Genetic Complementation between Two Mutant *unc* Alleles (*uncA401* and *uncD409*) Affecting the F₁ Portion of the Magnesium Ion-Stimulated Adenosine Triphosphatase of *Escherichia coli* K12

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(Received 9 August 1977)

A new mutant strain of *Escherichia coli* in which phosphorylation is uncoupled from electron transport was isolated. A genetic-complementation analysis, using partial diploid strains, showed that the new mutant allele, *uncD409*, is in a gene distinct from the other previously identified genes *uncA*, *uncB* and *uncC*. A strain carrying the *uncD409* allele has no Mg²⁺ ion-stimulated adenosine triphosphatase activity and is therefore phenotypically similar to strains carrying the *uncA401* mutant allele. Complementation between the *uncA401* and the *uncD409* alleles occurred, as indicated by growth of partial diploid strains on succinate and their growth yields on limiting concentrations of glucose. Complementation was confirmed by using membranes prepared from the above partial diploids. Such membranes were found to have Mg²⁺-stimulated adenosine triphosphatase activity, ATP-dependent transhydrogenase activity, ATP-induced atebirin-fluorescence quenching and low but significant amounts of oxidative phosphorylation.

Mutations in the *uncA*, *uncB* and *uncC* genes have been shown to affect oxidative phosphorylation in *Escherichia coli* K12 (Butlin *et al.*, 1971, 1973; Gibson *et al.*, 1977b). The *uncA* and *uncB* genes were originally distinguished on biochemical grounds in that a mutation in the *uncA* gene caused loss of Mg-ATPase activity (i.e. the mutation affected the F₁ portion of the Mg-ATPase complex), whereas a mutation in the *uncB* gene did not affect the Mg-ATPase activity. Reconstitution experiments showed (Cox *et al.*, 1973b) that a mutation in the *uncB* gene affected the F₀ portion of the Mg-ATPase complex. The observations that normal *unc* alleles are dominant over mutant *unc* alleles (Gibson *et al.*, 1977a) and the development of a method for incorporating *unc* alleles into plasmids (Gibson *et al.*, 1977b) allowed the characterization, by genetic complementation, of a new mutant allele, *uncC424*. Strains carrying the *uncC424* allele resembled strains carrying the *uncB402* allele in that the Mg-ATPase activity was retained, but in the former strains the membrane-associated Mg-ATPase activity remained sensitive to dicyclohexylcarbodi-imide (Cox *et al.*, 1977). It was concluded that the *uncC* gene coded for a second component of the F₀ portion of the Mg-ATPase complex.

The present paper describes complementation

Abbreviations used: Mg-ATPase, Mg²⁺-stimulated adenosine triphosphatase (EC 3.6.1.3); Tes, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

between the *uncA401* allele and the new mutant allele *uncD409*, which like the *uncA401* allele affects Mg-ATPase activity.

Materials and Methods

Chemicals

Chemicals were of the highest purity available commercially and were not further purified. Labelled P_i (carrier-free) was from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia, and used after appropriate dilution with carrier.

Organisms

All the bacterial strains used are derived from *E. coli* K12 and are described in Table 1.

Media and growth of organisms

The mineral-salts minimal medium used and additions, unless otherwise indicated, were as described previously (Gibson *et al.*, 1977b).

Cells for the preparation of membranes were grown in 14-litre New Brunswick Fermenters essentially as described by Cox *et al.* (1970). The inoculum was grown in 1 litre of glucose-mineral salts medium at 37°C overnight with shaking. The inoculum was then added to 10 litres of medium in the fermenter and

Table 1. *Strains of E. coli K12 used*

The gene designations follow those of Bachmann *et al.* (1976) and the plasmid nomenclature is that of Novick *et al.* (1976).

Bacterial strain	Relevant genetic loci	Other information
AB259	Hfr	Hfr Hayes
M54	<i>uncD409</i>	Isolated after treatment of strain AB259 with <i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine
AN346	F ⁻ , <i>ilvC</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i>	Gibson <i>et al.</i> (1977a)
AN817	F ⁻ , <i>uncD409</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i>	Isolated after transduction with strain M54 as donor and strain AN346 as recipient
KL163	Hfr, <i>nalA</i> , <i>recA</i>	Obtained from J. Pittard (University of Melbourne, Melbourne, Vic., Australia)
AN724	F ⁻ , <i>ilvC</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Isolated after mating between strains KL163 and AN346
AN730	F ⁻ , <i>uncA401</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Isolated after co-transduction of <i>uncA401</i> allele (Butlin <i>et al.</i> , 1971) with <i>ilvC</i> ⁺ into strain AN346 followed by mating with strain KL163
AN727	F ⁻ , <i>uncB402</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Isolated after co-transduction of <i>uncB402</i> allele (Butlin <i>et al.</i> , 1973; Gibson <i>et al.</i> , 1977a) with <i>ilvC</i> ⁺ into strain AN346 followed by mating with strain KL163
AN802	F ⁻ , <i>uncC424</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Isolated after co-transduction of <i>uncC424</i> allele (Gibson <i>et al.</i> , 1977b) with <i>ilvC</i> ⁺ into strain AN346 followed by mating with strain KL163
AN818	F ⁻ , <i>uncD409</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Isolated after co-transduction of <i>uncD409</i> allele (this paper) with <i>ilvC</i> ⁺ into strain AN346 followed by mating with strain KL163
Diploids		
AN862	F' (pAN11), <i>ilvC</i> , <i>argH</i> , <i>pyrE</i> , <i>purE</i> , <i>recA</i> , <i>nalA</i>	Strain with plasmid carrying normal <i>unc</i> alleles. Isolated as described by Gibson <i>et al.</i> (1977b)
AN780	F' (pAN2), <i>ilvC</i> , <i>argH</i> , <i>pyrE</i> , <i>purE</i> , <i>recA</i> , <i>nalA</i>	Strain with plasmid carrying <i>uncA401</i> allele. Isolated as described by Gibson <i>et al.</i> (1977b)
AN797	F' (pAN5), <i>ilvC</i> , <i>argH</i> , <i>pyrE</i> , <i>purE</i> , <i>recA</i> , <i>nalA</i>	Strain with plasmid carrying <i>uncB402</i> allele. Isolated as described by Gibson <i>et al.</i> (1977b)
AN805	F' (pAN6), <i>ilvC</i> , <i>argH</i> , <i>pyrE</i> , <i>purE</i> , <i>recA</i> , <i>nalA</i>	Strain with plasmid carrying <i>uncC424</i> allele. Isolated as described by Gibson <i>et al.</i> (1977b)
AN819	F' (pAN7), <i>ilvC</i> , <i>argH</i> , <i>pyrE</i> , <i>purE</i> , <i>recA</i> , <i>nalA</i>	Strain with plasmid carrying <i>uncD409</i> allele. Isolated as described by Gibson <i>et al.</i> (1977b)
AN821	F' (pAN7), <i>ilvC</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Isolated after mating between strains AN819 and AN724
AN823	F' (pAN7), <i>uncA401</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Isolated after mating between strains AN819 and AN730
AN829	F' (pAN2), <i>uncD409</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Isolated after mating between strains AN780 and AN818
Segregants		
AN822	F ⁻ , <i>ilvC</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Spontaneous segregant isolated from strain AN821
AN824	F ⁻ , <i>uncA401</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Spontaneous segregant isolated from strain AN823
AN830	F ⁻ , <i>uncD409</i> , <i>argH</i> , <i>pyrE</i> , <i>entA</i> , <i>recA</i> , <i>nalA</i>	Spontaneous segregant isolated from strain AN729
Plasmids		
pAN11	<i>unc</i> ⁺ , <i>ilvC</i> ⁺ , <i>argH</i> ⁺ , <i>pyrE</i> ⁺	
pAN2	<i>uncA401</i> , <i>ilvC</i> ⁺ , <i>argH</i> ⁺ , <i>pyrE</i> ⁺	
pAN5	<i>uncB402</i> , <i>ilvC</i> ⁺ , <i>argH</i> ⁺ , <i>pyrE</i> ⁺	
pAN6	<i>uncC424</i> , <i>ilvC</i> ⁺ , <i>argH</i> ⁺ , <i>pyrE</i> ⁺	
pAN7	<i>uncD409</i> , <i>ilvC</i> ⁺ , <i>argH</i> ⁺ , <i>pyrE</i> ⁺	

incubated at 37°C with aeration until the cells were in mid-exponential phase. The medium in the fermenter vessels was supplemented with 5% (v/v) Luria broth (Luria & Burrous, 1957).

Solid media were prepared by the addition of 2% (w/v) agar to the medium described above. For agar plates containing succinate as sole source of carbon, glucose was replaced by sodium succinate (30 mM) and,

in addition to the appropriate growth requirements at the concentrations given above, the medium was supplemented with 0.06% acid-hydrolysed casein (Difco, Detroit, MI, U.S.A.).

The nutrient broth used for genetic experiments was Luria broth, with glucose (10mm) included as indicated.

Growth yields were measured as described by Cox *et al.* (1970) in glucose-mineral salts medium supplemented with Luria broth (5%, v/v) as indicated. Turbidities of cultures were measured with a Klett-Summerson colorimeter.

Genetic techniques

The techniques used for genetic experiments were as outlined previously (Gibson *et al.*, 1977a,b). Tests for transfer of plasmids and for complementation were carried out as follows. Donor and recipient strains were grown to early exponential phase (about 100 Klett units) in Luria broth containing 5mm-glucose. The culture of the donor strain was centrifuged, and cells were resuspended in one-tenth the original volume of mineral-salts medium. Recipient cells were then cross-streaked against donor cells on glucose and succinate media and examined for the appearance of transconjugant colonies after 2 days incubation at 37°C.

Preparation of cell membranes

Membranes were prepared as described previously (Cox *et al.*, 1973a). Briefly, washed cells were disintegrated by using a Sorvall Ribi cell fractionator and the membranes were separated by differential centrifugation and resuspended in a 0.1M-Tes buffer system (adjusted to pH7.0 with NaOH) containing magnesium acetate, sucrose and EGTA.

Protein concentrations were determined by using Folin's phenol reagent (Lowry *et al.*, 1951), with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

Assay of Mg-ATPase

Assays for Mg-ATPase activity were carried out as described previously (Gibson *et al.*, 1977a).

Measurement of the ATP-dependent transhydrogenase activity

The reduction of NADP⁺ was assayed by coupling the reaction to the NADPH-dependent glutathione reductase and measuring the decrease in *A*₃₄₀ (Ernster & Lee, 1967). Details of the technique used are described by Cox *et al.* (1971).

Measurement of atebrin fluorescence

Atebrin fluorescence was measured at 30°C as described by Haddock & Downie (1974) by using an Aminco-Bowman fluorimeter with the excitation wavelength set at 450nm and emission wavelength set at 510nm.

Oxidative phosphorylation

P/O ratios were measured as described by Cox *et al.* (1973b).

Results

Isolation of a mutant strain carrying the *uncD409* allele

A mutant strain (M54) of *E. coli* K12, unable to carry out oxidative phosphorylation, was isolated after screening for strains unable to grow with a non-fermentable carbon source as described by Butlin *et al.* (1971). A mutant allele (*unc-409*) from strain M54 was transferred by co-transduction with the *ilv* locus into strain AN346 to form a new strain, AN817. The latter strain was phenotypically identical with the *uncA* mutant described previously (Butlin *et al.*, 1971). Thus strain AN817 gave low aerobic growth yields when grown in limiting concentrations of glucose, and membrane preparations showed lowered P/O ratios, no detectable Mg-ATPase activity, and no ATP-dependent energy-linked transhydrogenase activity. However, as shown below, the *unc-409* mutation is not in the *uncA* gene and therefore the *unc-409* allele has been designated *uncD409*.

Construction of partial diploids and genetic complementation

The method of transferring *unc* alleles to a plasmid has been described previously (Gibson *et al.*, 1977b). The procedure involves the insertion of a mutant *unc* allele into a plasmid covering the *pyrE,unc,ilv,argH* region of the chromosome, but in which the *ilv,unc* region has been deleted. Genetic complementation tests can then be performed by cross-streaking tests (see the Materials and Methods section) on a medium containing succinate as sole source of carbon. The assumption is made that, if complementation occurs, growth on succinate will be obtained. Genetic complementation was observed between the *uncD409* allele and the three mutant alleles *uncA401*, *uncB402* and *uncC424* (Table 2). In control tests no colonies were formed when the plasmid presumed to carry the *uncD409* allele was cross-streaked against either a *recA* or *recA*⁺ derivative of a female carrying the *uncD409* allele.

The following diploid strains were isolated as described by Gibson *et al.* (1977b): strain AN821,

Table 2. Formation of *Suc*⁺ transconjugants in matings between *recA*⁻ strains carrying various *unc* alleles

Growth obtained on succinate after cross-streaking of donor and recipient strains: +, transconjugant colonies formed; -, no transconjugant colonies formed. The matings between strains carrying the *uncA401* allele and the *uncD409* allele gave very small colonies.

Recipient carrying <i>unc</i> allele on chromosome	Donor carrying <i>unc</i> allele on plasmid				
	AN862 (<i>unc</i> ⁺)	AN780 (<i>uncA401</i>)	AN797 (<i>uncB402</i>)	AN805 (<i>uncC424</i>)	AN819 (<i>uncD409</i>)
AN724 (<i>unc</i> ⁺)	+	+	+	+	+
AN730 (<i>uncA401</i>)	+	-	+	+	+
AN727 (<i>uncB402</i>)	+	+	-	+	+
AN802 (<i>uncC424</i>)	+	+	+	-	+
AN818 (<i>uncD409</i>)	+	+	+	+	-

Table 3. Growth yields

Growth yields were measured as turbidities (Klett units) after growth in 5 mm-glucose-mineral-salts medium with or without the addition of 5% (v/v) Luria broth as indicated.

Segregant	Growth yields (Klett units)		Diploid	Growth yields (Klett units)	
	No addition	Plus 5% Luria broth		No addition	Plus 5% Luria broth
AN822 (<i>unc</i> ⁺)	181	209	AN821 (<i>uncD409/unc</i> ⁺)	152	200
AN824 (<i>uncA401</i>)	113	140	AN823 (<i>uncD409/uncA401</i>)	142	186
AN830 (<i>uncD409</i>)	108	137	AN829 (<i>uncA401/uncD409</i>)	141	189

carrying the *uncD409* allele on the plasmid and a normal *unc* allele on the chromosome (*uncD409/unc*⁺); strain AN823 (*uncD409/uncA401*); strain AN829 (*uncA401/uncD409*). When referring to a particular diploid strain the relevant alleles are included in parentheses with the first-mentioned allele being on the plasmid. Segregants (see Table 1) were isolated from each of these diploid strains as described previously (Gibson *et al.*, 1977a) and the properties of the partial diploids and the segregant strains examined.

Growth yields

Aerobic growth yields of the diploid and segregant strains were measured as turbidities after growth on mineral-salts medium containing 5 mm-glucose. The segregant strains gave growth yields (Table 3) similar to those recorded previously for strains carrying mutant *unc* alleles (see Cox & Gibson, 1974). The diploid strains had growth yields that were intermediate between those obtained from strains carrying a normal *unc* allele and those obtained from strains carrying mutant *unc* alleles. It was found that the addition of Luria broth (5%, v/v) to the glucose/mineral salt medium used for determining growth yields restored the growth yield of strain AN821 (*uncD409/unc*⁺) to about that of the normal strain (AN822) and increased the growth yields of the other diploid strains (Table 3). This observation led to the inclusion of 5% Luria broth in the glucose-mineral-salts medium used for growing cells for the preparation of membranes.

Energy-linked reactions

As mentioned above, mutant strains carrying the *uncD409* allele are phenotypically identical with those carrying the *uncA401* allele. Thus membranes prepared from segregant strains containing either of these alleles lack ATP-induced atebrin-fluorescence quenching, ATP-dependent transhydrogenase activity and oxidative phosphorylation (Table 4). In these strains, energization of the membrane by electron transport is normal as judged by the high amount of NADH-induced atebrin-fluorescence quenching. However, in the two diploid strains AN829 and AN823, which contain both the *uncD409* and *uncA401* mutant alleles, it is clear that complementation has occurred. The amounts of ATP-induced atebrin-fluorescence quenching and ATP-dependent transhydrogenase activity in membranes prepared from the two diploid strains AN829 and AN823 are restored to about 35% of those observed with membranes from the normal strain AN822. Low but significant amounts of oxidative phosphorylation were observed in membranes from the diploid strains, although the relative degree of complementation is difficult to assess in view of the low overall efficiency of oxidative phosphorylation in membranes prepared from *E. coli*.

The introduction of a plasmid carrying the *uncD409* allele into a normal strain results in a lowered efficiency of ATP-induced atebrin-fluorescence quenching, ATP-dependent transhydrogenase activity and oxidative phosphorylation (cf. strains AN822 and AN821, Table 4). This observation is similar to previous

Table 4. *Energy-linked reactions in membranes from diploid and segregant strains*

Details are given in the Materials and Methods section. NADH was the oxidizable substrate used when determining the P/O ratios.

Strain	Atebrin-fluorescence quenching (%)		ATP-dependent transhydrogenase (nmol of NADPH formed/min per mg of protein)	P/O ratio (nmol of glucose 6-phosphate formed/ng-atom of oxygen taken up)
	NADH	ATP		
Segregant				
AN830 (<i>uncD409</i>)	84	0	1	<0.01
AN824 (<i>uncA401</i>)	85	0	0	<0.01
AN822 (<i>unc</i> ⁺)	81	82	18	0.27
Diploid				
AN829 (<i>uncA401/uncD409</i>)	80	29	6	0.03
AN823 (<i>uncD409/uncA401</i>)	84	27	7	0.02
AN821 (<i>uncD409/unc</i> ⁺)	81	43	10	0.09

Table 5. *Mg-ATPase activities in diploid and segregant strains*

Segregant	Mg-ATPase (μmol/min per mg of protein)	Diploid	Mg-ATPase (μmol/min per mg of protein)
AN830 (<i>uncD409</i>)	0.02	AN829 (<i>uncA401/uncD409</i>)	0.20
AN824 (<i>uncA401</i>)	0.02	AN823 (<i>uncD409/uncA401</i>)	0.20
AN822 (<i>unc</i> ⁺)	0.74*	AN821 (<i>uncD409/unc</i> ⁺)	0.41

* This value is higher than that previously reported for a normal strain (Gibson *et al.*, 1977b), owing to the inclusion of Luria broth in the growth medium.

results obtained with partial diploid strains carrying the *uncA401* and normal *unc* alleles (Gibson *et al.*, 1977a).

Mg-ATPase activities

As shown in Table 5, membranes prepared from the segregant strain carrying either the *uncD409* allele (AN830) or the *uncA401* allele (AN824) have no significant Mg-ATPase activity. However, in membranes from the diploid strains containing both *uncA401* and *uncD409* alleles (strains AN829 and AN823) Mg-ATPase activity is restored to about 30% of the value found in membranes from the normal strain AN822. This degree of complementation is similar to that observed for the energy-linked reactions. Membranes from the diploid strain AN821 (*uncD409/unc*⁺) have a Mg-ATPase activity that is significantly lower than that found with the normal strain (AN822), but greater than that found with the diploid strains AN823 (*uncD409/uncA401*) and AN829 (*uncA401/uncD409*).

Discussion

From data described above it is clear that a mutation in the *uncD* gene causes a mutant phenotype similar to the phenotype of a strain carrying a mutation in the *uncA* gene. Apart from the loss of ATP-dependent energy-linked activities, which is a general characteristic of uncoupled mutants, the loss of

Mg-ATPase activity is characteristic of strains carrying the *uncA401* allele, as distinct from strains carrying the *uncB402* or *uncC424* alleles (Gibson *et al.*, 1977a,b). However, relating a mutation to a particular gene is dependent on genetic tests rather than on a biochemical phenotype.

The ability of *uncD409/uncA401*, *uncD409/uncB402* and *uncD409/uncC424* partial diploid strains to grow on succinate is indicative of genetic complementation occurring and that the *uncD* gene is indeed distinct from the *uncA*, *uncB* and *uncC* genes. The growth yields obtained for the diploid strains also indicate that partial complementation occurs between mutant *uncA* and *uncD* alleles, and the degree of complementation increases when Luria broth is added to the glucose-mineral-salts medium. This increase is presumably due to an increased mixing of the subunits of the Mg-ATPase before assembly of the aggregate occurs. It has been pointed out previously (Gibson *et al.*, 1977a) that, if mixing of the subunits does not occur before assembly then complementation will not occur, even though the mutations are located in different genes.

The results obtained by using membrane preparations from the partial diploid strains carrying both the *uncA401* and *uncD409* alleles confirm the conclusions drawn from the growth experiments that these mutations do, in fact, occur in different genes. Thus Mg-ATPase activity is present in membrane preparations from both strain AN823 (*uncD409/uncA401*) and strain AN829 (*uncA401/uncD409*). In

addition, ATP-dependent transhydrogenase activity, ATP-dependent fluorescence quenching and a low amount of oxidative phosphorylation are present in the membrane preparations from the *uncA/uncD* diploid strains.

It should also be emphasized that the *uncD409* allele described above bears no relationship to the UncD phenotype described previously (Thipayathasana, 1975). The latter strains had Mg-ATPase activity, but with altered metal specificity, and such biochemical tests cannot be used to ascribe a mutation to a particular gene.

There have now been four different *unc* genes characterized by genetic complementation between mutant alleles, namely the *uncB* and *uncC* genes affecting components of the F_0 portion of the Mg-ATPase complex and the *uncA* and *uncD* genes affecting components of the F_1 portion. Gene-polypeptide relationships are yet to be determined.

We thank Mr. B. Webb for skilled technical assistance and Ms. B. Homer and Mr. D. Abigail for assistance in the growth of cells and preparation of membranes.

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