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A Mutation Affecting a Second Component of the F_0 Portion of the Magnesium Ion-Stimulated Adenosine Triphosphatase of *Escherichia coli* K12

THE *uncC424* ALLELE

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A new mutant strain of *Escherichia coli* in which phosphorylation is uncoupled from electron transport was isolated. The new mutant strain has a similar phenotype to the *uncB* mutant described previously; results from reconstitution experiments *in vitro* indicate that the new mutation also affects a component of the F_0 portion of the Mg^{2+}-stimulated adenosine triphosphatase. A method was developed to incorporate mutant *unc* alleles into plasmids. Partial diploid strains were prepared in which the *uncB402* allele was incorporated into the plasmid and the new *unc* mutation into the chromosome, or vice versa. Complementation between the mutant *unc* alleles was indicated by growth on succinate, growth yields on glucose, ATP-dependent transhydrogenase activities, ATP-induced atebrin-fluorescence quenching and oxidative-phosphorylation measurements. The gene in which the new mutation occurs is therefore distinct from the *uncB* gene, and the mutant allele was designated *uncC424*.

The number of genes and polypeptides concerned with the coupling of phosphorylation to electron transport is not known. Mutants of *Escherichia coli* in which the Mg-ATPase* complex is affected (*unc* mutants) have been isolated in many laboratories (see Simoni & Postma, 1975), but gene–polypeptide relationships have not been established. It is possible to classify *unc* mutations broadly as affecting either the F_1 or F_0 portion of the Mg-ATPase complex by reconstitution experiments *in vitro* (see Cox & Gibson, 1974). The *uncA401* and the *unc-405* alleles are examples of the former class and the *uncB402* allele is an example of the latter class (see Cox & Gibson, 1974). The numbers of genes concerned in each of these classes can be determined either by a detailed study of the polypeptide composition of membranes, or by genetic complementation tests, using appropriate mutant strains.

It has recently been shown that the *unc* alleles mentioned above are recessive to the normal allele (Gibson *et al.*, 1977). Therefore an examination of partial diploids in which one of the mutant alleles under study is carried on a plasmid, and the second on the chromosome, should provide evidence as to the identity or otherwise of the two mutant alleles. In the present work a technique has been devised for transferring *unc* alleles to a plasmid and the partial diploids used to characterize a mutant allele (*uncC424*) affecting a second component of the F_0 portion of the Mg-ATPase complex.

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

Materials and Methods

Chemicals

Chemicals were of the highest purity available commercially and were not further purified. Labelled orthophosphate (carrier-free) was obtained 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 minimal medium used contained K_2HPO_4 (60mM), $NaH_2PO_4,2H_2O$ (40mM) and $(NH_4)_2SO_4$ (15mM), to which was added 1ml of a concentrated trace-element solution (containing 14mM-$ZnSO_4$, 1mM-$MnSO_4$, 4.7mM-H_3BO_3, 0.7mM-$CaSO_4$, 2.5mM-$CaCl_2$ and 1.8mM-$FeCl_3$)/litre. After sterilization 1ml of a sterile 1M-$MgSO_4$ solution was added/litre, together with other growth supplements, as required, at the following final concentrations: 30mM-glucose;

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
AN812	*uncC424*	Isolated after treatment of strain AB259 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
AN248	F$^-$, *ilvC7,argH1,entA*	Butlin *et al.* (1973)
AN283	F$^-$, *uncB402,argH1,ent*A	Butlin *et al.* (1973)
AN346	F$^-$, *ilvC,argH,pyrE,entA*	Gibson *et al.* (1977)
AN719	F$^-$, *uncB402,argH,pyrE,entA*	Isolated after transduction with strain AN283 as donor and strain AN346 as recipient
AN781	F$^-$, *uncC424,argH,pyrE,entA*	Isolated after transduction with strain AN812 as donor and strain AN346 as recipient
AN627	F$^-$, *argH,ilvC,pyrE,purE,recA,nalA*	Gibson *et al.* (1977)
KL163	Hfr, *nalA,recA*	Obtained from J. Pittard
AN727	F$^-$, *uncB402,argH,pyrE,entA,recA,nalA*	Isolated after mating between strains KL163 and AN719
AN802	F$^-$, *uncC424,argH,pyrE,entA,recA,nalA*	Isolated after mating between strains KL163 and AN781
Diploids		
AN800	F111(Δ*ilv-unc*),*argG6,metB1,his-1,leu-6,recA*	Culture of CGSC strain 4258 found to be carrying a deletion in the plasmid F111 (see the text)
AN797	F′ (pAN5), *ilvC,argH,pyrE,purE,recA,nalA*	Strain with plasmid carrying *uncB402* allele. Isolated as described in the text
AN805	F′ (pAN6), *ilvC,argH,pyrE,purE,recA,nalA*	Strain with plasmid carrying *uncC424* allele. Isolated as described in the text
AN803	F′ (pAN5), *uncC424,argH,pyrE,entA,recA,nalA*	Isolated after mating between strains AN797 and AN802
AN806	F′ (pAN6), *uncB402,argH,pyrE,entA,recA,nalA*	Isolated after mating between strains AN805 and AN727
AN760	F′ (pAN1), *ilvC,argH,pyrE,entA,recA,nalA*	Gibson *et al.* (1977)
Segregants		
AN804	F$^-$, *uncC424,argH,pyrE,entA,recA,nalA*	Segregant isolated from strain AN803
AN807	F$^-$, *uncB402,argH,pyrE,entA,recA,nalA*	Segregant isolated from strain AN806
AN786	F$^-$, *ilvC,argH,pyrE,entA,recA,nalA*	Gibson *et al.* (1977)
Plasmids		
F111(Δ*ilv-unc*)Δ(*ilv-unc*), *pyrE*$^+$, *argH*$^+$		
pAN1	*unc*$^+$, *ilvC*$^+$, *argH*$^+$, *pyrE*$^+$	Gibson *et al.* (1977)
pAN5	*uncB402*, *argH*$^+$, *pyrE*$^+$	
pAN6	*uncC424*, *argH*$^+$, *pyrE*$^+$	

0.2 μM-thiamin hydrochloride; 40 μM-2,3-dihydroxybenzoate; 0.8 mM-L-arginine hydrochloride; 0.2 mM-uracil; 0.15 mM-adenine hydrochloride; 0.3 mM-L-isoleucine; 0.3 mM-L-valine.

Cells for the preparation of membranes were grown in 14-litre New Brunswick fermenters as described by Cox *et al.* (1970).

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 as described by Luria & Burrous (1957), with glucose (10 mM) included as indicated.

Growth yields were obtained as described by Cox *et al.* (1970), with a Klett–Summerson colorimeter.

Genetic techniques

The techniques used for genetic experiments were as outlined previously (Gibson *et al.*, 1977), except that diploids were prepared after growing male and female cells in glucose/Luria broth each to a density of 2×10^8 cells/ml and mixing 1 ml of the culture of males with 4 ml of culture of females and incubating the mixture, with slow shaking, at 37°C for 1 h. Mating was then interrupted by vortex-mixing a

small volume of the mating mixture for 1 min and plating 0.1 ml of undiluted mixture, or a dilution of 1:100, on the appropriate selective media.

Preparation of cell membranes

Membranes were prepared as described previously (Cox *et al.*, 1973*a*). Briefly, washed cells were disintegrated by using a Sorvall Ribi cell fractionator and the membranes were separated by ultracentrifugation and resuspended in a 0.1 M-Tes buffer system (adjusted to pH 7.0 with NaOH) containing magnesium acetate, sucrose and EGTA.

Hybrid membranes were isolated after disruption of a mixture of equal quantities of two mutant types (Kanner *et al.*, 1975) and then following the procedure as outlined above.

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.*, 1977).

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 450 nm and emission wavelength set at 510 nm.

Oxidative phosphorylation

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

Results

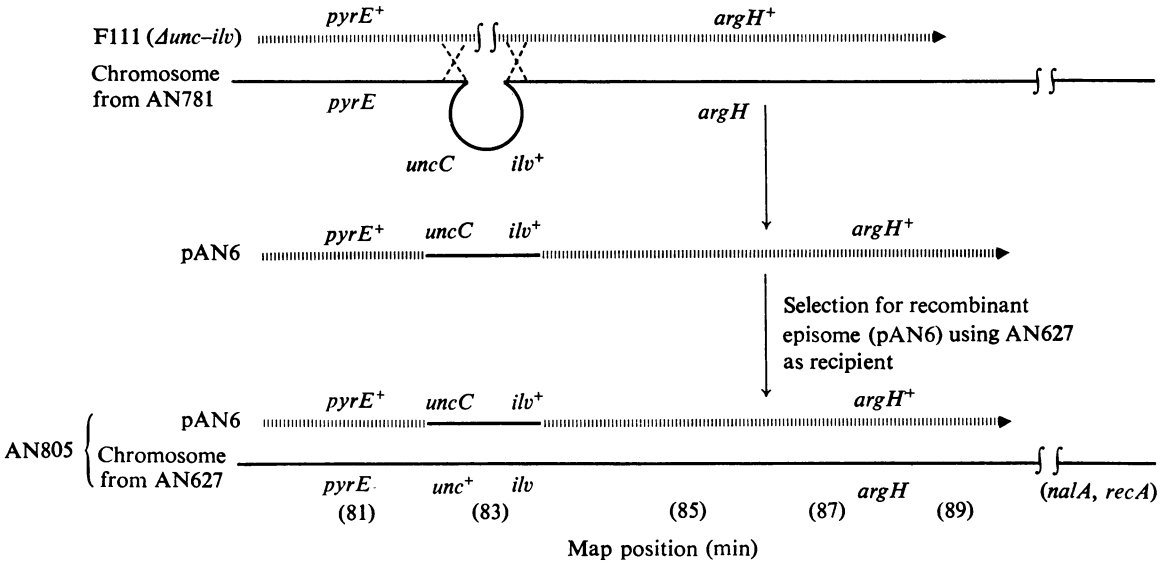
Isolation of a mutant strain carrying the uncC424 allele

A mutant strain (AN812) of *E. coli* K12 affected in oxidative phosphorylation was isolated as described previously (Butlin *et al.*, 1971). The mutant allele (*unc-424*) present in strain AN812 was co-transduced with the *ilv* locus and the transductant strain AN781 was phenotypically identical with the *uncB* mutant described previously (Butlin *et al.*, 1973). Thus strain AN781 gave low aerobic growth yields when grown in limiting concentrations of glucose, normal oxidase activities, lowered P/O ratios, no ATP-dependent energy-linked transhydrogenase activity, but normal Mg-ATPase activity. However, as shown below, the *unc-424* mutation is not in the *uncB* gene and accordingly the *unc-424* allele has been designated *uncC424*.

Construction of partial diploids carrying the uncB402 and uncC424 alleles

In early experiments designed to examine the properties of strains diploid for the *unc* region of the *E. coli* chromosome, the plasmid F111, which is reported to cover the *pyrE,ilv,argH* region of the chromosome (see Low, 1972), appeared to have the *ilv* genes deleted. After the observation that unc^+ alleles were dominant over *unc* alleles (Gibson *et al.*, 1977), the plasmid F111 was reinvestigated. A new culture was obtained from the *E. coli* Genetic Stock Culture Center (Yale University, U.S.A.), and it was found that this copy of the plasmid carried both ilv^+ and unc^+ alleles. Thus the introduction of this plasmid into female strains carrying unc^+, *uncA401*, *uncB402* and *unc-405* alleles gave similar results to those obtained with the plasmid pAN1 (see above). It seemed therefore that the copy of the F111 plasmid used in the earlier experiments (Gibson *et al.*, 1977) carries a deletion covering the *unc,ilv* region. Advantage was taken of this fact to provide a method for introducing *unc* alleles into this episome. The episome carrying the deletion was introduced into a $pyrE^-$,unc^-,ilv^+,$argH^-$ female (e.g. AN781). The resulting partial diploid was then mated with the $recA^-$,$pyrE^-$,ilv^-,$argH^-$ female AN627 selecting for pyr^+,ilv^+,arg^+ transconjugants and selecting against the male donor with naladixic acid. Thus a selection was made for recombinant plasmids in which the ilv^+ allele on the chromosome had been incorporated into the plasmid (e.g. pAN6). An outline of the procedure is depicted in Scheme 1. The incorporation of the ilv^+ allele into the episome should be accompanied by the incorporation of the *unc* region, since the original deletion covered both the *ilv* and *unc* genes. Transfer of the *unc* region could be demonstrated by testing the presumed diploid carrying the mutant *unc* allele on the episome in complementation tests with $recA^+$ females carrying various mutant *unc* alleles. No colonies were formed on succinate medium when the female strain carried the same mutant *unc* allele as presumed to be on the episome. The selected diploid strains (e.g. AN805) were tested as a routine for sensitivity to u.v. light to check for the presence of the mutant *recA* allele (Gibson *et al.*, 1977).

Diploids carrying the *uncB402* allele on the plasmid and the *uncC424* allele on the chromosome (strain AN803, *uncB402/uncC424*) or the converse (strain AN806, *uncC424/uncB402*) were prepared (see the Materials and Methods section). The selective media used contained 2,3-dihydroxybenzoate but not adenine, uracil or arginine, in order to select against the male donor and female recipient but permit



Scheme 1. *Construction of an episome carrying an unc mutation*
Details are described in the text. ||||||||||||, Original plasmid DNA; ——, original chromosomal DNA.

Table 2. *Growth yields*
Growth yields were measured as turbidities after growth on 5 mM-glucose.

Diploid	Growth yield (Klett units)	Segregants	Growth yield (Klett units)
AN803 (*uncB402/uncC424*)	152	AN804 (*uncC424*)	120
AN806 (*uncC424/uncB402*)	152	AN807 (*uncB402*)	125
AN760 (*unc*$^+$/*unc*$^+$)	193	AN786 (*unc*$^+$)	190

growth of transconjugants. Segregant strains were then isolated from both diploids as described previously (Gibson *et al.*, 1977).

Growth on succinate

Strains carrying mutant *unc* alleles will not grow on a medium containing succinate as sole source of carbon. The diploid strains AN803 (*uncB402/uncC424*) and AN806 (*uncC424/uncB402*) both grow on succinate as sole source of carbon, whereas the segregant strains AN804 (*uncC424*) and AN807 (*uncB402*) do not.

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 2) similar to those recorded previously for strains carrying mutant *unc* alleles (see Cox & Gibson, 1974). The diploid strains AN803 (*uncB402/uncC424*) and AN806 (*uncC424/uncB402*), however, had growth yields which, although higher than the growth yields obtained from the segregant strains, were lower than for normal strains (Table 2).

Energy-linked reactions

As shown in Table 3, membranes prepared from the segregant strain carrying the *uncC424* allele (AN804) are similar to those prepared from the segregant strain carrying the *uncB402* allele (AN807) in that there is no significant ATP-dependent transhydrogenase activity, ATP-induced atebrin-fluorescence quenching or oxidative phosphorylation. In these strains, energization of the membrane by the oxidation of NADH is normal, as judged by the high NADH-induced atebrin-fluorescence quenching.

However, in the diploid strains containing either *uncC424* on the chromosome and *uncB402* on the episome (AN803), or *uncB402* on the chromosome and *uncC424* on the episome (AN806), it is clear that complementation has occurred. Thus in membranes from these two diploid strains the ATP-induced atebrin-fluorescence quenching, ATP-dependent transhydrogenase and P/O ratios are restored to values similar to those measured in membranes

Table 3. *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				
AN804 (*uncC424*)	83	5	2	<0.01
AN807 (*uncB402*)	81	2	2	<0.01
AN786 (*unc*$^+$)*	74	66	18	0.14
Diploid				
AN803 (*uncB402/uncC424*)	80	69	11	0.15
AN806 (*uncC424/uncB402*)	77	78	11	0.10
AN760 (*unc*$^+$/*unc*$^+$)*	73	72	12	0.13

* The values for these strains, except for the atebrin-fluorescence quenching, differ from those reported previously (Gibson *et al.*, 1977), probably owing to the different growth medium used.

Table 4. *Mg-ATPase activities in diploid and segregant strains*

Strain	Mg-ATPase (μmol/min per mg of protein)		Distribution of Mg-ATPase (μmol/min per g dry wt. of cells)	
	Membrane-bound	Soluble	Membrane-bound	Soluble
Segregant				
AN804 (*uncC424*)	0.31	0.62	50	210
AN807 (*uncB402*)	0.30	0.59	50	200
AN786 (*unc*$^+$)*	0.34	—	55	—
Diploid				
AN803 (*uncB402/uncC424*)	0.70	0.34	110	120
AN806 (*uncC424/uncB402*)	0.58	0.54	95	180
AN760 (*unc*$^+$/*unc*$^+$)*	0.65	—	100	—

* The values for these strains differ from those reported previously (Gibson *et al.*, 1977), probably owing to the different growth medium used.

Table 5. *Reconstitution of ATP-dependent transhydrogenase activity*

Membranes prepared from	ATP-dependent transhydrogenase (nmol of NADPH formed/min per mg of protein)
Strains	
AN807 (*uncB402*)	2
AN804 (*uncC424*)	2
AN285 (*unc-405*)	0
Mixed strains	
AN807/AN285	12
AN804/AN285	17

prepared from the normal control diploid strain AN760 (Table 3).

Mg-ATPase

The Mg-ATPase activities of the membranes prepared from the various strains reflect the presence or absence of the plasmid, the diploid strains having twice the activity found in the segregant strains (Table 4). The segregant strains carrying either the *uncC424* allele or the *uncB402* allele both have comparatively high ATPase activity in the cytoplasmic fraction, the total activity in the soluble fraction being about four times that bound to the membrane (Table 4). The diploid strains also have ATPase activity in the soluble fraction, although less than that found in the segregant strains (Table 4).

Reconstitution of ATP-dependent transhydrogenase activity

To determine whether the Mg-ATPase formed by the *uncC* mutant is normal, the reconstitution method *in vitro* of Kanner *et al.* (1975) was used. A cell suspension of the *uncC* mutant (AN804) was mixed with a cell suspension of strain AN285 (*unc-405*), a strain lacking the Mg-ATPase (Cox *et al.*, 1974), and the mixed suspension was disrupted in a French pressure cell. The resultant membranes were then examined for ATP-dependent transhydrogenase activity. Either of the segregant strains when dis-

rupted with strain AN285 gave reconstitution of the transhydrogenase activity (Table 5).

Discussion

Results of reconstitution experiments *in vitro* have confirmed that mutant strains carrying the *uncB402* allele have a normal F_1 portion but a defective F_0 portion of the Mg-ATPase complex (Cox *et al.*, 1973*a*). The phenotype of mutant strains carrying the *uncC424* allele appears similar to that of strains carrying the *uncB402* allele. Thus in both mutant types, although Mg-ATPase activity is retained, ATP-dependent transhydrogenase activity, ATP-induced atebrin-fluorescence quenching and oxidative phosphorylation are lost. In addition, the ability of the *uncC* mutant to reconstitute energy-linked reactions *in vitro* with the mutant strain AN285 (*unc-405*) in which the Mg-ATPase activity is lacking indicates that, as in the *uncB* mutant, the F_0 portion of the Mg-ATPase is affected.

Genetic complementation tests using partial diploids have distinguished between the *uncB* and *uncC* genes. The partial diploids contained a mutation in the *recA* gene to prevent genetic recombination occurring. Thus membranes prepared from the partial diploids AN803 (*uncB402/uncC424*) and AN806 (*uncC424/uncB402*) have normal energy-linked activities. However, the growth yields obtained for these strains are intermediate between the values for fully uncoupled and normal strains, indicating that complete complementation *in vivo* has not occurred. During disruption of the cells, additional reconstitution presumably occurs, restoring the energy-linked activities in membrane preparations to normal values.

Attempts have been made, using such criteria as the effects of *NN'*-dicyclohexylcarbodi-imide on the energization of mutant membranes (Nieuwenhuis *et al.*, 1973) or altered metal specificity of the Mg-ATPase (Thipayathasana, 1975), to subdivide uncoupled mutant strains. However, it is clear that such criteria do not give definitive information about the genes or polypeptides affected in individual mutant strains. Caution, therefore, has to be observed not to infer that phenotypic differences result from mutations in different genes.

The results described in the present paper indicate that it should be possible to define the number of genes and therefore the number of polypeptides actually concerned in the ATP-synthesizing complex (F_1+F_0) in *E. coli*.

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