Biochimica et Biophysica Acta, 406 (1975) 50–59

BBA 77060

INDUCIBLE GLUCONATE PERMEASE IN A GLUCONATE KINASE-DEFICIENT MUTANT OF *ESCHERICHIA COLI*

ALINE ROBIN and ADAM KEPES

Centre National de la Recherche Scientifique, Université Paris VII, Institut de Biologie Moléculaire, Laboratoire des Biomembranes, Tour 43–2, place Jussieu, 75221 Paris Cedex 05 (France)

(Received April 17th, 1975)

SUMMARY

Gluconate-resistant mutants were isolated from *Escherichia coli* strain DF 1070 deficient in phosphogluconate dehydrogenase (EC 1.1.1.44) and in phosphogluconate dehydrogenase (EC 4.2.1.12) which is inhibited by gluconate. Among the resistant mutants, AR 13 has been identified as a gluconate kinase (EC 2.7.1.12)-deficient strain.

This mutant exhibits an inducible gluconate transport system capable of concentrating gluconate in the cytoplasm against a concentration gradient. The accumulated gluconate is subject to permanent turnover, and is not chemically modified.

The kinetics of induction and deinduction indicate a single inducible component, rate limiting for the transport function, and the distribution of transport capacity among non-induced progeny of induced parents indicates that the inducible protein is membrane bound.

INTRODUCTION

Among the large number of bacterial transport systems those transporting neutral nutrients, have received most attention [1, 2]. These include sugar permeases and neutral amino acid permeases. Uptake systems for ionized nutrients like C4 dicarboxylic acids, dicarboxylic or dibasic amino acids purine and pyrimidine bases have also been described, but seldom was sufficient emphasis put simultaneously on specificity, regulation, flux pattern and thermodynamics.

One particularly interesting point, the mechanism of maintenance of electrical neutrality during the transport has received very little attention. Cotransport of Mg^{2+} together with citrate anion in *B. subtilis* [3] and requirement of citrate for the function of one of the iron transport systems in *Escherichia coli* [4] have been described and proton translocation was implicated in the transport of some amino acids carrying a net charge [5].

Transport of gluconic acid, and of other carboxylic acids capable to support the growth of *E. coli* was not easily accessible to detailed study in the absence of a non-metabolizable analog. Studies carried out with protoplast ghosts [6] have shown that gluconic acid transport could be classified with the large number of transport

systems utilizing oxidative energy derived preferentially from the oxidation of D-lactate, but for many aspects of the transport system in physiological conditions, the study had to be made with a mutant unable to carry out any biochemical reaction to alter the transport substrate.

A gluconate kinase-deficient mutant AR 13 has been isolated and was utilized to demonstrate the existence of an inducible gluconate permease, with high specificity for inducer and for transport substrate.

In a short note [7] we reported the Michaelis-type substrate concentration dependence of the rate of transport, the attainment of high intracellular concentrations of gluconate and evidence that cotransport of H^+ with gluconate anion can account for the electric neutrality of the system.

During the preparation of the manuscript, Pouysségur et al. [8] reported the isolation from the same parental strain and a study of a mutant similar to AR 13 named R6. The many common characteristics will be very briefly summarized below, more emphasis will be put on differences and on independent information, particularly the kinetics of induction and deinduction which support the involvement of a stable inducible membrane-bound component in the transport.

MATERIALS AND METHODS

Bacterial strains. Strain AR 13 is a derivative of strain DF 1070, a double mutant deficient in 6-phosphogluconate dehydrogenase and 6-phosphogluconate dehydrase edd1 gnd1 constructed by Fraenkel [9] from *E. coli* K10 Hfr.

Mutagenesis was obtained by 2 h incubation at 37 °C in mineral medium with 0.035 vol. ethyl methane sulfone. The mutagenized culture was immediately plated on selective medium.

For uptake experiments, bacteria were grown on mineral medium 63 [10] with 4 g/l glycerol as the carbon source and 0.5 mM D-gluconate as the inducer in aerated conical flasks at 37 °C. Growth was followed by absorbance at 600 nm. Cells were harvested by centrifugation, washed in one volume of mineral medium at room temperature or in one volume of ice-cold distilled water when cells free from traces of gluconate accumulated during culture were desired. Cells were resuspended at a final density of 100–200 μg dry mass per ml in the uptake medium, which was the above mineral medium containing 50 μg/ml chloramphenicol and put in the shaking bath at 25 °C. [U-^{14}C]Gluconate (Amersham, U. K.) was added at the start and 0.5–1-ml aliquots were filtered on nitrocellulose filters (Millipore HA) of 0.45 μm pore size, rinsed with mineral medium [11] dried and counted in a scintillation counter with a toluene-based scintillation fluid.

Assay for gluconate kinase [*12*]. The reaction mixture contained in 1 ml 50 mM Tris · HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM sodium gluconate, 2 mM ATP, 0.4 mM NADP, and 10 μg of 6-phosphogluconate dehydrogenase (yeast, Boehringer). The reaction was started by the addition of bacterial extracts (40–150 μg protein) or a toluenized bacterial suspension, and the formation of NADPH was monitored at 340 nm.

Chromatography of sugars was made on Schleicher and Schull paper 2316 with solvent acetic acid/ammonium acetate, 1M (pH 3.8)/95 % ethanol (2 : 5 v/v).

RESULTS

Selection and phenotypic properties of gluconate kinase-deficient mutants

The double mutant DF 1070 cannot grow on gluconate as sole source of carbon and when growing on another utilizable carbon source, e.g. glycerol, growth is inhibited by the presence of gluconate. This inhibition is attributed to the accumulation of 6-phosphogluconate [13]. The loss of gluconate kinase should then result in a disappearance of gluconate inhibition without a reversal to the gluconate-utilizing phenotype.

A large number of gluconate-resistant strains have been isolated after growth on minimal agar/glycerol/gluconate plates. They have been reisolated, and replicated on minimal agar/gluconate medium. The gluconate-negative, gluconate-resistant strains were tried for gluconate uptake and gluconate kinase activity. They turned out to be of two kinds: permease negative, kinase negative, and permease positive, kinase negative. Among the second type strain AR 13 has served for the study below.

Fig. 1 shows the growth of the mutant strain AR 13 and the parental strain DF 1070 on liquid medium 63; (a) with 4 g/l glycerol, (b) with 4 g/l gluconate and (c) with the mixture of the two carbon sources. Both strains grew normally on glycerol, both were unable to grow on gluconate, but their behavior was different on a mixture. The parental strain when growing on glycerol was inhibited by the addition of gluconate, the inhibition was progressive, to reach more than 90 % after 3 h, while the mutant AR 13 showed a 25 % decreased growth rate in the presence of gluconate, but no further slow down.

In the mutant gluconate kinase activity (4–5 μmol per min per g dry weight) was less than 5 % of the parent strain (100–180 μmol per min per g dry weight).

Gluconate uptake in parent and mutant strain

As expected and as shown by Pouysségur et al. [8], the parent strain, when

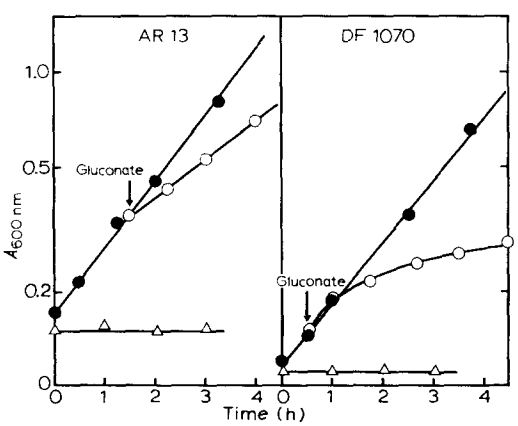

Fig. 1. Growth of mutant and parent strains on glycerol (4 g/l), gluconate (4 g/l) and a mixture of the two. Preculture was grown on medium 63/glycerol. After dilution in the same medium and resumption of exponential growth (●—●), part of the culture was centrifuged, washed, and resuspended in medium 63/gluconate (△—△). Another aliquot was transferred (arrow) to gluconate (○—○).

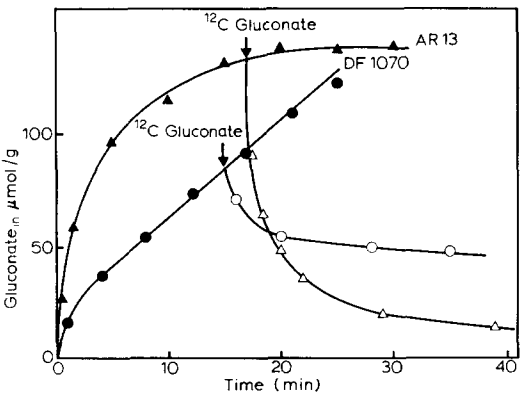

Fig. 2. [^{14}C]Gluconate uptake by mutant (triangles) and parent (circles) strains followed by chase with non-radioactive gluconate. Uptake: full symbols; chase: open symbols.

grown on medium containing glycerol and gluconate has a gluconate uptake system and a gluconate-metabolizing system, so that intracellular gluconate is transformed into 6-phosphogluconate which is strongly retained by the membrane permeability barrier. For this reason, as shown in Fig. 2, the intracellular pool of radioactivity derived from [^{14}C]gluconate increased steadily and the bulk of it could not be chased by added excess unlabelled gluconate. In contrast, mutant AR 13 after 3h induction exhibited an uptake with a higher initial rate than the parent. However, the intracellular concentration of gluconate reached a steady state instead of increasing continuously. With many other molecules accumulated by other permeases the steady state reflects a continuing uptake balanced by equivalent efflux. Accordingly Fig. 2 shows the complete renewal of the intracellular gluconate pool in AR 13.

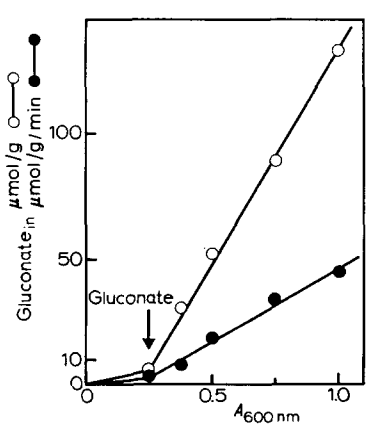

Fig. 3. Kinetics of induction of gluconate transport. Transport activity was measured after arrest of growth washing and resuspension of aliquots in the initial volume by adding 0.20 mM [^{14}C]-gluconate at 37 °C. Initial velocities were determined from the uptake at 30 s, plateau values are means of at least three measurements between 20 and 30 min.

Induction

As shown on Fig. 3, growth of AR 13 in the presence of gluconate resulted in an increase of the initial rate of gluconate uptake, proportionally to the increase of the cell density as expected in conditions of gratuity, where the induced activity is neither indispensable nor harmful to the general process which concurs to increase the cell mass [14]. Fig. 3 shows in addition, that the steady-state pool of gluconate reached after 25 min at 37 °C increased proportionally to the initial rate of uptake. A similar relation in the case of lactose permease was taken as indicating, that the exit process which balances uptake at the steady state was a non-inducible mechanism with first-order kinetics [11]. This contention regarding gluconate flux will be re-examined, in a second article.

Fig. 4 shows the differential rate of induction of gluconate permease as a function of the concentration of gluconate as the inducer. The optimal concentration of inducer was $2 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ M and a higher concentration diminished the inducing efficiency.

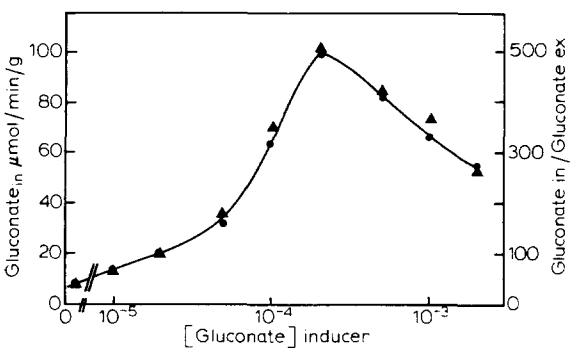

Fig. 4. Inducer concentration dependence of the induction of gluconate permease. Cultures of strain AR 13 were grown overnight at 37 °C with the indicated concentrations of gluconate. Transport was measured as described in Materials and Methods with 0.25 mM [^{14}C]gluconate at 25 °C. ● — ●, initial velocity; ▲ — ▲, final concentration ratio, assuming 2.9 ml intracellular water per g dry weight of bacteria.

TABLE I

INDUCTION OF GLUCONATE PERMEASE AND GLUCOSE EFFECT

Bacteria AR 13 were grown overnight in medium 63 with the indicated carbon source and inducer at 37 °C in aerated flasks, diluted in the morning in the same medium and incubated until exponential growth resumed. Gluconate uptake was measured with $2 \cdot 5 \cdot 10^{-4}$ M [^{14}C]gluconate at 25 °C in mineral medium with glycerol and chloramphenicol.

Inducer (M)	Gluconate uptake (µmol per g dry weight), additions to growth medium					
	Glycerol (4 g/l)		Glucose (4 g/l)		Glucose (4 g/l) cyclic AMP (5 mM)	
	30 s	25 min	30 s	25 min	30 s	25 min
0	4.5	15	3.7	16.6	—	—
$5 \cdot 10^{-5}$	16.3	105	5.0	35.0	7.7	30.0
$5 \cdot 10^{-4}$	45.6	350	13.2	110.0	12.8	92.0

Table I shows that the rate of induced synthesis of gluconate permease was inhibited by glucose. This glucose effect was not reversed by the presence of 2'-5' cyclic adenosine monophosphate (cyclic AMP) which usually counteracts the catabolite repression [15].

Deinduction of gluconate permease

The stability of gluconate permease with time irrespective of bacterial growth authorized the experiment aimed to find out the fate of gluconate permease in the progeny of the induced cells. Since such an experiment [16] is based on the penicillin selection against descendents capable to utilize gluconate as carbon source, it had to be performed with a wild type strain K12 3000. After one or several cell divisions the population is expected to become heterogeneous, part of the progeny containing parental permease in its membrane, another part has only membrane-synthesized posterior to the removal of inducer.

The results of two such experiments are represented on Figs 5 and 6. Fig. 5 shows the time course of lysis when populations of *E. coli* strain 3000 deinduced 0, 1, 2, 3 generation times after full induction with gluconate were incubated in medium 63/gluconate containing 1000 units/ml penicillin G. Until two cell divisions were completed all descendent cells behaved as gluconate utilizing by undergoing fast

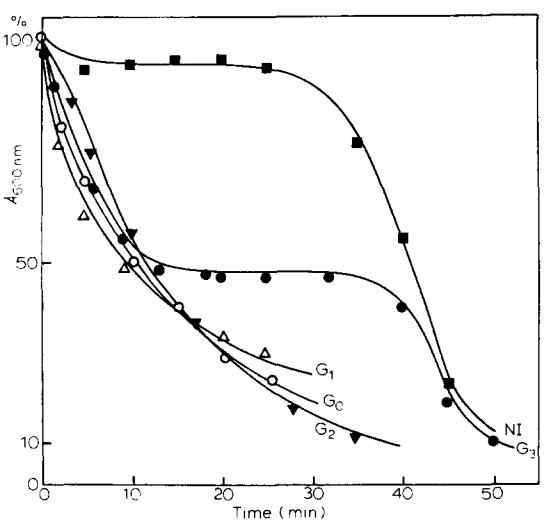

Fig. 5. Appearance of heterogeneity for gluconate utilization among the progeny of induced cells. Wild type strain 3000 K12 was grown overnight on medium 63 containing glycerol and gluconate. After resumption of exponential growth on the same medium, bacteria were filtered, washed and resuspended in medium 63/glycerol. Growth at 37 °C was monitored by absorbance readings at 600 nm. Samples were withdrawn immediately after resuspension: GO, and after 1, 2, 3 doubling times: G1, G2, G3, respectively. Each sample was submitted to an EDTA treatment according to ref. 17 and suspended in medium 63/gluconate containing 1000 units/ml penicillin G. The time course of lysis was monitored by absorbance readings at 600 nm here expressed in percent of initial absorbance at the time of addition of penicillin. A control culture not previously induced by gluconate was also submitted to the EDTA gluconate penicillin treatment. ■—■, non-induced; ○—○, induced GO; △—△, G1; ▼—▼, G2; ●—●, G3.

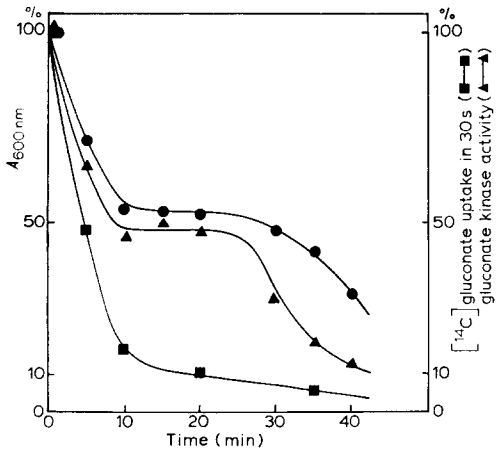

Fig. 6. Segregation of gluconate permease together with lactose permease after deinduction while soluble enzymes are distributed at random. The experiment was conducted as described in the legend of Fig. 5 except that the inducing medium also contained 0.2 mM isopropyl-β-D-galactopyranoside, that only the G3 population was further analyzed and the lysis was conducted in medium 63/lactose containing penicillin. ●—●, absorbance; ▲—▲, gluconate kinase activity and rate of gluconate uptake (■—■) per unit volume of the suspension are expressed as percent of the starting value. 100 % uptake was 11.5 nmol/min per ml suspension (42 μmol/g dry weight per min). 100 % gluconate kinase activity was 10.1 nmol of NADPH produced/min per ml toluenized suspension (39 μmol/min/per g dry weight).

lysis in the presence of penicillin. After three doubling times (G3) half the progeny had lost some parental component essential for gluconate utilization and its lysis by penicillin was delayed until the lost component was induced again.

Fig. 6 shows the analysis of two functions essential for gluconate utilization in the heterogeneous G3 population. Unlike in the experiment of Fig. 5, the parental cells were induced for the utilization of two carbon sources, for lactose utilization by adding 0.2 mM isopropyl-β-D-galactopyranoside and for gluconate utilization by 4 g/l D-gluconate. Deinduction for three generation times was made with glycerol as carbon source without inducers. Final selection represented on the figure was made with penicillin and lactose, so that gluconate-utilizing enzymes were not induced during this phase. The absorbance of the culture decreased as a result of penicillin lysis in two distinct steps. It was shown earlier, that the half population lysed after a delay was devoid of lactose permease activity, while it still contained half of the total β-galactosidase. The figure shows, that the half population which survived 10–25 min had lost the capacity for gluconate uptake, while it contained about half of the initial gluconate kinase.

Search for phosphotransferase and/or for a periplasmic component of gluconate permease

A shock according to Neu and Heppel [18] had no major inactivating effect on gluconate uptake (Fig. 7) (contrary to the observations of Pouysségur et al. [8]). The control suspension underwent the same procedure as the shocked suspension, except that the resuspension after the sucrose-EDTA incubation and centrifugation

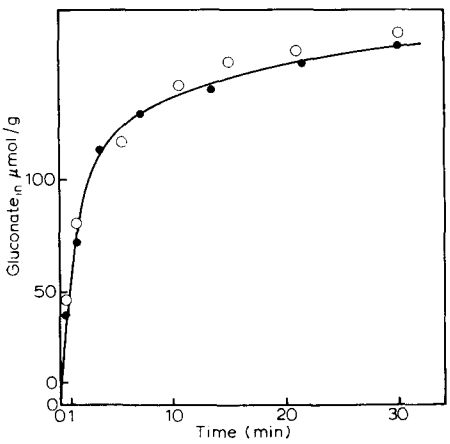

Fig. 7. Gluconate uptake in cells submitted to a cold osmotic shock according to Neu and Heppel [18]. A culture of strain AR 13 induced for gluconate permease was washed with Tris · HCl buffer incubated in Tris · HCl buffer containing 0.1 mM EDTA and 0.5 M sucrose and then divided in two parts. These were centrifuged and resuspended, respectively, in medium 63 (unshocked) or in 0.5 mM $MgCl_2$ solution (shocked). After centrifugation both aliquots were resuspended in medium 63 and assayed for gluconate transport with 0.25 mM [^{14}C]gluconate at 25 °C. ○ — ○, shocked; ● — ●, unshocked.

was made in medium 63 instead of in distilled water containing $5 \cdot 10^{-4}$ M $MgCl_2$. In the same conditions, a galactokinase-negative mutant 108 K showed an inactivation of galactose uptake of at least 95 % compared to a similar control (results not shown).

The existence of a phosphotransferase system was scrutinized by using differential energy inhibitors such as NaN_3 or 2,4-dinitrophenol which do not inhibit α-methyl glucoside uptake in *E. coli* including in strain AR 13 and iodoacetate which strongly and rapidly inhibits phosphoenolpyruvate-dependent uptake. Gluconate up-

TABLE II

EFFECT OF ENERGY INHIBITORS ON GLUCONATE UPTAKE AND ON α-METHYL GLUCOSE UPTAKE

For the measurement of initial rates bacteria were preincubated with the inhibitor 5 min before the addition of [^{14}C]gluconate. For measurement of the steady-state level the inhibitor was added after 15 min uptake, when the control steady state was reached and the new steady state was measured 10 min later.

Inhibitor	Activity percentage of uninhibited control		
	Gluconate uptake		α-Methyl glucose uptake
	Initial rate	Steady-state level	Steady-state level
2,4-Dinitrophenol (2 mM)	15	25	150
Iodoacetate (2 mM)	90	95	12.5

take was strongly inhibited by the former and very slightly by the latter inhibitor (Table II).

Chromatographic analysis of the intracellular radioactivity in AR 13 showed more than 95 % of free gluconate and negligible amounts of 6-phosphogluconate.

DISCUSSION

Deficiency of gluconate kinase completely prevents metabolic transformation of gluconate by *E. coli*. The appropriate mutation therefore allows to study gluconate transport without any side effect.

Gluconate kinase is inducible, the induction process delays the formation of 6-phosphogluconate and this explains the delay of growth inhibition by gluconate in strain DF 1070. Growth stops only after about 3 h and at this point about one duplication of the cell mass has occurred. The arrest of growth prevents the induction to proceed to its theoretical maximum.

The exact target of growth inhibition by the accumulation of phosphate esters is not known. The 25 % inhibition by the addition of gluconate in the gluconate kinase-deficient mutant might be a milder form of the same effect, since the mutant accumulates non-physiological amounts of an anion. Since this amount levels off, inhibition is not progressive. An alternate or complementary explanation might be the energy expenditure necessary to bring about the accumulation.

Gluconate permease is also inducible, and its induced synthesis only occurs when conditions for protein synthesis are fulfilled. Gluconate permease was not fully induced in the parental strain used in the experiment of Fig. 2 since gluconate inhibition by stopping growth prevented further induced synthesis. This could explain why in Fig. 2 initial rate of uptake in the parental strain is lower than in AR 13.

Gluconate uptake is a thermodynamically active process, the ratio of cytoplasmic to external gluconate concentration can be over 2000 and intracellular pools as high as 460 μmol per g dry weight [7] (about 150 mmol per l of cell water) can be achieved.

The maximal rate of gluconate uptake in fully induced AR 13 is 220 μmol/g per min.

The doubling time of a wild type *E. coli* on gluconate as sole carbon source is approx. 55 min and the yield of production is 0.2 g bacterial dry mass per g of sodium gluconate so that the rate of gluconate uptake required is 286 μmol per g dry bacterial mass per min. Therefore gluconate uptake is closely adjusted to satisfy the carbon source requirements of *E. coli* and makes it likely that the uptake observed in AR 13 is representative of the uptake system used by the wild type and suggests that like with many other carbon sources, gluconate uptake is the major rate-limiting step in gluconate utilization. It must be noted here that the maximal rate of uptake found by Pouysségur et al. [8] in mutant R6 and 8–10 times lower than the above figures.

The simple induction kinetics seem to indicate that a single inducible protein (or several coordinately induced proteins) is (are) rate limiting for the transport function. The unequal distribution of the permease among the progeny in contrast to the random distribution of the soluble enzyme gluconate kinase, indicates that the rate-limiting protein(s) is (are) membrane bound.

The presence of a membrane-bound protein does not rule out the requirement of a periplasmic protein for the transport function. Maltose permease of *E. coli* [19] and histidine permease of *Salmonella* [20] exemplify situations in which a periplasmic and a membrane-bound protein are essential. The absence of inactivation after a shock according to Neu and Heppel [18] and the presence of the transport system in empty membrane vesicles [6] provide rather strong evidence against this hypothesis.

Also in accordance with Pouysségur et al. [8] we report data difficult to fit with a phosphotransferase-linked transport system. In conclusion, the features reported so far do not suggest fundamental differences in transport mechanism between lactose permease and gluconate permease.

Due to the anionic charge of gluconate some differences should be expected, and a more detailed kinetic study was undertaken in order to assess analogies and differences.

ACKNOWLEDGEMENTS

This work was supported by the Délégation Générale à la Recherche Scientifique et Technique and the Commissariat à l'Energie Atomique. The authors wish to thank Françoise Autissier-Descos for her help with the experiments of penicillin selection of permease-negative progeny.

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