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OXIDATIVE PHOSPHORYLATION IN *ESCHERICHIA COLI* K-12: THE GENETIC AND BIOCHEMICAL CHARACTERISATION OF A STRAIN CARRYING A MUTATION IN THE *uncB* GENE

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SUMMARY

1. A mutant strain of *Escherichia coli* unable to grow with succinate as sole carbon source was isolated. This mutant was found to carry a mutation in a gene (designated *uncB*) mapping at about minute 73.5 on the *E. coli* chromosome and close to the *uncA* gene which is probably the structural gene for (Mg^{2+},Ca^{2+})-stimulated ATPase.

2. The *uncB401* allele was transduced into two other strains of *E. coli* and the transductants compared with the parent strains.

3. Strains carrying the *uncB401* allele have low aerobic growth yields when grown on limiting concentrations of glucose, but unlike mutations in the *uncA* gene, mutations in the *uncB* gene do not impair anaerobic growth on a glucose–mineral salts medium.

4. Oxidase activities in membranes from the normal strains and strains carrying the *uncB401* allele were similar.

5. Measurement of P/O ratios indicated that a mutation in the *uncB* gene causes uncoupling of phosphorylation associated with electron transport with D-lactate as substrate.

6. (Mg^{2+},Ca^{2+})-stimulated ATPase activities in the normal strains and in strains carrying the *uncB401* allele are similar.

7. Estimation of the energy-linked and non-energy-linked transhydrogenase activities in membrane preparations from both the normal and mutant strains indicated that the protein affected by a mutation in the *uncB* gene is essential for the functioning of the ATP-dependent energy-linked transhydrogenase.

8. It is concluded that two proteins, specified by the *uncA* and *uncB* genes, are essential for phosphorylation coupled to D-lactate oxidation and also for the energy-linked transhydrogenase activity using ATP as the energy source.

INTRODUCTION

A mutant strain of *Escherichia coli* K-12 has been described in which phosphorylation is uncoupled from electron transport with D-lactate as substrate[1]. This

Abbreviation: TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethane-sulphonic acid.

strain was shown to carry a mutation in the *uncA* gene causing a loss of (Mg^{2+}, Ca^{2+})-stimulated ATPase activity. It was concluded that a functional (Mg^{2+},Ca^{2+})-stimulated ATPase was essential for oxidative phosphorylation in *E. coli*.

The present paper describes an investigation of another mutant strain in which phosphorylation is also uncoupled from D-lactate oxidation but retains a normal (Mg^{2+},Ca^{2+})-stimulated ATPase activity.

MATERIALS AND METHODS

Chemicals and enzymes

Lactate dehydrogenase and hexokinase were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Glutathione reductase was obtained from Calbiochem (Australia) Pty. Ltd., Carlingford, N.S.W. Chemicals generally were of the highest purity available commercially and were not further purified.

Organisms

All the bacterial strains used in this work were derived from *E. coli* K-12 and are described in Table I.

TABLE I

STRAINS OF *E. coli* K-12 USED

Strain	*Relevant genetic loci**	*Other information*
AB3311	*metB*–, *thi*–	Hfr Reeves, Hfr I
AB313	*thi*–, *leu-6*, *thr-1*, *str*^{R}	Hfr male
JP58	*ilvC7*, *argE3*, *thi-1*, *str*^{R}	
AN231	*metB*–, *uncB401*, *thi-1*	Derived from strain AB3311 after MNNG** treatment
AN180	*argE3*, *thi-1*, *str*^{R}	See ref. 1
AN120	*argE3*, *thi-1*, *str*^{R}, *uncA401*	See ref. 1
AN232	*uncB401*, *argE3*, *thi-1*, *str*^{R}	Isolated after transduction with AN231 as donor and JP58 as recipient
AN248	*ilvC7*, *argH1*, *entA*–	
AN237	*uncA401*, *argH1*, *purE*–	Isolated after transduction with AN119 (ref. 1) as donor and AN236 as recipient
AN283	*uncB401*, *argH1*, *entA*–	Isolated after transduction with AN231 as donor and AN248 as recipient
AN259	*argH1*, *entA*–	Derived by transduction from AN248

* Genes coding for enzymes in various biosynthetic pathways are denoted as follows: *thi*, thiamine; *ilv*, isoleucinevaline; *arg*, arginine; *met*, methionine; *leu*, leucine; *thr*, threonine; *ent*, enterochelin; *pur*, purine. Streptomycin resistance is denoted by *str*^{R}. The mutant allele *uncB401* is described in this paper.

** MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Media

The mineral-salts medium 56 described by Monod *et al.*[2] was used. Sterile solutions of various carbon sources to give a final concentration of 30 mM were

added to the sterilized mineral-salts base. Additional amino acid supplements (0.2 mM), thiamine (0.2 μM) and 2,3-dihydroxybenzoate (10 μM) were added as required. The cells used for genetic experiments were grown on a tryptone–yeast extract broth[3]. The nutrient broth was solidified with 2% (w/v) Difco agar as required.

Transduction techniques

The generalised transducing bacteriophage Plkc was used for transduction experiments as described by Pittard[4].

Conditions for conjugation experiments

The conditions under which mating was carried out were similar to those described by Taylor and Thoman[5].

Growth conditions

Growth yields of various strains on media containing limiting glucose were measured as turbidities in a Klett–Summerson colorimeter as described by Cox *et al.*[6]. A reading of 200 Klett units is equivalent to about 0.4 mg dry weight of cells per ml of culture. For growth under anaerobic conditions, the cultures were incubated in an atmosphere of H_2 as described previously[1].

For the preparation of cell extracts, the growth from 5 nutrient agar plates, incubated at 37 °C overnight, was suspended in medium 56 and added to 5 l of medium 56 in a 7-l New Brunswick fermenter. The fermenter was kept at 37 °C, aerated at 12 l/min, stirred at 600 rev./min and the culture harvested in mid-exponential phase at about 0.6 mg dry weight of cells/ml.

Preparation of "cell envelope" fraction

The cells were washed, disrupted by passage through a Sorvall Ribi Cell Fractionator, and the cell envelope fraction separated by $(NH_4)_2SO_4$ precipitation, as described by Cox *et al.*[6].

Preparation of membrane fraction

The cells were washed in a buffer consisting of 0.1 M *N*-tris-(hydroxymethyl)-methyl-2-aminoethane-sulphonic acid (TES) containing 0.02 M magnesium acetate, 0.25 M sucrose and 0.25 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetra-acetic acid. The cells were then resuspended in fresh buffer (1 ml per 0.5 g wet weight cells) and disrupted by passage through a Sorvall Ribi Cell Fractionator at 20000 lb/inch^2. The cell extract was then centrifuged at 25000 × *g* for 20 min and the supernatant fraction separated. The supernatant fraction was then centrifuged at 100000 × *g* for 4 h and the pellet retained. The pellet consisted of two layers, a pale-yellow translucent heavy layer and a red-brown light layer. The red-brown layer was separated and resuspended in 1 ml of the TES buffer system for each original 1 g wet weight of cells. This preparation was used for both the adenosine triphosphatase and the transhydrogenase assays.

The preparation of cell membranes for the assay of oxidative phosphorylation differed from the above in that 0.1 M sodium–potassium phosphate buffer, pH 7.0, was used throughout.

All operations on the harvested cells were conducted at 0–4 °C. The protein

content of the membrane preparations, determined using Folin's phenol reagent[7], with bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.) as standard, was 10–15 mg/ml.

Determination of fermentation products

The supernatants after centrifuging cultures grown in the New Brunswick fermenters were examined by gas–liquid chromatography for fermentation products[8].

Measurement of oxygen uptake

Oxygen uptakes at 30 °C were measured polarographically as described by Cox *et al.*[6].

Difference spectra

Difference spectra for the determination of cytochromes b_1, a_1, a_2 and *o* and the flavoproteins were recorded as described previously[6] except that an Aminco-Chance spectrophotometer was used.

Determination of quinones

The ubiquinone and menaquinone content of 2 g wet weight samples of cells were determined as described previously[9].

Measurement of ATPase activity

The reaction mixture contained 0.1 M Tris–HCl buffer (pH 9), 20 mM ATP, 10 mM $MgCl_2$ and 0.75 mM EDTA in a final volume of 1 ml. The reaction was initiated by the addition of 20–50 µl of membrane preparation. After incubation for 10 min at 30 °C in a water bath, the tubes were transferred to an ice bath, and 0.5-ml samples of the reaction mixture added to 9.5 ml of King's reagent[10]. After 15 min the absorption at 660 nm was measured. The assay was always checked to ensure that the activity was proportional to the amount of membrane protein added. Controls without substrate or without membranes were also included.

P/O ratios

Determinations of P/O ratios with D-lactate as substrate were carried out as described previously[1].

Measurement of transhydrogenase activities

Both the energy-linked and non-energy-linked transhydrogenase activities were measured as described previously[11].

RESULTS

Mutants unable to grow on succinate but able to grow on glucose as sole source of carbon (Suc^- phenotype) were screened to detect strains giving low aerobic growth yields in media containing limiting concentrations of glucose. Such strains were then examined for (Mg^{2+},Ca^{2+})-stimulated ATPase activity. One strain (AN231) was found to have a normal level of (Mg^{2+},Ca^{2+})-stimulated ATPase activity and was therefore examined in detail.

Genetic mapping

Strain AN231, although derived from an Hfr male, was found to be female. The Hfr strain AB313, was used in interrupted mating experiments with AN231 in order to map the mutation causing the Suc^- phenotype. The Suc^+ recombinants were selected on a medium containing succinate as sole source of carbon and one of the amino acid requirements of the Hfr strain was omitted to prevent growth of the male cells. A time of entry for the wild type allele of about 10 min was obtained. It appeared therefore that the mutation causing the Suc^- phenotype was close to the *uncA* gene at about minute 74 on the *E. coli* chromosome[1].

The mutation causing the Suc^- phenotype was located more precisely by tests for co-transduction with the *ilvC* gene. The generalised transducing bacteriophage, Plkc was grown on strain AN231 and used for co-transduction experiments with strain JP58 (*ilvC*$^-$). Of the *ilvC*$^+$ transductants examined, 24 out of 72 were able to grow on succinate and the *ilvC* gene was therefore co-transducible with the mutation causing the Suc^- phenotype at a frequency of about 66%. The co-transduction results are consistent with the mutation present in strain AN231 being located at about minute 74 on the *E. coli* genome. The gene carrying the mutation preventing growth on succinate was designated *uncB* for reasons outlined below. The mutant allele was designated *uncB401*.

The *uncB* gene was apparently close to the *uncA* gene. A bacteriophage lysate prepared on strain AN232 (*uncB*$^-$) was therefore used as donor and strain AN237 (*uncA*$^-$) as recipient in a transduction experiment and a selection was made for Suc^+ transductants. The numbers of Suc^+ transductants obtained were normalised with respect to the number of Pur^+ transductants obtained by using the same bacteriophage lysate. The Suc^+ transductants were detected at frequencies of about 13% of the frequencies obtained by using a bacteriophage lysate prepared on a wild-type strain (Table II). These results confirmed that the *uncA* and *uncB* genes are close to each other and may be contiguous.

TABLE II

TRANSDUCTION CROSSES INVOLVING STRAINS AN232 (*uncB401*) AND AN237 (*uncA401*)

The transducing bacteriophage Plkc was grown on either strain JP58 (Pur^+, Suc^+) or on strain AN232 (Pur^+, Suc–) and used as donor with strain AN237 (Pur–, Suc–) as recipient. Either Pur^+ or Suc^+ transductants were selected.

Donor	*Recipient*	*Number of Pur$^+$ transductants*	*Number of Suc$^+$ transductants*
JP58	AN237 (*uncA*–)	1006	6800
AN232 (*uncB*–)	AN237 (*uncA*–)	1766	1511

A study of the biochemical effects of the mutation in the *uncB* gene was made by a comparison of strain AN232 (*uncB*$^-$), derived from strain JP58 by transduction, and strain AN180, a normal strain also derived from strain JP58 by transduction[1]. Strains AN283 (*arg*$^-$, *entA*$^-$, *uncB*$^-$) and AN259 (*arg*$^-$, *entA*$^-$), both derived by

transduction from strain AN248 (*ilv*$^-$, *arg*$^-$, *entA*$^-$), were also used as a suitable pair of strains for biochemical comparison. Strains AN180 and AN259 will be referred to as normal strains and strains AN232 and AN283 will be referred to as *uncB* mutants.

Growth characteristics of normal and mutant strains

The *uncB* mutants grew well aerobically on a glucose–mineral salts medium with a mean generation time approximately 1.5 times that of the normal strain. However the mutant strains were unable to grow on either solid or liquid media with succinate, malate or D-lactate as sole source of carbon.

The culture supernatant from cells grown for the preparation of membranes were examined by gas–liquid chromatography for products of glucose metabolism[8]. The concentrations of lactate, ethanol and acetate were similar for both the normal and mutant strains (Table III). These results are in marked contrast to those obtained using a ubiquinone-deficient strain of *E. coli* K-12 grown under similar conditions[6].

TABLE III

PRODUCTS OF GLUCOSE METABOLISM FORMED BY A NORMAL STRAIN AND AN *uncB* MUTANT STRAIN GROWN UNDER AEROBIC CONDITIONS

Determinations were made by gas–liquid chromatography on supernatants from cultures of cells grown for the preparation of membranes.

Strain	*Product (mM)*		
	Ethanol	*Acetate*	*Lactate*
AN180	1.1	9	3.0
AN232 (*uncB*−)	1.3	11	2.8

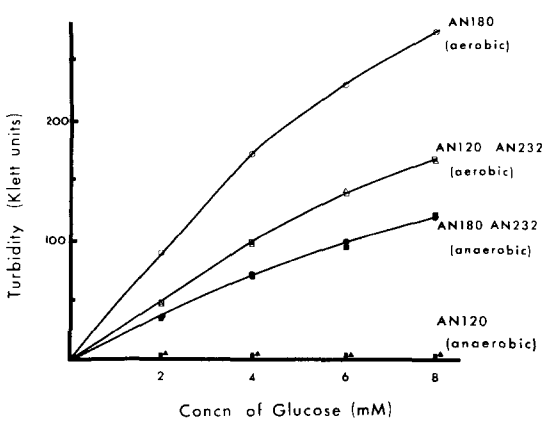


Fig. 1. Growth yields (turbidity) for strains AN180, AN120 (*uncA*$^-$) and AN232 (*uncB*$^-$) grown on limiting concentrations of glucose. ○, strain AN180, aerobic; ●, strain AN180, anaerobic; △, strain AN120, aerobic; ▲, strain AN120, anaerobic; □, strain AN232, aerobic; ■, strain AN232, anaerobic.

Succinate was also present in the supernatants from both normal and mutant strains, but the variable results obtained with the method used made accurate determinations difficult.

Aerobic growth yields of the normal and mutant strains were measured as turbidities after growth on mineral-salts media containing limiting concentrations of glucose. The aerobic growth yields of the *uncB* mutants were similar to those obtained for the *uncA* mutants in that the aerobic growth yield was intermediate between those values obtained for the normal strain grown under aerobic and under anaerobic conditions (Fig. 1) (see also ref. 1). The *uncB* mutants grew normally under anaerobic conditions with glucose as the sole source of carbon in contrast to the *uncA* mutants which are unable to grow under these conditions (Fig. 1).

Concentration of membrane components

Concentrations of total flavin and cytochromes in cell envelope preparations from the normal and mutant strains were determined from the reduced *minus* oxidised difference spectra. It was found (Table IV) that the concentrations of these components did not differ significantly between the normal and mutant strains. Similarly, the concentrations of ubiquinone and menaquinone in whole cells, determined after acetone extraction and partial purification, did not vary significantly between the normal and mutant strains (Table IV).

TABLE IV

CONCENTRATIONS OF SOME CELL ENVELOPE COMPONENTS IN NORMAL AND MUTANT STRAINS

Cell envelope component	*Concentrations of components (nmoles/mg of protein)*	
	AN180	*AN232 (uncB−)*
Total flavin	1.3	1.1
Cytochrome b_1	0.54	0.54
Cytochrome a_2	0.02	0.02
Cytochrome *o*	0.24	0.23
Cytochrome a_1*	+	+
Ubiquinone	6.1	5.1
Menaquinone	2.0	2.6

* Cytochrome a_1 was present but the quantities were too low for determination.

Oxidase systems

The NADH and D-lactate oxidase systems have been shown to be quantitatively the most significant in cell envelope preparations from *E. coli* K-12 grown with glucose as carbon source[6]. Comparisons of these oxidase systems in cell envelope preparations from the *uncB* mutants and the normal strains showed that there was no significant difference in NADH oxidase activity (950–1050 ngatoms O per min per mg protein). The D-lactate oxidase activity however was greater in the mutant strains (350 ngatoms O per min per mg protein) than in the normal strains (255

ngatoms O per min per mg protein). There was some variation in the succinoxidase activities of different batches of membrane preparations but a similar range of activities was found in membranes from both normal and mutant strains.

Oxidative phosphorylation

There was no detectable phosphorylation coupled to D-lactate oxidation by membrane preparations from the *uncB* mutants whereas the normal strains gave P/O ratios of about 0.2 (Table V). It therefore appears that a mutation in the *uncB* gene has a similar effect to a mutation in the *uncA* gene in that phosphorylation coupled to D-lactate oxidation could not be detected.

TABLE V

OXIDATIVE PHOSPHORYLATION BY MEMBRANES FROM NORMAL AND *uncB* MUTANT STRAINS

Experimental details are given in Materials and Methods.

Source of membranes	*Oxygen uptake* (ngatoms/min per mg of protein)*	*Glucose-6-phosphate formed (nmoles/min per mg of protein)*	*P/O ratio***
AN180	189	34.5	0.18
AN232 (*uncB*−)	220	< 0.1	< 0.001

* The presence of AMP and ADP in the reaction mixture used for the determination of P/O ratios inhibits the lactate oxidase system.

** Similar results were found for the pair of strains AN259 and AN283 (*uncB*−).

ATPase activity

The Mg^{2+}-stimulated ATPase activity of membrane preparations from the *uncB* mutants and the normal strains were determined. The values obtained for the normal strains were 160–167 nmoles P_i released per mg of protein per min, and those obtained for the *uncB* mutants were slightly higher at 172–184 nmoles P_i released per mg of protein per min. The activities obtained for the Ca^{2+}-stimulated ATPase activities were similar (about 90 nmoles of P_i released per mg of protein per min) in membrane from normal or mutant strains. The effect of a mutation in the *uncB* gene on the (Mg^{2+}, Ca^{2+})-stimulated ATPase activity is therefore in marked contrast to the effect of a mutation in the *uncA* gene[1]. (Values for Mg^{2+}, Ca^{2+}-stimulated ATPase activities given previously[1] should be divided by 10 to correct for an arithmetical error).

Transhydrogenase activities

The energy-linked and non-energy-linked transhydrogenase activities were measured in membrane preparations from the *uncB* mutants and the normal strains. The non-energy-linked transhydrogenase activities of the membrane preparations from both the *uncB* mutants and the normal strains were similar (Table VI). However the energy-linked transhydrogenase activities in the *uncB* mutant strains were

TABLE VI

TRANSHYDROGENASE ACTIVITIES OF MEMBRANES FROM NORMAL AND *uncB* MUTANT STRAINS

Experimental details are given in Materials and Methods.

Strain	*Energy-linked transhydrogenase (nmoles of NADPH formed/min mg of protein)**	*Non-energy-linked transhydrogenase (nmoles of NADH formed/min per mg of protein)**
AN259	72	119
AN283 (*uncB*−)	4.4	109

* Similar results were found for the pair of strains AN180 and AN232 (*uncB*−).

much lower than those obtained for membranes from the normal strains (Table VI) but similar to the value previously reported for the *uncA* mutants[11].

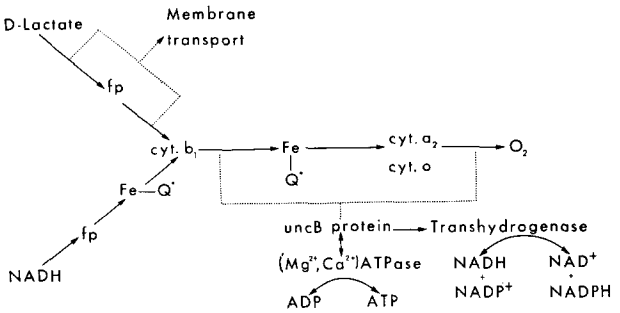
DISCUSSION

The studies reported above indicate that mutations in the *uncB* gene have many similar effects to mutations in the *uncA* gene, the latter gene probably being the structural gene for the (Mg^{2+},Ca^{2+})-stimulated ATPase. Thus the *uncB* mutants are unable to grow on succinate as the sole source of carbon but are able to grow on glucose, although at a somewhat slower rate than normal cells. The aerobic growth yields obtained with *uncB* mutants are similar to those obtained for the *uncA* mutants and lie in between those obtained for normal cells grown under aerobic and under anaerobic conditions. Mutations in both the *uncA* and *uncB* genes do not inhibit the rate of electron transport but apparently cause complete uncoupling of phosphorylation with D-lactate as substrate. The energy-linked transhydrogenase activities are lost or greatly reduced in membranes from both the *uncA* and *uncB* mutants whereas the non-energy-linked transhydrogenase activities are either derepressed or unaffected.

There are, however, two points of difference between the *uncA* and *uncB* mutants. Whereas mutations in the *uncA* gene essentially eliminate the (Mg^{2+},Ca^{2+})-stimulated ATPase activity, mutations in the *uncB* gene have no effect on this activity. The second point of difference is in the ability of the *uncB* mutants to grow anaerobically with glucose as sole source of carbon. The *uncA* mutants are unable to grow under these conditions. These differences in phenotype make it extremely likely that the gene designated *uncB* is distinct from the *uncA* gene described previously. Further work is in progress to compare the effects of the two mutant alleles concerned.

The *uncA* and *uncB* genes map close together at about minute 73.5 on the *E. coli* chromosome and may be contiguous. The significance of the clustering of these two genes remains to be determined.

Scheme 1 is advanced as a basis for further work and combines the present results with those obtained previously[1,6,11]. The scheme also indicates the important role apparently played by lactate oxidation in transport across membranes[12]. The



Scheme 1. The involvement of the uncB protein in oxidative phosphorylation and ATP-dependent transhydrogenase activity. fp= flavoprotein.

scheme does not take into account the requirement for a functional (Mg^{2+},Ca^{2+})-stimulated ATPase for anaerobic growth on glucose as carbon source. Phosphorylation coupled to electron transport between NADH and cytochrome *b*, has not yet been studied using the *uncA* or *uncB* mutants. The understanding of these two areas is the subject of continuing work.

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REFERENCES

1 Butlin, J. D., Cox, G. B. and Gibson, F. (1971) *Biochem. J.* 124, 75–81
2 Monod, J., Cohen-Bazire, G. and Cohn, M. (1951) *Biochim. Biophys. Acta* 7, 585–599
3 Luria, S. E. and Burrous, J. W. (1957) *J. Bacteriol.* 74, 461–476
4 Pittard, J. (1965) *J. Bacteriol.* 89, 680–686
5 Taylor, A. L. and Thoman, M. S. (1964) *Genetics*, 50, 659–677
6 Cox, G. B., Newton, N. A., Gibson, F., Snoswell, A. M. and Hamilton, J. A. (1970) *Biochem. J.* 117, 551–562
7 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
8 Doelle, H. W. (1969) *J. Chromatogr.* 39, 398–406
9 Newton, N. A., Cox, G. B. and Gibson, F. (1972) *J. Bacteriol.* 109, 69–73
10 King, E. J. (1932) *Biochem. J.* 26, 292–297
11 Cox, G. B., Newton, N. A., Butlin, J. D. and Gibson, F. (1971) *Biochem. J.* 125, 489–493
12 Kaback, H. R. (1972) *Biochim. Biophys. Acta*, 265, 367–416