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THE REQUIREMENT FOR ENERGY TRANSDUCING ATPase FOR ANAEROBIC MOTILITY IN *ESCHERICHIA COLI*

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SUMMARY

A sensitive radioactive assay for motility based on infection with ^{32}P-labeled, flagella-specific phage χ is described. Anaerobic infection with phage χ, which requires a motile host, is blocked in energy transducing ATPase (*unc* A) mutants. Anaerobic infection is restored by addition of NO_3^- which functions as a terminal electron acceptor for anaerobic respiration.

The isolation and properties of energy transducing ATPase (coupling factor) mutants of *Escherichia coli* have recently been described by several workers [1–11]. The best studied class of mutants, called *unc* A (uncoupled), produce little detectable membrane-associated Mg^{2+}-ATPase activity [2, 6, 7, 10]. ATPase defective mutants are blocked in a variety of energy transducing steps of the membrane including oxidative phosphorylation [2, 11], ATP-dependent reverse electron flow [2], anaerobic active transport [4, 8, 10], and anaerobic motility [12]. Also anaerobic growth is completely blocked [3, 6]. In a previous publication we mentioned that anaerobic growth is restored to *unc* A mutants by addition of nitrate as terminal electron acceptor; likewise NO_3^- was shown to restore anaerobic active transport of proline in one of the *unc* A strains [6]. This paper summarizes our studies on anaerobic motility in *E. coli*.

For these studies an *unc* A mutant (No. 45 of our collection) of *E. coli* K12 (1100), a motile strain, was derived by the Kanamycin enrichment procedure described earlier [6]. The pertinent properties of Mutant 45 which characterize it as an *unc* A mutant are: (a) a lack of detectable ATPase activity [2]; (b) map position near *ilv* (isoleucine–valine) on the genetic linkage map of *E. coli* as determined by Pl transductional analysis [2]; (c) lack of anaerobic growth with restoration by NO_3^- [6]. The strain yielded succinate-utilizing revertants at a frequency of about 10^{-6}, suggesting that a single point mutation is responsible for the *unc* phenotype. Flagellar mutants derived from *E. coli* K12 (1350) were kindly supplied by Dr Melvin Simon [13].

Flagella-specific phage χ [14–16] was grown and titered according to the procedures of Schade and Adler [14] except *E. coli* K12 (1100) served as host. For preparing ^{32}P-labeled virus *E. coli* 1100 was grown with shaking at 37 °C in 20 ml of

low phosphate medium [17] (with 0.4 % glycerol replacing glucose)containing 5 mCi $^{32}P_i$. The exponentially growing culture, previously adapted to low phosphate growth by several consecutive transfers in cold medium, was infected at a cell density of about 10^8 cells/ml with a multiplicity of infection of about 1. After 3–4 h for virus replication, unlysed bacteria were removed by centrifugation at $10\,000 \times g$ for 10 min and NaCl (to 0.5 M) and polyethylene glycol (to 6 %) were added to the crude phase lysate. After 2 h at 0 °C the virus fraction was collected from the polyethylene glycol solution by centrifugation at $20\,000 \times g$ for 20 min; 2 ml of potassium phosphate buffer (0.05 M at pH 7.0) was added and the polyethylene glycol pellet containing virus was slowly resuspended over a period of several hours. Virus (1 ml) was next layered over a CsCl step gradient composed of 3 CsCl bands of density, 1.34 (1 ml on top), 1.45 (1.5 ml middle) and 1.56 (1.0 ml bottom). Centrifugation, using an SW-50.1 rotor, was at $40\,000 \times g$ for 2 h at 4 °C. Tubes were punctured and the phage band collected dropwise. Phosphate buffer (0.1 M at pH 7.0) was added to make a 5-fold dilution of the CsCl solution and the sample was dialyzed at 4 °C against 3 changes of 0.05 M phosphate buffer (pH 7.0). Radioactive phage was stored at 0 °C. In one experiment starting with $5 \cdot 10^{10}$ phage in the crude lysate, 70 % were recovered immediately after CsCl centrifugation and about 20 % after dialysis. One cycle of CsCl banding gave sufficiently purified virus for our purposes; about 10^7 cpm in phage, enough for about 100 penetration assays, was recovered.

E. coli 1100 and *unc* A mutant 45 were grown in maltose (0.1 %)-minimal salts medium [6] supplemented with 0.2 % KNO_3 and 10 μg/ml L-methionine in stationary filled 250-ml flasks at 37 °C. The cells, grown to $1 \cdot 10^8$ ml, were collected by centrifugation at $10\,000 \times g$ for 5 min followed by an additional cycle of washing of the cell pellet with minimal salts solution without sugar in order to remove endogenous NO_3^-. Cells, freed of NO_3^-, were resuspended in glucose (0.4 %)–salts medium to a cell density of about $2 \cdot 10^8$–$3 \cdot 10^8$/ml and 1.8 ml of the suspension was transferred to a 5-ml erlenmeyer flask fitted with a tight-fitting rubber serum stopper. The flasks were flushed via needles connected to a vacuum pump using 4–5 cycles of evacuation and flushing with argon to remove traces of air. Radioactive phage (about $3 \cdot 10^4$–$5 \cdot 10^4$ cpm per $2 \cdot 10^8$ phage) were added by microsyringe through the serum stopper to start the assay. After suitable times of incubation at 37 °C cell samples of 0.3 ml were removed by syringe and added to pre-chilled tubes (0 °C) containing 10 ml of 10^{-4} M EDTA at pH 7.0. The sample was next blended in the small bucket of a Sorvall Omnimixer at 2/3 maximum speed for 2 min at 0 °C to remove flagella and phage that had not infected. The blended cell suspension was filtered directly on Millipore holders fitted with glass filter pads (Whatman, GFC-24), washed with 5 ml of water, and counted using a toluene liquid-scintillation system (Nuclear Chicago).

Schade et al. [14] have shown that the extracellular stage of χ infection is a complicated process with most phages having injected their DNA within 5 min after infection. This unique phage winds its tail fibers around the flagellum in a reversible reaction that does not require a moving flagellum. Next, the phage travels along the flagellum to the base, a reaction requiring movement. Finally the irreversible attachment and injection steps take place at the base of the flagellum as seen by the occurrence of "empty heads" at the flagellar base [14]. χ was found to normally infect a "curly" flagellar mutant which spins but shows no translational movement [14].

TABLE I

REQUIREMENT OF ACTIVE FLAGELLA FOR PENETRATION OF PHAGE χ-DNA

For the aerobic penetration assay the various flagella mutants were grown aerobically in Tryptone broth (1 % Tryptone) with shaking at 37 °C to a cell density of about $2 \cdot 10^8$–$4 \cdot 10^8$ cells/ml. Radioactive phage was added to 2 ml of bacterial culture in a small flask; after 10 min for penetration 0.3-ml aliquots were blended and counted as described in the text.

E. coli strain	Flagellar defect	χ-DNA penetration (cpm; ^{32}P)
1350	Wild type	2440
fla (*mot*) 92	Paralyzed flagella	0
fla E 1011	Polyhook	74
fla E 694	Polyhook	21
fla (*hag*) 912	Hook	0
fla D 691 A	No flagellar apparatus	0

These workers concluded that bacteria with moving flagella are necessary for χ infection. We have confirmed these findings using radioactive phage χ labeled with ^{32}P (DNA) (Table I); note from the table that radioactive χ is unable to infect a paralyzed strain or strains with defective flagellar components [13]). A trace of activity was noted with the "polyhook" mutants, strains producing unusually long hook components [13]. In this experiment a blending procedure (see above) was used to remove phage reversibly adsorbed to flagella, a reaction which does not require moving flagella [14]. For convenience we use the term "penetration" in reference to the phage DNA remaining with the cell fraction after vigorous blending. There is the possibility that some "full" particles which have migrated down the flagellum to the base may not be removed by blending; radioactive DNA in such tightly bound particles would not be distinguished in our assay from "penetrated" intracellular phage DNA. However, since migration of phage particles to the binding sites located at the flagellar base require a moving flagellum, radioactivity remaining associated with the blended cell in both instances is a measure of motility.

For the experiments reported here blended cells were collected directly on spun glass filter pads as described above. An alternate procedure which we found suitable is to collect the blended cells as a pellet by centrifugation, suspend the cells in a small portion of water, add trichloroacetic acid to precipitate the cells, and collect on a glass pad, and count as above.

The radioactive phage assay was found to be well suited for studies of motility of energy transducing ATPase mutants. As shown in Fig. 1, anaerobic motility is completely blocked in *unc* A Mutant 45; motility is restored by addition of 0.2 % NO_3^-. Since penetration as measured in this experiment is a measure of the overall extracellular stages of infection, we cannot rule out the possibility that cellular energy generated via energy transducing ATPase is necessary for not only motility but other undefined step(s) as well, such as phage DNA penetration. In this regard we are currently carrying out experiments on the energy requirements and possible essential function of ATPase for anaerobic transport of RNA, DNA and nucleoproteins during bacterial mating and phage infection. Larsen et al. [12] have recently carried out

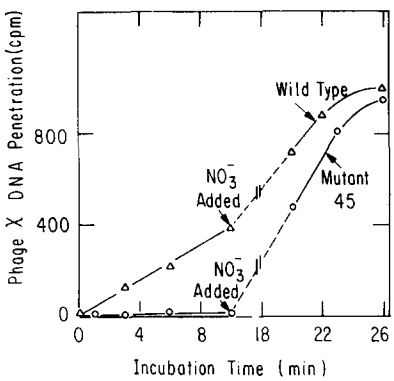

Fig. 1. Essential role of ATPase for anaerobic motility as measured by penetration of radioactive phage χ DNA. NO_3^- (0.2 %) was added at the times indicated by the arrows. Details of the χ assay procedure are given in the text.

a detailed study of the energy requirements for bacterial motility and the reader is referred to this interesting article for further discussion.

Our major conclusions from these experiments are that energy transducing ATPase is essential for anaerobic motility but is not necessary for respiratory-dependent motility.

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