JOURNAL OF BACTERIOLOGY, Feb. 1977, p. 606-615

Vol. 129, No. 2
Printed in U.S.A.

Escherichia coli K-12 Structural *kdgT* Mutants Exhibiting Thermosensitive 2-Keto-3-Deoxy-D-Gluconate Uptake

ALAIN E. LAGARDE* AND FRANÇOIS R. STOEBER

Laboratoire de Microbiologie (406), Institut National des Sciences Appliquées de Lyon, 69621 Villeurbanne Cedex, France

Received for publication 7 June 1976

A specific method is described for selecting thermosensitive mutants of *Escherichia coli* K-12 able to grow on 2-keto-3-deoxy-D-gluconate (KDG) and D-glucuronate at 28 but not at 42°C. The extensive analysis of one such mutant is consistent with the conclusion that the carrier molecule responsible for KDG and glucuronate uptake becomes thermolabile. (i) Growth on a variety of carbon sources is perfectly normal at 28 and 42°C, whereas in the same temperature range it gradually diminishes on KDG and glucuronate. (ii) The apparent K_m value for KDG is about twofold higher for the mutant than for the wild-type strain, and the K_m for glucuronate increases about threefold in the range 25 to 40°C. In the same temperature range, the V_{max} values for KDG influx are higher for the mutant compared with those of the wild-type strain, but the optimum temperature is 34°C instead of 38°C. On the contrary, the V_{max} values for glucuronate influx are lower for the mutant than for the parental strain, and the optimum temperature for both strains is shifted beyond 40°C. (iii) The activation energies for KDG and glucuronate uptake are about twofold higher in the mutant than in the wild-type strain. (iv) Kinetics of counterflow under de-energized conditions (overshoot) at different temperatures indicate that the defect is located in the translocation step rather than in the processes involved in energy coupling. (v) The first-order rate constants for thermal denaturation are, respectively, 2.5- and 5-fold higher at 40 and 30°C in the mutant than in the wild-type strain, and the activation energy for thermal denaturation is lower. (vi) The carrier molecule in the mutant is also much more sensitive to denaturation by *N*-ethylmaleimide. (vii) Four independent thermosensitive mutations and one revertant were located by transduction in or near the *kdgT* locus, defined previously as the site of nonconditional KDG transport-negative mutations. These results support the conclusion that *kdgT* represents the structural gene coding for the KDG transport system.

A transport system able to take up 2-keto-3-deoxy-D-gluconate (KDG) and, to a lesser extent, D-glucuronate has been described in *Escherichia coli* K-12 (7-9, 17) (step 3 of Fig. 1). Glucuronate and galacturonate can penetrate the cell through the specific hexuronate transport system (13) (step 1 of Fig. 1). These three acidic sugars may serve as unique carbon sources since they are degraded intracellularly by the enzymes of the hexuronate pathway (Fig. 1) (21).

Experiments performed in whole cells, as well as in isolated membrane vesicles (7-9), indicated that the KDG transport system is similar to the well-known β-galactoside system (6, 22): it is not sensitive to osmotic shock, and unidirectional fluxes (influx and efflux) are mediated by a mobile carrier embedded in the cytoplasmic membrane. The expression of the KDG transport system activity was shown to be under the control of an operator gene, *kdgP* (17; A. Lagarde, unpublished data), and a regulatory gene, *kdgR*, which codes for the repressor of the *kdg* regulon (controlling steps 3, 5, and 6 of Fig. 1) (19). Since the synthesis of the KDG transport system is not inducible, it is strictly dependent upon constitutive mutations in *kdgP* or *kdgR* (7).

Point mutations that reduce or abolish KDG uptake and revertant mutations that restore it were located by transduction in a single locus, called *kdgT*, adjacent to the operator gene *kdgP* (17). Hitherto the proximity of the two loci and other indirect evidence were taken to indicate that *kdgT* represents the structural gene coding for the component(s) of the KDG transport system (17). Since the *kdgT* gene product has not yet been identified biochemi-

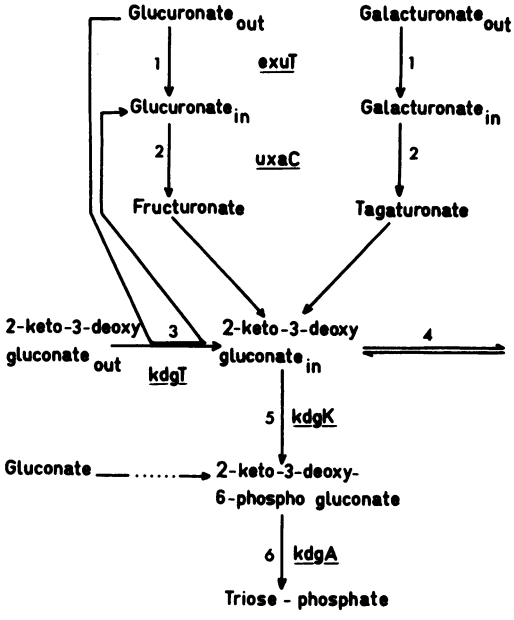

FIG. 1. *Simplified metabolic pathways for the degradation of hexuronates, KDG, and gluconate in E. coli K-12. For details see reference 21. Structural genes are indicated within brackets: (1) hexuronate transport system; (2) hexuronate isomerase; (3) KDG transport system; (4) KDG oxidoreductase, first enzyme of the KDG bypass (15); (5) KDG-kinase; (6) KDG-phosphate aldolase.*

cally, we decided to seek new classes of mutants in order to find further correlations between the state of the allele *kdgT* and the functional properties of the KDG transport system. We succeeded in selecting mutants exhibiting a conditional growth phenotype on KDG and glucuronate (positive at 28°C and negative at 42°C). Kinetic and genetic evidence is given pointing to the conclusion that the mutations leading to the observed thermosensitive growth phenotype are located close to, or more likely in, *kdgT* and are responsible for the synthesis of a thermolabile KDG carrier protein.

MATERIALS AND METHODS

Bacterial strains. The relevant genetic markers and origin of the strains used are listed in Table 1. They are all *E. coli* K-12 derivatives (thiamine auxotrophy). Genetic symbols are according to Bachmann et al. (2). In addition, *exuT* is the presumed structural gene for the hexuronate transport system (step 1, Fig. 1) (13), and *kdgT*(Ts) designates the *kdgT* allele responsible for the synthesis of the thermosensitive KDG transport system.

Medium and growth conditions. Bacteria were grown aerobically in medium 63 (20) supplemented with thiamine-hydrochloride(0.5 μg/ml) and amino acids (100 μg/ml). Since growth temperature is a critical parameter, it is specified for each experiment. Except when stated otherwise, glycerol (4 mg/ml) was used as a carbon source. Growth on solid media was as reported previously (17). Bacterial

TABLE 1. *Bacterial strains*

Strains	Sex	Genotype	Origin or derivation
P4X	Hfr	*metB1*	E. Wollman
PA3	Hfr	*metB1 kdgP3 kdgA2*	J. Pouyssegur and A. Lagarde (17)
FU9	F⁻	*exuT9 argG his str*	G. Nemoz et al. (13)
PA3U9	F⁻	*kdgP3 kdgA2 exuT9 metB1 argG str*	His⁺ (*str*) recombinant from PA3 × FU9 (this paper)
P146	F⁻	*kdgP2 glpK1 argH1 ilvD16 his-1 lac malA1 gal-6 mtl-2 tsx-7 str*	J. Pouyssegur (this laboratory)
TH9	Hfr	*exuT9 metB1*	G. Nemoz et al. (13)
TH9Y	Hfr	*exuT9, glpK1 argH1*	Met⁺ transductant from P1(P146) × TH9 (this paper)
PS393	Hfr	*kdgP3 metB1 fadD88*	J. Pouyssegur and A. Lagarde (17)
PU9	F⁻	*kdgP3 exuT9 metB1 argG str*	KdgA⁺ transductant from P1(P4X) × PA3U9 (this study)
PA3K	Hfr	*kdgP3 kdgA2 kdgK3 metB1*	Spontaneous mutant from PA3 (this laboratory)
PAT1 to PAT3	Hfr	*kdgP3 kdgA2 kdgT1*(Ts) to *kdgT3*(Ts) *metB1*	Spontaneous mutants from PA3 (this paper)
PAUT4	F⁻	*kdgP3 kdgA2 exuT9 kdgT4*(Ts) *metB1 argG str*	Spontaneous mutant from PA3U9 (this paper)
PUT4	F⁻	*kdgP3 exuT9 kdgT4*(Ts) *metB1 argG str*	KdgA⁺ transductant from P1(P4X) × PAUT4 (this paper)
PAUK1	F⁻	*kdgP3 kdgA2 exuT9 kdgK3 metB1 argG str*	KDG⁺ transductant from P1(PA3K) × PA3U9 (this paper)
PAUKT4	F⁻	*kdgP3 kdgA2 exuT9 kdgK3 kdgT4*(Ts) *metB1 argG str*	KDG⁺ transductant from PI(PA3K) × PAUT4 (this paper)
T4-r3	F⁻	*kdgP3 exuT9 kdgT4*(Ts)r3 *metB1 argG str*	KDG⁺ (42C) spontaneous revertant from PUT4 (this paper)

density was assayed spectrophotometrically at 600 nm.

Genetic techniques. Conditions for crosses between Hfr and F-minus strains and for the transduction with phage P1*kc* were as described by Miller (12) and are detailed elsewhere (16).

Uptake experiments. Glycerol-grown cells in the exponential phase were washed free from the source and suspended in medium 63 (pH 7.0) supplemented with chloramphenicol (50 μg/ml). Details for conducting uptake studies were as given previously (7-9). Specific modifications concerning the assay temperature and substrate concentrations are described in the figure legends. Care was taken to wash the membrane filters with isotonic and isothermal medium 63.

Selection for thermosensitive transport mutants and derivative strains. The principle governing the selection of *kdgT* mutants unable to take up KDG was detailed previously (17). We adopted the same method for selecting *kdgT*(Ts) mutants. Strains PA3 (*kdgP3 kdgA2*) and PA3U9 (*kdgP3 kdgA2 exuT9*) synthesize the KDG constitutively as a result of a mutation in the operator gene *kdgP* (17). However, because of the additional lack of KDG-phosphate aldolase, these strains (genotype: *kdgA*) are unable to grow on glycerol plus KDG because KDG is converted into KDG-phosphate, which accumulates inside the cells and is toxic (17, 19). Spontaneous mutants able to escape the growth stasis were obtained as cells exhibiting the phenotype glycerol$^+$ KDG$^+$ at 42°C, by methods published previously (17). Among them, the presumed thermosensitive mutants PAT1, PAT2, PAT3, and PAUT4 (Table 1) were selected as exhibiting the "poisoned" phenotype glycerol$^-$ KDG$^-$ at 28°C.

Strains PUT4 and PU9 were derived from strains PAUT4 and PA3U9, respectively, by transducing the *kdgA*$^+$ allele (step 6, Fig. 1), using phage P1 made on the wild-type strain P4X, and selecting gluconate$^+$ transductants (15, 17).

To prevent the subsequent conversion of accumulated substrate through step 5 (Fig. 1) during uptake experiments, strains PAUKT4 and PAUK1 were derived from strains PAUT4 and PA3U9, respectively, by transducing the *kdgK* allele with phage P1 made on strain PA3K (Table 1) (19).

Revertants from the thermosensitive mutant PUT4. About 10^{10} cells from the thermosensitive mutant PUT4 [*kdgT4*(Ts)] were spread onto plates containing KDG and incubated at 42°C. Revertants arose at a frequency of about 10^{-8}. After purification, clones were analyzed at 28 and 42°C on KDG, glucuronate, and galacturonate. All revertants selected on KDG exhibit the phenotype KDG$^+$ glucuronate$^+$ at 28 and 42°C, and galacturonate$^-$ at any temperature. One of them was designated T4-r3 (Table 1).

Chemicals. Potassium [^{12}C]KDG and [U-^{14}C]KDG (5 mCi/mmol) were prepared as described previously (16, 18). Potassium D-[U-^{14}C]glucuronate (76 mCi/mmol) was from the Radiochemical Centre, Amersham, U.K. Glucuronate, galacturonate, and gluconate were purchased from B.D.H. Fructuronate and tagaturonate were a gift from J. Robert-Baudouy of our laboratory. Other reagents and substrates were from Sigma Chemical Co., St. Louis, Mo., Calbiochem, Los Angeles, Calif., and K & K Laboratories, Inc., Jamaica, N.Y.

RESULTS

Growth temperature dependence of the mutants. The selection for thermosensitive KDG transport mutants depends primarily upon the spontaneous emergence, in strains carrying the *kdgP* and *kdgA* alleles, of a conditional mutation preventing the synthesis of the toxic derivative KDG-phosphate at high temperature (42°C) but not at low temperature (28°C) (Fig. 1). *kdgT*(Ts) as well as *kdgK*(Ts) mutations can lead to such a phenotype (Fig. 1) (17), but both classes can be differentiated on the basis of their growth phenotypes on glucuronate and galacturonate (17, 19) as well as by the KDG-kinase assay. Four presumed *kdgT*(Ts) mutants were selected, but we analyzed only the one that carried the mutation *kdgT4*(Ts) and which was derived from strain PA3U9 (Table 1).

We compared the ability of the wild-type strain PU9 (*kdgT*$^+$), the thermosensitive mutant PUT4 [*kdgT4*(Ts)], and the revertant T4-r3 [*kdgT4*(Ts)r3] to grow on KDG and glucuronate at different temperatures in liquid cultures. The three strains are *kdgA*$^+$ transductants derived from strains PA3U9 and PAUT4, respectively (Table 1), so that the enzymes KDG-kinase (step 5, Fig. 1) and KDG-phosphate aldolase (step 6, Fig. 1) are functional. The doubling times on KDG and glucuronate decreased for the wild-type PU9 but increased for the thermosensitive mutant PUT4 when the growth temperature was raised from 25 to 40°C (Fig. 2). The behavior of revertant strain T4-r3 was similar to that of parental strain PU9. In contrast, the three strains grew well at 28 or 42°C on the following carbon sources: (i) lactose, glucose, galactose, rhamnose, fructose, maltose, mannose, xylose, and mannitol; (ii) gluconate, fructuronate, and tagaturonate; (iii) glycerol, succinate, fumarate, malate, acetate, and lactate. These results indicate that the inability of thermosensitive mutant PUT4 to grow at high temperature is restricted to KDG and glucuronate, the two substrates that are specifically transported by the KDG transport system (7, 8). The absence of pleiotropic effects caused by the mutation in the mutant PUT4 excludes the possibility of damage in the bacterial cell envelopes leading to unusual leakiness or of an enzyme of the hexuronate pathway, or alternatively for some step in the energy-yielding metabolism, becoming thermosensitive. As a result of this preliminary screen-

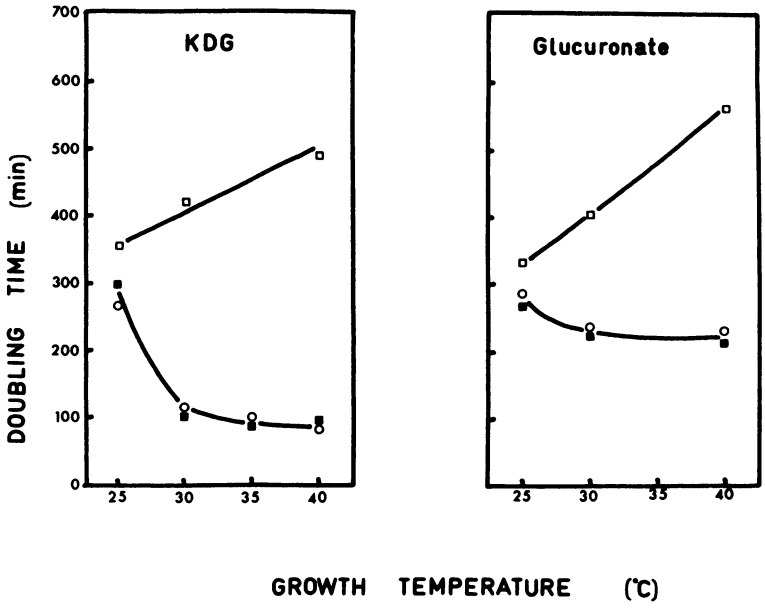

FIG. 2. *Comparison of growth of the parental strain PU9, the thermosensitive mutant PUT4, and the revertant T4-r3, as a function of temperature. Cells grown overnight at 25°C in medium 63 with either KDG (10 mg/ml) or glucuronate (5 mg/ml) served to inoculate fresh cultures with the same substrates at the indicated temperatures. Growth was followed by turbidimetry at 600 nm over a 10-h period. The doubling times were plotted as a function of growth temperature. Symbols: ■, PU9; □, PUT4; ○, T4-r3.*

ing, made necessary when a complex function such as transport is concerned, it is concluded that the thermosensitive phenotype of mutant PUT4 on KDG and glucuronate results from a thermosensitivity in the first step (the uptake process) of the metabolism of these sugars.

Mapping of the thermosensitive mutations. All negative mutations leading to the loss of KDG transport activity were previously located in a single locus, *kdgT*, adjacent to the operator gene *kdgP*, cotransducible with the markers *rhaD*, *pfk*, *glpK*, *metB*, and *argH* (17). The cotransduction frequencies between *glpK* and the thermosensitive phenotype of mutants PAT1, PAT2, PAT3, and PUT4, and the wild-type phenotype of the parent PU9 and of the revertant T4-r3, are listed in Table 2. Since the recipient P146 carries the wild-type *exuT*$^+$ allele and is thus able to use glucuronate through the specific hexuronate transport system (13), the thermosensitive phenotype of the transductants can only be observed on KDG (experiments 1 through 3). The analysis of the classes of unselected markers is in agreement with the thermosensitive mutations being located counterclockwise to *glpK*. The analysis of the classes in experiments 4 through 9 deserves preliminary comments. It must be recalled that the transduction of the phenotype KDG$^+$ is strictly dependent upon the simultaneous transfer of the operator-constitutive mutation *kdgP* into the recipient TH9Y (*kdgP*$^+$ *kdgT*$^+$ *exuT*). Consequently, the characteristic phenotype of *kdgP* transductants is KDG$^+$ at 28°C, whereas the phenotype of the thermosensitive transductants is KDG$^-$ at 42°C. Results given in Table 2 point to the following facts. (i) All *kdgT*(Ts) mutations were transduced with the same frequency as the operator gene *kdgP3*, so that it is clear that they are most likely located between *glpK* and *kdgP*. Furthermore, the transduction frequencies between *glpK* and *kdgT*(Ts) are of the same order of magnitude (32 to 44%) and are similar to the transduction frequency between *glpK* and *kdgP* (49%). (ii) In experiments 4 through 7, the thermosensitive phenotype of all *kdgT*(Ts) transductants was observed both on KDG and on glucuronate, suggesting that a single carrier component bears the recognition sites for both substrates. (iii) The *kdgT4*(Ts)r3 allele of revertant strain T4-r3 cotransduces with the marker *glpK* at about the same frequency as the *kdgT*(Ts) alleles.

Thermosensitive uptake of KDG and glucuronate. To assay KDG and glucuronate uptake under conditions in which the transported substrates are not converted inside the cells, the *kdgK* derivatives (Fig. 1) PAUK1 (wild type) and PAUKT4 (thermosensitive mutant) were

TABLE 2. *Cotransduction frequencies between the mutations kdgT(Ts) and glpK*

Expt	Donor[a]	Recipient	No. of *glpK*$^+$ analyzed[b]	% Inheritance of the unselected markers						
				kdgP3[c]	*kdgT*(Ts)	*metB*$^-$	*argH*$^+$	*metB*$^-$ *argH*$^+$	*kdgT*(Ts) *metB*$^-$	*kdgt*(Ts) *argH*$^+$
1	PAT1	P146 (*kdgP2 glpK1 argH1*)	100		29[d]	35	18	17	11	5
2	PAT2	P146 (*kdgP2 glpK1 argH1*)	100		15[d]	48	19	13	9	3
3	PAT3	P146 (*kdgP2 glpK1 argH1*)	100		22[d]	49	32	28	14	9
4	PAT1	TH9Y (*glpK1 exuT9 argH1*)	156	32	32[e]	59	19	15	27	5
5	PAT2	TH9Y (*glpK1 exuT9 argH1*)	156	30	30[e]	51	17	16	23	3
6	PAT3	TH9Y (*glpK1 exuT9 argH1*)	156	39	39[e]	53	15	15	24	5
7	PUT4	TH9Y (*glpK1 exuT9 argH1*)	222	44	44[e]	61	26	24	26	6
8	T4-r3	TH9Y (*glpK1 exuT9 argH1*)	152	38	38[f]	55	22	21	25	5
9	PU9	TH9Y (*glpK1 exuT9 argH1*)	301	49	49[f]	60	21	20	30	6

[a] All donors carry the *kdgP3* and *metB1* alleles.
[b] Phenotype: Glycerol$^+$ (37°C).
[c] Phenotype: KDG$^+$ (28°C).
[d] Phenotype: KDG$^-$ (42°C).
[e] Phenotype: KDG$^-$ (42°C) and glucuronate$^-$ (42°C).
[f] Phenotype: KDG$^+$ (42°C) and glucuronate$^+$ (42°C).

obtained by transduction from PA3U9 and PAUT4, respectively. Cells were grown on glycerol at a permissive temperature (30°C), and the kinetics of KDG and glucuronate uptake were followed at several assay temperatures (Fig. 3). Initial rates of uptake were measured to obtain V_{max} and K_m values. At 25°C the parental strain PAUK1 and the thermosensitive mutant PAUKT4 took up KDG at about the same rate, indicating that the synthesis and insertion of the carrier into the membrane occurred normally during growth at low temperature (30°C). When the assay temperature was raised, V_{max} values for KDG increased up to 34°C and then decreased abruptly in the mutant; for the parental strain the optimum temperature was 38°C. Between 25 and 40°C, V_{max} values for KDG in the mutant were generally found to range above the corresponding V_{max} values for the parent. The accuracy of the method did not permit detection of variation of the K_m for KDG with temperature, but it was slightly higher in the mutant PAUKT4 (K_m = 1.2 mM) than in the wild-type PAUK1 (K_m = 0.8 mM).

The situation with respect to glucuronate uptake appears somewhat different. The optimal temperature for uptake is shifted beyond 40°C in both strains. Evidence for a reduced ability to take up glucuronate in the mutants PAUKT4 compared with that of the parent PAUK1 can be deduced from the fact that the V_{max} values are lower and the K_m values increase with temperature. The experimental activation energies calculated from Fig. 3 are 19 and 10 kcal/mol for strains PAUKT4 and PAUK1, respectively, for KDG uptake and 14 kcal/mol for strains PAUKT4 and PAUK1, respectively, for glucuronate uptake.

The differences in V_{max} and K_m values and activation energies between the parental strain PAUK1 and the thermosensitive mutant PAUKT4 can be explained if it is assumed that for increasing temperature the carrier molecule involved in the translocation of KDG and glucuronate gradually loses its native structure and, consequently, its affinity for ligands and/or its mobility within the membrane.

Thermosensitive overshoot. Counterflow in the absence of energy, called overshoot (6, 22) is a classical experiment that strongly suggests the participation of a mobile carrier in the overall translocation of a substance. Homo- and hetero-overshoot were demonstrated previously for KDG and glucuronate in strains carrying the wild-type allele *kdgT* (9). Similar experiments were performed with the mutant PAUKT4 and the wild-type PAUK1, the assay temperature being the only varying parameter. Two main differences can be observed between the strains (Fig. 4). (i) When the temperature is raised, the initial rates of KDG uptake increase in the wild type but decrease in the mutant. However, the maximal internal substrate concentrations that can be reached are similar in both strains. (ii) The overall plots of kinetics

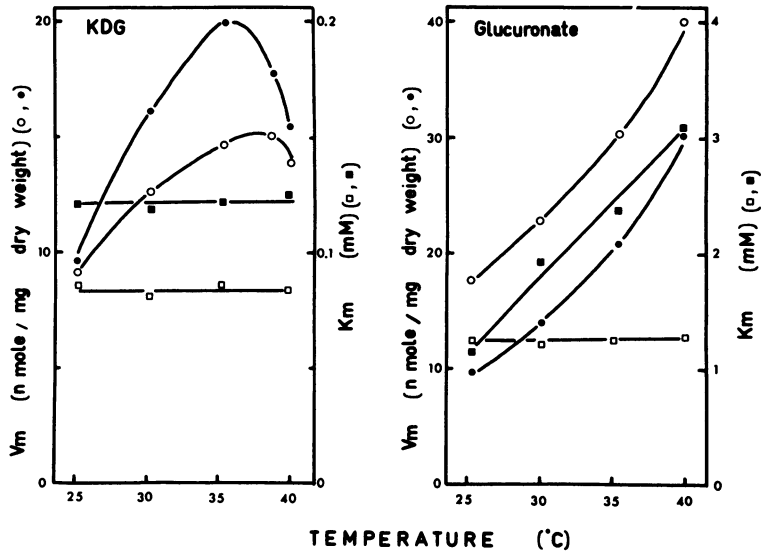

FIG. 3. *Dependence of the kinetics parameter V_{max} and K_m for the influx of KDG and glucuronate upon temperature, as compared in the parental strain PAUK1 and the mutant PAUKT4. Cells were grown at 30°C and washed. Initial rates of uptake (1-min period) were measured at the indicated temperature in a reaction medium containing: cells, 100 μg (dry weight) per ml; [^{14}C]KDG, 40 μM to 0.5 mM; or [^{14}C]glucuronate, 0.25 to 3 mM. V_{max} and K_m values were calculated from Lineweaver-Burk plots and were then plotted as a function of assay temperature. Symbols: ●, ■, PAUKT4; ○, □, PAUK1.*

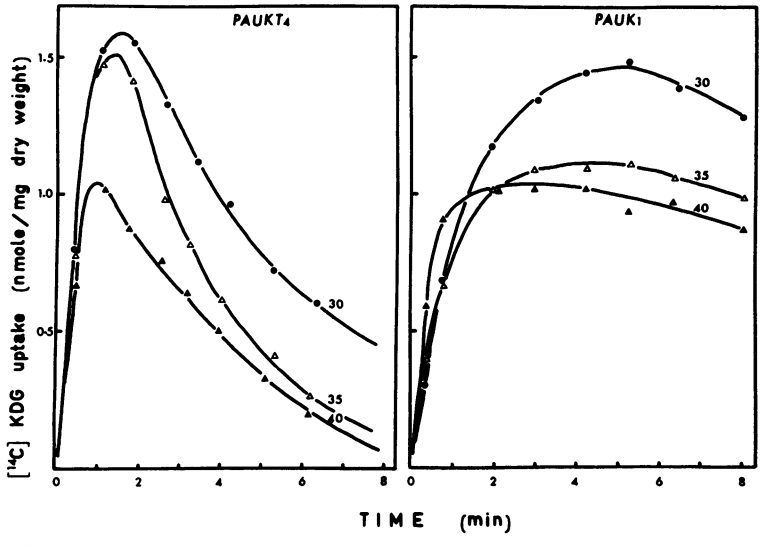

FIG. 4. *Dependence of KDG overshoot kinetics upon temperature in the parental strain PAUK1 and the mutant PAUKT4. Cells were grown at 30°C and washed as described in the legend of Fig. 3. Cells (200 μg [dry weight] per ml) were incubated at 25°C with unlabeled KDG (100 mM) and azide (50 mM). After a 60-min period, they were centrifuged and the supernatant was pipetted off. At time zero, the pellet was suspended in medium 63 containing [^{14}C]KDG (42 μM) and azide (50 mM), at the indicated temperature. Samples were filtered at various time intervals.*

(the ascending and decay portions) are sharper for the mutant than for the wild type. According to Wong and Wilson (22), in the case of β-galactosides, sharper plots of overshoot kinetics are correlated with an increased concentration of carrier within the membrane. Although such an interpretation would be compatible with the higher V_{max} values found for mutant PAUKT4

compared with those for the parent PAUK1 (Fig. 3), we have no a priori indication that it is correct (see Discussion). Nonetheless, the occurrence of marked thermosensitive overshoot in mutant PAUKT4 is in agreement with the view that the responsible mutation does affect the translational movement of the carrier and not the components or mechanisms involved in the coupling to energy under normally energized conditions.

Kinetics of thermal denaturation. In experiments depicted in Fig. 5, the kinetics of thermal denaturation at several temperatures were followed in the mutant PAUKT4 and the wild-type PAUK1 (at pH 7.0). Rates of denaturation follow classical first-order kinetics up to 10% residual transport activity. At 40°C, the half-life in the mutant PAUKT4 (3 ± 0.5 min) is about 2.5-fold shorter than in the parent PAUK1 (7.5 ± 0.5 min). The first-order rate constants (k_{denat}) have the same value whether the transport activity is measured with KDG or glucuronate as a substrate. This finding strongly suggests that a single component mediates the fluxes of both substrates and is similarily denaturated at high temperature. Denaturation is irreversible since the incubation of any strain at 40°C for 20 min followed by a 60-min incubation at 0°C never leads to the recovery of the initial transport activity.

Arrhenius plots shown in Fig. 6 give the experimental values for the activation energies of the denaturation process: 42 kcal/mol for the mutant PAUKT4 and 62 kcal/mol for the wild-type PAUK1. The difference is consistent with the conclusion that the structure of the carrier in the mutant is more susceptible to heat denaturation.

Inactivation by *N*-ethylmaleimide. We showed previously that the KDG transport system can be inactivated by thiol reagents such as *p*-chloromercuribenzoic acid and *N*-ethylmaleimide (7, 8). When cells from strain PAUK1 or PAUKT4 are incubated at 25°C in the presence of *N*-ethylmaleimide and KDG

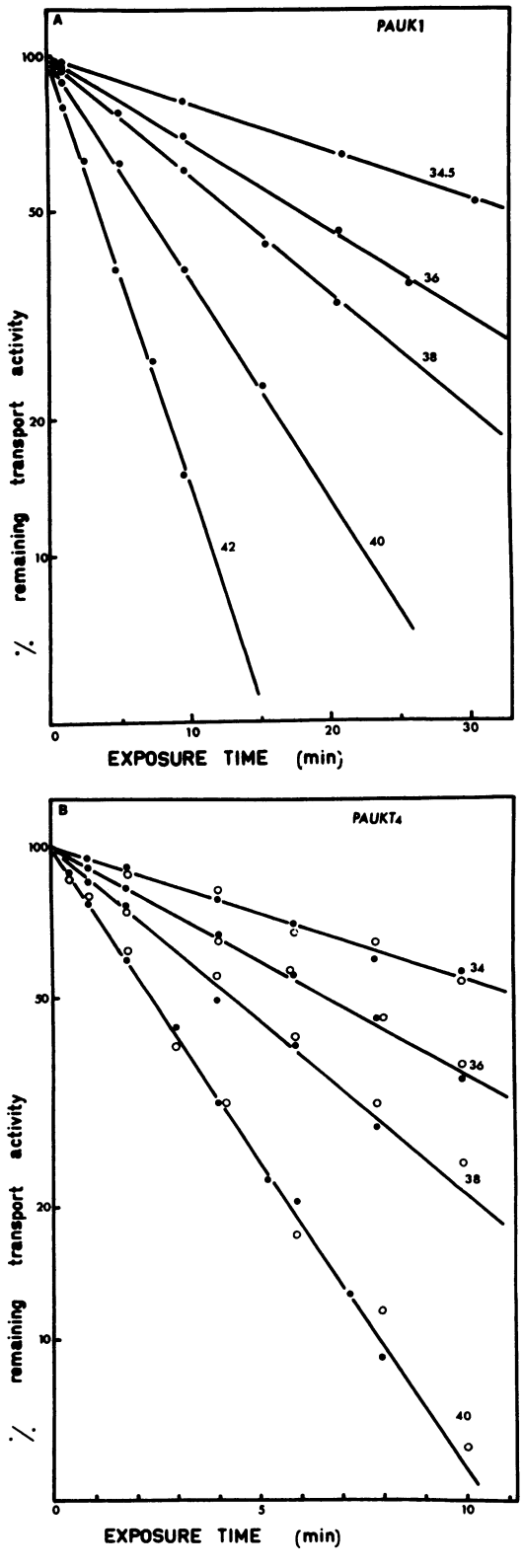

FIG. 5. *Kinetics of thermal denaturation at different temperatures in the parental strain PAUK1 and the mutant PAUKT4. Cells were grown at 30°C and washed as described in the legend of Fig. 3. Cells (60 μg [dry weight] per ml) were incubated in a shaking bath at the indicated temperature. At various time intervals, samples were withdrawn and immediately chilled in ice. Uptake was assayed at 25°C with either [^{14}C]KDG (42 μM) or [^{14}C]glucuronate (0.5 mM). The percent remaining transport activity was calculated by using initial rates of uptake (1-min period) and plotted on a semilogarithmic scale versus time of exposure at the indicated temperature. Symbols: ●, KDG; ○, glucuronate.*

uptake is measured in the same medium, inactivation occurs. As shown in Table 3, the transport activity is reduced more in the mutant PAUKT4 than in the wild-type PAUK1 when the concentration of *N*-ethylmaleimide is increased up to 3 mM.

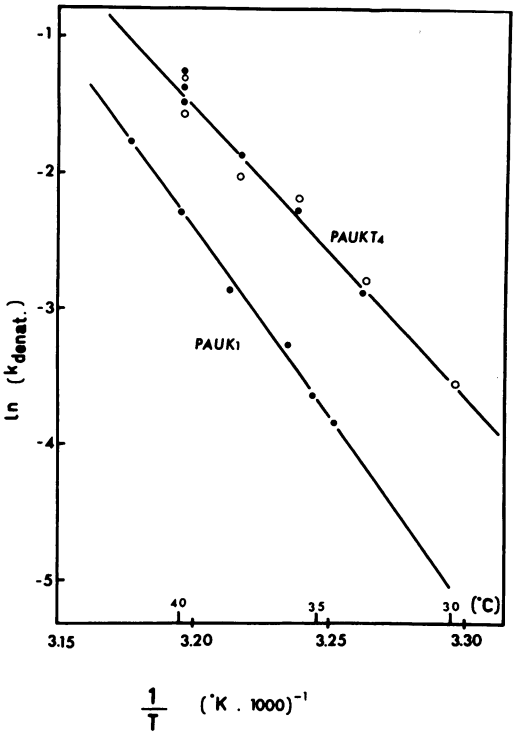

FIG. 6. *Dependence of the rate constants for thermal denaturation (k_{denat}) upon temperature as compared in the parental strain PAUK1 and the mutant PAUKT4. The first-order rate constants (expressed in minutes $^{-1}$) were calculated from Fig. 5. They were plotted according to the Arrhenius equation: ln(k_{denat}) versus 1/T (°K). Symbols: ●, KDG; ○, glucuronate.*

TABLE 3. *Inactivation by N-ethylmaleimide*[a]

N-ethylmaleimide concn (mM)	% Residual transport activity in strain:	
	PAUK1 (*kdgT*$^+$)	PAUKT4 *kdgT4*(Ts)
0	100	100
0.5	69	42
1.0	55	36
2.0	41	36
3.0	38	24

[a] Glycerol-grown cells (30°C) were incubated (100 μg [dry weight] per ml) at 25°C in the presence of *N*-ethylmaleimide at the indicated concentration for exactly 2 min. [^{14}C]KDG (40 μM final concentration) was then added to start the uptake. Initial rates of uptake (after a 1-min incubation) were compared to the standard without *N*-ethylmaleimide.

DISCUSSION

Selecting thermosensitive mutations affecting the structure of a soluble enzyme or a regulatory protein is a classical step in molecular biology when one wishes to ascertain the nature of the protein coded for by a gene. The correlation between a well-characterized genetic lesion and some properties of a purified molecule is straightforward. When the same approach is adopted for membrane-bound proteins, especially transport systems, several difficulties arise. (i) A qualitative difference is that the mutation cannot be correlated to in vitro properties. Biochemical techniques devised to identify and isolate the membrane components of the transport systems are available in few restrictive cases (1, 4). (ii) The function of a carrier protein cannot be studied outside its normal environment (in a membrane separating two compartments), so that the mutation can indirectly affect the function of the carrier through the modification of the structure of the neighboring components in contact with it. For instance, the lipid composition of the membrane from unsaturated fatty acid auxotroph mutants was shown to influence both the insertion into the membrane phase of the newly synthesized carrier molecules and the shuttling of the active sites (11, 14). Unspecific thermosensitive mutations affecting some part of the membrane structure were demonstrated to modify the transport characteristics of several substrates (3). (iii) When active transport is involved, translocation of a substrate against a concentration gradient is strictly dependent upon various energy-supplying processes taking place in the membrane (electron transfer, adenosine 5'-triphosphate synthesis, and hydrolysis). Thermosensitive uptake can then result from thermosensitive energy-transducing mechanisms, as already demonstrated (5, 10).

All of the above-mentioned peculiarities concerning transport systems may explain why mutations leading to the synthesis of a thermosensitive carrier were rarely obtained. It has clearly been shown that the binding properties of a particulate fraction containing the M protein are in accord with the thermosensitive uptake of β-galactosides found in vivo in one *lacY*(Ts) mutant selected by Fox et al. (4). Thermosensitive *hisJ* mutants in *Salmonella* were also shown by Ames and Lever (1) to synthesize a thermolabile histidine periplasmic binding protein responsible for thermosensitive histidine uptake in whole cells.

Results presented in this paper demonstrate unequivocally that the mutation leading to the thermosensitive growth phenotype on KDG and glucuronate altered the structure of the

sole KDG transport system. The lability of the carrier protein was demonstrated by thermal denaturation experiments (Fig. 5 and 6). In addition, the transport properties of the wild-type PAUK1 and the mutant PAUKT4 appeared to differ with respect to a number of other points: activation energies for KDG and glucuronate uptake, optimum temperature for KDG uptake, overshoot kinetics, activation energies for denaturation, sensitivity to *N*-ethylmaleimide, variation of the V_{max} and K_m values for KDG, and glucuronate influx as a function of temperature. Another distinctive feature is that a symport, KDG^-: H^+ and $glucuronate^-$:H^+, was demonstrated in strain PAUKT4 grown at 30°C but not at 42°C (A. Lagarde and B. Haddock, Biochem. J., in press), whereas the symport occurred in the wild-type strain PAUK1 at all growth temperatures.

Four independent thermosensitive mutations and one revertant were localized by transduction in or close to *kdgT* (Table 2), which was defined previously as the site of totally negative mutations (17). As the dual thermosensitive phenotype (on KDG and glucuronate) was transferred in all cases examined, the results attest the uniqueness of the KDG transport system. The evidence, as a whole, supports the conclusion that *kdgT* is the structural gene coding for the components of the KDG transport system.

The sharper plots of overshoot kinetics (Fig. 4) and the higher V_{max} values for KDG influx (Fig. 3) found in the mutant PAUKT4 compared with those of the wild-type PAUK1 are difficult to interpret. We studied isogenic strains with respect to the operator-constitutive *kdgP* mutation and auxotrophies; it appears likely that the difference cannot be ascribed to a modification of the carrier concentration due to a different level of constitutivity, or to sensitivity to catabolite repression. Although the V_{max} parameter is proportional to the carrier concentration, it also depends upon the rate constants for diffusion of the free carrier and of the carrier-substrate complex within the membrane. Therefore one plausible reason for the enhancement of the V_{max} values found in the mutant with KDG as a substrate would be an increased mobility of the carrier protein in the membrane when complexed to KDG but not to glucuronate. This intriguing point deserves additional analysis. In addition, the use of unsaturated fatty acid auxotrophs (11) in which the lipid phase and the fluidity of the membrane can be monitored should facilitate the understanding of the carrier-lipids interactions.

ACKNOWLEDGMENTS

We thank G. Couchoux and M. Mata for their skillful technical assistance, and G. Nemoz and J. Robert-Baudouy for providing strains and substrates.

This work was supported by the Centre National de la Recherche Scientifique (ERA no. 177), the Délégation Générale à la Recherche Scientifique et Technique (Action Complémentaire Coordonée "Interaction Moléculaires en Biologie"), and the Fondation pour la Recherche Médicale Francaise. This paper will be included in the Doctorat-es-Sciences thesis to be held by A. L. at INSA de Lyon.

LITERATURE CITED

1. **Ames, G. F., and J. E. Lever.** 1972. The histidine-binding protein J is a component of the histidine transport. Identification of its structural gene *hisJ*. J. Biol. Chem. 247:4309-4316.
2. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. 40:116-167.
3. **Crandall, M., and A. L. Koch.** 1971. Temperature-sensitive mutants of *Escherichia coli* affecting β-galactoside transport. J. Bacteriol. 105:609-619.
4. **Fox, C. F., J. R. Carter, and E. P. Kennedy.** 1967. Genetic control of the membrane protein component of the lactose transport system of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 57:698-705.
5. **Hong, J. S., and H. R. Kaback.** 1972. Mutants of *Salmonella typhimurium* and *Escherichia coli* pleiotropically defective in active transport. Proc. Natl. Acad. Sci. U.S.A. 69:3336-3340.
6. **Kepes, A.** 1969. Carrier properties of the β-galactoside permease: the role of permease in the leak of β-galactosides from *E. coli*, p. 353-389. *In* D. C. Tosteson (ed.), The molecular basis of membrane function. Prentice-Hall Inc., Englewood Cliffs, N.J.
7. **Lagarde, A., J. Pouysségur, and F. Stoeber.** 1973. A transport system for 2-keto-3-deoxy-D-gluconate uptake in *Escherichia coli* K-12. Biochemical and physiological studies in whole cells. Eur. J. Biochem. 36:328-341.
8. **Lagarde, A. E., and F. R. Stoeber.** 1974. Transport of 2-keto-3-deoxy-D-gluconate in isolated membrane vesicles of *Escherichia coli* K-12. Eur. J. Biochem. 43:197-208.
9. **Lagarde, A. E., and F. R. Stoeber.** 1975. The energy-coupling controlled efflux of 2-keto-3-deoxy-D-gluconate in *Escherichia coli* K-12. Eur. J. Biochem. 55:343-354.
10. **Liebermann, N. A., and J. S. Hong.** 1974. A mutant of *Escherichia coli* defective in the coupling of metabolic energy to active transport. Proc. Natl. Acad. Sci. U.S.A. 71:4395-4399.
11. **Linden, C. D., and C. F. Fox.** 1973. A comparison of characteristic temperatures for transport in two unsaturated fatty acid auxotrophs of *Escherichia coli*. J. Supramol. Struct. 1:535-544.
12. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Nemoz, G., J. Robert-Baudouy, and F. Stoeber.** 1976. Physiological and genetic regulation of the hexuronate transport system in *Escherichia coli* K-12. J. Bacteriol. 127:706-718.
14. **Overath, P., F. F. Hill, and I. Lamnek-Hirsch.** 1971. Biogènesis of *E. coli* membrane: evidence for randomization of lipid phase. Nature (London) New Biol. 234:264-267.
15. **Pouyssegur, J.** 1971. Localisation génétique de mutations 2-céto-3-désoxy-6-P gluconate aldolase négatives chez *E. coli* K-12. Mol. Gen. Genet. 113:31-42.
16. **Pouyssegur, J.** 1973. Préparation microbiologique du 2-

céto-3-désoxy-D-gluconate 1-^{14}C ou U-^{14}C. J. Labelled Compd. 9:3–13.

17. **Pouyssegur, J., and A. Lagarde.** 1973. Systeme de transport du 2-céto-3-désoxy-gluconate chez *E. coli* K-12: localisation d'un gène de structure et de son opérateur. Mol. Gen. Genet. 121:163–180.
18. **Pouyssegur, J., and F. Stoeber.** 1970. Synthèse enzymatique du 2-céto-3-désoxy-D-gluconate. Bull. Soc. Chim. Biol. 52:1419–1428.
19. **Pouyssegur, J., and F. Stoeber.** 1974. Genetic control of the 2-keto-3-deoxy-D-gluconate metabolism in *Escherichia coli* K-12: *kdg* regulon. J. Bacteriol. 117:641–651.
20. **Sistrom, W. R.** 1958. On the physical state of the intracellularly accumulated substrates of β-galactoside permease in *Escherichia coli*. Biochim. Biophys. Acta 29:579–587.
21. **Stoeber, F., A. Lagarde, G. Nemoz, G. Novel, M. Novel, R. Portalier, J. Pouyssegur, and J. Baudouy-Robert.** 1974. Le métabolisme des hexuronides et des hexuronates chez *Escherichia coli* K-12. Aspects physiologiques et génétiques de sa régulation. Biochimie 56:199–213.
22. **Wong, P. T. S., and T. H. Wilson.** 1970. Counterflow of galactosides in *Escherichia coli*. Biochim. Biophys. Acta 196:336–350.