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

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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, 1988*a, 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, 1987).

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 *Bam*HI 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^R). 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^R 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 *Eco*RI. Hybridizations were carried out in 50% (v/v) formamide at 42 °C (*T*_m – 14 °C) overnight with the denatured labelled probe and the filters were washed in 2 × SSC/0.1% SDS at room temperature for 15 min and then at 65 °C (*T*_m – 25 °C) for 2 h with four changes of buffer (1 × 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. The subunit molecular masses (kDa) of the protein products found in maxicells transformed with cloned DNA fragments are shown. The maximum size of each gene (kb) is also shown. The figure was drawn from data presented by Sankar *et al.* (1985), Chaudhuri & Krasna (1987) and Sankar & Shanmugam (1988b), and in this paper.

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, *Bam*HI–*Sau*3A junction of vector and insert; H, *Hind*III; N, *Nru*I; RI, *Eco*RI; S, *Sal*I. There are no sites in the insert for *Cla*I, *Eco*RV, *Nde*I, *Nhe*I, *Pst*I, *Pvu*II, *Sca*I and *Sph*I. The insert in pSE3-1Chr has one *Bal*I site, one *Kpn*I site, two *Ava*I sites, five *Sty*I sites and five *Pvu*I sites which have not been mapped precisely.

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

Recipient strain	<i>hyd</i> genotype	Plasmid	Hydrogenase specific activity* [units (mg protein) ⁻¹]	
			Glucose grown	Fumarate + H ₂ grown
K12W6	Wild-type	None	0.7	4.8
SE-31	<i>hydB</i>	None	< 0.02	NG
SE-31	<i>hydB</i>	pSE3-1Chr	0.56	4.6
SE-19-1	<i>hydA</i>	None	< 0.02	NG
SE-19-1	<i>hydA</i>	pSE3-1Chr	0.38	4.4
SE-31	<i>hydB</i>	pSE3-1PV	0.33	3.7
SE-19-1	<i>hydA</i>	pSE3-1PV	0.73	4.4
SE-31	<i>hydB</i>	pSE3-1PVSp	0.3	4.0
SE-19-1	<i>hydA</i>	pSE3-1PVSp	0.4	4.7

NG, No growth.

* A unit of activity is defined as the appearance of 0.1% ²H₂ h⁻¹ 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 (pSE3-1ChrH) 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 *Bam*HI/*Sau*3A–*Eco*RI insert fragment contains a single promoter for two genes with the first gene extending past the first *Nru*I 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.

Fig. 3. Restriction digest maps of plasmids from *P. vulgaris*. The restriction sites and their abbreviations are: B/Sa, *Bam*HI–*Sau*3A junction of insert and vector; Nd, *Nde*I; PvI, *Pvu*I; RV, *Eco*RV; Sp, *Sph*I. There are no sites in the insert for *Ava*I, *Ba*II, *Cl*aI, *Eco*RI, *Hind*III, *Kpn*I, *Nhe*I, *Nru*I, *Pst*I, *Pvu*II, *Sal*I, *Sca*I and *Sty*I.

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 *Eco*RV gave a 2.7 kb fragment containing the 0.4 kb *Sph*I–*Eco*RV fragment of pBR322. Treatment of pSE3-1PV with *Pvu*I gave a 2.6 kb fragment containing the 1.1 kb *Pvu*I–*Bam*HI/*Sau*3A fragment of pBR322. Ligation of these fragments to pBR322 cut with *Eco*RV or *Pvu*I 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 *Sph*I–*Sph*I 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 (pSE3-1PVPvI) 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 *Sph*I–*Sph*I 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,

Table 2. Southern hybridization among different DNA fragments

Probe	Hybridization* to restriction-enzyme-digested DNA from:						
	pSE-130	pSE-201	pSE3-1PV	pSE3-1Chr	<i>E. coli</i>	<i>P. vulgaris</i>	<i>C. vinosum</i>
<i>E. coli</i> <i>hydB</i> †	+	—	—	—	+	—	—
<i>E. coli</i> <i>hydA</i> ‡	—	+	—	—	+	—	—
<i>P. vulgaris</i> <i>hydAB</i> §	—	—	+	—	—	+	—
pSE3-1Chr	ND	ND	ND	+	—	—	+

* +, 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*).

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* *hydB* 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, pSE3-1PV, restored FHL activity in both mutant strains, whereas the *C. vinosum* plasmid, pSE3-1Chr, 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 viologen 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

Table 3. FHL activity in mutant strains transformed by plasmids

Recipient strain	<i>hyd</i> genotype	Plasmid	FHL activity* [nmol H ₂ evolved min ^{−1} (mg protein) ^{−1}]
K12W6	Wild-type	None	42
SE-31	<i>hydB</i>	None	0
SE-31	<i>hydB</i>	pSE-128	154
SE-31	<i>hydB</i>	pSE3-1Chr	94
SE-31	<i>hydB</i>	pSE3-1PV	116
SE-19-1	<i>hydA</i>	None	0
SE-19-1	<i>hydA</i>	pSE-201	94
SE-19-1	<i>hydA</i>	pSE3-1Chr	10
SE-19-1	<i>hydA</i>	pSE3-1PV	148

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

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 (1988*a*, *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.

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.

experiment.) Sankar *et al.* (1988) reported that the 72 and 78 kDa proteins were products of the *fdv* and *fhlA* 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.*

Fig. 5. Western blot of solubilized membrane preparations with antibodies to *E. coli* anti-hydrogenase 1. A and B, *E. coli* strain SE-31 (*hydB*) transformed by plasmid pSE-128 (A, Lubrol solubilized; B, Triton solubilized); C, *P. vulgaris* solubilized with Lubrol; D and E, *E. coli* strain SE-31 transformed by plasmid pSE3-1PV (D, Lubrol solubilized; E, Triton solubilized); F, *C. vinosum* solubilized with Lubrol; G and H, *E. coli* strain SE-31 transformed by plasmid pSE3-1Chr (G, Lubrol solubilized; H, Triton solubilized); I, purified *E. coli* hydrogenase 1; J, wild-type *E. coli* cells. Sample A, 10 µg; B and F, 5 µg; all others 2 µg.

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 1 while the *E. coli hydB* mutant transformed by the cloned DNA fragment from *C. vinosum* synthesizes *E. coli* hydrogenase 1. 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 *fhlA* 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 pSE3-1Chr 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 hydrogenlyase activity in a *fhlA* 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 *fhlA* gene (2 kb) lies immediately to the right of the *hydB* gene.

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