The *hoxZ* Gene of the *Azotobacter vinelandii* Hydrogenase Operon Is Required for Activation of Hydrogenase

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The roles of the product of the *hoxZ* gene immediately downstream of the hydrogenase gene (*hoxKG*) in *Azotobacter vinelandii* were investigated by constructing and characterizing a mutant with the center of the *hoxZ* gene deleted. The strain lacking the functional *hoxZ* gene product exhibited a low rate of H₂ oxidation with O₂ as the electron acceptor relative to that of the wild-type strain. Nevertheless, when the enzyme was exogenously activated and methylene blue was used as the electron acceptor from hydrogenase, rates of H₂ oxidation comparable to those in the wild-type strain were observed. These results suggest that the gene product of *hoxZ* plays a role in activating and maintaining hydrogenase in a reduced active state. The product of *hoxZ* could also be the link necessary for transfer of electrons from H₂ to the electron transport chain.

Hydrogenase in *Azotobacter vinelandii* catalyzes H₂ oxidation. The electrons released from H₂ travel through the electron transport chain to O₂. Little is known about the roles of the genes in the hydrogenase operon. The genes in the *hox* loci of *A. vinelandii* that code for the large and small subunits of hydrogenase have been sequenced (11). They occur as the first two open reading frames (ORFs) of the *hox* operon (11). A third ORF was identified immediately downstream from the genes that code for the hydrogenase subunits. The function of the gene product from this ORF was not determined. The sequence of these three genes in *A. vinelandii* is similar to the hydrogenase gene sequences in other microorganisms (7, 11, 12, 15). In *Escherichia coli*, the operon containing these genes is designated *hya* (12). It is speculated, on the basis of amino acid sequence similarities, that the gene immediately downstream from the hydrogenase gene in *E. coli* (*hyaC*) codes for a protein involved in electron transfer or proton translocation. A role of this peptide in anchoring the structural subunits of hydrogenase to the membrane is also hypothesized (11). The gene *hoxZ* in *Alcaligenes eutrophus*, which lies immediately downstream of its hydrogenase genes as well, encodes a protein that has a role in the processing of hydrogenase to the catalytically active form (8). In *Rhodobacter capsulatus*, the product of the *hupM* gene was reported to be essential for hydrogenase activity in intact cells, apparently by anchoring the enzyme to the membrane and possibly by transferring electrons from hydrogenase to the respiratory chain (7). The sequence of the first ORF downstream of the hydrogenase of *A. vinelandii* is similar to the genes of *E. coli* (12), *R. capsulatus* (7), and *Bradyrhizobium japonicum* (15) and has nucleotide sequences consistent with those that code for transmembrane proteins as well (4, 11). We have designated this ORF *hoxZ* (hydrogen oxidation Z) on the basis of the findings described here and the general nomenclature previously reported in the literature (7, 8).

To test the roles of the *hoxZ* gene product in *A. vinelandii*, a mutant was constructed by deleting a DNA fragment from the center of the *hoxZ* ORF. The results revealed that the strain lacking the *hoxZ* gene product was capable of only a low rate of electron transfer from H₂ to O₂; however, upon exogenous activation and use of methylene blue as the electron acceptor, the mutant expressed levels of hydrogenase activity similar to those in the wild-type strain. Methylene blue, unlike O₂, can accept electrons directly from hydrogenase. The findings support the hypothesis that the *hoxZ* gene product has a role in activating hydrogenase and, very possibly, linking hydrogenase to the electron transport chain. The main role for the *hoxZ* gene product appears to be to maintain hydrogenase in the reduced active state by transferring electrons from the electron transport chain back to hydrogenase. The product of *hoxZ* apparently also stabilizes and partially anchors the hydrogenase moiety to the membrane of *A. vinelandii*.

**MATERIALS AND METHODS**

Plasmids, bacterial strains, and DNA handling. Plasmids, bacterial strains, and their relevant characteristics are listed in Table 1. Starter DNA was pALMZ'1 (kindly provided by R. L. Robson, University of Georgia). It was constructed in pTZ19R (10) containing a 9-kb insert with the *hox* loci starting approximately at 5,000 bp (11). Plasmid pAVhoxZ was constructed by excising a 231-bp *KpnI* DNA fragment from the center of the *hoxZ* gene locus in pAVH100. Plasmid pAVH100 was derived from pALMZ'1 by deletion of the *KpnI* site at its polylinker cassette (see Fig. 1). The deletion in plasmid pAVhoxZ was corroborated by lack of hybridization to OAV1 (TGTCGCTGATGCCGCGGAGT, complementary to the left side of the deleted DNA fragment) before transformation. Isolation of plasmid DNA from *E. coli* JM109 cultures grown in ampicillin-LB medium and genomic DNA from *A. vinelandii* cultures grown in rifampin-basal medium (1) were performed as previously described (14). *A. vinelandii* DJ (hoxKG*+*), a strain with a high rate of transformation, was kindly supplied by Dennis Dean (Virginia Polytechnic Institute and State University). Plasmid pDB303 contains a 1.7-kb EcoRI *A. vinelandii* genomic DNA fragment that confers rifampin resistance. pDB303 was cloned into the EcoRI site of pUC8 and was also supplied courtesy of Dennis Dean. The negative control for H₂ uptake was the deletion mutant *A. vinelandii* hoxKG, which was derived from *A. vinelandii* DJ after transformation with...
TABLE 1. Plasmids and bacterial strains

<table>
<thead>
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<th>Plasmid or strain</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td>pTZ19R</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt; LacZ</td>
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<tr>
<td>pALMZ/1</td>
<td>pTZ19R with the structural genes cloned into XbaI and BamHI sites</td>
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<td>pDB303</td>
<td>A. vinelandii Rif&lt;sup&gt;+&lt;/sup&gt; genomic DNA fragment in pUC8; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Dennis Dean*</td>
</tr>
<tr>
<td>pAVhox&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pALMZ&lt;sup&gt;+&lt;/sup&gt; with deletion of the SplI DNA fragment at the hoxKG loci</td>
<td>This work</td>
</tr>
<tr>
<td>pAVH100</td>
<td>pALMZ&lt;sup&gt;+&lt;/sup&gt; with deletion of the SplI DNA fragment at the hoxKG loci</td>
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<tr>
<td>pAVhoxZ&lt;sup&gt;+&lt;/sup&gt;</td>
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Strains

<table>
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<td>lac-proAB</td>
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<tr>
<td>A. vinelandii DJ</td>
<td>High-transformation strain</td>
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<td>A. vinelandii hoxKG mutant</td>
<td>Strain with nonfunctional hoxKG genes derived from A. vinelandii DJ</td>
<td>This work</td>
</tr>
<tr>
<td>A. vinelandii hoxZ mutant</td>
<td>Strain with nonfunctional hoxZ gene derived from A. vinelandii DJ</td>
<td>This work</td>
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</table>

* Virginia Polytechnic Institute and State University.

pAVhox<sup>+</sup>, pAVhox<sup>+</sup> was constructed by deleting a 1.5-kb SplI DNA fragment at the center of the hoxKG loci. DNA handling was done as previously described (14).

Production, screening, and corroboration of the A. vinelandii mutant. Mutations were transferred to the chromosome as previously described (5), except that cells were taken directly from 10 colonies which were made competent on a plate with no added Fe<sup>3+</sup> and MoO<sub>4</sub><sup>2-</sup> ions (5). Plasmid pDB303 was used as a selection marker (on basal medium agar plates containing 20 mg of rifampin per liter) by cotransformation. The cells were allowed to recover overnight after transformation in 3 ml of basal medium supplemented with NH<sub>4</sub>Cl before plating onto rifampin-containing plates.

Hydrogenase activity was detected by one of three methods. (i) The H<sub>2</sub> content in the gas phase was determined by gas chromatography with an MS5A column at 40°C and a thermal conductivity detector (2). Approximately 8.9 μmol of H<sub>2</sub> gas was injected into stoppered 10-ml test tubes containing approximately 2 ml of a culture grown to an A<sub>600</sub> of 1.0 and incubated at 30°C with agitation (150 rpm in a 5-cm orbit shaker). The amounts of H<sub>2</sub> oxidized were normalized with respect to the N<sub>2</sub> content of the injection. Analyses were performed with at least three replicates and five independently isolated clones. (ii) H<sub>2</sub> in solution was determined amperometrically by using a Clark-style electrode (Yellow Springs Instruments). The reaction chamber (1.6 ml) contained 0.05 M phosphate buffer (pH 7.0), 0.2 mM methylene blue as an alternate electron acceptor, 0.15 mM NaF as a respiratory inhibitor, and 4 mM EDTA. Sodium dithionite (Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>) from a stock solution (2 mM) was used to activate hydrogenase when necessary. (iii) Methylene blue reduction linked to H<sub>2</sub> oxidation was determined spectrophotometrically at 690 nm in a stopped cuvette in 0.05 M morpholineethanesulfonic acid buffer (pH 6.0) (2, 3). The optical density of the reaction mixture was adjusted to approximately 3 with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> from a stock solution (2 mM).

Primary screening of mutants was by gas chromatography. Mutants exhibiting deficient hydrogenase activity were isolated. The introduced mutation was corroborated by the lack of hybridization to the deleted fragment in digested genomic DNA from the mutant and control strains (A. vinelandii DJ and the hoxKG mutant) with restriction enzymes HindIII, PstI, and KpnI. Hybridizations were performed at 65°C as previously described (14). The probe used to detect the mutation was the OAV1 oligonucleotide. Independently isolated mutant clones of A. vinelandii exhibited identical phenotypes.

Membrane preparation and determination of the membrane-associated and soluble forms of hydrogenase. The cells from 1 liter of 3-day-old cultures of A. vinelandii were harvested by centrifugation, and the membranes were prepared either aerobically or anaerobically. When prepared aerobically, the cells were suspended in 0.1 M Tris--10 mM EDTA (pH 7.5) and disintegrated by passage through a French cell pressure cell disruptor (9 tons [about 8,165 kg/cm<sup>2</sup>]). The suspensions were immediately chilled, and the debris and unbroken cells were sedimented by centrifugation (15,000 × g). The supernatant containing the membranes was subjected to centrifugation for 15 h at 90,000 × g to ensure complete pelleting of hydrogenase in membrane particles. The pellets were suspended in 20 mM Tris--10 mM EDTA. Hydrogenase activity was assayed with the amperometric method after treatment with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (to activate hydrogenase and scavenge O<sub>2</sub>). Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (2 mM stock solution) was added until a slight reduction of the methylene blue was observed, and then additional methylene blue was added to restore the original level of oxidized methylene blue. When prepared anaerobically, the harvested cells were equilibrated in 20 mM Tris--2 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>--4 M glycerol (pH 7.5) and broken by osmotic shock in anaerobic, N<sub>2</sub>-purged 20 mM Tris--2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (pH 7.5) as previously described (16). The membranes were subjected to centrifugation and suspended as described above but anaerobically and including 2 mM Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>. Hydrogenase activity was assayed with the spectrophotometric assay immediately after suspension.

Detection of hydrogenase protein in the membranes was performed by Western blotting (immunoblotting) as previously described (14), by using rabbit antiserum against the large subunit of hydrogenase of B. japonicum and horseradish peroxidase goat antiserum as the secondary antibody. To confirm whether hydrogenase in a given sample was membrane associated or dissociated from the membranes, the sample was passed through a gel filtration column (Sephadex CL-4B; range, 60 to 2,000 kDa) equilibrated and eluted with 0.1 M NaCl, 0.1 M Tris-HCl (pH 7.5), and 0.001 M EDTA. Membrane-associated and purified hydrogenases from the wild-type strain were used to determine the elution volumes corresponding to membrane-associated or dissociated hydrogenase in samples from mutant strains. The void volume, corresponding to membrane-associated hydrogenase, was determined with dextran blue.

RESULTS

Screening of mutant strains. Deficient H<sub>2</sub> oxidation mutants were clearly differentiated from the wild-type phenotype (rifampin resistant but with normal rates of H<sub>2</sub> oxida-
VOL. 174, PSTI digests produce the deleting the KpnI restriction sites loci. The letters was pAVhoxZ- with A fragments phisms tentative mutants were DNAs genomic poration and that membranes and in controls and that were digested with PSTI, KpnI, and HindIII. Lanes 1 to 3 contained hoxZ and hoxKG mutant and wild-type (hoxKGZ) DNAs, respectively. Fragments A and B are indicated to account for the differences in intensity of the hoxZ fragments (darker bands; see text).

FIG. 1. (Top) Construction of pAVhox- and pAVhoxZ-. Clone pAVhoxZ- was constructed by deleting a 231-bp DNA fragment at the KpnI sites. Construction of clone pAVhox- was carried out by deleting the DNA fragment between the SphI sites of the hoxKG loci. These two plasmids were transformed into A. vinelandii DJ to produce A. vinelandii hoxKG and hoxZ mutant strains, respectively. Restriction sites pertinent to the blot in panel B are indicated (four PstI sites are designated 4X). The genes hoxKGZ are indicated by the letters K, G, and Z, respectively. (Bottom) Genomic DNAs of the strains of A. vinelandii hybridized to probe OAVH. DNA was digested with HindIII, PstI, and KpnI. Lanes 1 to 3 contained hoxZ and hoxKG mutant and wild-type (hoxKGZ) DNAs, respectively. Fragments A and B are indicated to account for the differences in intensity of the hoxZ fragments (darker bands; see text).

H2 oxidation in the wild-type and the mutant strains. The hox mutants were detected by their lowered H2 consumption rates. During the screening, the hydrogenase activity observed in independently isolated hoxZ mutant clones was less than 20% of that in the wild-type clone. To characterize the hydrogenase activity in hoxZ mutant clones further, the H2 consumption rates were monitored for up to 20 h with cells (A660 of 1.0) of the hoxZ and hoxKG mutants and the wild type (hoxKGZ) by using gas chromatography (Fig. 2). The hoxZ mutant cells consistently consumed H2 at rates above those observed for hoxKG mutant cells (all of the loss could be accounted for as leakage from the vials). In the positive control (hoxKGZ), H2 was completely consumed in less than 12 h, whereas in the hoxZ deletion mutant, 44% remained at 12 h and 33% remained at the point of complete O2 depletion (at 20 h; data not shown). The difference in activity was even more dramatic after 3.5 h, when the wild type had only 36% of the H2 in the hoxKG mutant control while the hoxZ mutant still had 98% of the H2 in the hoxKG.
mutant control. It should be noted that in Fig. 2 the two independently isolated hoxZ mutant clones shown gave almost identical activities. In summary, the hoxZ mutant showed hydrogenase activity but at a considerably lower rate than the wild-type strain (hoxKG\(^+\)).

**Amperometric and spectrophotometric measurements of \(\text{H}_2\) oxidation in the wild-type and mutant strains.** For determinations of hydrogenase activity over several hours, the gas chromatographic assay described above is most suitable. However, for short-term assays that take a few minutes, the amperometric assay is more convenient. The wild type (hoxKG\(^+\)) rapidly consumed \(\text{H}_2\) with either methylene blue or \(\text{O}_2\) as the terminal electron acceptor (Fig. 3, first trace) in the amperometric assay. In contrast to the wild-type, the hoxZ mutant strain showed no detectable activity in the amperometric assay with methylene blue or \(\text{O}_2\) as the electron acceptor (Fig. 3, second trace, prior to addition of \(\text{Na}_2\text{S}_2\text{O}_4\)). A low rate of activity was expected on the basis of the gas chromatographic assay, but it is not surprising that this low rate of activity was not detected above the rate of background \(\text{H}_2\) loss observed in the amperometric assay. However, when the \(\text{O}_2\) in the reaction chamber was depleted by addition of \(\text{Na}_2\text{S}_2\text{O}_4\) and the alternate electron acceptor (methylene blue) was replenished, a high rate of hydrogenase activity, nearly equivalent to that observed in the wild-type strain, was observed (Fig. 3, second trace). This result demonstrated that the hoxZ mutant strain expressed a functional hydrogenase when coupled with an artificial electron acceptor, even though electrons could not be transferred to \(\text{O}_2\). The levels of activity obtained in the hoxZ mutant strain following activation with \(\text{Na}_2\text{S}_2\text{O}_4\) were consistently 50 to 80% of the levels of activity obtained with the hoxKG\(^+\) wild-type strain. No activity was detected in the hoxKG mutant strain, with either \(\text{O}_2\) or methylene blue as the electron acceptor.

The second trace of Fig. 3 shows that addition of \(\text{Na}_2\text{S}_2\text{O}_4\) served not only to remove \(\text{O}_2\), thereby providing an unambiguous assignment of methylene blue as the electron acceptor, but also to activate the hydrogenase. Addition of methylene blue alone did not result in \(\text{H}_2\) consumption. When a concentrated (40×) culture was assayed in the presence of methylene blue and no respiration inhibitor (NaF), the \(\text{O}_2\) was depleted by respiration and a portion of the methylene blue was reduced. This was followed by initiation of hydrogenase activity (data not shown). This result indicates that \(\text{Na}_2\text{S}_2\text{O}_4\) addition was not the only means to activate hydrogenase and suggests that an artificial reductant was required. The requirement of an artificial reductant to activate isolated NiFe hydrogenases is common (6, 13, 17, 19), but the need for such a reductant in intact cells has not been observed in wild-type strains.

The results of the amperometric assay were confirmed with a spectrophotometric assay that monitored the \(\text{H}_2\)-coupled reduction of methylene blue without addition of \(\text{Na}_2\text{S}_2\text{O}_4\). In this assay, the cells were made devoid of \(\text{O}_2\) by displacement with \(\text{N}_2\) in a vacuum manifold. Hydrogenase activity was observed in the hoxZ mutant strain with methylene blue as the acceptor (after a lag period of 1 to 2 min). The rates of methylene blue reduction were comparable to the rates of \(\text{H}_2\) oxidation observed in the amperometric assay after reductive activation of the hydrogenase. The wild-type hoxKG\(^+\) strain showed a lag period of less than 30 s. Apparently, the small amount of reduced methylene blue that the cells were able to produce was enough to activate the hydrogenase in the hoxZ mutant strain.

**Detection of the membrane-associated and soluble forms of hydrogenase in the hoxZ mutant and hoxKG\(^+\) strains.** To determine whether hydrogenase was membrane associated in the hoxZ deletion mutant, membranes were aerobically prepared from both hoxZ mutant and hoxKG\(^+\) cells and the hydrogenase activities of the membranes and supernatant were determined amperometrically. Membranes isolated from either hoxZ mutant or hoxKG\(^+\) cells required activation with \(\text{Na}_2\text{S}_2\text{O}_4\) even though intact cells of the wild type did not require activation by \(\text{Na}_2\text{S}_2\text{O}_4\). Apparently the pathway by which electrons are transported from cellular pools of reductant to hydrogenase were disrupted in the preparation of the membranes. To ensure that the hydrogenase activity isolated in the supernatant was due to unbound hydrogenase and not to small membrane particles or complexes that sedimented poorly, samples were passed through a Sepharose CL-4B gel filtration column. The hydrogenase in the supernatant eluted from the column at the same elution volume as purified hydrogenase (16). Membrane-associated hydrogenase eluted in the void volume. Thus, hydrogenase activity in the supernatant was clearly not associated with the membrane or any fragment of the membrane, while the hydrogenase activity in the pellet was associated with the membranes.

We consistently observed a greater proportion of the
TABLE 2. Hydrogenase activities observed in cells, membranes, and soluble fractions of *A. vinelandii* DJ (hoxKGZ<sup>−</sup>) and hoxZ and hoxKG mutants

<table>
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<th>Fraction</th>
<th>Total activity (% initial activity)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>hoxKGZ&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cell</td>
<td>8,600</td>
</tr>
<tr>
<td>Membrane</td>
<td>3,950 (46)</td>
</tr>
<tr>
<td>Soluble</td>
<td>1.6 (0.01)</td>
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<sup>a</sup> Total activity of cells from 1-liter 3-day-old cultures (<i>A<sub>wox</sub></i> of 1.0) in nanomoles of H<sub>2</sub> per minute.

<sup>b</sup> Aerobically prepared.

<sup>c</sup> Anaerobically prepared.

<sup>d</sup> Upon activation with Na<sub>2</sub>S<sub>5</sub>O<sub>4</sub>.

<sup>*</sup> ND, not determined.

Whole-cell activity in the membranes isolated aerobically from wild-type cells than in the membranes isolated aerobically from hoxZ mutant cells. In five experiments, the specific activity of hydrogenase varied substantially among different batch cultures and membrane preparations. The data presented in Table 2 are from a single experiment which is representative of the other experiments. We also observed a higher proportion of the whole-cell activity in the soluble fraction of hoxZ mutant cells than in the wild-type cells (Table 2). However, the total activity recovered in the supernatant and membranes was much lower in the hoxZ mutant-derived fractions than in the wild-type-derived fractions. Because of this low recovery of activity, it could not be concluded whether or not the hoxZ deletion had altered the partitioning of hydrogenase between the membranes and the cytoplasm.

To examine further the possibility that the hoxZ deletion had altered the partitioning of total hydrogenase protein (active plus inactive), membranes from hoxZ mutant and wild-type cells were isolated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the separated proteins were transferred to a nylon membrane. The blot was probed with antibody raised against the large subunit of *B. japonicum* hydrogenase. The amounts of hydrogenase detected were similar in membranes isolated from the hoxZ mutant and the wild type (data not shown). Furthermore, activity in anaerobically prepared hoxZ mutant membranes was higher than in aerobically prepared hoxZ mutant membranes but still lower than in similarly prepared wild-type membranes (16 and 72% of the initial activity, respectively) (Table 2). This indicates that the low activity observed in the membranes of hoxZ mutant cells is due to instability of the hydrogenase in these cells and not to substantially decreased association of the protein with the membranes.

**DISCUSSION**

The ORF immediately downstream of the genes that code for the small and large subunits of hydrogenase is essential for hydrogenase activity in the presence of O<sub>2</sub> in cells of *A. vinelandii*. In *R. capsulatus*, inactivation of the equivalent gene resulted in a hydrogenase that was active when assayed with artificial electron acceptors but was not physiologically active (7). The mutants were unable to grow autotrophically, to recycle electrons from H<sub>2</sub> to nitorgenase, or to respire on H<sub>2</sub>. To examine the function of the product of the comparable gene in *A. vinelandii*, a mutant with a hoxZ deletion in this ORF was constructed. As with the *R. capsulatus* insertion mutants, the *A. vinelandii* deletion mutant was capable of hydrogenase activity with an artificial electron acceptor (methylene blue) but required an exogenous reductant for activation (Fig. 3). This indicates that a functional hydrogenase was expressed in the mutant and rules out a role for hoxZ in the production of active hydrogenase (e.g., processing of the hydrogenase polypeptides to form active hydrogenase). However, the hydrogenase that was expressed could not transfer electrons to O<sub>2</sub> at rates comparable to those observed in wild-type cells. Therefore, the physiological activity of hydrogenase was impaired by disruption of the hoxZ gene product. We have considered four possible roles for the hoxZ gene product, as discussed below.

**Role 1, activation of hydrogenase.** *A. vinelandii* hydrogenase, as well as other NiFe hydrogenases (6, 13, 19), can be isolated in a stable, reversibly inactive state. Activation of *A. vinelandii* hydrogenase requires removal of O<sub>2</sub> and supply of a reductant (19). Presumably, maintenance of hydrogenase activity in the cell also requires that the hydrogenase remain in a reduced state. The results of this work suggest that one role of the hoxZ gene product is to maintain hydrogenase in an active reduced state. Perhaps the hoxZ gene product fulfills this role by supplying reductant from the cell to hydrogenase. In the absence of the hoxZ gene product, hydrogenase remains in the inactive oxidized state in the cells (Fig. 3). Activation of hydrogenase in the mutant required a supply of an exogenous reductant, Na<sub>2</sub>S<sub>5</sub>O<sub>4</sub> or reduced methylene blue, which could donate electrons directly to hydrogenase. Perhaps the hoxZ gene product provides the conduit for transfer of cellular reductant to hydrogenase. In the absence of hoxZ, an artificial reductant is required to obtain full activity. It is interesting that H<sub>2</sub> does not seem to be able to provide the reductant necessary for hydrogenase activation in the hoxZ mutant strain.

**Role 2, mediation of electron flow from hydrogenase to the electron transport chain.** The immediate acceptor of electrons from *A. vinelandii* hydrogenase has not been identified. The possibility of a mediator unique to hydrogenase has been suggested for microorganisms with similar hydrogenases (7, 12). If the product of the hoxZ gene serves as a mediator of electron flow from hydrogenase to the electron transport chain, then disruption of this gene product could lead to a phenotype in which hydrogenase was still produced but oxidation of H<sub>2</sub> could not be coupled to reduction of O<sub>2</sub>. This was precisely the phenotype we observed when a portion of the hoxZ gene was deleted. Therefore, the results support a role for the mediation of electron flow from hydrogenase to the electron transport chain.

A low rate of oxidation of H<sub>2</sub> coupled to O<sub>2</sub> was observed in the hoxZ mutant strain (Fig. 2). It appears that alternate, albeit much less efficient pathways for transport of electrons from H<sub>2</sub> to O<sub>2</sub> were still present in the cells. Given the ability of hydrogenase to transfer electrons to a number of acceptors (18), it seems reasonable that hydrogenase could transfer electrons to a nonspecific electron mediator in the cells (e.g., cytochrome or quinone) which could in turn transfer electrons to the electron transport chain. Another possibility is that additional genes are present in *A. vinelandii* that produce gene products that can substitute for the hoxZ gene product.

**Role 3, stabilization of hydrogenase.** In strains of *R. capsulatus* with a defective *hapM* gene product, hydrogenase was very unstable in cell extracts (7). We also observed that hydrogenase activity in aerobically prepared cell extracts of *A. vinelandii* hoxZ mutant cells was very unstable. In
wild-type cells, 46% of the activity observed in whole cells was accounted for in aerobically prepared cell extracts. In contrast, in the hoxZ mutant strain, less than 1% of the activity observed in whole cells was accounted for in similarly prepared cell extracts. When cell extracts were prepared anaerobically, higher activities were observed in the hoxZ mutant (Table 2). Therefore, it appears that one role of the hoxZ gene product is in stabilizing hydrogenase. The mechanism by which this occurs is not clear. Perhaps the product of hoxZ is involved in stabilizing hydrogenase towards O₂. Hydrogenase in wild-type cells is stable towards O₂ while bound to the membrane (9, 19). In experiments with *R. capsulatus*, in contrast to those with *A. vinelandii*, no increase in hydrogenase stability in extracts of hupM-defective cells was found when O₂ was excluded (7).

**Role 4, attachment of hydrogenase to the membrane.** The derived amino acid sequence of the hoxZ polypeptide product contains several regions that are consistent with membrane-spanning regions. Hydrogenase in *A. vinelandii*, as with several other microorganisms, is tightly bound to the membrane and requires treatment with a detergent for solubilization. However, once solubilized the continued presence of a detergent is not required to maintain the hydrogenase in a soluble state. This would be consistent with the attachment of hydrogenase to another polypeptide that is embedded in the membrane. Such a role was proposed for the hupM gene in *R. capsulatus* (7) and the hycA gene in *E. coli* (12) and was considered for hoxZ in *A. vinelandii* in this work. Unfortunately, the straightforward test of this proposal—fractionation of cells and determination of the location of hydrogenase activity—did not provide definitive results because of the instability of the hydrogenase in cell extracts of hoxZ mutant cells (Table 2). However, Western blots revealed that comparable levels of hydrogenase were present in membrane extracts of wild-type and hoxZ mutant cells. While this appears to rule out binding of hydrogenase to the gene product of hoxZ as the only means of anchoring the hydrogenase to the membrane, it does not rule out the possibility that hydrogenase forms a stable, tight complex with the hoxZ gene product. It is noteworthy that we consistently observed more hydrogenase activity in the soluble fraction of hoxZ mutant cells than in wild-type cells. The possibility that a truncated product of the defective hoxZ gene still anchors the hydrogenase cannot be discarded.

The various roles proposed for the gene product of hoxZ are not necessarily mutually exclusive. For example, the same protein which serves to mediate electron flow from the cell to hydrogenase to maintain the enzyme in the reduced active state (role 1) could also mediate electron flow from hydrogenase to the electron transport chain (role 2). Likewise, the mechanism by which the hoxZ gene product stabilizes hydrogenase (role 3) could involve its ability to transfer electrons to and from hydrogenase. If hydrogenase forms a complex with the product of hoxZ, then this could form at least a part of the mechanism by which hydrogenase is anchored to the membrane (role 4).

A scheme which summarizes the results of this study and the putative roles of the hoxZ gene product is presented in Fig. 4. To activate hydrogenase, a flow of electrons from the cell through hoxZ to hydrogenase is proposed. The predominant direction of electron flow, possibly through hoxZ, is from hydrogenase to the electron transport chain. In the absence of hoxZ, H₂ oxidation coupled to O₂ still occurred, albeit at a much lower rate (Fig. 2). Therefore, an alternate electron transport is proposed, although it is not clear whether this alternate pathway has any physiological significance. Determination of whether the hoxZ gene product and hydrogenase form a complex in the membrane awaits further experimentation.

**REFERENCES**


