Isolation of a Gene Required for Growth of *Escherichia coli* on Fumarate and H₂

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Two mutant strains of *Escherichia coli*, AK11 and AK22, express normal levels of hydrogenase activity, assayed by deuterium exchange, when grown on glucose or complex medium but cannot reduce methyl viologen by H₂ nor grow on fumarate plus H₂. The mutant strains also lack formate hydrogenlyase and formate dehydrogenase activities. The mutation in these strains was located near minute 17 of the genome map and a single mutation was shown to be responsible for loss of both hydrogen uptake and formate-related activities. Membrane vesicles and solubilized membranes of strains AK11 and AK22 were capable of methyl viologen reduction by H₂ and had the normal complement of hydrogenase isoenzymes 1 and 2. Intact cells of the mutant strains could reduce fumarate by H₂ but could not grow under these conditions. A plasmid, pAK11, was isolated, as well as smaller plasmids derived from it, which restored the hydrogen uptake activities in the two mutant strains, the smallest active DNA fragment being 1.4 kb. The formate activities were partially restored by some of the plasmids. The plasmids which restored hydrogen uptake activities led to synthesis of a polypeptide of subunit molecular mass 30 kDa.

INTRODUCTION

Anaerobic growth of *Escherichia coli* cells on glucose, in the absence of added electron acceptors, leads to H₂ production. Glucose is degraded via pyruvate to acetate and formate, and the latter is then cleaved to CO₂ and H₂ by 'formate hydrogenlyase', composed of formate dehydrogenase and hydrogenase. Cells can grow on glucose even in the absence of hydrogenase or when hydrogenase activity is inhibited (Krasna, 1980, 1984). Maximal levels of hydrogenase activity are expressed when hydrogenase is essential for anaerobic growth, and such growth ceases when hydrogenase is inhibited (Krasna, 1980, 1984). These conditions are achieved when cells are grown on fumarate as the sole carbon source in the presence of H₂ gas. In this case, H₂ is used as electron donor and fumarate, the electron acceptor, is reduced to succinate (Macy *et al.*, 1976; Yamamoto & Ishimoto, 1978) in a pathway which generates ATP by oxidative phosphorylation. This pathway is dependent on fumarate reductase as well as electron carriers which couple this enzyme to H₂ as electron donor (Cole *et al.*, 1985; Lambden & Guest, 1976; Spencer & Guest, 1973). There are three distinct hydrogenase isoenzymes in *E. coli* required for H₂ evolution and uptake under different conditions of growth (Sawers *et al.*, 1985).

The reduction of fumarate can also be coupled to the oxidation of glycerol-3-phosphate, NADH, lactate, formate and dihydroorotate, with a specific enzyme being required for each substrate (Cole *et al.*, 1985). This occurs via a membrane-linked electron-transport chain composed of a *b*-type cytochrome, a lipid-soluble quinone, fumarate reductase, and perhaps electron carriers specific for each oxidizable substrate. Mutations in any of the components of the pathway would inhibit growth on the specific substrate and fumarate.

Abbreviations: FDH-1, formate dehydrogenase 1; FDH-2, formate dehydrogenase 2; FHL, formate hydrogenlyase.

E. coli strains with mutations leading to the loss of all hydrogenase activity (genotype *hyd*⁻) fail to grow on fumarate plus H₂ (Krasna, 1984; Lee *et al.*, 1985; Sankar *et al.*, 1985; Chaudhuri & Krasna, 1987) but grow on glycerol plus fumarate, indicating the presence of active fumarate reductase. This laboratory (Krasna, 1984) has isolated two mutant strains of *E. coli* (AK11 and AK22) which show a hydrogen-uptake-negative phenotype, Hup⁻ (failure to reduce methyl viologen with H₂ and failure to grow on H₂ plus fumarate), but express normal levels of hydrogenase, as measured by the deuterium exchange assay, when grown anaerobically on glucose or complex medium (genotype *hup*). These mutant strains grow on glycerol plus fumarate. The unique characteristic of these strains is that the hydrogenase activity is only detected in intact cells by the exchange assay.

Failure to grow on fumarate plus H₂, in the presence of fumarate reductase and hydrogenase, could be due to a number of possible mutations in the H₂-fumarate pathway. Lee *et al.* (1985) isolated mutant strains of *E. coli* (called class I *hup* mutants) with a Hup phenotype similar to strains AK11 and AK22. The class I *hup* mutant strains mapped near minute 65 of the *E. coli* genome map.

This report describes a DNA fragment isolated from wild-type *E. coli* cells which restores the normal hydrogen uptake phenotype to mutant strains AK11 and AK22. The genotypic and phenotypic characteristics of the mutation are defined.

METHODS

Organisms and growth conditions. The wild-type strain used in this investigation was *E. coli* K12W6 *met bio* (ATCC 25019); the two mutant strains AK11 and AK22 were derived from this strain by mutagenesis with ultraviolet light and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, respectively (Krasna, 1984). *E. coli* strains MV10 *trpE* and N99 *galK* were obtained from Drs D. Figurski and M. Gottesman of the Department of Microbiology, Columbia University, and were used as recipients for P1 *c100* transduction.

Bacteria were grown in complex or minimal medium, the composition of which was given by Chaudhuri & Krasna (1987). The complex medium was supplemented with glucose (0.5%) or formate (0.2%) and the minimal medium contained glucose (27 mM) or fumarate (43 mM) as sole carbon source. Where appropriate, ampicillin or tetracycline was added to a final concentration of 60 µg ml⁻¹ or 15 µg ml⁻¹, respectively. For enzyme assays, cells were grown anaerobically in completely filled bottles. Growth on fumarate plus H₂ was achieved by bubbling H₂ into the growth medium. Protein was determined by the Lowry method.

Enzyme assays. The enzyme activities of interest (see below) were measured at 25 °C in washed cell suspensions, crude extracts, membrane vesicles and solubilized membranes. Washed cells were suspended in 75 mM-sodium phosphate buffer, pH 6.7, and broken by ultrasonic treatment for 3 min. Crude extracts were prepared by centrifugation at 13000 *g*. The membrane fraction was prepared by centrifugation of the crude extracts at 130000 *g* and the membranes in the pellet were solubilized as described by Ballantine & Boxer (1985).

Hydrogenase (EC 1.12.1.2) was assayed by the deuterium exchange method (Krasna, 1978) in 10% ²H₂O. One unit of activity is defined as the appearance of 0.1% ²H¹H h⁻¹ in the gas phase, which is equivalent to reaction with 0.287 µmol H₂ min⁻¹. Methyl viologen reduction by H₂ was measured spectrophotometrically at 600 nm in the presence of small quantities of sodium dithionite to maintain anaerobiosis. The assay mixture contained cells, membrane vesicles or solubilized membranes, and methyl viologen (10 mM), in 0.33 M-phosphate buffer, pH 6.7. The mixture was gassed with H₂ and the reaction started by addition of sodium dithionite to raise the absorbance at 600 nm to 0.2 and the increase in absorbance was then measured. A unit of activity is defined as the H₂-catalysed reduction of 1 nmol methyl viologen min⁻¹. Formate dehydrogenase 1 (FDH-1; EC 1.2.2.3), formate dehydrogenase 2 (FDH-2; EC 1.2.1.2) and 'formate hydrogenlyase' (FHL, comprising hydrogenase and FDH-2) were assayed as previously described (Chaudhuri & Krasna, 1987), one unit of activity in all cases being defined as the oxidation of 1 nmol formate min⁻¹. Reduction of fumarate (17 mM) by H₂ was measured manometrically, one unit of activity being the uptake of 1 nmol H₂ min⁻¹.

Native gel electrophoresis. Polyacrylamide electrophoresis was done on solubilized membranes with a 6.5% (w/v) separating gel and a 3% (w/v) stacking gel (Ballantine & Boxer, 1985) at pH 9.5 and 8.0 (Maurer, 1971). The pH 9.5 system maintained stacking at pH 8.3 and separation at pH 9.5 with a Tris/glycine running buffer at pH 8.3. The pH 8.0 system maintained stacking at pH 7.0 and separation at pH 8.0 with a Tris/barbital running buffer at pH 7.0. The gels were placed in 100 mM-sodium phosphate buffer, pH 6.7, containing 1 mM-methyl viologen and 1 mM-2,3,5-triphenyltetrazolium chloride and placed in a H₂ atmosphere overnight. Hydrogenase activity was revealed as intense red zones.

Genetic techniques. Genetic mapping was done by hybridizing cloned DNA fragments to a *NotI* macro-restriction digest of *E. coli* DNA separated by pulsed-field gel electrophoresis as described by Smith *et al.* (1987).

We are indebted to Dr C. L. Smith of the Department of Genetics and Development, Columbia University, for these hybridization studies. Fine mapping was carried out by P1 transduction (Miller, 1972). Transposon Tn10 was introduced into strain AK11 by generalized transduction with bacteriophage P1 c100. The P1 c100 lysate was prepared from *E. coli* strain MC4100 (F⁻ *araD139* Δ *lacU169* *rpsL150* *relA1* *flbB5301* *thi* *ptsF25* *deoC1* *rbsR*) containing random Tn10 insertions (provided by Dr H. Shuman of the Department of Microbiology, Columbia University). Tetracycline-resistant transductants of the recipient strains were plated on selective media plus tetracycline (20 μ g ml⁻¹) and screened for the genetic marker of interest.

Screening methods for hydrogenase. The symbols Hup⁻ and *hup* (hydrogen uptake) designate the phenotype and genotype of cells which have hydrogenase activity when assayed by the exchange reaction but do not reduce viologen dyes by H₂ nor grow on fumarate plus H₂. 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, and growth on fumarate plus H₂ (Krasna, 1984; Lee *et al.*, 1985). The details of the screening methods were described by Chaudhuri & Krasna (1987). Strains AK11 and AK22 are Hup⁻ by these screening methods and when the mutation is complemented by a cloned DNA fragment they show the Hup⁺ phenotype. To prove the complementation, the transformed cells are then grown on fumarate plus H₂ and show high levels of hydrogenase activity by the deuterium exchange assay.

Cloning the gene which restores activity. The *E. coli* chromosomal DNA library prepared from strain K12W6 was described by Chaudhuri & Krasna (1987). This recombinant plasmid library was prepared by ligating plasmid pBR322 treated with *Bam*HI to chromosomal DNA partially digested with *Sau*3A. This DNA library was used to transform strains AK11 and AK22 with good efficiencies. A large number (about 40000) of Ap^R colonies were grown in fumarate plus H₂ to enrich for Hup⁺ cells (Chaudhuri & Krasna, 1987). These cells were then plated on agar and further tested for the Hup phenotype.

None of the transformants from strain AK22 were Hup⁺. From the transformation of strain AK11, a large number of Hup⁺ colonies were detected. Fifteen separate isolates were then selected for further study. Plasmid DNA was prepared from each isolate and used to transform strain AK11 to the Hup⁺ phenotype. In each case, all Ap^R transformants were Hup⁺ by all criteria, including high levels of deuterium exchange in cells grown on fumarate plus H₂. The separate plasmids were mapped by restriction enzyme digestion and found to be identical; the plasmid was designated as pAK11.

Synthesis of plasmid-coded proteins. The maxicell procedure of Sancar *et al.* (1979) was followed except that a mixture of ¹⁴C-labelled amino acids was used in place of labelled methionine. *E. coli* strain CSR603 (Sancar *et al.*, 1979) was obtained from the *E. coli* Genetic Stock Center, Yale University.

RESULTS AND DISCUSSION

Restoration of the Hup⁺ phenotype in strain AK11 by cloned DNA fragments

E. coli mutant strains AK11 and AK22 (Krasna, 1984) are not hydrogenase-negative since when grown anaerobically on glucose they have the same hydrogenase activity, as measured by deuterium exchange, as the wild-type parent, strain K12W6. They are Hup⁻ when screened for reduction of viologen dyes and cannot grow on fumarate plus H₂, although they grow on glycerol plus fumarate. None of the plasmids described previously (Sancar *et al.*, 1985; Chaudhuri & Krasna, 1987), which restored activity in hydrogenase-negative strains with mutations in *hydA*, *hydB* and *hydE*, restored activity in strains AK11 and AK22. A plasmid, pAK11 (10.9 kb, with a 6.5 kb chromosomal DNA insert), was isolated from strain AK11 transformed by an *E. coli* DNA library which restored the Hup⁺ phenotype in strain AK11. This plasmid also restored the hydrogen uptake activities in strain AK22, suggesting that the mutations in the two strains are close to each other, if not in the same gene. This plasmid did not complement *hydA*, *hydB* or *hydE* mutations.

Table 1 reports the hydrogenase activity, as measured by deuterium exchange, for strain AK11 transformed by pAK11. There was little change in this activity in cells grown anaerobically on glucose, but the transformants grew on fumarate plus H₂ and expressed high levels of hydrogenase activity. Thus, the cloned DNA fragment restores the ability of the cells to grow on fumarate plus H₂ as well as the ability to reduce viologen dyes by H₂ (see below, Table 3). The restriction digest map of pAK11 is shown in Fig. 1. The insert contains single cleavage sites for *Eco*RV, *Hind*III, and *Bal*I, three sites for *Eco*RI, and four sites for *Pvu*II. pAK11 was treated with these enzymes to give smaller fragments containing different parts of the insert DNA fragment and these were used to transform strain AK11. The results are summarized in Fig. 1 and Table 1.

Fig. 1. Restriction digest maps of plasmids studied in strain AK11. The circular plasmids are written in linear form for ease of comparison. The heavy line is the vector plasmid and the light line is the chromosomal DNA insert. The numbers beneath the line indicate the size, in kb, of the fragment between the two indicated restriction sites. The letters indicate the restriction sites and the abbreviations are: B/Sa, *Bam*HI-*Sau*3A junction of vector and insert; Ba, *Bal*I; H, *Hind*III; N, *Nru*I; Pv, *Pvu*II; RI, *Eco*RI; RV, *Eco*RV. There are additional sites in pAK11 for *Nru*I and *Pvu*II to the right of the indicated sites. There is an additional *Eco*RI site in the insert fragment between the two sites indicated.

Plasmid pAK11H did not restore the Hup⁺ phenotype in strain AK11, suggesting that the 1.3 kb insert fragment to the left of the *Hind*III site in pAK11 is required for activity. pAK11B (Fig. 1), with an insert of 2.5 kb, was as active as pAK11 (Table 1). pAK11P, with a chromosomal DNA insert of 1.4 kb, also restored the Hup⁺ phenotype. pAK11P was then digested with *Eco*RV or *Eco*RI to give the smaller plasmids pAK11PRV and pAK11PRI (Fig. 1). Both were inactive, suggesting that the 0.3 kb fragment at the extreme left (as drawn) of the 1.4 kb insert in pAK11P is essential for activity. Similar results to those reported in Table 1 were obtained with mutant strain AK22.

Lee *et al.* (1985) isolated mutant strains of *E. coli* (called class I hup mutants) which mapped near minute 65 of the *E. coli* genome map and had a Hup phenotype similar to strains AK11 and AK22. One strain of this class, strain SE-29 (a Tet^s derivative of strain SE-8, kindly provided by Dr K. T. Shanmugam), was transformed by plasmids pAK11, pAK11B and pAK11P. None of these plasmids complemented the mutation in strain SE-29, suggesting that the mutation in that strain is different from the mutation(s) in strains AK11 and AK22. This conclusion will be confirmed by experiments reported below.

Table 1. *Hydrogenase exchange and formate-related activities of E. coli strain AK11 transformed by plasmids*

Recipient strain	Plasmid	Exchange specific activity* [units (mg protein) ⁻¹]		Formate activities† [nmol formate oxidized min ⁻¹ (mg protein) ⁻¹]		
		Glucose grown	Fumarate + H ₂ grown	FHL	FDH-2	FDH-1
K12W6	None	0.6	1.3	55	87	67
AK11	None	0.6	NG	0	8	3
AK11	pAK11	0.6	2.0	12	9	7
AK11	pAK11H	ND	NG	ND	ND	ND
AK11	pAK11B	0.8	3.1	15	27	8
AK11	pAK11P	0.9	2.7	3	0	5

ND, Not determined.

NG, No growth.

* These values are the means of two or three separate determinations with different samples of intact cells. The maximum deviation from the mean was $\pm 10\%$.

† Cells were grown in LB broth plus 0.2% formate. The values reported are for intact cells and are the means of four separate determinations. The maximum deviation from the mean was $\pm 15\%$. With crude extracts the values were zero for all samples except K12W6 where the values were 80–90% of those found in intact cells.

Growth in high concentrations of $NiCl_2$, which restores hydrogenase activity to *hydE* mutant strains of *E. coli* (Vaugh & Boxer, 1986; Chaudhuri & Krasna, 1987) did not change the Hup phenotype of strains AK11 and AK22.

Formate-related activities of strain AK11 transformed by plasmids

Mutant strain AK11 was assayed for H_2 evolution from formate (FHL) and for two formate dehydrogenase activities, FDH-2 (benzyl-viologen-linked activity; the FDH associated with FHL) and FDH-1 (the FDH associated with nitrate reduction; Ingledew & Poole, 1984). The results summarized in Table 1 show that the mutant strain is devoid of these activities even though it has normal hydrogenase activity as measured by deuterium exchange. The class I *hup* mutant strains isolated by Lee *et al.* (1985) were reported to have normal FHL and FDH activities and are, therefore, different from mutant strain AK11.

Plasmid pAK11, which restored growth on fumarate plus H_2 and reduction of methyl viologen, restored only 22% FHL activity and no FDH-2 activity in strain AK11 (Table 1). pAK11B restored 27% and 22% of the FHL and FDH-2 activities, respectively. pAK11P failed to restore FHL or FDH-2 activities. None of the plasmids restored FDH-1 activity. Similar results to those reported in Table 1 were obtained with mutant strain AK22. Thus, although the three plasmids pAK11, pAK11B and pAK11P restore the Hup⁺ phenotype completely in strains AK11 and AK22, they vary in their ability to restore the formate activities. A similar observation of variable ability of different plasmids to restore formate-related activities in a *hydE* mutant of *E. coli* (though they all restored hydrogenase activity) has been previously reported (Chaudhuri & Krasna, 1987).

Synthesis of plasmid-coded proteins

The size and number of polypeptides synthesized by the pAK11 plasmids was determined by the maxicell procedure of Sancar *et al.* (1979) (Fig. 2). The plasmid vector pBR322 coded for three polypeptides of molecular masses 43, 33 and 28 kDa, which correspond to the three proteins reported to be encoded by this plasmid (Sancar *et al.*, 1979). The 33 and 28 kDa polypeptides are products of the *amp* gene and the 43 kDa polypeptide is the product of the *tet* gene. Plasmids pAK11, pAK11B and pAK11P, all of which restored the hydrogen uptake activities in strains AK11 and AK22, led to the synthesis of a new polypeptide of molecular mass 30 kDa. This polypeptide is readily distinguishable from the 28 kDa polypeptide synthesized by the plasmid vector because it is observable after very short autoradiography whereas the 28 kDa

Fig. 2. Autoradiograph of an SDS-polyacrylamide gel of polypeptides expressed in maxicells (*E. coli* CSR603) by pAK11-derived plasmids: A, pBR322; B, pAK11; C, pAK11B; D, pAK11P; E, pAK11H; F, pAK11PRV.

polypeptide is not. This polypeptide was not observed with pAK11H, which did not restore the Hup⁺ phenotype. pAK11PRV, while inactive in restoring the Hup⁺ phenotype, led to the synthesis of the 30 kDa polypeptide, but at a much reduced level compared to the other plasmids. Thus the gene product which restores hydrogen uptake activities in strains AK11 and AK22 may be a protein with subunits of molecular mass 30 kDa. The molecular mass reported may not be the actual size of the subunit. The protein band observed could be a hybrid protein created by fusion of the insert and plasmid DNA.

Mapping the mutation in strain AK11

To confirm that the mutation in strain AK11 was different from the class I *hup* mutant strains described by Lee *et al.* (1985), it was necessary to map the mutation. Since a cloned DNA fragment which restores the Hup⁺ phenotype to the mutant strain was available, the mutation could be mapped by hybridization of the cloned DNA to a macro-restriction map of the entire genome of *E. coli*. Smith *et al.* (1987) have prepared such a map by *NotI* macro-restriction digestion of *E. coli* DNA and size separation of the resulting DNA fragments by pulsed-field gel electrophoresis followed by hybridization to cloned DNA fragments of known genetic locations.

Plasmid pAK11 was hybridized to a separated *NotI* digest of *E. coli* DNA and did not bind to band A (1000 kb), which represents minutes 58–82 of the *E. coli* genome map. As a control, plasmid pAK23, which restores activity to a *hydE* mutation that maps at minute 59 (Chaudhuri & Krasna, 1987), did hybridize to band A. This clearly establishes that the mutation in strain AK11 is not at minute 65, a conclusion already suggested by experiments described above. pAK11 hybridized to a band consisting of band P (100 kb, minutes 14–17) and band R (95 kb, minutes 25–27).

To determine whether the mutation was at minutes 14–17 or 25–27, the wild-type gene was isolated and mapped by P1 transduction. A P1 c100 lysate was prepared from an *E. coli* strain

Table 2. *Hydrogen uptake and formate phenotypes of P1 transductants*

Recipient strain and phenotype	Donor strain and phenotype	Selected phenotype (and frequency†)	Activity* [nmol formate oxidized min ⁻¹ (mg protein) ⁻¹]		
			FHL	FDH-2	FDH-1
K12W6 (Tet ^S Hup ⁺)	—	—	55	87	67
AK11 (Tet ^S Hup ⁻)	—	—	0	8	3
AK11 (Tet ^S Hup ⁻)	MC4100 (Tet ^R Hup ⁺)	Tet ^R Hup ⁺ (45%)	98	107	55
AK11 (Tet ^S Hup ⁻)	AK11 (Tet ^R Hup ⁺)	Tet ^R Hup ⁻ (40%)	0	0	2
K12W6 (Tet ^S Hup ⁺)	AK11 (Tet ^R Hup ⁻)	Tet ^R Hup ⁻ (65%)	0	0	0

* The values were obtained with intact cells and are the means of four separate determinations on each of two selected transductants of each phenotype. The maximum deviation from the mean was $\pm 15\%$.

† The frequency of the selected phenotype was determined in 200–300 transductants in each case.

(MC4100) containing random *Tn10* insertions (Tet^RHup⁺). Transposon *Tn10* was introduced into strain AK11 (Tet^SHup⁻) by generalized transduction with P1 c100. Tet^R transductants were plated and screened for the Hup⁺ phenotype. Of 200 transductants screened, 9% were Hup⁺. Lysates were prepared from these AK11 (Tet^RHup⁺) transductants by infection with P1 c100. These lysates, which contained the wild-type gene not expressed in strain AK11, were used to transduce a *trpE* mutant (minute 27.5) and a *galK* mutant (minute 16.7) to Tet^RTrp⁺ and Tet^RGal⁺, respectively. Of 200 transductants from the *trpE* mutant, all were Tet^RTrp⁻, establishing that the mutation in strain AK11 is not near minute 27. Transduction of the *galK* mutant gave Tet^R transductants which were also Gal⁺ (45% of 200 transductants), showing that the AK11 mutation is near minute 17 on the *E. coli* genome map.

None of the reported hydrogenase-related or formate-related genes map near minute 17. *frdA*, *B*, *C* and *D*, genes involved with fumarate reductase, map at minute 94.4 (Cole *et al.*, 1985). The *fnr* gene, which pleiotropically regulates hydrogenase synthesis, maps near minute 29 (Ingledew & Poole, 1984; Tait, 1982) and mutations in this gene lead to complete loss of hydrogenase activity. Various formate dehydrogenase genes map at minutes 38, 80, 82, 87 and 92.4 (Ingledew & Poole, 1984; Tait, 1982). The succinate dehydrogenase gene maps at minute 16.7 and is induced aerobically and repressed anaerobically (Ingledew & Poole, 1984). Strains AK11 and AK22 have the normal succinate-positive phenotype. We propose that the *hup* gene at minute 65 be designated as *hupA* and the *hup* gene at minute 17 as *hupB*.

With the *hup* mutation in strain AK11 mapped, it was necessary to establish whether the formate-related mutation was due to the same mutation or to a second unrelated mutation. This was accomplished by a series of P1 transductions and analysis of the transductants for both the hydrogen uptake and the formate-related phenotypes. The results are presented in Table 2. Transduction of strain AK11 (Tet^SHup⁻) by a P1 lysate prepared from a Tet^RHup⁺ wild-type donor yielded strain AK11 with a Tet^RHup⁺ phenotype (described above). These transductants all had high levels of the three formate-related activities (Table 2), suggesting that the hydrogen uptake and formate mutations in strain AK11 may be due either to the same mutation or to two very close mutations. Strain AK22 (Tet^SHup⁻) was also transduced to the Tet^RHup⁺ phenotype and all transductants had recovered the formate-related activities along with the hydrogen uptake activities. Strains AK11 and AK22 have very similar properties although they were isolated by different mutagenic procedures (AK11 by ultraviolet and AK22 by nitrosoguanidine mutagenesis). The restoration of both hydrogen uptake and formate phenotypes in both strains by transduction from a wild-type donor suggests that there is a single very similar mutation in both strains rather than multiple mutations.

This was further confirmed by transferring the *hup* mutation to the wild-type parent K12W6 (Table 2). First the Tet^R marker was introduced next to the *hup* mutation in strain AK11 by P1 transduction of strain AK11 (Tet^SHup⁻) with DNA from strain AK11 (Tet^RHup⁺) and selection of transductants with the Tet^RHup⁻ phenotype. As expected, the transductants lacked both hydrogen uptake and formate activities (Table 2). This strain was then used as donor to

Table 3. *Reduction of methyl viologen by H₂ in strain AK11*

Strain	Plasmid	Activity* [nmol methyl viologen reduced min ⁻¹ (mg protein) ⁻¹]		
		Intact cells	Membrane vesicles	Solubilized membranes
K12W6	None	6	6	17
AK11	None	2	17	49
AK11	pAK11	9	22	89
AK11	pAK11B	8	ND	ND
AK11	pAK11P	7	ND	ND

ND, Not determined.

* The values reported are the means of two separate determinations on each of three separate preparations. The maximum deviation from the mean was $\pm 10\%$.

strain K12W6 and the Tet^RHup⁻ transductants were found to lack all the formate-related activities (Table 2).

It is thus clear that the mutation in strains AK11 and AK22 which leads to a Hup⁻ phenotype also leads to loss of all formate-related activities. In spite of the single mutation (or perhaps two very close mutations), the cloned DNA fragments which restore the Hup⁺ phenotype do not restore the formate phenotypes completely (see Table 1). Plasmid pAK11 hybridized to the chromosomal DNA of strains AK11 and AK22, showing that the mutant strains did not have large deletions in the genes involved.

Hydrogen uptake activities in strain AK11

Strains AK11 and AK22 clearly synthesize active hydrogenase when grown on glucose or complex medium. However, in whole cells the enzyme is active only for deuterium exchange, and not for reduction of viologen dyes by H₂. The relationship between the loss of this catalytic activity and the failure to grow on fumarate plus H₂ is not clear. Failure to grow on fumarate plus H₂, in the presence of hydrogenase and fumarate reductase, could be due to a number of possible mutations. The mutation could be in a gene regulating synthesis of isoenzyme 2, the uptake hydrogenase (Ballantine & Boxer, 1985; Sawers *et al.*, 1985), with isoenzymes 1 and 3 (required for H₂ evolution under different conditions of growth) being produced normally when cells are grown on glucose. The mutation could be in a gene coding for an electron-transport protein required for the H₂-fumarate pathway but not for the glycerol-fumarate pathway, with all hydrogenase isoenzymes being synthesized normally. Alternatively, the H₂-fumarate electron transport pathway may be normal, the mutation being in a gene coding for a protein product required for energy production during electron transport in the H₂-fumarate pathway.

The different hydrogenase activities of strain AK11 as well as the isoenzyme profile were therefore examined. The first question to be addressed is why the active hydrogenase isoenzymes in this strain grown on glucose or complex medium do not catalyse reduction of methyl viologen by H₂. Is this an inherent property of these isoenzymes or is it only manifested in intact cells? Membrane vesicles and solubilized membranes were prepared from the strains of interest and tested for reduction of methyl viologen. The membrane vesicles and solubilized membranes prepared from cells of strain AK11 (cells did not reduce methyl viologen by H₂) reduced methyl viologen quite well, demonstrating that the hydrogenase isoenzymes released from the intact cell are fully capable of reducing methyl viologen. This is similar to the finding of Glick *et al.* (1980) with a different mutant strain; however, the latter strain grew on fumarate plus H₂ (tested in this laboratory) and is different from strain AK11.

Reduction of methyl viologen by H₂, in the absence of small quantities of sodium dithionite, gave rates (even in active preparations) that were too low to give accurate quantitative data. By addition of small quantities of sodium dithionite to remove residual oxygen, quantitative data were obtained as shown in Table 3. The small activity in strain AK11 could be real or due to some changes caused in the cell wall or membrane by the dithionite. In all cases, the activity in

Fig. 3. Native gel electrophoresis of solubilized membranes. The gels were stained for hydrogenase activity by reduction of methyl viologen by H_2 as outlined in Methods. Panel (a) is from electrophoresis with the pH 8.0 system, panel (b) with the pH 9.5 system. A, B and C, glucose-grown cells; D and E, cells grown on fumarate plus H_2 . A, K12W6; B, AK11; C, AK11 with plasmid pAK11; D, K12W6; E, AK11 with plasmid pAK11.

intact cells was restored by the cloned DNA fragments. The membrane vesicles and solubilized membranes from the mutant strain were fully capable of methyl viologen reduction and were even more active than those of the parental strain K12W6. Similar results to those reported in Table 3 were obtained with mutant strain AK22. The differences reported in Table 3 for reduction of methyl viologen by H_2 in intact cells, membrane vesicles and solubilized membranes may reflect differences in the permeability of methyl viologen to hydrogenase in cells, vesicles and solubilized membranes. Jones & Garland (1977) reported that oxidized or reduced methyl viologen, as well as dithionite anion, did not rapidly cross the *E. coli* cytoplasmic membrane, whereas reduced benzyl viologen did.

Since the solubilized membranes are capable of methyl viologen reduction, they could be analysed for the isoenzyme profile. The results are presented in Fig. 3 for strain AK11; identical results were obtained for strain AK22. The bands are referred to by the designation used by Ballantine & Boxer (1985), band 1 representing isoenzyme 1 (required for H_2 evolution) and bands 2 and 3 together representing isoenzyme 2 (required for H_2 uptake). At pH 8.0, all three bands are observed in the parent strain K12W6 (lane A), the mutant strain AK11 (lane B), and strain AK11 transformed by the plasmid pAK11 (lane C), all grown on glucose. At pH 9.5, only band 1 is observed since isoenzyme 2 is irreversibly inactivated at high pH (Ballantine & Boxer, 1985). In cells grown on fumarate plus H_2 (lanes D and E), only isoenzyme 2 is observed at pH 8.0 (bands 2 and 3), isoenzyme 1 content being negligible under these conditions (Sawers *et al.*, 1985). At pH 9.5, no bands are observed (Fig. 3).

It is thus clear that strains AK11 and AK22 contain the normal complement of hydrogenase isoenzymes 1 and 2, and the failure to grow on fumarate plus H_2 cannot be ascribed to a lack of isoenzyme 2. To determine whether there is a deficiency in electron transport from H_2 to fumarate in these strains, reduction of fumarate by H_2 was measured in intact cells. Surprisingly, strains AK11 and AK22 showed about half the activity of the parental strain [9 nmol H_2 uptake $\text{min}^{-1}(\text{mg protein})^{-1}$ in mutant strains versus 19 in parental strain]. The activity was increased in cells transformed by plasmid pAK11 (to a specific activity of 15). Crude extracts of all cells could not reduce fumarate by H_2 , suggesting that disruption of the cells destroys the integrity of the electron-transport chain. Lee *et al.* (1985) reported that cells of class I *hup* mutant strains completely lack the ability to reduce fumarate by H_2 . This is yet another indication that the class I mutant strains are different from strains AK11 and AK22.

In spite of the ability of strains AK11 and AK22 to reduce fumarate by H_2 , they show no growth under these conditions. The lower levels of this activity (about half that of the parent or of cells transformed by plasmid pAK11) may be insufficient to support growth. Alternatively, the electron-transport rate may be adequate but the mutation could be in a gene required to couple this electron-transport pathway to production of ATP required as an energy source for growth. The interesting observation is that this mutation also leads to the inability of intact cells to reduce methyl viologen by H_2 although the hydrogenase isoenzymes 1 and 2 are all present and capable of carrying out this reaction. This mutation also leads to loss of formate-related activities.

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