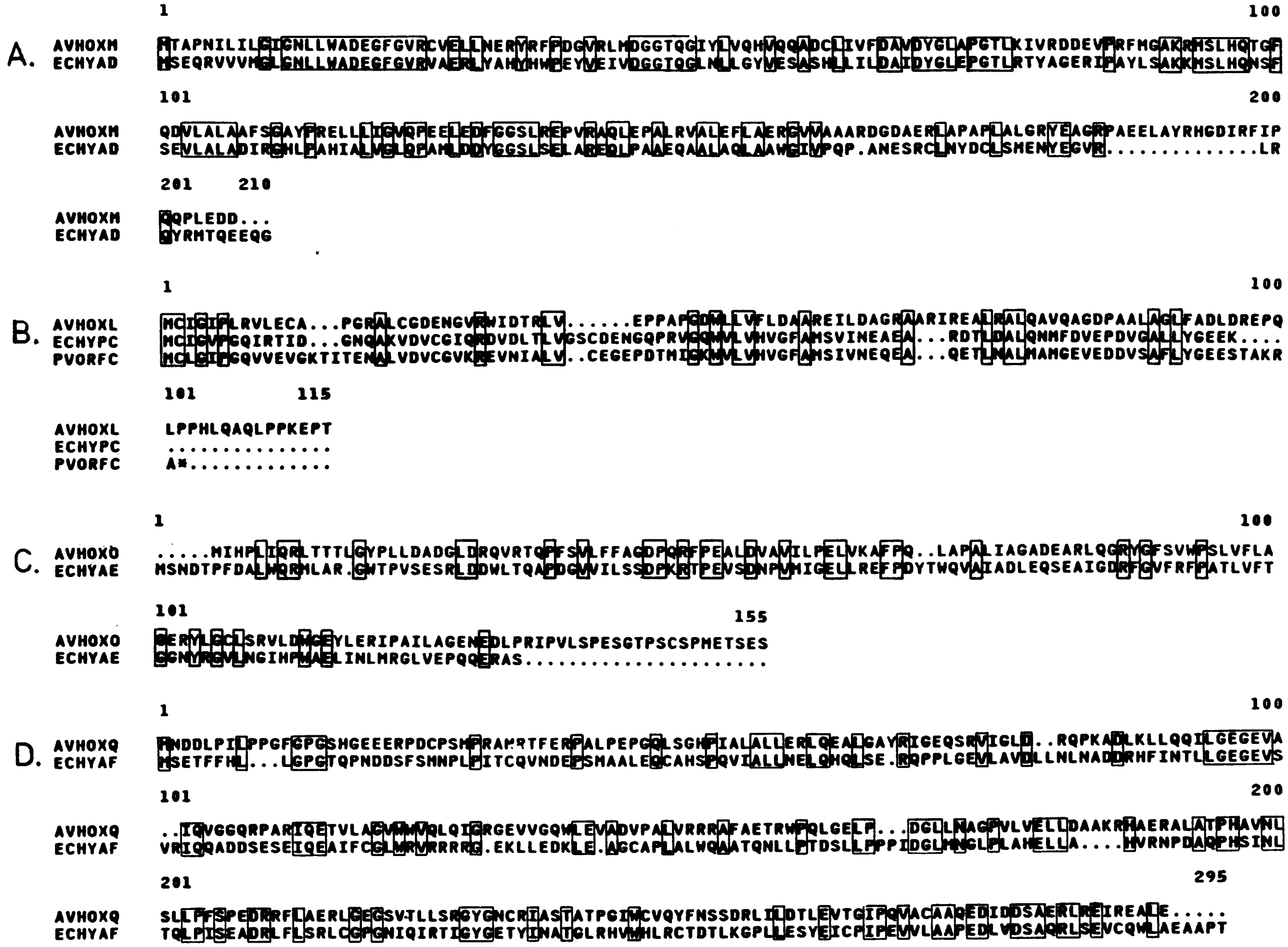

FIG. 6. Comparison of the deduced amino acid sequences of *A. vinelandii hox* gene products with those of other known gene products. Identical residues are boxed. (A) *A. vinelandii* HoxM (AVHOXM) and *E. coli* HyaD (ECHYAD) (38). (B) *A. vinelandii* HoxL (AVHOXL), *E. coli* HypC (ECHYPC) (31), and *P. vulgaris* ORFC (PVORFC) (12). (C) *A. vinelandii* HoxO (AVHOXO) and *E. coli* HyaE (ECHYAE) (38). (D) *A. vinelandii* HoxQ (AVHOXQ) and *E. coli* HyaF (ECHYAF) (38). Numbers serve as reference points and are not indicative of absolute amino acid residue positions.

identity exists between genes for the [NiFe]hydrogenase subunits. Furthermore, *hoxZ*, *hoxM*, *hoxO*, and *hoxQ* are homologous and colinear with *hyaC*, *hyaD*, *hyaE*, and *hyaF*, respectively. No *hoxL* equivalent appears to be present in the *hya* operon. However, it is interesting that apparently similar genes occur both in the *E. coli* HYD2 gene cluster (*hybG*) and in the pleiotropic *hyp* gene cluster (*hypC*).

Similarities in the organization of membrane-bound [NiFe] hydrogenase genes shows that a core of hydrogenase-specific genes has been conserved in different bacteria. In *A. vinelandii*, the functions of only *hoxK* and *hoxG* are known (40). Mutagenesis of these genes not only confirms their role but also establishes that, at least under the growth conditions tested, this organism has only one enzyme capable of oxidizing H_2.

The functions of *hoxZ* through *hoxQ* in H_2 oxidation are not known. Comparative analysis of the potential gene products has provided some clues as to the potential roles of these genes. In *A. vinelandii*, oxidation of H_2 is linked to the respiratory chain and coupled to ATP synthesis (22). Thus, genes within the *A. vinelandii hox* gene cluster may be components of a membrane complex involved in energy conservation, e.g., they may encode a hydrogenase-specific proton-translocating loop and/or electron transfer chain. The *hoxZ* and, to a lesser extent, the *hoxM* and *hoxO* gene products are rich in hydrophobic residues, consistent with their having a membrane location and being involved in the formation of a larger complex. *hoxZ*, which potentially encodes a cytochrome, may be involved in electron transfer.

One or more of the *hox* genes may be required for maturation of the enzyme, e.g., membrane translocation, complex assembly, and/or incorporation of Ni and Fe into the protein. Insertion mutations in *hoxZ*, *hoxM*, *hoxL*, hoxO, and *hoxQ* abolish hydrogenase activity and lead to a form(s) of HoxG with slightly lowered mobility in SDS-PAGE. The nature of the alteration in HoxG is unknown, although N-terminal cleavage can be ruled out (40). It is noteworthy that these results are consistent with altered forms of hydrogenase subunits arising in accessory gene mutants of *E. coli*. For example, mutations in *hyaD* (which resembles *hoxM*) does not prevent HYD1 from associating with the membrane but does lead to altered forms of both the α and β subunits (39). Also, mutations in *hypC* (which resembles *hoxL*) and *hypB* (31) produce apparently larger forms of HYD1 and HYD2 subunits. Mutations in *hypB* (31) and *hyaF* (39), which is similar to *hoxQ* in *A. vinelandii*, are overcome by supplementing the growth medium with high levels of nickel. Potentially, mutations in *hoxQ* may be overcome by supplying increased levels of Ni, though this has not been tested.

In future, the construction of in-frame deletions, which should minimize polar effects on distal genes, and the overexpression, purification, and characterization of individual gene products should provide new insights into the roles of the genes identified in this study.

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