Restoration of hydrogenase activity in hydrogenase-negative strains of Escherichia coli by cloned DNA fragments from Chromatium vinosum and Proteus vulgaris

ALPANA CHAUDHURI and ALVIN I. KRASNA*

Department of Biochemistry and Molecular Biophysics, The Cancer Center/Institute for Cancer Research, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

(Received 1 September 1989; revised 29 January 1990; accepted 15 February 1990)

DNA fragments from Proteus vulgaris and Chromatium vinosum were isolated which restored hydrogenase activities in both hydA and hydB mutant strains of Escherichia coli. The hydA and hydB genes, which map near minute 59 of the genome map, 17 kb distant from each other, are not structural hydrogenase genes, but mutation in either of these genes leads to failure to synthesize any of the hydrogenase isoenzymes. The smallest DNA fragments which restored hydrogenase activity to both E. coli mutant strains were 4.7 kb from C. vinosum and 2.3 kb from P. vulgaris. These fragments were cleaved into smaller fragments which did not complement either of the E. coli mutations. The cloned heterologous genes also restored formate hydrogenlyase activity but they did not restore activity in hydE, hupA or hupB mutant strains of E. coli. The cloned genes, on plasmids, did not lead to the synthesis of proteins of sufficient size to be the hydrogenase catalytic subunit. The hydrogenase proteins synthesized by hydA and hydB mutant strains of E. coli transformed by cloned genes from P. vulgaris and C. vinosum were shown by isoelectric and immunological methods to be E. coli hydrogenase. Thus, these genes are not hydrogenase structural genes.

Introduction

The synthesis of the enzyme hydrogenase in Escherichia coli requires a number of genes which have been characterized by mutational analysis. Four of these genes, hydA, hydB, hydE and hydF, have been mapped near minute 59 of the E. coli genome map and DNA fragments have been isolated which complemented the individual mutations (Lee et al., 1985; Sankar et al., 1985; Waugh & Boxer, 1986; Chaudhuri & Krasna, 1987; Sankar & Shanmugam, 1988a, b). Strains with mutations in these genes are devoid of all hydrogenase activities (reduction of viologen dyes with H₂, exchange reaction, growth on fumarate plus H₂, evolution of H₂ from formate) and lack the three hydrogenase isoenzymes (Ballantine & Boxer, 1985; Sawers et al., 1985; Waugh & Boxer, 1986). The genetic map of the hydrogenase genes near minute 59 is shown in Fig. 1. The hydA and hydB genes are 17 kb distant from each other. The hydC and hydD genes, which map at minute 77, are also required for synthesis of all three isoenzymes (Wu & Mandrand-Berthelot, 1986). Mutations in the hydC and hydE genes can be overcome by growing cells in the presence of high concentrations of nickel (Waugh & Boxer, 1986; Wu & Mandrand-Berthelot, 1986; Chaudhuri & Krasna, 1987). The role of the hydA, hydB and hydF genes is not known. None of these genes are structural genes for hydrogenase but they are required for the synthesis of all hydrogenase isoenzymes.

To elucidate the role of the cloned E. coli hydrogenase genes near minute 59, DNA fragments were isolated from DNA libraries of hydrogenase-containing Chromatium vinosum and Proteus vulgaris and tested for their ability to restore the hydrogenase phenotype in hydA and hydB mutant strains of E. coli.

Methods

Organisms and growth conditions. Hydrogenase-positive C. vinosum and P. vulgaris were grown as previously described (Gitlitz & Krasna, 1975; Schengrund & Krasna, 1969) and the DNA isolated from these cells was used to prepare genomic libraries (Chaudhuri & Krasna, 1987, 1988). The wild-type hydrogenase-positive E. coli strain was K12W6 met bio (ATCC 25019). The hydrogenase-negative E. coli strains were SE-31 (hydB) and SE-19-1 (hydA) (Lee et al., 1985), kindly supplied by

Abbreviation: FHL, formate hydrogenlyase
Dr K. T. Shanmugam. As positive controls, the hydA strain was transformed by plasmid pSE-201 and the hydB strain by plasmid pSE-128 (Sankar et al., 1985).

Bacteria were grown in complex or minimal medium containing appropriate antibiotics when required for selection as described previously (Chaudhuri & Krasna, 1987).

Enzyme assays. The enzyme activities of interest were measured at 25 °C in washed cell suspensions. Hydrogenase activity was assayed by the deuterium exchange method and reduction of viologen dyes; formate hydrogenlyase (FHL) activity was determined by manometric measurements of evolution of H2 from formate as described previously (Chaudhuri & Krasna, 1985).

Screening methods for hydrogenase. The symbols Hyd+ and hyd designate the phenotype and genotype of cells that lack all demonstrable hydrogenase activity: exchange reaction, reduction of viologen dyes by H2, growth on fumarate plus H2 and evolution of H2 from formate. The details of the screening methods were described by Chaudhuri & Krasna (1987) and are based on reduction of methyl viologen by H2 and growth on fumarate plus H2. Strains SE-31 (hydB) and SE-19-1 (hydA) are Hyd+ and become Hyd+ when transformed by the cloned E. coli DNA fragments on plasmids (Sankar et al., 1985). The same screening methods were used when these strains were transformed by plasmid DNA libraries from C. vinosum or P. vulgaris. Hyd+ transformants were then grown on minimal medium plus glucose or on fumarate plus H2 and assayed by deuterium exchange.

Preparation of and transformation by genomic DNA libraries. The methodology was essentially that described earlier (Chaudhuri & Krasna, 1987) for the cloning of and transformation by an E. coli DNA library. Chromosomal DNA from C. vinosum or P. vulgaris was partially digested with the restriction enzyme Sau3A, subjected to electrophoresis on 0.6% agarose, and fragments in the size range 4–20 kb isolated. These fragments were ligated to pBR322 which had been cleaved with BamHI and treated with calf intestinal phosphatase followed by purification by agarose electrophoresis. These plasmid libraries transformed E. coli strain CC114 with good efficiency (Chaudhuri & Krasna, 1987) and all the transformed colonies were collected, grown in large volume and plasmid DNA isolated. These purified plasmid DNA preparations were the C. vinosum and P. vulgaris libraries used for gene selection.

These two libraries were used to transform hydA and hydB mutant strains of E. coli to ampicillin resistance (Ap+). A large number of these colonies were then grown on fumarate plus H2 to enrich for Hyd+ cells. These cells were plated on agar and screened for the Hyd+ phenotype. A number of positive isolates were selected, and DNA was prepared from each and used to transform the mutant strains to the Hyd+ phenotype. In each case all Ap+ transformants were Hyd+ by all criteria including high levels of deuterium exchange in cells grown on fumarate plus H2. The plasmids were mapped by restriction enzyme digestion. All the Hyd+ transformants from the respective genomic libraries were identical.

Hybridization studies. The cloned plasmid DNA fragments isolated from the different organisms were treated with appropriate restriction enzymes and purified by agarose gel electrophoresis to obtain unique fragments from each plasmid (free of vector plasmid DNA) that could be used as probes for hybridization to other plasmid digests or to chromosomal DNA digested with EcoRI. Hybridizations were carried out in 50% (v/v) formamide at 42 °C (Tm - 14 °C) overnight with the denatured labelled probe and the filters were washed in 2 x SSC/0.1% SDS at room temperature for 15 min and then at 65 °C (Tm - 25 °C) for 2 h with four changes of buffer (1 x SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0). Autoradiography was used to locate the bands. Washing the filters at 50, 37 or 25 °C gave the same results as washing at 65 °C.

Synthesis of plasmid-coded proteins. The maxicell procedure of Sancar et al. (1979) was followed, using a mixture of 14C-labelled amino acids and 35S-labelled methionine. E. coli strain CSR603 (Sancar et al., 1979) was obtained from the E. coli Genetic Stock Center, Yale University.

Characterization of hydrogenase proteins. Membranes were isolated as previously described (Chaudhuri & Krasna, 1988) from parental strains of E. coli, P. vulgaris and C. vinosum as well as from hydA and hydB mutant strains of E. coli (SE-31 and SE-19-1) transformed by the different plasmids. The membranes were solubilized with Triton or Lubrol, and analysed by isoelectric chromatofocusing between pH 4 and pH 6 using Polybuffer PBE94 (Pharmacia). The solubilized membranes were also tested for reactivity with antibodies to E. coli hydrogenase isoenzyme 1 (Sawers et al., 1985). The antibodies were prepared by Dr K. T. Shanmugam and were a mixture of monoclonal antibodies to the separated 59 kDa subunit and the 28 kDa subunit. The membrane preparations were analysed by ELISA and by Western blotting. The proteins were treated with anti-hydrogenase 1 antibodies and then with anti-mouse-IgG antibodies conjugated with alkaline phosphatase and the colour developed with suitable substrates (Blake et al., 1984).

Results and Discussion

Restoration of the Hyd+ phenotype by a cloned DNA fragment from C. vinosum

From the C. vinosum library a single plasmid was isolated which restored hydrogenase activity in both hydA and hydB mutant strains. The plasmid, pSE3-1Chr (9-6 kb) had a chromosomal DNA insert of 5-2 kb; the restriction digest map is shown in Fig. 2. The results in Table 1 show that this plasmid restored deuterium exchange activity in both mutant strains. The deuterium exchange activity in the wild-type E. coli strain K12W6 was not affected by transformation by pSE3-1Chr. This plasmid did not restore activity to a hydE mutant strain of E. coli. An E. coli DNA fragment has been isolated (Waugh & Boxer, 1986; Chaudhuri & Krasna, 1987) which restored activity to both hydB and hydE mutant strains of E. coli. No DNA fragment from E. coli has been isolated which complements both hydA and hydB mutations. In E. coli the distance between these two genes is about 17 kb, based on reported genetic maps near minute 59 (Yerkes et al., 1984; Karube et al., 1984; Sankar et al., 1985; Kohara et al., 1987).

![Fig. 1. Location of the hyd genes near minute 59 in the E. coli genome map.](image-url)
Hydrogenase genes from P. vulgaris and C. vinosum

Fig. 2. Restriction digest maps of plasmids from C. vinosum. The circular plasmids are represented in linear form, the heavy line being the vector plasmid and the light line the chromosomal DNA insert. The numbers beneath the line indicate the size (kb) of the fragment between the two indicated restriction sites. The letters indicate the restriction sites: B/Sa, BamHI–Sau3A junction of vector and insert; H, HindIII; N, NruI; RI, EcoRI; S, Sall. There are no sites in the insert for Clal, EcoRV, Ndel, NheI, PstI, PvuII, Scal and Sphi. The insert in pSE3-1Chr has one Ball site, one Kpnl site, two Aavr sites, five StyI sites and five PslI sites which have not been mapped precisely.

Table 1. Hydrogenase activity of E. coli mutant strains transformed by cloned DNA fragments

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>hyd genotype</th>
<th>Plasmid</th>
<th>Glucose grown</th>
<th>Fumarate + H2 grown</th>
</tr>
</thead>
<tbody>
<tr>
<td>K12W6 Wild-type</td>
<td>None</td>
<td>0.7</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>SE-31 hydB</td>
<td>None</td>
<td>&lt;0.02</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td>SE-31 hydB</td>
<td>pSE3-1Chr</td>
<td>0.56</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>SE-19-1 hydA</td>
<td>None</td>
<td>&lt;0.02</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td>SE-19-1 hydA</td>
<td>pSE3-1Chr</td>
<td>0.38</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>SE-31 hydA</td>
<td>pSE3-1PV</td>
<td>0.33</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>SE-19-1 hydA</td>
<td>pSE3-1PV</td>
<td>0.73</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td>SE-31 hydA</td>
<td>pSE3-1PVSp</td>
<td>0.3</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>SE-19-1 hydA</td>
<td>pSE3-1PVSp</td>
<td>0.4</td>
<td>4.7</td>
<td></td>
</tr>
</tbody>
</table>

NG, No growth.

* A unit of activity is defined as the appearance of 0.1% 2H2H2O in the gas phase from 10% 2H2O and is equivalent to reaction with 0.287 μmol H2 min⁻¹. These values are the mean of two separate determinations with different samples of intact cells. The maximum deviation from the mean was ±10%.

A single plasmid complementing both hydA and hydB mutations could be due to the plasmid containing both the individual hydA and hydB genes from C. vinosum which are homologous to those in E. coli. To decide whether pSE3-1Chr contained individual hydA and hydB genes, the plasmid was subcloned into smaller fragments by restriction enzyme digestion of the plasmid and self-ligation (Fig. 2). Removal of the 0.5 kb fragment at the right of the insert gave a plasmid with a 4.7 kb insert (pSE3-1ChrSS) which was as active as the original plasmid in restoring activity in both hydA and hydB mutant strains. The 2.2–2.5 kb insert at the left of the fragment (pSE3-1ChrS and pSE3-1ChrN) and the 2.9 kb insert at the right of the fragment (pSE3-1ChrRI) failed to complement either the hydA or the hydB mutant strain, suggesting that the original 4.7 kb DNA fragment did not contain independently active hydA and hydB genes. Removal of the 0.4 kb fragment at the extreme left of the original insert (pSE3-1ChrRI) led to the loss of complementation for both hydA and hydB mutant strains. It is possible that the 0.4 kb BamHI/Sau3A–EcoRI insert fragment contains a single promoter for two genes with the first gene extending past the first NruI site.

The smallest active DNA fragment, 4.7 kb, could code for a protein of molecular mass 157 kDa; this is sufficiently large to be the hydrogenase protein, which was reported to be a dimer of two 50 kDa subunits (Gitlitz & Krasna, 1975). Sawers & Boxer (1986) reported that the subunit molecular masses of hydrogenase isoenzyme 1 were 64 and 35 kDa, and Ballantine & Boxer (1986) found that the subunit molecular masses of isoenzyme 2 were 61 and 35 kDa. The catalytic subunit was the one of higher molecular mass (Harker et al., 1986). It is, of course, possible that the DNA fragment cloned is a gene or genes whose effect in E. coli is the same as the combined effect of the individual hydA and hydB genes. This will be further discussed below.
Restoration of the Hyd+ phenotype by a cloned DNA fragment from P. vulgaris

From the P. vulgaris library a single plasmid was isolated which restored hydrogenase activity in both hydA and hydB mutant strains. The plasmid, pSE3-1PV (8.4 kb) had a chromosomal DNA insert of 4.0 kb; the restriction digest map is shown in Fig. 3. The results in Table 1 demonstrate that this plasmid restored deuterium exchange activity in both mutant strains. The activity in E. coli strain K12W6 (wild-type) was not affected by the P. vulgaris plasmid and this plasmid did not restore activity to a hydE mutant strain of E. coli.

The plasmid was subcloned into smaller fragments by restriction enzyme digestion, purification of the fragment of interest, and ligation to pBR322 treated with the same restriction enzyme. Treatment of pSE3-1PVSp with EcoRV gave a 2.7 kb fragment containing the 0.4 kb SphI-EcoRV fragment of pBR322. Treatment of pSE3-1PV with PvuI gave a 2.6 kb fragment containing the 1.1 kb PvuI-BamHI/Sau3A fragment of pBR322. Ligation of these fragments to pBR322 cut with EcoRV or PvuI gave plasmids containing a repeat of 0.4 kb or 1.1 kb pBR322 fragment, respectively.

The restriction maps of the subclones are shown in Fig. 3. The 2.7 kb SphI-SphI insert (pSE3-1PVSp) was as active as the 4.0 kb insert in restoring activity in both hydA and hydB mutant strains (see Table 1). Removal of a 0.4 kb fragment at the left of this insert (pSE3-1PVSpRV) did not affect the complementation. Removal of the 2.5 kb DNA fragment at the right of the original insert (pSE3-1PVSpV) led to the loss of all activities. The smallest active DNA fragment, 2.3 kb, could code for a protein of molecular mass 77 kDa. The native molecular mass of the P. vulgaris hydrogenase was reported to be 115 kDa (Schengrund & Krasna, 1969); the subunit composition has not been determined.

Hybridization studies

To determine whether the cloned DNA fragments from C. vinosum and P. vulgaris which restored hydrogenase activity to both hydA and hydB mutant strains of E. coli contained individual hydA or hydB genes homologous to those of E. coli, Southern blot hybridizations were carried out among the different plasmids as well as to chromosomal DNA digests of the three organisms of interest (see Methods for details). [The restriction digest maps of hydA and hydB are given by Sankar et al. (1985) and the map of hydE by Chaudhuri & Krasna (1987).] The results are summarized in Table 2. The E. coli hydB probe did not hybridize to the E. coli hydA probe nor to plasmids pSE3-1Chr or pSE3-1PV. It only hybridized to E. coli chromosomal DNA. The E. coli hydA probe did not hybridize to plasmids pSE3-1Chr or pSE3-1PV; it only hybridized to E. coli chromosomal DNA. A probe prepared from plasmid pSE3-1PV (2.7 kb SphI-SphI fragment) did not hybridize to pSE3-1Chr and only hybridized to P. vulgaris chromosomal DNA. Plasmid pSE3-1Chr only hybridized to C. vinosum chromosomal DNA. None of these plasmids hybridized to the cloned hydE gene (Chaudhuri & Krasna, 1987) or hupB gene (Chaudhuri & Krasna, 1988).

It is clear that the cloned DNA fragments from C. vinosum and P. vulgaris which complement E. coli strains with mutations in hydA and hydB did not hybridize to each other, nor to the cloned E. coli hydA and hydB genes,
and it is unlikely that they are simply carrying individual genes homologous to hydA and hydB genes. The cloned genes could be structural genes for hydrogenase or genes whose effect on E. coli is the same as the combined effect of hydA and hydB genes, though there is no strong homology in the DNA sequences.

Restoration of FHL activity

hydA and hydB mutant strains of E. coli lack all hydrogenase isoenzymes and are devoid of FHL activity, which has been ascribed to isoenzyme 3 (Ballantine & Boxer, 1985; Sawers et al., 1985). The transformants described above were assayed for FHL activity (Table 3). Plasmids pSE-128 and pSE-201 contain cloned E. coli hydE genes and hydA genes (Sankar et al., 1985) and serve as controls for the restoration of FHL activity in the two mutant strains. The P. vulgaris plasmid, pSE-1PV, restored FHL activity in both mutant strains, whereas the C. vinosum plasmid, pSE-3Chr, restored FHL activity in the hydB mutant but not in the hydA mutant.

Lack of complementation of Hup− mutants

Hup− mutant strains of E. coli show a hydrogen-uptake-negative phenotype (failure to reduce violigen dyes with H2 and to grow on fumarate plus H2), but express normal hydrogenase activity as measured by the deuterium exchange reaction. The cloned DNA fragments from C. vinosum or P. vulgaris did not complement hupA mutations at minute 65 (Lee et al., 1985) or hupB mutations at minute 17 (Chaudhuri & Krasna, 1988) (data not shown).

Synthesis of plasmid-encoded proteins

Since the molecular masses of the polypeptides encoded by the P. vulgaris and C. vinosum plasmids might indicate whether the cloned genes were structural genes, the maxicell procedure of Sancar et al. (1979) was used to identify the subunit molecular masses of these polypeptides. We had previously shown (Chaudhuri & Krasna, 1987) that a plasmid containing the E. coli hydB, hydE and hydF genes led to the synthesis of polypeptides of molecular masses 41, 36 and 30 kDa, the 36 kDa protein being the product of hydE. Sankar & Shanmugan (1988a, b) identified the 30 kDa protein as the product of the hydB gene and the 41 kDa protein as the product of the hydF gene.

Table 2. Southern hybridization among different DNA fragments

<table>
<thead>
<tr>
<th>Probe</th>
<th>pSE-130</th>
<th>pSE-201</th>
<th>pSE3-1PV</th>
<th>pSE3-1Chr</th>
<th>E. coli</th>
<th>P. vulgaris</th>
<th>C. vinosum</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli hydB†</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>E. coli hydA‡</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>P. vulgaris hydAB§</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>pSE3-1Chr</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, Good hybridization under the conditions outlined in Methods; −, no hybridization; ND, not done.
† 2.8 kb SalI–SalI fragment from plasmid pSE-130 (hydB).
‡ 3.9 kb EcoRI–PstI fragment from plasmid pSE-201 (hydA).
§ 2.7 kb SphI–SphI fragment from plasmid pSE3-1PV (hydAB).

Table 3. FHL activity in mutant strains transformed by plasmids

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>hyd genotype</th>
<th>Plasmid</th>
<th>FHL activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>K12W6</td>
<td>Wild-type</td>
<td>None</td>
<td>42</td>
</tr>
<tr>
<td>SE-31</td>
<td>hydB</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>SE-31</td>
<td>hydB</td>
<td>pSE-128</td>
<td>154</td>
</tr>
<tr>
<td>SE-31</td>
<td>hydB</td>
<td>pSE3-1Chr</td>
<td>94</td>
</tr>
<tr>
<td>SE-31</td>
<td>hydB</td>
<td>pSE3-1PV</td>
<td>116</td>
</tr>
<tr>
<td>SE-19-1</td>
<td>hydA</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>SE-19-1</td>
<td>hydA</td>
<td>pSE-201</td>
<td>94</td>
</tr>
<tr>
<td>SE-19-1</td>
<td>hydA</td>
<td>pSE3-1Chr</td>
<td>10</td>
</tr>
<tr>
<td>SE-19-1</td>
<td>hydA</td>
<td>pSE3-1PV</td>
<td>148</td>
</tr>
</tbody>
</table>

* These values are the mean of two separate determinations with different samples of intact cells. The maximum deviation from the mean was ±10%.

Fig. 4 shows the maxicell results for the plasmids used in the present study. The plasmid vector, pBR322, coded for three polypeptides of molecular masses 43, 33, and 28 kDa (Sancar et al., 1979); the former two were seen only in longer exposures. Plasmid pSE-201, containing the hydA gene, led to the synthesis of a protein of subunit molecular mass 44 kDa. Plasmid pSE-128, used as the source of the hydB gene, led to the synthesis of proteins of molecular masses 72 and 78 kDa. (The 30 kDa protein product of the hydB gene was not discernible in this
Fig. 4. Autoradiograph of an SDS-polyacrylamide gel of polypeptides expressed in maxicells (*E. coli* CSR603) by plasmids derived from *E. coli*, *P. vulgaris* and *C. vinosum*, A, pBR322; B, pSE-201; C, pSE-128; D, pSE3-1Chr; E, pSE3-1ChrSS; F, pSE3-1PV; G, pSE3-1PVSpRV.

Sankar et al. (1988) reported that the 72 and 78 kDa proteins were products of the *fde* and *fhfA* genes. Plasmid pSE3-1Chr led to the synthesis of polypeptides of molecular masses 33, 38 and 44 kDa (the last seen only in longer exposures) while the smaller plasmid pSE3-1ChrSS led to the synthesis of proteins of molecular masses 38 and 44 kDa. Plasmid pSE3-1PV led to the synthesis of polypeptides of molecular masses 33 and 40 kDa and the smaller plasmid pSE3-1PVSpRV led to the synthesis of polypeptides of the same molecular masses (seen only in longer exposures).

**Characterization of hydrogenase in mutant strains transformed by plasmids**

If the hydrogenase genes isolated from *P. vulgaris* and *C. vinosum* are genes which restore the ability of *hydA* and *hydB* mutant strains of *E. coli* to synthesize the hydrogenase isoenzymes, then the enzymes synthesized should be characteristic of those of *E. coli*. On the other hand, if the cloned genes are structural genes, then the hydrogenases synthesized should be characteristic of those found in *P. vulgaris* or *C. vinosum*. The active enzymes synthesized by the mutant *E. coli* strains transformed by the cloned genes from the two heterologous organisms were thus studied in detail.

Solubilized membranes were prepared from wild-type *P. vulgaris*, *C. vinosum* and *E. coli*, and from *hydA* and *hydB* mutant strains of *E. coli* transformed by plasmids pSE3-1PV and pSE3-1Chr; the preparations were analysed by isoelectric chromatofocusing and reactivity with antibodies to *E. coli* hydrogenase isoenzymes. On isoelectric chromatofocusing most of the hydrogenase preparations had pI values of 5.1–5.3, the only exception being the enzyme from *C. vinosum* which had a pI value of 4.5–4.7. This would suggest that the *E. coli* mutant strains transformed by plasmid pSE3-1Chr synthesize *E. coli* hydrogenases.

Sawers et al. (1985) and Ballantine & Boxer (1985) showed that *E. coli* has at least two hydrogenase isoenzymes and that antibodies to the individual isoenzymes do not cross-react with the different isoenzymes. To conclusively demonstrate that the hydrogenase protein synthesized in *E. coli* mutants transformed by the cloned genes from *P. vulgaris* and *C. vinosum* is *E. coli* hydrogenase, the solubilized membranes were tested for reactivity with antibodies to *E. coli* hydrogenase 1. The results from a Western blot are shown in Fig. 5; similar results were obtained by ELISA methods. The *hydB* mutant strain does not react with the antibodies (data not shown). The hydrogenase from *P. vulgaris* cross-reacts with antibodies to the *E. coli* hydrogenase as does the *E.
coli hydB mutant transformed by the P. vulgaris cloned DNA fragment. Thus, no definite conclusion can be drawn as to the nature of the hydrogenase synthesized by the E. coli mutant strain transformed by the P. vulgaris DNA fragment. However, from the data presented above on the restoration of all hydrogenase activities, and the relatively small size of the proteins encoded by the P. vulgaris DNA fragment, it seems probable that the hydrogenase protein synthesized by the E. coli hydB mutant transformed by the P. vulgaris DNA fragment is E. coli hydrogenase.

It is also clear from Fig. 5 that the proteins in C. vinosum do not cross-react with antibodies to E. coli hydrogenase I while the E. coli hydA mutant transformed by the cloned DNA fragment from C. vinosum synthesizes E. coli hydrogenase I. This demonstrates conclusively that the cloned DNA fragment is not a structural hydrogenase gene of C. vinosum.

**Concluding remarks**

This study has shown that DNA fragments from P. vulgaris and C. vinosum (which do not hybridize to each other) restore activity to two distinct mutant strains of E. coli (hydA and hydB) with mutant genes 17 kb distant from each other. The individual hydA and hydB genes have been shown to be required for the synthesis of all hydrogenase isoenzymes in E. coli. It was also demonstrated that the two heterologous DNA fragments do not contain individual hydA and hydB genes homologous to those of E. coli. Rather they contain a gene (or genes) whose protein product (or products) in E. coli has the same positive effect as the combined effects of the products of the E. coli hydA and hydB genes. The precise mechanism of this effect is not known, but it is likely that both the hydA and hydB gene products (as well as those of the genes cloned from P. vulgaris and C. vinosum) affect another gene or genes which lead to synthesis of all hydrogenase isoenzymes. A similar conclusion was arrived at by Sankar & Shanmugam (1988b) with regard to the E. coli hydF and jhlA genes.

DNA fragments from Clostridium butyricum and Citrobacter freundii have been shown to complement E. coli hydA mutations (Karube et al., 1983; Kanayama et al., 1986). In the former case the DNA fragment hybridized to E. coli DNA and in the latter case hybridization results were not reported.

**Note added in proof**

It has recently been demonstrated in this laboratory that plasmids pSE3-IChr and pSE3-1PV also restore all hydrogenase activities in a hydF mutant strain of E. coli (Sankar & Shanmugam, 1988b). These plasmids, however, do not restore formate hydrogonylase activity in a jhlA mutant strain of E. coli (Sankar et al., 1988). The position of the hydF gene, immediately to the left of the hydB gene, is shown in Fig. 1. The jhlA gene (2 kb) lies immediately to the right of the hydB gene.

This research was supported by the US Department of Energy Grant no. DE-FG02-85ER13374 and a Biomedical Research Support Grant from the National Institutes of Health. However, any opinions, finding, conclusions or recommendations expressed herein are those of
the authors and do not necessarily reflect the view of the DOE or the NIH.

References


