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A Mutant of *Escherichia coli* Defective in the Coupling of Metabolic Energy to Active Transport

(temperature-sensitive mutant/energy coupling factor/cystathionase)

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ABSTRACT **The isolation of a temperature-sensitive mutant of *E. coli* K12 whose active transport of amino acids and sugars is not coupled to metabolic energy at 42° is described. This mutant cannot grow on succinate, fumarate, malate, or D-lactate as sole carbon source at 42° and grows on glucose at 42° with a reduced rate and yield. Efflux of accumulated substrate is also demonstrated upon heat inactivation. The defect of this mutant in both growth and transport is not due to a failure in electron transport through the respiratory chain nor the absence of Mg, Ca-ATPase activity. The mutant is thus distinct from the other energy-uncoupled mutants *uncA*, *uncB*, or *etc.* Analysis of spontaneous revertants indicates that the transport defect is caused by two mutations, one in the energy coupling factor gene and the other in the *metC* gene. The *ecf*^{ts} mutation has been mapped to be in the 54.5- to 60-min region of the *E. coli* chromosome map. Possible interactions between the *metC* mutation and the mutated energy coupling factor protein are discussed.**

The molecular mechanisms by which metabolic energy is coupled to active transport in bacteria have been a subject of considerable interest (1–5). In the *Escherichia coli* membrane vesicle system the oxidation of D-lactate to pyruvate facilitates the transport of a wide variety of amino acids and sugars against a concentration gradient (1, 6). In whole cells the main energy source utilized for aerobic transport comes from respiration and the generation or utilization of ATP does not seem to be involved (7, 8). On the other hand, cells apparently utilize the energy derived from the hydrolysis of ATP for anaerobic transport, as demonstrated in studies on mutants defective in the Ca,Mg-ATPase (7, 9). The form of the metabolic energy, whether it be a high-energy intermediate of oxidative phosphorylation, an energized membrane state, or some other form, has not yet been determined (2, 10–12).

In this paper we describe a mutant that is temperature-sensitive in a pleiotropic manner for the active transport of amino acids and sugars and is altered in a component energy coupling factor (ECF) that is necessary for the coupling of metabolic energy to transport. We also present evidence that a derivative of methionine may be interacting with the altered protein.

MATERIALS AND METHODS

Bacterial Strains. All strains used were of *E. coli* K12 and are listed in Table 1.

Media. Nutrient broth or minimal salts medium E (13) containing 0.5% carbon source and 40 μM vitamin B1 was used. When required, amino acids were added at 0.4 mM. Solid media contained 1.5% agar.

Phage. P1kc was used for transductions. Phage lysates were prepared by the plate method (15) and stored at 4°. Transductions were carried out using a modification (16) of the procedure of Luria *et al.* (17).

Isolation of Mutants. Nitrosoguanidine mutagenesis was done according to Adelberg *et al.* (18). Auxotrophs were enriched with the use of penicillin. Neomycin-resistant mutants were selected at 42° in the presence of 20 μg/ml of neomycin. *thyA*$^-$ mutants were selected by resistance to trimethoprim as described by Miller (19). HfrH strains were made female by treatment with 10% sodium dodecyl sulfate–Penassay broth as described (20) with a slight modification: cells were incubated with sodium dodecyl sulfate for 5 days at 18° instead of for 3 days at 37°. Survivors were checked for loss of male-

TABLE 1. *Bacterial strains used*

	Relevant genotype	Comments
JSH1	HfrH: *thi*	From R. F. Schleif.
JSH4	HfrH: *thi, ecf, metC**	JSH1 by nitroso-guanidine.
JSH197	F$^-$: *thi, purH47, asn, ilv, his, str-117*	From PC0132 (obtained as CGSC 4496 from B. Bachmann) and made *ilv, his, asn* by diethylsulfate.
JSH18	HfrH: *thi, ecf, metC* str-117*	From JSH4 by transduction with P1 lysate of JSH197.
JSH122	HfrH: *thi, ecf, ilv, metC*, str-117*	From JSH18 by diethylsulfate.
MAL60	F$^-$: *thi, ecf, ilv, metC*, str-117*	From JSH122 by sodium dodecyl sulfate–Penassay broth treatment.
MAL66	F$^-$: *thi, ecf, ilv, thyA, metC*, str-117*	From MAL60 by trimethoprim resistance.
4257	F′: *argG*$^+$, *thy*$^+$/*argG6, metB1, his-1, leu-6, thy-23, recA1*	Episome F′122, from B. Bachmann.
4291	F′: *lysA*$^+$, *tyrA*$^+$/*tyrA2, pyrD34, thi-1, his-68, trp-45, thyA33, recA1*	Episome F′143 from B. Bachmann.
4254	F′: *metC*$^+$, *fuc*$^+$/*argG6, metB1, his-1, thyA23, leu-6, recA1*	Episome F′116 from B. Bachmann.

*metC** is discussed in the text.

Abbreviations: ECF, energy coupling factor; SFML, succinate, fumarate, malate, and D-lactate; Suc, the ability to grow on succinate, fumarate, malate, or D-lactate as sole carbon source; Met, Thy, the ability to grow without exogenous methionine or thymine, respectively; *ts*, temperature-sensitive.

ness by their ability to become prototrophic after mating with an appropriate episome-carrying strain.

Matings. Episome matings were performed as described (21). Episome-carrying strains were always grown in minimal media to insure that the presence of the episome was necessary for satisfaction of at least one nutritional requirement of the host strain.

Preparation of Membrane Vesicles. Membrane vesicles were prepared according to Kaback (22).

Transport Assays. Amino acid, sugar, and α-methylglucoside transport were assayed in membrane vesicles as previously described (22) and in whole cells as described in the legend to Fig. 1. Unless otherwise indicated the specific activities and final concentrations of the radioactive compounds used were as follows: L-[*U*-^{14}C]proline (255 Ci/mol), 7.7 μM; β-methyl-D-[^{14}C]thiogalactoside (14.4 Ci/mol), 100 μM; L-[*1*-^{14}C]arabinose (9.78 Ci/mol), 220 μM; L-[*U*-^{14}C]-alanine (157 Ci/mol), 13 μM; L-[*U*-^{14}C]serine (156 Ci/mol), 12.8 μM; L-[*U*-^{14}C]tyrosine (460 Ci/mol), 4.4 μM; methyl-α,D-[^{14}C]glucopyranoside (52.2 Ci/mol), 36 μM.

Oxygen Uptake. Rates of oxygen uptake were measured with a Clark electrode as previously described (23).

Cystathionase. Cystathionase (EC 4.4.1.1) activity was assayed on either toluenized cells or sonic-disrupted cell-free extracts as previously described (24).

Protein Determinations. Protein was determined as described by Lowry *et al.* (25) with bovine serum albumin as standard.

Materials. Radioactive amino acids, L-[*1*-^{14}C]arabinose, [γ-^{32}P]ATP, and β-methyl-D-[^{14}C]thiogalactoside were obtained from New England Nuclear Corp. Cystathionine was purchased from Calbiochem. Methyl-α,D-[^{14}C]glucopyranoside was the generous gift of Dr. H. R. Kaback. All other materials were reagent grade and purchased from commercial sources.

RESULTS

Isolation and Transport Properties of the Mutant. A temperature-sensitive (*ts*) neomycin-resistant mutant was isolated from nitrosoguanidine-induced JSH1 cells. This mutant, JSH4, grows as well as wild type on glucose, succinate, malate, fumarate, and D-lactate as sole carbon source at the permissive temperature (25°). However, at the nonpermissive temperature (42°) the mutant does not grow on succinate, fumarate, malate or D-lactate (SFML), and grows on glucose with a markedly reduced rate and cell yield compared to wild type.

This mutant is found to be pleiotropically defective in active transport at 42°. Fig. 1 shows the transport of various amino acids and sugars by aerobically grown (25°) wild-type and mutant cells at both 25° and 42°. Clearly the transport ability of the mutant is sensitive to high temperature, and essentially no transport substrate is taken up under these

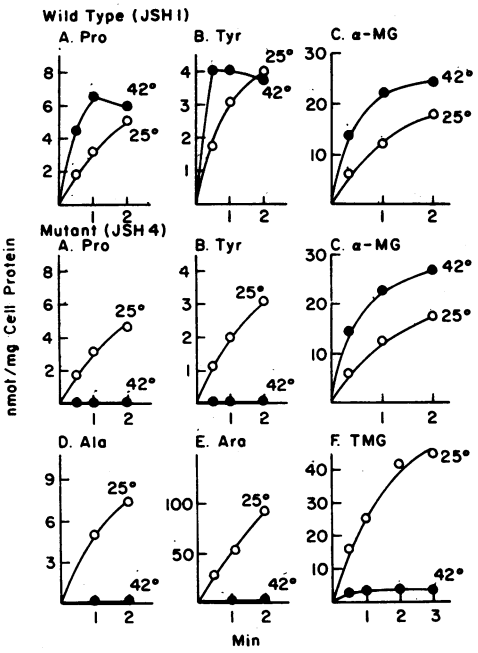

FIG. 1. Uptake of amino acids and sugars by intact cells. Cultures were grown with vigorous shaking by diluting overnight cultures 100-fold with fresh medium (100 ml in a 500-ml Erlenmeyer flask) and were harvested in mid-log phase (OD_{660} = 0.3–0.7). To harvest, the cells were washed once with and resuspended in carbon-free salts medium (14) containing 100 μg/ml of chloramphenicol to an OD_{660} = 5.0. Transport was assayed in the absence of added carbon source, and performed as follows. Cells (50 μl) in 12 × 75 mm test tubes were preincubated at 25° or 42° for 2 min before the addition of radioactive substrate. At various times thereafter a tube was removed from the water bath, 2 ml of carbon-free salts medium were added, and the cells were collected and washed once with 2 ml of the same medium on a Millipore filter (0.45-μm pore size). The filter was then dried and the radioactivity was measured in a gas-flow counter. For the assays described above cells were grown in minimal glucose medium at 20° with the following exceptions: to measure arabinose uptake cells were induced for the arabinose transport system by growth in 0.5% arabinose, and thiomethylgalactoside (TMG) uptake was assayed on cells grown in 0.5% galactose minimal media containing 0.5 mM isopropylthiogalactoside (IPTG). Transport was assayed at 25° and 42°. α-Mg is α-methylglucoside.

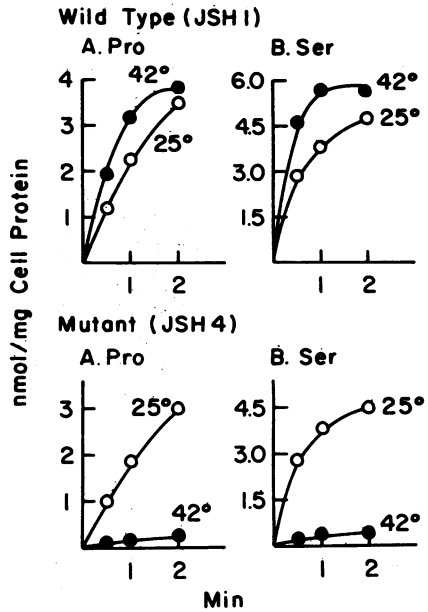

FIG. 2. Uptake of proline and serine by anaerobically grown (20°) cells. Cells were grown in 250-ml Erlenmeyer flasks containing 240 ml of media and 25 mM $NaHCO_3$ without shaking. Thirty minutes prior to harvesting (OD_{660} = 0.3–0.7) cultures were chilled in ice and chloramphenicol was added to 100 μg/ml. Cells were harvested and transport assays were performed as described in the legend to Fig. 1.

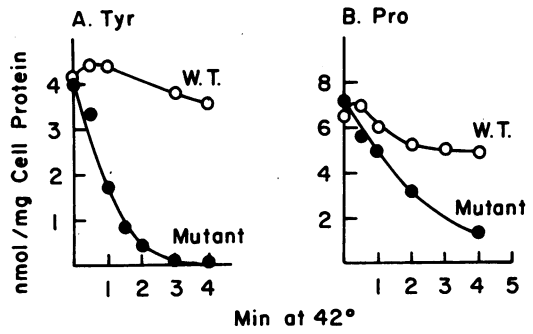

FIG. 3. Efflux of amino acids from cells. Washed cells were loaded with either proline or tyrosine for 8 minutes at 25°, then rapidly immersed in a 42°-water bath. At various times after the temperature shift the retention of substrate was assayed as described in the legend to Fig. 1. Cells were grown in glucose minimal medium at 20°. W.T. is wild type.

assay conditions. In addition to the data shown, the uptake of glutamine, serine, lysine, methionine, and D-galactose were also found to be sensitive to high temperature. No other amino acids or sugars were tested. Anaerobically grown mutant cells, as shown in Fig. 2, manifest the same defect in transport as aerobically grown mutant cells. The heat inactivation of the ability to transport is rapid and follows first order kinetics with a half-life ($t_{1/2}$) of approximately 1 min at 42°. The inactivation of transport by heat is irreversible; mutant cells either partially or completely inactivated for transport at 42° cannot regain the lost transport ability at 25°, even after cooling in ice or at 25° for as long as 3 hr. Fig. 3 shows that when mutant cells are preloaded with transport substrate at 25° a temperature shift to 42° causes a rapid efflux of the substrate. Thus, not only the ability of the mutant cells to *transport*, but also their ability to *retain* the transport substrate accumulated inside the cells is sensitive to high temperature.

To ensure that the *ts* defect in transport is not due to a nonspecific membrane lesion, we also studied the transport of α-methylglucoside (Fig. 1). This glucose analogue is transported via the phospho*enol*pyruvate–phosphotransferase system (26) by a mechanism that has no direct relationship to respiration-dependent transport (1). As shown, the mutant transports α-methylglucoside at the same rate as does the wild type at both 25° and 42°, thus indicating that the mutation specifically affects the respiration-driven transport systems.

TABLE 2. *Rate of oxygen uptake by intact cells*

Cells	Stimulation of oxygen uptake, ng-atoms/min per mg of cell protein, in the presence of: D-lactate (20 mM)	Succinate (20 mM)	Glucose (20 mM)
Wild Type (JSH1)			
unheated	83	196	328
heated	141	151	336
Mutant			
unheated	62	202	393
heated	57	123	587

Cells were grown in minimal glucose medium and harvested in late log phase as described in Fig. 1. Oxygen uptake was measured at 25.5°. The vessel contained 2.9 ml carbon-free salts medium containing 100 μg/ml of chloramphenicol and 100 μl of cells (OD_{660} = 50) which were allowed to equilibrate for 5 min. The probe was then inserted and an endogenous rate was determined. To measure the rate in the presence of added carbon source, 60 μl of an 1 M solution was then added. The endogenous rate of oxygen uptake has been subtracted in the values given here. The heated cells were prepared by placing the cells in a 42° water bath for 3 min and cooling in ice before using.

TABLE 3. *Mg^{++}-activated ATPase activities*

Strain		P_i released, nmol/mg protein per min $-Mg^{++}$	$+Mg^{++}$
JSH1	25°	30	258
	42°	32	630
JSH4	25°	19	215
	42°	37	480

The activity was assayed in a reaction mixture (100 μl) containing 0.1 M Tris· HCl (pH 8.0), 2 mM $MgSO_4$ (when present), membrane particles (3–6 μg of protein), and 0.05 M [γ-^{32}P]ATP (2.0×10^6 cpm/μmol). [^{32}P]P_i released was extracted by the method described (28) and measured in a scintillation spectrometer. Membrane particles were prepared according to the method of Fisher *et al.* (29) except that $MgSO_4$ and bovine serum albumin were omitted throughout the entire procedure.

The *ts* defect of the mutant in transport and growth is not attributable to a defect in the respiratory chain, as the rate of oxygen uptake of the heated mutant cells in the presence of various carbon sources is similar to that of unheated mutant cells or wild-type cells (Table 2). The oxygen utilization of these cells in the presence of these carbon sources is sensitive to cyanide (3 mM), as expected. The slightly lowered rates of oxygen uptake by the heated mutant cells in the presence of D-lactate or succinate as compared to the wild type may be partially due to their inability to efficiently transport these compounds, as Matin and Konings (27) have shown that the transport of these substrates occurs via a respiration-dependent system.

The data presented in Table 3 indicate that the *ts* defect in transport is not caused by the lack of a functional Ca,Mg-ATPase, as mutant membrane particles show normal ATPase activity at 42°.

Transport Properties of Membrane Vesicles. As shown in Fig. 4, membrane vesicles prepared from JSH4 cells grown at 24° were able to transport normally at both 25° and 42°. Normal transport was also observed with membrane vesicles prepared from JSH4 cells that had been inactivated for transport by heating for 10 min at 42° (data not shown). Thus, vesicles prepared from this mutant behave like wild-type vesicles in their transport ability, even if transport has been inactivated prior to the vesicle preparation.

Two Distinct Classes of Revertants of JSH4. To determine whether there was a genetic relationship between the inability of the mutant cells to grow on SFML and their inability to transport at 42°, we isolated and studied revertants. Revertants able to grow on succinate at 42° appeared spontaneously (at a frequency of 6×10^{-6}). These secondary mutants (Class I Suc$^+$ revertants) regained not only the ability to grow on SFML as sole carbon source, but also the ability to transport amino acids and sugars at 42°. Revertants were also isolated under the same conditions but with a limiting amount of amino acids and vitamins (in the form of 0.01% nutrient broth) present. Under these selection conditions, in addition to

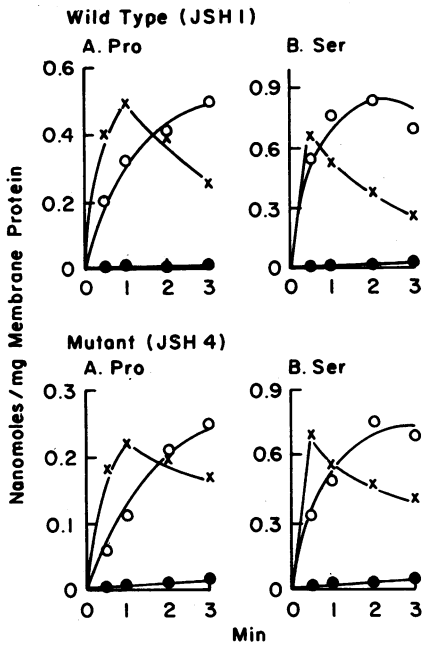

FIG. 4. Uptake of proline and serine by membrane vesicles. Vesicles were prepared (22) from cells grown in minimal glucose medium at 20°. No D-lactate added, ●; 20 mM D-lactate added, 25°, O; 20 mM D-lactate added, 42°, ×.

Class I, a distinct class of Suc$^+$ revertants (Class II) was found that required methionine for growth. Class II revertants regained simultaneously the ability to grow on SFML and the ability to transport at 42°. No other auxotrophic revertants were found. These results demonstrate that the defects of JSH4 cells, both in growth and in transport at 42°, are a consequence of the same mutation (or mutations) and that the return to functionality can be effected by secondary mutations in either of two ways, one resulting in a requirement for methionine and the other not.

Two Mutations Affecting Active Transport. The reversion study described above strongly suggests that the defect of JSH4 cells in growth and transport probably results from two mutations. To determine whether this was the case, we used P1 lysates of JSH1 and JSH4 to transduce Class II revertants to methionine independence (Met$^+$) on minimal glucose plates at 25°. The resulting transductants were then tested for both the ability to grow on SFML and the ability to transport amino acids and sugars at 42°. When a P1 lysate of JSH1 was used to transduce 13 independently isolated Class II revertants to Met$^+$, the following results were obtained. In each case all transductants (105/105) were found to be unable to grow on SFML at 42°, and of those assayed (one transductant from each of 10 different Class II revertants) all were found to be able to transport at 42°. Thus these Met$^+$ transductants are similar to the mutant JSH4 in their inability to grow on SFML but are different in that they are able to transport at 42°. These results demonstrate clearly (*i*) that the *ecf* (energy coupling factor) mutation conferring the *ts* defect in growth is not linked to the *met* mutation by P1 transduction and hence is still present in the Met$^-$ revertants, and (*ii*) that the *ecf*^{ts} mutation alone is sufficient to render the mutant a *ts* Suc$^-$ phenotype. When a P1 lysate of JSH4 was used for the transductions, a different result was obtained. Although in each case all transductants (136/136) were again found to be unable to grow on SFML at 42°, of those assayed (one tranductant from each of eight Class II revertants) all were found unable to transport at 42°. Thus these transductants phenotypically (and undoubtedly genotypically as well) are identical with the mutant JSH4 in their defect in transport and growth on SFML at 42°. These results indicate that a mutation (*met**) is reintroduced into the Met$^+$ transductants which is present in JSH4 but absent in JSH1 and that two mutations are needed to phenotypically express the transport defect, one in the *ecf* gene and the other in a *met* gene (identified below as *metC*). If only the *ecf*^{ts} mutation is present, as in the Met$^+$ transductants obtained with phage lysate of JSH1, the ability to grow on SFML is temperature-sensitive but transport is normal. If only the *met** mutation is present, as in Class I revertants, a wild-type phenotype is expressed. The nature of the interaction between the two mutations is not yet fully understood.

Identification of the met Auxotrophic Mutations. The *met* mutations of Class II revertants were found to map in the *metC* gene, which codes for cystathionase. This conclusion is reached on the basis of the following results: (*i*) Homocysteine thiolactone satisfied the methionine requirement for the growth of all 13 independently isolated revertants and cystathionine did not. (*ii*) All revertants tested (six out of six) were deficient in cystathionase activity as determined with toluenized cells or cell-free extracts. (*iii*) Merodiploids of these revertants harboring episome F'116, which carries a functional *metC*$^+$ allele, were found to be Met$^+$.

Mapping of ecf. The mapping of *ecf* was accomplished with the use of episomes. Episomes F'122, F'116, and F'143 (Fig. 5) were introduced into strain MAL66 and thymine-independent (Thy$^+$) merodiploids were selected for on succinate at 42°. Episomes F'116 and F'122 permitted both growth on succinate and transport at 42°. Episome F'143 did not. These results indicate that the *ecf* gene lies in the 54.5- to 60-min region of the *E. coli* chromosome map.

DISCUSSION

Mutants of *E. coli* and *Salmonella typhimurium* that are energy-uncoupled for oxidative phosphorylation have been described previously (7, 9, 11, 12, 30–32), and are defective in the Mg,Ca-ATPase (*uncA* mutants) or energy coupling proteins (*uncB* in *E. coli*, *etc* in *S. typhimurium*). These mutants cannot grow on succinate, fumarate, malate, or D-lactate as sole carbon and energy source and grow on glucose with a markedly reduced rate and growth yield. The mutant described in this paper manifests the same growth defect as these energy-uncoupled mutants. However, in striking contrast to these mutants, which have normal *aerobic* transport capability (7, 8; J.-s. Hong, manuscript in preparation)†,

† There have been three reports in which *uncA* mutations were isolated and shown to result in defective aerobic transport (9, 12; 32). However, the involvement of a functional ATPase in aerobic transport remains controversial (7–9, 12). Unpublished results from our laboratory indicate that when the *uncA* mutations described by Yamamoto *et al.* (9) were transduced into other strains, no defect in aerobic transport was found. Furthermore, in a study of over 50 independently isolated ATPase mutations, including 15 amber mutations, none was found to be defective in aerobic transport (J.-s. Hong, manuscript in preparation). It seems unlikely that the hydrolysis of ATP is necessary for aerobic transport; however, the structural integrity of the membrane might be affected by a mutated ATPase protein, as suggested by Rosen (32).

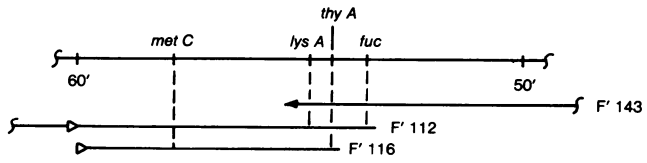

FIG. 5. A partial genetic map of the *E. coli* chromosome indicating the regions covered by episomes F'116, F'112, and F'143.

our mutant is completely defective in the active transport of amino acids and sugars. On this basis alone it is apparent that the *ecf* mutant is distinct from these mutants. Our mapping data clearly establish that this is indeed the case. Moreover, the normal Ca,Mg-ATPase activity in this mutant at 42° rules out the possibility that the ECF protein is one of the components of the multi-component, membrane-bound ATPase which is necessary for hydrolytic activity; however, the possibility of its being one of the components necessary for the synthetic activity cannot be ruled out at this time. The normal oxygen consumption rate of this mutant indicates that the ECF protein is not involved in electron transport through the respiratory chain.

We have shown that both the *ecf* and *metC** mutations are required, although neither of them alone is sufficient, to express the defect in active transport. This suggests that there is a profound effect of the *metC** mutation on the ECF protein which results in a complete loss of transport capability. Although the double mutant (*ecf metC**) and the single mutant (*ecf*) are unable to grow on SFML at 42°, a significant difference in growth properties does exist between these two mutants. We have found that the double mutant grows at a markedly reduced growth rate and cell yield on glucose or enriched media at 42° as compared to the single mutant. This result implies that the mutated ECF protein, in the absence of the *metC** mutation, still retains sufficient activity for active transport and efficient growth on glucose at 42°. This activity is apparently greatly reduced when the *metC** mutation is present, however, so that both transport and efficient growth are no longer possible. On the other hand, although the *ecf* mutation confers a *ts* Suc$^-$ phenotype in the presence of either a *metC*$^+$ gene or a *metC** mutation, it appears that the *ts* Suc$^-$ phenotype is suppressible by *metC* mutations, as observed with the Class II Suc$^+$ revertants. The complexity of the effect of various genetic conditions in the *metC* gene on the mutated ECF protein is not yet understood.

The normal transport activity at 42° by the membrane vesicles prepared from JSH4 is surprising. Two possible explanations are (*i*) an extensive dilution during the vesicle preparation of a possible cytoplasmic inhibitor of the mutated ECF protein and (*ii*) the mutated ECF protein is altered during the vesicle preparation such that it is active and no longer sensitive to high temperature.

One conclusion that emerges from our studies of this mutant is that the ECF protein is essential for *both* aerobic and anaerobic transport, since anaerobically grown mutant cells exhibit the same temperature-sensitivity in transport as do aerobically grown cells. This implies that the mechanism of energy coupling to active transport for both situations is likely to be the same, regardless of the energy source, as suggested previously by others (5, 12, 33).

The ECF protein thus appears to play a pivotal role in energy coupling to active transport, and possibly in oxidative phosphorylation. This mutation should prove useful in attempting to elucidate the energy transduction processes in *E. coli*.

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1. Kaback, H. R. (1972) *Biochim. Biophys. Acta* **265**, 367-416.
2. Harold, F. M. (1974) *Ann. N.Y. Acad. Sci.* **227**, 297-311.
3. Parnes, J. R. & Boos, W. (1973) *J. Biol. Chem.* **248**, 4429-4435.
4. Berger, E. A. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1514-1518.
5. Klein, W. L. & Boyer, P. D. (1972) *J. Biol. Chem.* **247**, 7257-7265.
6. Reeves, J. P., Hong, J.-s. & Kaback, H. R. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1917-1921.
7. Schairer, H. V. & Haddock, B. A. (1972) *Biochem. Biophys. Res. Commun.* **48**, 544-551.
8. Prezioso, G., Hong, J.-s., Kerwar, G. K. & Kaback, H. R. (1973) *Arch. Biochem. Biophys.* **154**, 575-582.
9. Yamamoto, T. H., Mével-Ninio, M. & Valentine, R. C. (1973) *Biochim. Biophys. Acta* **314**, 267-275.
10. Lombardi, F. J., Reeves, J. P., Short, S. A. & Kaback, H. R. (1974) *Ann. N.Y. Acad. Sci.* **227**, 312-327.
11. Hong, J.-s. & Kaback, H. R. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3336-3340.
12. Simoni, R. D. & Shallenberger, M. K. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2663-2667.
13. Vogel, H. J. & Bonner, D. M. (1956) *J. Biol. Chem.* **218**, 97-106.
14. Gutnick, D., Calvo, J. M., Klopotowski, T. & Ames, B. N. (1969) *J. Bacteriol.* **100**, 215-219.
15. Adams, M. H. (1959) in *Bacteriophages* (Interscience, New York), p. 456.
16. Hill, C. W., Foulds, J., Soll, L. & Berg, P. (1969) *J. Mol. Biol.* **39**, 563-581.
17. Luria, S. E., Adams, J. N. & Ting, R. C. (1960) *Virology* **12**, 348-390.
18. Adelberg, E. A., Mandel, M. & Chen, G. C. C. (1965) *Biochem. Biophys. Res. Commun.* **18**, 788-795.
19. Miller, J. H. (1972) in *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), pp. 218-220.
20. Inuzuka, N., Nakamura, S., Inuzuka, M. & Tomoeda, M. (1969) *J. Bacteriol.* **100**, 827-835.
21. Miller, J. H. (1972) in *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), pp. 82-85.
22. Kaback, H. R. (1971) in *Methods in Enzymology*, ed. Jakoby, W. B. (Academic Press, New York), Vol. XXII, pp. 99-120.
23. Barnes, E. M., Jr. & Kaback, H. R. (1971) *J. Biol. Chem.* **246**, 5518-5522.
24. Holloway, C. T., Greene, R. C. & Su, C. H. (1970) *J. Bacteriol.* **104**, 734-747.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. J. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
26. Kaback, H. R. (1970) *Annu. Rev. Biochem.* **39**, 561-598.
27. Matin, A. & Konings, W. N. (1973) *Eur. J. Biochem.* **34**, 58-67.
28. Martin, J. B. & Doty, D. M. (1949) *Anal. Chem.* **21**, 965-967.
29. Fisher, R. J., Lam, K. W. & Sanadi, D. R. (1970) *Biochem. Biophys. Res. Commun.* **39**, 1021-1025.
30. Butlin, J. D., Cox, G. B. & Gibson, F. (1973) *Biochim. Biophys. Acta* **292**, 366-375.
31. Butlin, J. D., Cox, G. B. & Gibson, F. (1971) *Biochem. J.* **124**, 75-81.
32. Rosen, B. P. (1973) *J. Bacteriol.* **116**, 1124-1129.
33. Kashket, E. R. & Wilson, T. H. (1972) *J. Bacteriol.* **109**, 784-789.