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The lactose system in *Klebsiella aerogenes* V9A

2. Galactoside permeases which accumulate lactose or melibiose

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SUMMARY

Klebsiella aerogenes V9A possesses at least three galactoside permeases, the lactose permease (LacP), the melibiose permease (MelP) and a third permease (GPIII), and also a galactose permease. It is shown that the first three of these permeases all accumulate both lactose and melibiose efficiently, while the galactose permease takes up neither sugar. Lactose only induces LacP and melibiose only induces MelP. D-fucose, isopropyl-β-D-thiogalactoside (IPTG) and methyl-β-D-thiogalactoside (TMG) all induce GPIII, but galactose does not. GPIII takes up both lactose and melibiose but cannot accumulate lactose in the presence of IPTG. Thus substantial differences are found between the galactoside permeases of *Klebsiella* and *Escherichia coli*.

1. INTRODUCTION

V9A is a wild strain of *Klebsiella aerogenes* with several unexpected properties. It contains at least two separate plasmids, one determining resistance to tetracycline and the other carrying the genes of a *lac* operon, and the strain is also resistant to ampicillin, possibily determined by a third plasmid. These plasmids do not determine transfer systems, but the plasmids carrying tetracycline and *lac* genes can be transferred to *Escherichia coli* K12 if the host strain is first infected with one of a variety of sex factors. The *lac* genes are always associated with a sex-factor repressor gene of *fi*$^+$-type, and both appear to be on the same plasmid (Reeve & Braithwaite, 1970; Reeve, 1970).

The most surprising property of V9A is that it mutates spontaneously to a particular Lac$^-$ phenotype, which can grow very slowly in minimal medium on 0·2 % lactose as carbon source, but more rapidly as the concentration of lactose in the medium is increased, growth being almost as fast as the wild-type on 1 % lactose. A number of mutants of this type have been isolated, and they all still carry the sex factor repressor gene and can revert spontaneously to a Lac$^+$ state indistinguishable from the wild-type. These ML$^-$ mutants are also able to grow rapidly on low levels of lactose as carbon source (0·05–0·2 %) after induction by growth on galactose, melibiose or raffinose, or by growth on glycerol in the presence

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of TMG* or D-fucose. This induced ability is lost two or three generations after the inducer has been removed, suggesting that a permease (other than the lactose permease) has been induced which accumulates but is not induced by lactose (Reeve & Braithwaite, 1972).

In this paper we present evidence that V9A possesses two permeases, apart from the lactose permease, which can accumulate both lactose and melibiose. One, presumably the melibiose permease, is induced by melibiose, and the other is induced by neither lactose nor melibiose.

2. MATERIALS AND METHODS

Abbreviations: IPTG, isopropyl-β-D-thiogalactoside; Lac, lactose; Mel, melibiose MgP, methyl-β-galactoside permease of *Escherichia coli*; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; ONPG, *o*-nitrophenyl-β-D-galactoside; TMG, methyl-β-D-thiogalactoside.

Bacterial strains: *Klebsiella aerogenes* V9A Thy3 and its derivative ML^{-}18 were described by Reeve & Braithwaite (1972). The other strains were mutants of ML^{-}18, obtained as described in the text.

Media and chemicals were as described by Reeve & Braithwaite (1972). Minimal medium was M9 medium, supplemented with thymine at 80 μg/ml since all the bacterial strains were Thy^{-}. Carbon sources were added at 0·2 % unless otherwise specified. IPTG was obtained from Sigma London Chemical Co.

Diauxie growth tests: bacteria were grown overnight in minimal medium on 0·05 % glycerol as carbon source, and were then diluted directly about 1/12 into the various growth media, previously warmed, to give a starting optical density of 0·1, measured with a Beckman DB spectrophotometer at 550 mμ with 1 cm light path. Incubation was at 37 °C, in 500 ml metal-capped flasks with 60–100 ml medium, rotated in a Gallenkamp orbital incubator. Dilution from cultures grown overnight with a limiting carbon source, such as 0·05 % glycerol, gave fresh growth without any lag and without any carry-over of a source of carbon from the parent culture. At the end of each test the cultures were checked by streaking on MacConkey plates containing lactose or melibiose.

Assay of β-galactosidase: cells growing in log phase in minimal glycerol were diluted to about 5×10^7 cells/ml in the same medium and grown for two further cell generations with or without 1 mM TMG as inducer. The OD_{550} was then read and at the same time 2 ml of the bacteria were added to 3 ml chilled medium containing 200 μg chloramphenicol. The chilled mixture was sonicated for four periods of 30 sec, in ice, with an M.S.E. 150 watt Ultrasonic Disintegrator (at medium power and amplitude 4). 2 ml of each sonicate were transferred to a reaction tube and a blank tube in a water bath at 28 °C, and after temperature equilibration 1 ml ONPG solution (0·9 mg/ml in 0·25 M sodium phosphate buffer pH 7) was added to the reaction tube and 1 ml buffer alone to the blank. The reaction was stopped by adding 2 ml of 1 M K_2CO_3 to both tubes and the colour in the reaction tube was read

* For abbreviations see Materials and Methods section.

against the blank at OD_{420}. Enzyme activity was calculated as usual and corrected to the value for bacteria at an optical density (550 mμ) of 1·0.

3. RESULTS

Two hypotheses can explain the growth behaviour of the Lac⁻(ML⁻) mutants of *Klebsiella* V9A described above.

(1) The mutation results in lactose becoming a poor inducer of the *lac* operon, which is still efficiently induced by melibiose, TMG, etc. This theory is difficult to reconcile with the fact that ML⁻ strains can grow rapidly on 1 % lactose but immediately revert to slow growth when transferred to 0·2 % lactose, while induction by melibiose or TMG results in rapid growth on 0·2 % lactose even when the inducer is removed (Reeve & Braithwaite, 1972).

(2) The mutation results in the production of a defective but not completely inactive lactose permease, and the ability to grow normally on lactose after induction by TMG, melibiose etc. is the result of induction of other permease(s).

The second hypothesis requires that, in these ML⁻ mutants, galactose, melibiose, raffinose, TMG and D-fucose induce one or more permeases which accumulate lactose. To test this theory, strain ML⁻18 was treated with NG and selected for failure to grow on raffinose. Most of the mutants obtained were unable to utilize a number of carbon sources and appeared to be of the *ctr* type (Wang, Morse & Morse, 1969), but three raffinose-negative mutants with normal ability to grow on galactose and glucose were obtained. These all had similar properties, and tests on one of them, ML⁻18Raf⁻31 (referred to below as R⁻31) will be described.

Figure 1 shows a diauxie test on R⁻31 in which cells previously grown in glycerol minimal medium were transferred to minimal medium containing 0·01 % glycerol with or without the addition of lactose or melibiose at the concentrations shown, and subsequent growth was followed by optical density (OD) readings at 550 mμ. Cells receiving glycerol alone exhausted the supply in 2 h and the OD of the culture then slowly declined. Lactose at 0·2 % gave no growth for at least 3 h after exhaustion of the glycerol, but very slow utilization of the lactose may then have started (ML⁻18 generally shows very slow growth on 0·2 % lactose following a lag of several hours, after growing on glucose or glycerol). There was rapid growth, without any lag, on 1 % lactose, showing that strain R⁻31 retains the curious leaky behaviour of ML⁻18 on lactose. In contrast, the deficiency in melibiose utilization was absolute, since the strain was unable to grow on 1 % melibiose, and raffinose was equally unable to support growth when tested in the same way (data not shown).

A further analysis of R⁻31 is given in Fig. 2. Cells grown in glycerol minimal medium were transferred to minimal medium containing 0·01 % glycerol, as before, and either melibiose at 0·2 % (graphs on the left) or lactose at 0·1 % (graphs on the right) was added. Separate cultures received also either TMG or IPTG at 1 mM, all additions being made at the time of transfer and start of incubation. Growth of a

control culture which received only the glycerol is plotted on both sides of the figure, and shows that 0·01 % glycerol was again exhausted in 2 h.

The curves on the left show that, although there was no growth on melibiose alone (curve M), the presence of either TMG or IPTG enabled strain R−31 to grow rapidly on melibiose (curves MT and MI). In similar tests these two inducers also enabled R−31 to grow rapidly on raffinose. These results can be explained in two ways: either the raffinose-minus mutation in R−31 results in the production of

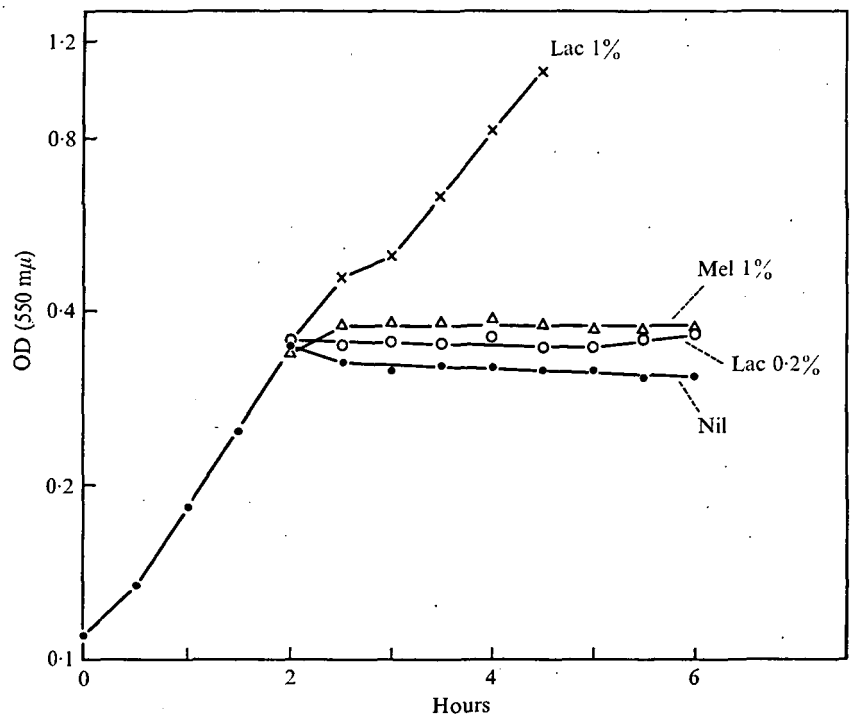

Fig. 1. Strain R−31 grown overnight in minimal medium on 0·05 % glycerol as carbon source was diluted into fresh warm minimal medium containing 0·01 % glycerol and the additions shown, and was incubated at 37 °C. Vertical scale: optical density at 550 mμ, plotted logarithmically. Horizontal scale: incubation time in hours. Incubation started immediately additions were made. Lac, Mel: lactose, melibiose added at the final concentrations shown. Nil: no addition.

an inactive melibiose permease which is also the permease for raffinose, and TMG and IPTG switch on another permease which is able to accumulate both melibiose and raffinose efficiently but is not inducible by these sugars; or the mutation has changed the presumed regulatory system for this permease so that it is no longer induced by melibiose or raffinose. The second hypothesis is difficult to maintain in view of the facts that (1) many mutations with the properties of R−31 have recently been obtained, whereas a mutation changing in such a specific way the regulatory protein should occur very rarely, (2) neither TMG nor IPTG are able to induce the melibiose permease, and (3) Lac$^+$ revertants of R−31 with the *lac*

operon induced can grow well on melibiose, so that melibiose which has entered cells of R⁻31 by the lactose permease can then induce the structural gene for α-galactosidase (the evidence for points 2 and 3 will be given later). We conclude from these facts that the mutation in R⁻31 results in a defective melibiose permease.

Figure 2 also shows the behaviour of R⁻31 on lactose. Clearly there was no growth on 0·1 % lactose alone, once the glycerol had been exhausted (curve L), but TMG induced rapid growth on lactose (curve LT), just as it did on melibiose. This suggests that TMG induces a third permease able to accumulate lactose, melibiose and raffinose, but not induced by any of these sugars. However, IPTG

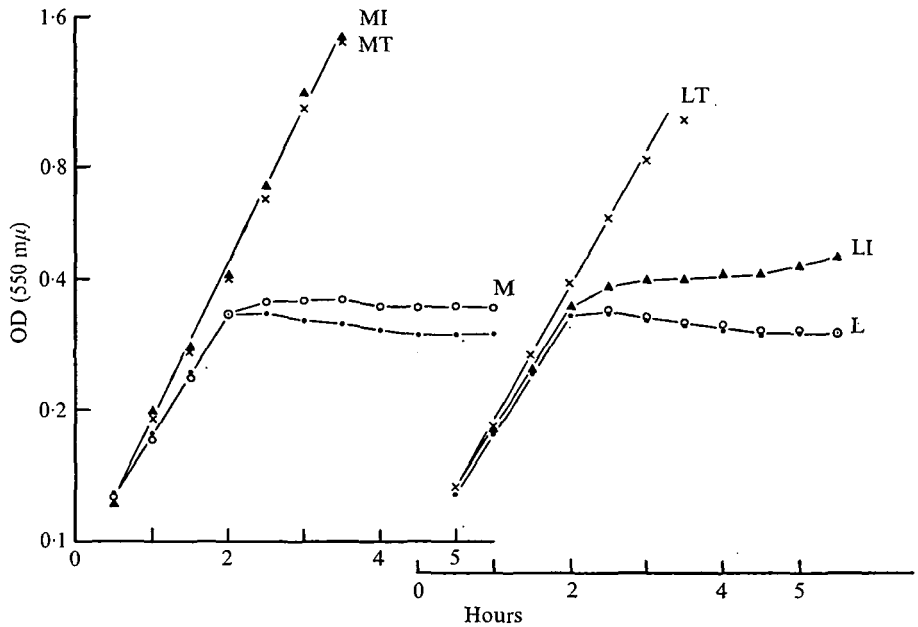

Fig. 2. Diauxie test on R⁻31, with same conditions as for Fig. 1. All cultures received 0·01 % glycerol and the following additions at time 0. ●—● none, ○—○ melibiose (M) or lactose (L), ×—× TMG + melibiose (MT) or lactose (LT) ▲—▲ IPTG + melibiose (MI) or lactose (LI). Melibiose was added at 0·2 %, lactose at 0·1 %, and IPTG at 1 mM.

failed to induce rapid growth on lactose, although there was very slow growth on 0·1 % lactose in the presence of this inducer. We attribute this slow growth to the fact that IPTG induces a normal (wild-type) level of β-galactosidase in ML⁻ mutants, and the leaky lactose permease of these mutants will therefore be maximally induced and will be able to accumulate lactose slowly from an external concentration of 0·1 or 0·2 %.

It thus appears that strain R⁻31, defective in both the lactose and melibiose permeases, is induced to grow efficiently on both lactose and melibiose by TMG and on melibiose alone by IPTG. This leads to the hypothesis that V9A possesses two galactoside permeases in addition to those of the *lac* and *mel* operons, of which

one (GPIII) can accumulate both melibiose and lactose and is induced by TMG, while the other (GPIV) accumulates melibiose alone and is induced by IPTG – or, alternatively, that GPIII accumulates melibiose and is induced by both TMG and IPTG, while GPIV accumulates lactose and is induced by TMG but not by IPTG. In either event it should be possible to select two classes of mutant from R^{-}31, with permeases III and IV inactivated, respectively, which will show different induction responses to TMG and IPTG.

Selection of these two mutant types was made possible by the fact that R^{-}31 gives colourless colonies on MacConkey melibiose agar, but gives pink 'melibiose-positive' colonies on the same agar in which TMG or IPTG has been incorporated at 1 mM. Pale colonies on these plates can thus be assumed to carry a mutation inactivating either the α-galactosidase gene or the permease by which melibiose enters in the induced R^{-}31 cells. R^{-}31 was therefore mutagenised with NG, grown to log phase in minimal medium containing melibiose and either TMG or IPTG, enriched for the required mutant type by incubating for 90 min with ampicillin at 1 mg/ml before washing out the antibiotic, and then plated on MacConkey melibiose agar containing the same inducer (TMG or IPTG). Pale colonies on these plates should include mutants of the desired types.

Two mutants of each class – those failing, respectively to respond on melibiose plates to induction by TMG and by IPTG, were isolated in this way and were subjected to a number of tests. They grew normally on glucose and galactose, and showed the same rapid growth on 1 % but not on 0·1 % lactose as ML^{-}18 and R^{-}31, and thus retained the original Lac^{-} state. The four mutants were labelled R^{-}31MelT^{-}, nos. 1 and 2, and R^{-}31 MelI^{-}, nos. 1 and 2, where T and I stand for the inducers used in their selection. Diauxie tests of the kind illustrated in Fig. 2 were used to test the ability of TMG, IPTG and D-fucose to induce in each mutant the ability to grow on melibiose and on 0·1 % lactose, and the results of these tests are summarized in table 1, which gives the corresponding behaviour of the wild-type strain (ML^{+}) and the single and double permease mutants ML^{-}18 and R^{-}31.

Much to our surprise, the four triple mutant strains were all unable to grow on either melibiose or on low levels of lactose when induced by IPTG, TMG or D-fucose: i.e. the growth in minimal medium containing 0·01 % glycerol plus one of the three inducers and either melibiose or lactose as in Fig. 2, was identical with the growth pattern found when the inducer was omitted, ceasing after 2 h with the exhaustion of the glycerol.

The third mutation in each of these strains cannot have been in the structural gene for α-galactosidase, since they would then have retained the ability to grow on lactose after TMG induction, and a pleiotropic *ctr* mutation with an effect on carbohydrate transport (Wang *et al.* 1969) can also be ruled out because the two MelT^{-} and two MelI^{-} mutants all grew normally on glucose and galactose. There seem to be only two ways of explaining these results: either all four mutations in R^{-}31 involve a deletion covering the genes of both GPIII and GPIV, or only a single permease is involved in the induced growth of R^{-}31 on lactose and meli-

biose, which is inactivated in all four mutants. In the latter case, this permease must be inducible by both TMG and IPTG and must be able to accumulate both lactose and melibiose, and we have to assume that its ability to take up lactose is inhibited by the presence of IPTG.

This hypothesis was tested in the experiment shown in Fig. 3. Strain ML⁻18, previously grown in minimal glycerol, was transferred to minimal medium containing 0·01 % glycerol and 0·1 % lactose, together with either TMG or IPTG at 1 mM. After exhaustion of the glycerol there was rapid growth on lactose in the presence of TMG but not of IPTG. An hour after the glycerol had been exhausted, the culture now growing rapidly on 0·1 % lactose + TMG (curve LT) was diluted

Table 1. *Growth behaviour of permease-negative mutants*

	Permease genes*					Growth on:†	
Strain	Lac	Mel	III	IV	Inducer	Lac	Mel
ML⁺	+	+	+	+	None	+	+
ML⁻18	−	+	+	+	None	−	+
					Mel	+	+
					TMG	+	+
R⁻31	−	−	+	+	None	−	−
					TMG	+	+
					IPTG	−	+
R⁻31MelT⁻1, 2	−	−	−	+	Any‡	−	−
R⁻31MelI⁻1, 2	−	−	+	−	Any‡	−	−

Based on glycerol-lactose and glycerol-melibiose diauxie tests, using the protocol of Fig. 2, with lactose at 0·1 %, melibiose at 0·2 %. Two mutants each of the classes R⁻31MelT⁻ and MelI⁻ were tested.

* For permease genes, + means normal, − means mutant. III and IV are the hypothetical permeases GPIII and GPIV referred to in the text.

† + means rapid growth, − means very slow or no growth.

‡ 'Any' includes galactose, melibiose, TMG, IPTG and D-fucose.

into minimal medium containing 0·1 % lactose and 1 mM-TMG, and IPTG was added at 1 mM (curve labelled LT → LTI). The growth rate at once dropped sharply to that characteristic of cells growing on lactose in the presence of IPTG (see Fig. 2). At the same time the LI culture, growing very slowly on lactose + IPTG, was centrifuged and resuspended in minimal medium containing 0·1 % lactose without IPTG (curve labelled LI → L). Rapid growth, characteristic of TMG-induced cells, started immediately and continued for the remaining 3 h of the test. Precisely similar results were obtained when the same experiment was done with strain R⁻31.

These experiments make it clear that ML⁻18 and R⁻31 possess a single permease (GPIII) able to accumulate both lactose and melibiose after induction by TMG or IPTG. The permease can accumulate lactose and melibiose efficiently enough to give rapid growth on low external concentrations of either sugar. In the presence of IPTG, however, this permease cannot accumulate lactose, presumably because

IPTG has a higher affinity than lactose for the permease membrane protein. A further deduction can be made from the fact that IPTG inhibited lactose uptake by cells of both R⁻31 and ML⁻18, when these had been induced by TMG or IPTG: since ML⁻18 possesses an intact melibiose system, this system cannot be inducible by either TMG or IPTG, because otherwise its permease would take up lactose and so permit growth on lactose in the presence of IPTG. The wild-type strain, ML⁺, it may be noted, grew well on lactose in the presence of IPTG, so this

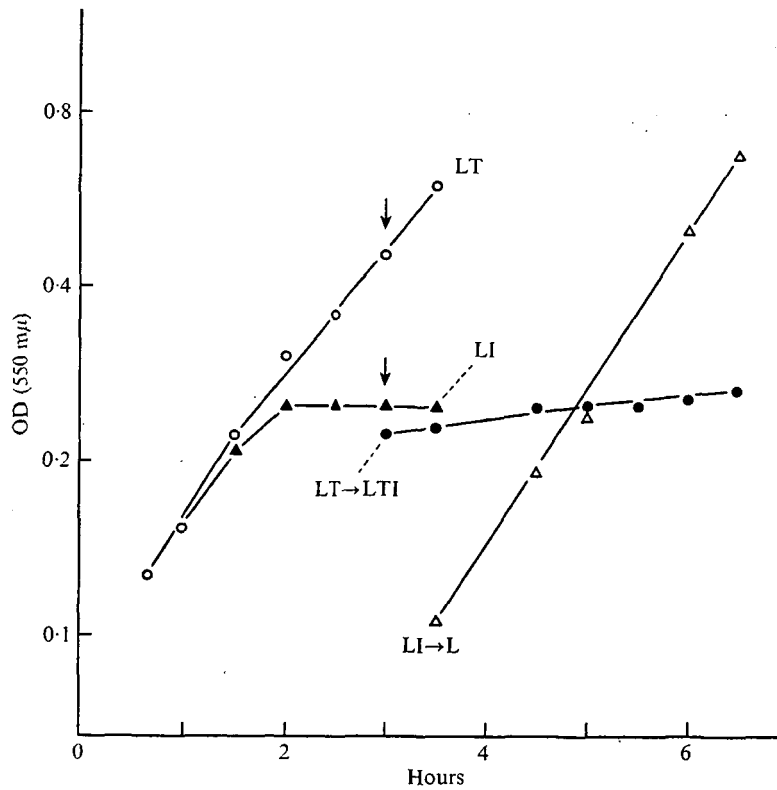

Fig. 3. Strain ML⁻18 treated as LT and LI cultures in Fig. 2. Vertical arrows indicate the time at which samples of these cultures were taken to form the LT → LTI and LI → L cultures. Further explanation in the text.

substance did not interfere with either the entry of lactose by the lactose permease, or with its metabolism by β-galactosidase.

Escherichia coli K12 possesses three galactoside permeases (Lac, Mel and MgP), all able to accumulate galactose, and in addition a 'galactose permease' which is only able to accumulate galactose of the substances so far tested (Rotman, Ganesan & Guzman, 1968). We have not yet tested the abilities of the three galactoside permease in *Klebsiella* V9A to take up galactose, but this strain evidently possesses a galactose permease in addition to these, since the four triple permease-minus strains referred to in table 1 can grow normally on galactose. They

do not acquire the ability to grow on lactose, melibiose or raffinose after growing on galactose, so the galactose permease cannot take up these sugars.

Two further points will be considered here, the first being whether melibiose can induce, or get in by, the lactose permease. This can be tested in the following way: ML^{-}18 reverts spontaneously to the wild-type lactose positive state (Reeve & Braithwaite, 1972), and both R^{-}31 and the triple permease-minus mutants retain the same ability – when colonies of these strains are aged several days on MacConkey lactose agar and then spread on the same medium, a small proportion of Lac^{+} revertant clones is always obtained. Tests on such a Lac^{+} revertant of one

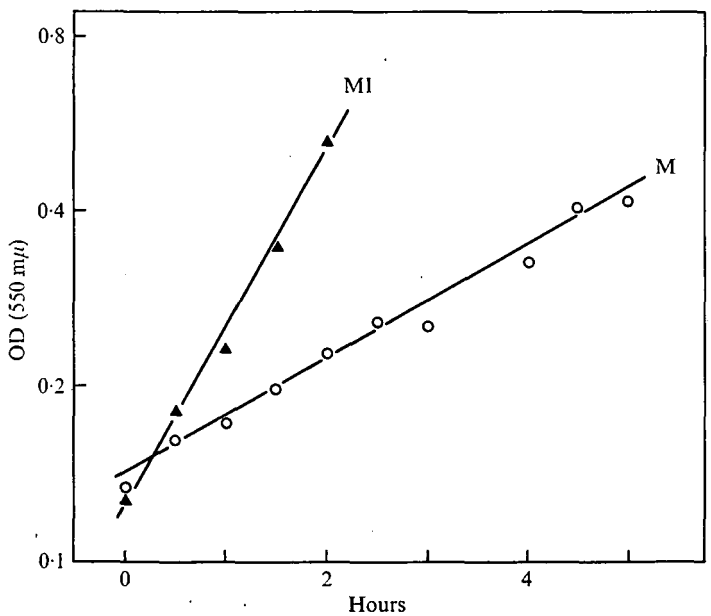

Fig. 4. A Lac^{+} revertant of the triple permease-minus strain R^{-}31MelT^{-}1 was grown in minimal glycerol 0·05% until the glycerol was exhausted, then diluted into warm minimal medium containing 0·2% melibiose alone (M), or together with 1 mM IPTG (MI). Additions at time 0, incubation and growth measurements as previously.

of the triple permease-minus strains (R^{-}31MelT^{-}1) showed it to have a normal inducible lactose operon while the other two permeases were still inactive. Figure 4 shows the effect of transferring this strain from glycerol to melibiose minimal medium, with or without the presence of TMG. Growth was slow on transfer from glycerol to melibiose alone, showing that melibiose is at best a very poor inducer of the lactose system, but rapid growth occurred when TMG was added, showing that melibiose is taken up by the lactose permease. This experiment also provides further evidence for our conclusion that R^{-}31 has an inactive melibiose permease and not a changed regulator system for the melibiose genes such that it can no longer be induced by melibiose. Melibiose, having entered the Lac^{+} revertant cell

by the lactose permease, must be able to induce the α-galactosidase gene in order to support growth.

The second point is the ability of TMG to induce the lactose system in the different mutant strains. Table 2 gives the levels of β-galactosidase activity obtained in cells of the various types, growing in minimal glycerol alone and after induction for 2 h by 1 mM TMG. The enzyme level is in each case standardised to cells at an OD_{550} reading of 1·0, the assays being made on cells growing in log phase at OD about 0·2. Clearly the four strains all have a normal inducible *lacZ* gene. IPTG has been found to produce similar enzyme levels, and D-fucose also induces substantial levels of β-galactosidase in all four strains.

Table 2. *β-galactosidase activity in strains of* Klebsiella *V9A*

	Peremease genes			β-galactosidase activity	
Strain	Lac	Mel	GPIII	Not induced	Induced
L+3	+	+	+	0·55	94
L−18	−	+	+	0·51	100
R−31	−	−	+	0·57	97
R−31MelT1	−	−	−	1·14	74

The bacteria were grown in minimal glycerol, and induction was with 1 mM TMG for two cell generations. Cell sonicates were assayed and adjusted to enzyme activity at OD_{550} 1·0 for living cells (see Methods).

4. DISCUSSION

Escherichia coli possesses three galactoside permeases, a galactose permease (Rotman, Genezan & Guzman, 1968), and possibly a further permease for galactose mediated by the P-enolpyruvate-P-transferase system (Roseman, 1969). We shall leave out of account the last two systems and compare the characteristics of the three galactoside permeases in *E. coli* and *Klebsiella* V9A. It is obviously reasonable to assume homology between, respectively, the lactose and the melibiose permease systems in the two species, and the Klebsiella permease GPIII may be compared with the *E. coli* methyl-β-galactoside permease (MgP).

Table 3 summarizes the characteristics of the three permeases in V9A, and indicates those which differ from the properties of the corresponding *E. coli* permeases, by a circle surrounding the + or − symbol in question. The differences between the two species are surprisingly extensive, and include more than a third of all the reactions tested. Thus lactose and melibiose are both substrates for all three permeases in V9A, while in *E. coli* MelP does not take up lactose and MgP takes up neither sugar. There are also marked differences between the two species in the induction profiles of the three permeases, particularly those of LacP and of GPIII compared with MgP.

The striking differences between GPIII and MgP raise doubts whether these two permeases have a common evolutionary origin. MgP appears to be the main permease for galactose in *E. coli*, since it is associated with a galactose-binding

protein which possesses the properties expected of a membrane carrier molecule (Anraku, 1967; Boos & Gordon, 1971), and this protein also forms an essential part of the receptor system responsible for chemotaxis towards galactose in *E. coli* (Hazelbauer & Adler, 1971). It is not possible to test our mutants for chemotaxis activity, since V9A is not motile, but further comparison between GPIII and MgP should be of interest.

In *E. coli* lactose must be converted to allolactose by an active β-galactosidase before it can induce the *lac* operon (Jobe & Bourgeois, 1972), so that mutations in the *lacZ* gene which prevent lactose being converted to allolactose, or in the *lacI* gene such that allolactose is no longer an inducer, would mimic Lac-permease

Table 3. *The galactoside permeases of* Klebsiella *V9A*

		LacP	MelP	GPIII
Substrates:	Lactose	+	⊕	⊕
	Melibiose	+	+	⊕
Inducers:	Lactose	+	−	−
	Melibiose	⊖*	+	−
	TMG, IPTG	+	−	⊕
	D-fucose	⊕	−	+
	Galactose	⊕	⊕	⊖
Competitive inhibition by:	TMG	−	−	−
	IPTG	−	−	Lactose†

Circles round + or − symbols indicate those reactions which differ in V9A and *E. coli* K12 (Gal$^+$ strains), according to the data of Rotman *et al.* (1968) and Schmitt (1968). GPIII is compared with the methylgalactoside permease of *E. coli*.

* Melibiose gives slight induction of the *lac* operon in V9A (scored as minus), and good induction in *E. coli*.

† The methylgalactoside permease of *E. coli* does not accumulate lactose, hence no inhibition by IPTG can be measured.

mutants in some respects. Similar possibilities must also be considered for melibiose-negative mutants. Uptake studies with lactose and melibiose would provide the most direct evidence on whether the ML$^-$ and R$^-$31 strains carry specific permease defects, but our results appear to rule out any other explanation of their behaviour. Thus, only a defective lactose permease would account for the facts that ML$^-$ cells grow rapidly on 1% lactose but revert immediately to slow growth when transferred to 0·2% lactose: if the high lactose concentration was necessary to induce a normal lactose permease, then rapid growth would continue on 0·2% lactose until the permease membrane sites had been diluted out.

The Mel$^-$ mutation in R$^-$31 can only be a permease-negative mutation, since melibiose allowed to enter by another permease (induced by a substance which does not induce the *mel* operon) promotes rapid growth and so must be able to induce this operon. In *E. coli* K12, it can be assumed that melibiose is the direct inducer of the *mel* operon, since Schmitt (1968) has shown that melibiose induces the melibiose permease in mutants with an inactive structural gene for

α-galactosidase. Whether lactose and melibiose are direct inducers of their operons in *Klebsiella* strains remains to be determined.

Klebsiella V9A is unusual in carrying a complete *lac* operon on a plasmid, F_K*lac*, and it is not yet clear what chromosomal genes of the *lac* system are also present in V9A, owing to the failure of conventional methods to cure the host of the plasmid. Very recently we have isolated variants of V9A which appear to have lost the complete F_K*lac* plasmid with its associated sex-factor repressor gene, and these variants give uninduced and induced levels of β-galactosidase activity of about 10% the corresponding levels in the parent strain. They thus resemble the Lac^L variants of another *Klebsiella* strain which Brenchley & Maganasik (1972) found had also lost a resident Lac^+ plasmid. It should be emphasized that V9A which has lost the complete F_K*lac* plasmid has quite different characteristics from those of the ML^- mutants described by Reeve & Braithwaite (1972) and in this paper. These mutants retain the sex-factor repressor gene, are only defective in the lactose permease, and readily revert to a fully Lac^+ state from which F_K*lac* can again be transferred to *E. coli* with the help of an introduced sex factor. The nature of the ML^- state is still obscure, but it could be the result of a reversible association between the chromosomal and plasmid *lac* genes which affects the two permease genes, or a change in one of these genes might lead to the production of a partially inactive permease through the association of subunits from the two genes. Further papers on these problems are in preparation.

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