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Membrane Mutation Affecting Energy-linked Functions in *Escherichia coli* K 12

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ABSTRACT. A small-colony forming variant of *Escherichia coli* with a mutation in the *ncf* gene was analysed. The alternation of the protein composition in the cytoplasmic membrane and the interaction with K and E group colicins indicated a membrane mutation. The effect of this mutation on some membrane-bound processes, the activity of Mg^{2+}-activated ATPase, the growth on different carbon sources and the active transport of amino acids, is described. This mutation does not exert any effect on the electron transport system.

Mutational changes in the bacterial cell surface mostly exhibit a pleiotropic effect (Rolfe and Onodera, 1972; Rolfe *et al.*, 1973; Ohki and Mitsui, 1974; Osborn *et al.*, 1974) and such mutants differ in their sensitivity to various detergents (Onodera *et al.*, 1970; Bernstein *et al.*, 1972), antibiotics (Ennis, 1971; Ennis and Bloomstein, 1974) and colicins (Nagel de Zwaig and Luria, 1967; Rolfe and Onodera, 1971; Buxton and Holland, 1973, 1974). Some membrane mutations are known to have an altered activity of membrane-bound enzymes (Butlin *et al.*, 1971; Rolfe and Onodera, 1972; Nieuwenhuis *et al.*, 1973). The pleiotropic effect observed in the formation of several membrane components and in cell growth may be caused by a single mutation or these properties may be the result of multiple mutations with different cross interactions.

In our previous work we have analyzed the effect of a mutation in the *ncf* gene on the growth and some other properties of *Escherichia coli*. The bacterial strains differ in their colony-forming ability, in cell density and in accumulation of reserve material (Hubáček *et al.*, 1972), and in ATP and cAMP metabolism (Braná *et al.*, 1973). This study was undertaken to analyze the effect of the *ncf* mutation on some structural and functional properties of the cell membrane.

MATERIALS AND METHODS

Bacterial strains. Escherichia coli HK 26 and HK 66 were described previously (Hubáček *et al.*, 1972). *Escherichia coli* K 12 strains carrying col K(K49), E1(K30)

and E3(CA38) plasmids and *Salmonella typhimurium* LT_2 strain with E2(P9) plasmid were used for colicin production.

Media. M9 synthetic medium, Difco L-broth (LB) and L-agar (LA) were used throughout the experiments. Difco Nutrient broth (NB) and agar (NA) were used for the determination of the effect of detergents and antibiotics. For the isolation of membrane proteins and enzyme preparations bacteria were grown in Difco Penassay broth (PNS).

Determination of cell sensitivity to detergents and antibiotics. The effect of sodium dodecylsulphate (SDS), vancomycin and bacitracin was tested as described by Bernstein *et al.* (1972). Sensitivity to sodium deoxycholate (NaDOC) was determined on NA with appropriate concentrations of NaDOC at 37 °C for 24 h.

Preparation of membrane fractions for Mg-ATPase assay. The membrane fractions were obtained from spheroplasts prepared by the lysozyme method (Spizizen, 1962). Spheroplasts were lyzed and membranes were processed according to Evans (1969).

Enzyme assay. Mg-ATPase was determined according to Evans (1969), using 2 mM Mg^{2+} at pH 9.0. Proteins were determined by the procedure of Lowry *et al.* (1951).

Preparation of membrane proteins for gel electrophoresis. Membranes were isolated and lipids were extracted as described by Onodera *et al.* (1970). Membrane proteins were solubilized according to Schnaitman (1971) with the exception that 10 mM Tris buffer of pH 7.1 was used.

Polyacrylamide gel electrophoresis. The electrophoresis was done in 5×90 mm tubes using 8.33% acrylamide and 0.22% bis(acrylamide) in 0.1 M Tris-phosphate buffer (pH 7.1) in the presence of 0.2% Triton X-100. The duration of the run was 20 min at 2 mA/tube (50 V) and then for 100 min at 7 mA/tube (140 V). The same amounts of protein were applied in 50—100 µl per tube with 1.5% β-mercaptoethanol and 22.5% glycerol; proteins were monitored with bromophenol blue. The detection of protein bands was done with Amido black 10B in 7% acetic acid.

Isolation of colicin-tolerant mutants. The production and assay of colicin, isolation of spontaneous colicin K-resistant mutants and the receptor test for detection of colicin resistance or tolerance were done according to Hill and Holland (1967).

Transport of amino acids. Aerated cells (2 h on a magnetic stirrer in a growth medium without carbon source) were incubated at 25 °C at a density of about $3 \cdot 10^{10}$ cells ml^{-1} in the presence of 10 mM D-glucose or D-lactate. After 10 min labelled amino acid (L-proline or L-arginine) was added to a final concentration of 0.1 mM and 0.1 ml samples were withdrawn after 15 s, 1, 2, 3, and 4 min and squirted into 2 ml ice-cold water in the funnel of a membrane filtration apparatus (Synpor 6 filters with average pore diameter of 0.45 µm were used). The filter with cells was then placed directly into a toluene scintillation liquid and counted in a Nuclear Chicago Mark I scintillation counter.

RESULTS

Antibiotic and detergent sensitivity of HK 26 and HK 66 mutants

To study alterations of the cell surface in *Escherichia coli* strains we tested the effect of NaDOC and SDS on the viability of the cells, the results being given in Table I. The same effect of SDS was found in both strains whereas the strains were insensitive to NaDOC. Bacitracin and vancomycin are supposed to affect the bio-

TABLE I. Sensitivity of *Escherichia coli* HK 26 and HK 66 to NaDOC and SDS, expressed as viable cell counts per ml

Detergent	Strain	Concentration of the detergent in %			
		0	0.1	0.5	1
SDS	HK 26	1.8×10^{8}	6.3×10^{6}	1.0×10^{6}	3.8×10^{5}
	HK 66	1.1×10^{8}	5.9×10^{6}	1.5×10^{6}	8.8×10^{5}
NaDOC	HK 26	1.1×10^{9}	1.4×10^{9}	7.1×10^{8}	7.4×10^{8}
	HK 66	1.3×10^{9}	1.4×10^{9}	7.2×10^{8}	8.5×10^{8}

The sensitivity to SDS was determined as described in the Methods. The effect of NaDOC was measured by spreading NB overnight-grown cells on NA plates with NaDOC; the number of colonies was estimated after 24 h cultivation at 37 °C.

synthesis of the cell envelope and their action might be correlated with the effect of detergents (Bernstein *et al.*, 1972). Both mutants were found to be resistant to bacitracin (Table II) but sensitive to vancomycin, HK 66 being more sensitive than HK 26, but in no case could a decisive difference between the two strains be found.

Response of HK 26 and HK 66 mutants to colicins and analysis of their membranes

The strains were sensitive to colicins E1, E2, E3 and K to the same degree except that HK 66 was found to be more resistant to colicin K. The reciprocal of the highest dilution of colicin K preparation giving minimum inhibition of growth on LA was 10^4 and 10^3 for HK 26 and HK 66, respectively. For a quantitative estimation, overnight cultures in LB were mixed in equal proportion with serial dilutions of colicin K, incubated for 45 min at 35 °C and immediately diluted and plated on LA. An approximately 10 times higher concentration of colicin K was needed for a 50% killing effect on HK 66 in comparison with the HK 26 strain. To distinguish between receptor loss or tolerance, mixtures of the bacteria and colicin K in saline were incubated for 1 h at 37 °C, the bacteria were removed by centrifugation and residual

TABLE II. Effect of vancomycin and bacitracin on *Escherichia coli* HK 26 and HK 66, expressed as viable cell counts per ml

Antibiotic	Concentration μg/ml	Strain	
		HK 26	HK 66
Vancomycin	0	2.4×10^{8}	2.3×10^{8}
	5	1.2×10^{8}	2.1×10^{8}
	25	2.3×10^{7}	1.3×10^{4}
	50	1.3×10^{4}	1.0×10^{3}
Bacitracin	0	1.3×10^{8}	1.5×10^{8}
	80	6.8×10^{7}	7.1×10^{7}
	160	6.0×10^{7}	9.0×10^{7}
	320	7.8×10^{7}	9.0×10^{7}

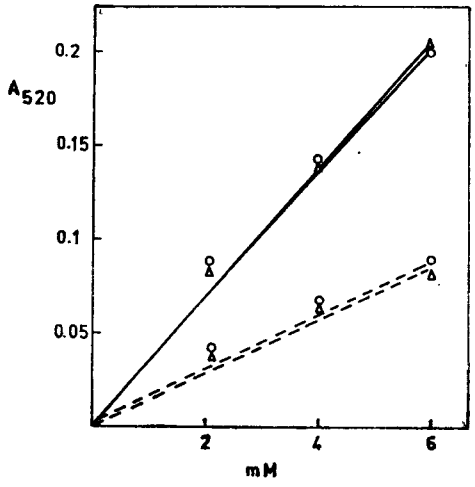

FIG. 1. The growth of *Escherichia coli* strains HK 66 (△) and HK 26 (○) under aerobic (——) and anaerobic (----) conditions in M9 minimal medium with the required supplements and limited concentrations of glucose (mM) A_{520}, Absorbance at 520 nm.

colicin assayed. The HK 66 cells adsorbed colicin K less efficiently and the partial resistance of this strain is thus due to receptor loss.

For further analysis of the cell surface we selected colicin K tolerant mutants of both strains and determined their response to colicins E1, E2 and E3. Table III demonstrates that the HK 26 colK tolerant colonies belong mostly to phenotype C and are resistant to colicins E1 and E2, whereas among HK 66 colonies there pre-

TABLE III. The response of col K tolerant colonies of *Escherichia coli* HK 26 and HK 66 strains to colicins E1, E2 and E3. The results are expressed as percent of *col* K-tol mutants analyzed for their sensitivity (S) or resistance (R) to colicins E1, E2 and E3. (In HK 26 53 and in HK 66 54 mutants were tested.)

Phenotype	Colicin			*E. coli* KH 26	*E. coli* HK 66
	E1	E2	E3		
A	S	S	S	0	33.3
B	S	R	S	13.2	42.7
C	R	R	S	86.8	22.2
D	R	R	R	0	1.8

dominate those that are sensitive either to all E colicins (phenotype A) or resistant only to colicin E2 (phenotype B).

On the basis of different response of the mutant strains to colicins and the alteration in the energy metabolism of the cells found previously (Hubáček *et al.*, 1972; Braná *et al.*, 1973) we assumed that the *ncf* mutation might excert its effect on the protein composition of the cytoplasmic membrane. Proteins from cytoplasmic membranes were examined by polyacrylamide gel electrophoresis. As demonstrated on Plate 1, the samples differ in two bands: one band is missing in HK 26, another in HK 66.

Table IV. Growth of HK 26 and HK 66 strains on different carbon sources. The relative growth yields were obtained by measuring the absorbance of cultures as they entered the stationary phase

Carbon source	Mean generation time min		Relative growth yield 66/26
	HK 26	HK 66	
Glucose, 3.5 mM	43.8	41.0	1.01
Fructose, 3.5 mM	65.7	65.2	0.70
Lactate, 20 mM	76.7	82.1	0.76
Succinate, 20 mM	60.1	77.0	0.84
Acetate, 20 mM	no growth	no growth	—

Effect of the ncf *mutation on some membrane-bound processes*

To learn more about membrane processes, the uncoupling of which may be the result of an alteration in membrane structure, we tried to define the efficiency of the electron transport systems in our mutants. We studied the growth ability of the bacteria on limited concentrations of glucose under aerobic or anaerobic conditions, the ability of the cells to utilize other substrates as the sole source of carbon (fructose, succinate, lactate and acetate) and the active transport of proline and arginine. It was found that both strains grown anaerobically give approximately 50% lower growth yields than the same strains growing under aerobic conditions (Fig. 1). The strains utilize all of the substrates tested for growth with the exception of acetate. The mean generation times and the relative growth yields of both mutants are given in Table IV.

Table V. Uptake of 0.1 mM L-proline and L-arginine by *Escherichia coli* strains, expressed in nmol amino acid taken up per min by mg dry weight (means from 2—4 experiments are shown)

Amino acid	Energy source	Inhibitor	HK 26	HK 66
L-Proline	glucose	none	1.85	5.0
		30 mM KCN	0.09	0.12
	lactate	none	1.00	1.1
		30 mM KCN	0.06	0.08
L-Arginine	glucose	none	1.74	1.66
	lactate	none	1.09	1.05

The transport of L-proline and of L-arginine was chosen for study since it has been reported that L-proline requires functioning oxidative phosphorylation and proton circulation to be transported (*e.g.* Harold, 1975) while L-arginine (like L-glutamine, L-ornithine and L-histidine) can be supported by substrate-level ATP (Berger, 1973). Table V shows that both amino acids were transported at a higher rate when glucose rather than lactate served as energy substrate but that the uptake

was completely abolished in the presence of 30 mM KCN. There is, however, a striking difference in the uptake of proline by the two strains, the HK 66 transporting much more rapidly (the same holds for the uptake of glycine, not reported here in detail). On the other hand, the uptake of arginine does not differ significantly in the two.

The activity of Mg-ATPase which is involved in the processes coupled to electron transport, such as oxidative phosphorylation and active transport was found to be similar in the two: 0.24 μmol P_i min^{-1} (mg protein)$^{-1}$ in HK 26 and 0.30 μmol P_i min^{-1} (mg protein)$^{-1}$ in HK 66.

DISCUSSION

Strain HK 66, a small-colony variant of *Escherichia coli*, does not grow on acetate. As the utilization of acetate requires ATP in this organism, cells with a defect in energy metabolism might not possess sufficient energy to permit cell growth on the carbon source in question. In mating experiments with the HK 66 mutant as the recipient we succeeded in obtaining normal-colony forming recombinants and the gene controlling this phenotype, called *ncf*, was mapped and located close to the *pro* marker (Hubáček *et al.*, 1972). The *ncf* mutation exhibits a pleiotropic effect on some cell properties such as the colony-forming character, cell density and the accumulation of reserve glycogen-like material (Hubáček *et al.*, 1972) and the ATP and cyclic 3′,5′-AMP levels in the cell (Braná *et al.*, 1973). These findings, together with the alteration in the protein composition of the cytoplasmic membrane (Plate 1) and the different response of the colicin K-tolerant mutants to E group colicins indicate a membrane mutation in the HK 66 strain, which might be associated with a defect in its energy metabolism.

As to the differences in the membrane protein composition it is not possible to decide on the basis of the electrophoretic pattern whether only one or two different proteins are involved.

The nature of the deficiency in the energy-linked reactions in our mutant is not clear, yet it does not seem to be caused by the absence of a component of the electron transport chain. An ubiquinone-deficient mutant of *Escherichia coli*, defective in the electron transport, isolated by Cox *et al.* (1970), exhibited the same growth yields on limited concentrations of glucose under aerobic as well as anaerobic conditions and was able to grow with glucose but was unable to utilize malate or succinate as the sole carbon sources. Our strain utilizes succinate and, when grown anaerobically, it gives a much lower growth yield as compared with aerobic growth (Table IV, Fig. 1). Alterations in membrane structure could be registered as changes in reactions coupled to electron transport, such as oxidative phosphorylation and active transport. Butlin *et al.* (1971) have isolated *unc* A mutants unable to use succinate or lactate as carbon source which have lost the enzyme essential for oxidative phosphorylation, the Mg-activated ATPase. Our mutant utilizes the above substrates and its unchanged activity of Mg-ATPase distinguishes it from the type of *unc* A mutant strains. Nevertheless, the results obtained here with the growth yield under anaerobic conditions and the unchanged activity of Mg-activated ATPase do not exclude the *unc* B mutation in our strain (Butlin *et al.*, 1973).

The rationale for studying the uptake of amino acids was to establish whether the HK 66 mutant is able to generate a high-energy membrane state which may be required for certain energy-driven processes in the cell.

The uptake of proline appears to be energized by respiratory reactions in both strains tested (complete suppression by KCN) but glucose serves as a better energy source than lactate. This may be associated with differences in the level of available ATP and it tallies with the previous results (Hubáček *et al.*, 1972; Braná *et al.*, 1973) that the HK 66 strain contains a higher ATP level than the HK 26 one. However, the uptake of arginine, presumably depending on ATP specifically, was practically the same in both mutants. At present, we have no explanation for the diverse phenomena.

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The plate will be found at the end of the issue.

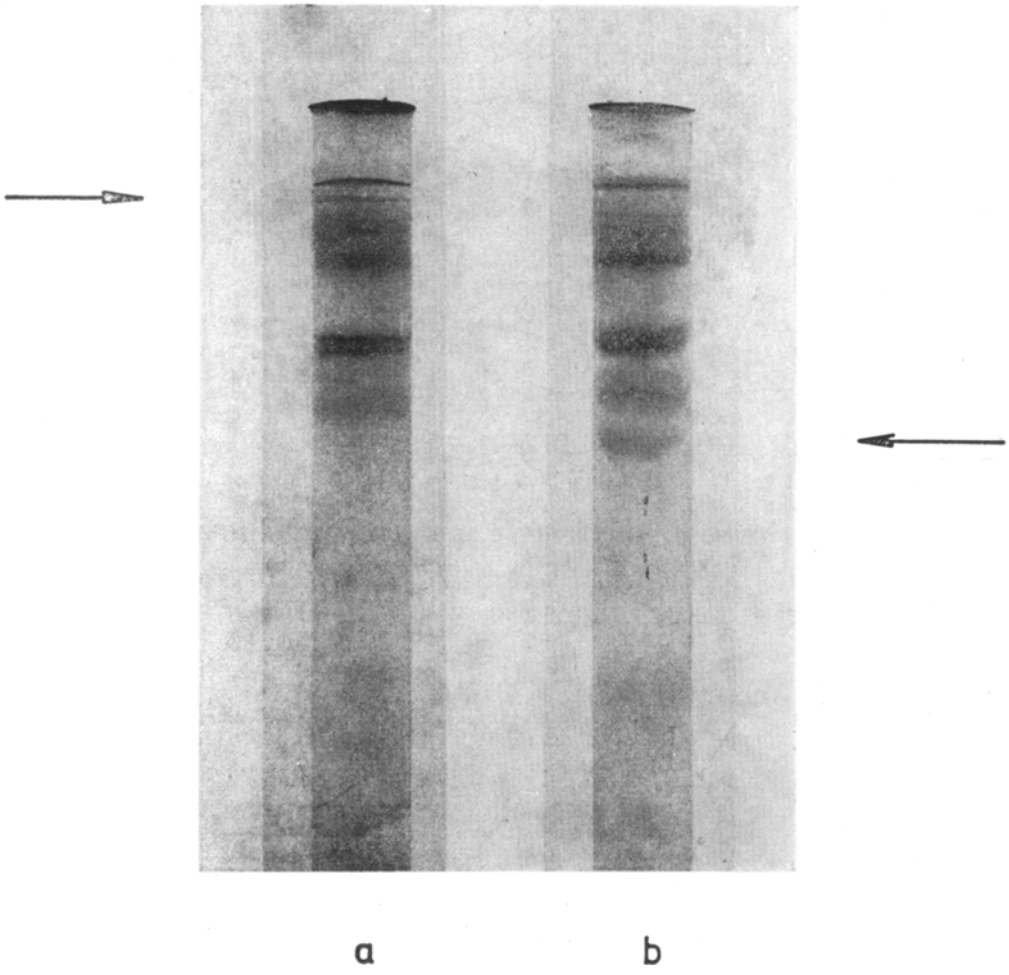

PLATE 1. Polyacrylamide gel electrophoresis of membrane proteins of *Escherichia coli* HK 66 (a) and HK 26 (b). The two differing bands are marked with arrows.