# The lactose system in *Klebsiella aerogenes* V9A

## 1. Characteristics of two Lac mutant phenotypes which revert to wild-type

BY E. C. R. REEVE* AND J. A. BRAITHWAITE*

*Institute of Animal Genetics,*
*West Mains Road, Edinburgh, EH9 3JN, Scotland*

### SUMMARY

A wild strain of *Klebsiella aerogenes* (V9A), which contains a plasmid $F_K lac$ carrying the genes of the *lac* operon, gives two mutant cell types, recognized by the appearance of their colonies on MacConkey lactose agar. These are referred to as ML⁻ and ML⁻/⁺, wild-type being ML⁺. ML⁻ cells can grow rapidly on 1 % lactose as carbon source but very slowly on 0·2 % unless induced by TMG or D-fucose, or by previous growth on galactose, melibiose or raffinose, which enables them to grow rapidly on 0·2 % lactose for 2–4 cell generations. Previous growth on 1 % lactose does not induce the ability to grow rapidly on 0·2 % lactose. It is concluded that ML⁻ cells have a defect in the lactose permease gene which allows slow uptake of lactose when the external concentration is 0·2 % and more rapid uptake when the external concentration is increased. In addition, TMG, D-fucose, galactose, melibiose and raffinose induce one or more other galactoside permeases which can accumulate lactose efficiently but are not induced by lactose. ML⁻/⁺ cells can grow normally on 0·2 % lactose as carbon source, but only after a substantial lag when transferred from glucose, glycerol or sucrose, and after an even longer lag when transferred from melibiose or raffinose. Wild-type cells (ML⁺) grow normally on 0·2 % lactose after a short lag of less than a cell generation time, when transferred from any other carbon source. Cells of each of the three phenotypes ( +, −/+ and − ) can mutate to give both of the other two phenotypes. Incomplete genetic evidence suggests that the ML⁻ mutation is a result of a reversible change in the $F_K lac$ plasmid.

## 1. INTRODUCTION

V9A is a wild strain of *Klebsiella aerogenes*, originating from vole faeces, which has been found to harbour a plasmid, $F_K lac$, with unusual properties. $F_K lac$ carries the genes of the *lac* operon and an active $fi^+$-type repressor gene for sex factor activity, but is unable to mediate cell conjugation. Its transfer to *Escherichia coli* is made possible by first introducing a sex factor into the donor strain, and $F_K lac$ is then found to have the compatibility system of F in *E. coli* (Reeve &

* A.R.C. Unit of Animal Genetics.

Braithwaite, 1970). Recently two variant phenotypes of the host *Klebsiella* have been recognized in which the lactose utilization system has been modified, and studies on these phenotypes are described below.

## 2. MATERIAL AND METHODS

### (i) *Bacterial strains*

The origin of *Klebsiella aerogenes* V 9 A has been described (Reeve & Braithwaite, 1970). A thymine-requiring strain, V 9 A Thy 3, derived from the original isolate by selection in minimal medium containing aminopterin plus thymine, together with mutant lines obtained from this strain, were used in the present study. The thymine requirement was used as a marker to help distinguish the strain. *E. coli* K 12 wild-type (our stock RE 15), obtained originally from W. Hayes, was used as a control in enzyme assays.

### (ii) *Media*

Broth was L-broth consisting of 10 g Difco Bacto Tryptone, 5 g Difco Yeast Extract, 5 g NaCl and 1 g glucose/l. distilled water; ML agar was MacConkey Lactose agar (Oxoid MacConkey agar No. 3); minimal medium was M 9 minimal medium (Adams, 1959) with a carbon source added at 0·2 % (w/v) unless otherwise specified. LZ agar contained 25·5 g Difco Bacto-Penassay Base Agar (dehydrated), 0·05 g 2,3,5-triphenyltetrazolium chloride (B.D.H.) and 10 g lactose/l., the lactose being added after autoclaving as a sterile 20 % solution. These media were all supplemented with thymine at 80 μg/ml for growing the Thy⁻ *Klebsiella* strains.

### (iii) *Chemicals*

The carbon sources used in the growth tests were D-galactose, D-glucose, α-lactose, melibiose, raffinose and sucrose, all from Sigma London Chemical Co., and glycerol (AnalaR grade) from B.D.H. Chemicals Ltd., Poole, England. D-Fucose, ONPG (*o*-nitrophenyl-β-D-galactopyranoside) and TMG (methyl-β-D-thiogalactopyranoside) were obtained from Sigma. Cetab (cetyltrimethylammonium bromide) was obtained from B.D.H., and other chemicals were of AnalaR grade.

### (iv) *Growth tests*

It was necessary to establish the growth rates of a number of V 9 A strains in minimal media containing various carbon sources, including diauxie tests in which two carbon sources were added together. For this purpose cells grown overnight or to log phase on a particular carbon source in liquid minimal medium were diluted into, or where necessary centrifuged and resuspended in, the test medium at an optical density of usually about 0·1 (550 mμ and 1 cm light path), corresponding to approximately $10^8$ bacteria/ml. Either 25–30 ml samples in 100 ml metal-capped flasks were incubated, with shaking, in a water bath, or 100 ml samples in 500 ml metal-capped flasks were rotated in a Gallenkamp orbital incubator. Incubation was at 37 °C, but was more accurately controlled in the water

bath than in the orbital incubator, since sampling at 30 min intervals meant frequent opening of the lid of the latter. A serious problem in growth tests with *Klebsiella* V9A substrains is that the cells often form clumps whose optical density cannot be measured accurately, and occasionally these clumps stick together to form large flocculi. Clumping was more frequent with cultures shaken in the water bath than in those rotated in the orbital incubator, but we have not yet been able to identify the conditions which keep clumping to a minimum. In long growth experiments cell clumps, once detected, were dispersed by hourly homogenization with an MSE Microhomogenizer, and this resulted in reasonably linear growth trends in spite of the short periods of reduced temperature caused by the treatment. Growth was measured by optical density (OD) change at 550 m$\mu$ (1 cm light path) read in a Beckman DB spectrophotometer.

### (v) *Assay of $\beta$-galactosidase*

The cells were grown at 37 °C in liquid M9 medium on glycerol as carbon source and were assayed at 0·2–0·5 $OD_{550}$, corresponding to about $2$–$5 \times 10^8$ cells per ml. Induced cells were grown for two generations in the presence of $10^{-3}$ M TMG* before assay. For the assay, 2 ml cells was added to a tube containing 0·1 ml of a solution in water of 2000 $\mu$g/ml chloramphenicol and 800 $\mu$g/ml Cetab, in a water bath at 28 °C. After 3 min, 1 ml ONPG solution (0·9 mg/ml in 0·25 M sodium phosphate buffer at pH 7) was added, and the reaction was stopped with 2 ml of 1 M-$K_2CO_3$ when sufficient colour had developed. The quantity of enzyme was measured as $OD_{420} - 1·56 \times OD_{550}$ (read against a water blank), and was standardized by dividing by reaction time (min) $\times 0·0075$. These standardized values were divided by the $OD_{550}$ of the cells at the time the assay began, to give the estimated enzyme concentration corresponding to OD 1·0.

Cetab was found to give very similar results to toluene in making the cells porous to the substrate, and is much less trouble to use.

### 3. RESULTS

### (i) *Three lactose phenotypes*

*Klebsiella* V9A, when plated on MacConkey lactose (ML) agar, gives typically lactose-positive colonies after overnight growth at 37 °C; they are about 1·5 mm in diameter, slightly mucoid and a deep pink in colour. The agar becomes turbid round the larger colonies and near areas of confluent growth, because of precipitation of bile salts in the medium. However, on further incubation at 37 °C or room temperature, the colonies gradually lose all trace of pink colouring and become a pale opaque yellow, while the turbidity fades from the agar. These changes are evidently the result of the conversion to acetyl-methyl-carbinol of the acids formed early on by lactose utilization, with a consequent rise in pH (cf. Thiman, 1955; Eddy, 1961). The wild-type strain described above will be labelled ML+, to signify its Lac+ phenotype on ML agar. During routine

* For abbreviations see Material and Methods, (iii) Chemicals.

plating on ML agar, a pale variant colony was observed which when purified on ML agar gave pale yellowish-white colonies and pale areas of confluent growth. A number of colonies having the same appearance on ML agar have now been isolated and purified, and the resulting clones are labelled ML⁻. When ML⁻ clones are streaked with a loop on ML agar, areas of confluent growth often contain red specks, and when these specks are picked and purified on ML agar they give either clones of wild-type (ML⁺) appearance or a third phenotype which appears intermediate between the (−) and (+) strains, and will be labelled ML⁻/⁺. On ML agar this phenotype gives colonies that are pink like those of ML⁺ but have a pale rim, and no misting of the agar occurs even near areas of thick confluent growth. These differences are quite sufficient to distinguish the three phenotypes consistently, and single colonies on ML agar can almost always be classified correctly, as judged by their appearance when purified on ML agar. After further incubation the three colony types gradually all come to look the same.

### (ii) *Interconversion between the ML phenotypes*

As we have seen, ML⁻ cells revert to the other two phenotypes, and, in order to get an indication of the reversion frequency, seven freshly repurified ML⁻ lines were aged for 1, 4 or 6 days on ML agar or MacConkey agar containing glucose, and colonies from each were then grown up in broth and plated on ML agar to measure the proportions of revertants. Table 1 shows the result of ageing the lines on ML agar. Fresh colonies (aged 1 day) contain very few revertants, the overall frequency being about 1 in 5000, but the frequency rises to about 3 % at 4 days and 10 % at 6 days. On glucose agar the frequency does not increase noticeably with age of colony and remains very low, the 18 000 colonies tested including only two (+) and four (−/+) colonies. Clearly the overall mutation frequency is low, but the revertant types appear with increased frequency on old ML plates, doubtless because the presence of lactose allows revertant cells in the colony to grow when other carbon sources, available to the (−) cells, have been largely exhausted.

All seven (−) lines were able to revert to (−/+), and additional plating tests have shown that they can all revert to (+) as well. ML⁻ 18, and possibly ML⁻ 21, gave a higher proportion of (+) to (−/+) than the other lines. The fact that all seven ML⁻ lines have the same general reversion pattern suggests that we are dealing with a single phenomenon and not a number of mutants of different types, and evidence presented later supports this belief.

Six revertant clones have been tested for their ability to change phenotype a second time, by growing up colonies in broth and plating on ML agar, and the results are given in Table 2. The (−) and (−/+) clones derived from ML⁻ 18 each gave colonies of both the other phenotypes, and only ML⁻ 21(+) gave no colonies of changed phenotype in a rather small test. Larger-scale tests of this kind are needed, but it is clear that cells which have switched phenotype once usually retain the ability to change again among the three phenotypes. In fact, five of the (−) clones listed in Table 2 were able to give revertants once more so that they

Table 1. *Reversion frequencies of* $ML^-$ *lines aged on ML agar*

Revertants per 1000 cells

| Age: | 1 day | | 4 days | | 6 days | |
|---|---|---|---|---|---|---|
| Line | $(-/+)$ | $(+)$ | $(-/+)$ | $(+)$ | $(-/+)$ | $(+)$ |
| ML$^-$ 1 | 0 | 0 | 25 | 0 | * | |
| ML$^-$ 3 | 0 | 0 | 33 | 0 | * | |
| ML$^-$ 4 | 0 | 0 | 6 | 0 | * | |
| ML$^-$ 11 | 0 | 0 | 21 | 2 | 87 | 5 |
| ML$^-$ 18 | 0 | 0 | 3 | 6 | 12 | 15 |
| ML$^-$ 20 | 0 | 0 | 110 | 3 | 160 | 1 |
| ML$^-$ 21 | 0 | 2 | 2 | 0 | 75 | 8 |
| Total no. of revertants | 0 | 1 | 245 | 13 | 1199 | 74 |
| Total no. of cells plated | 4940 | | 9270 | | 12400 | |

The seven lines freshly repurified were streaked to give single colonies on ML agar, incubated at 37 °C overnight, and then kept at room temperature. At 1, 4 and 6 days after plating, several colonies of each line were grown up in broth, diluted and plated to give 200–300 colonies per plate on ML agar. The frequencies of revertant colonies on these plates are given in the body of the table. The numbers of cells tested per line were 600–900 on day 1, 1100–1500 on day 4 and 2300–4600 on day 6. $(-/+)$ and $(+)$ indicate the two revertant colony types.

* Lines which were not tested on day 6.

Table 2. *Change of phenotype in revertant lines*

| | | Numbers found | | |
|---|---|---|---|---|
| Line | Colonies tested | $(+)$ | $(-/+)$ | $(-)$ |
| ML$^-$ 18(+) | 20 000 | — | 3 | 2 |
| ML$^-$ 18($-/+$) | 10 500 | 5 | — | 2 |
| ML$^-$ 21(+) | 9 000 | — | 0 | 0 |
| ML$^-$ 20($-/+$) | 20 500 | 5 | — | 1* |
| ML$^-$ 20(+) | 20 500 | — | 0 | 1 |
| ML$^-$ 11($-/+$) | 17 500 | 9 | — | 0 |

Colonies of each line from fresh MacConkey glucose plates were grown up in L-broth and plated on MacConkey lactose agar to give 200–300 colonies per plate. These were scored for phenotype, and any with changed phenotype were streaked on the same medium to check the new phenotype.

* This colony was found to be temperature-sensitive for lactose utilization, and is not a normal $(-)$ type. It does not revert to other phenotypes.

had undergone four successive changes of phenotype. The sixth clone, a $(-)$ line obtained from a $(-/+)$ derivative of ML$^-$ 20, did not revert again in large-scale tests and was found to be temperature-sensitive for lactose utilization, growing well on lactose at 31 °C (and giving pink colonies on ML agar at this temperature), growing very slowly on lactose at 37 °C and not at all at 41 °C. It seems probable

that this is in fact a $(-/+)$ line with a temperature-sensitive mutation in the *lacZ* gene, a conclusion supported by the fact that we found it very difficult to demonstrate the presence of any $\beta$-galactosidase in this strain, even when it was grown and assayed at 28 °C (cf. Table 3).

A curious feature of strain ML⁻ 20 and all the lines derived from it is that they do not produce gas from glucose or any other sugar when tested in the standard way (Durham, 1898), in contrast to V 9 A and all other lines derived from it. ML⁻ 20 and its derivatives, including the non-reverting $(-)$ strain referred to above, are all thymine-requiring and carry tetracycline and low-level ampicillin resistance like the parent V 9 A from which they originated, and it must be assumed that a mutation eliminating gas production from acid occurred at the time ML⁻ 20 was isolated.

The actual mutation rates from one phenotype to another cannot be derived from the mutant frequencies given in Tables 1 and 2, since the mutant frequency depends on the previous history of the colonies tested. Bacterial mutation rates are particularly difficult to measure accurately for characters such that non-mutated cells cannot be eliminated from a culture when measuring the number of mutated cells. The original ML⁺ strain has been found to give $(-/+)$ as well as $(-)$ mutants, both occurring with a frequency of about 1 in 5000–7000 when colonies of ML⁺ are grown up in broth and plated on ML agar or LZ agar. Tests are now in progress to measure the actual mutation rates from one phenotype to another, using the method of Stocker (1949).

### (iii) *Nature of Lac variant phenotypes*

Failure to give red colonies on MacConkey-lactose agar does not necessarily imply inability to utilize lactose, since a more rapid conversion of acid to acetyl-methyl-carbinol would have the same effect by preventing the accumulation of sufficient acid to affect the indicator in the medium. Assays for $\beta$-galactosidase were therefore carried out on the three phenotypes. Table 3 shows that both the original wild-type $(+)$ strain and the three $(-)$ variants tested contain a low basal level of enzyme when grown in minimal glycerol medium and the same much higher level when induced with TMG. The $(-/+)$ lines have very much lower enzyme levels in both induced and uninduced cells than the $(+)$ and $(-)$ strains, but still show clear evidence of possessing an inducible system for $\beta$-galactosidase, as seen from the induction ratio (last column in the table), which is at least as high in $(-/+)$ as in the other *Klebsiella* strains. The $(-/+)$ lines listed in the table were derived by reversion from $(-)$ strains 18, 20 and 21, and it is interesting that two lines of $(-/+)$ phenotype derived directly from the parent ML⁺ gave assay results precisely similar to those of the $(-/+)$ lines in the Table. This supports the idea that the $(-/+)$ clones of different origin are all the result of the same genetic change. Possibly this genetic change affects the metabolic state of the cells in such a way that the efficiency of the assay for $\beta$-galactosidase is reduced.

We conclude that all three phenotypes have an inducible system for $\beta$-galacto-sidase, and inability to make this enzyme is not responsible for their phenotypic

Table 3. *Levels of β-galactosidase in strains of* Klebsiella *V 9 A*

| | Enzyme units per ml of cells at $OD_{550}$ 1·0 | | |
| --- | --- | --- | --- |
| Strain | Uninduced cells (U) | Induced cells (I) | Induction ratio (I/U) |
| ML⁺ | 1·6 | 22·3 | **14** |
| ML⁻ 18 | 0·8 | 27·0 | 34 |
| ML⁻ 20 | 1·3 | 20·0 | 15 |
| ML⁻ 21 | 1·2 | 23·0 | 18 |
| ML⁻ 18(−/+) | 0·15 | 4·8 | 32 |
| ML⁻ 20(−/+) | 0·06 | 3·3 | 55 |
| ML⁻ 21(−/+) | 0·06 | 2·4 | 40 |
| *E. coli* K 12 | 0·45 | 90 | 200 |

Cells were assayed as described under Methods, when growing in minimal glycerol medium. Induced cells had been growing in the presence of $10^{-3}$ M TMG for two cell generations when assayed. Each value is the average of at least two independent assays on cell samples grown up from different colonies.
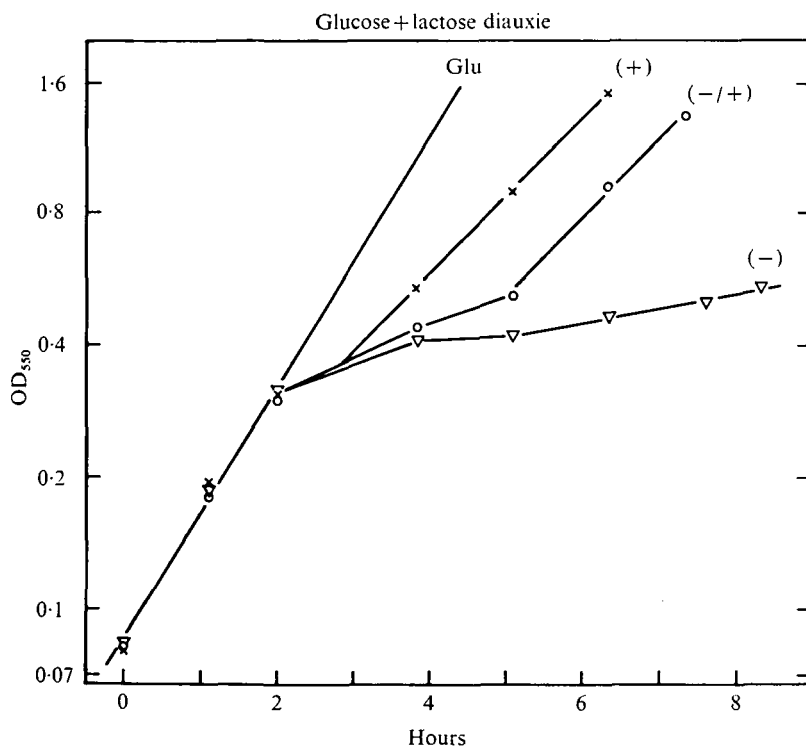


Fig. 1. Glucose–lactose diauxie test on ML⁻ 18 and a (−/+) and a (+) revertant of this strain. Cells grown in minimal medium plus glucose were resuspended in minimal medium containing 0·03 % glucose plus 0·2 % lactose, and incubated from time 0 in 100 ml flasks shaking in a water bath at 37 °C.
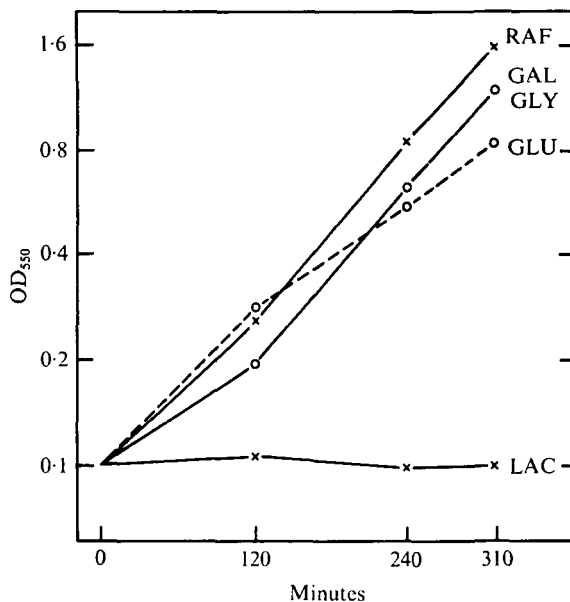
Fig. 2. Effect of transferring ML⁻ cells from glucose to other carbon sources in minimal medium. Cells grown to stationary phase in minimal glucose medium were centrifuged and resuspended in minimal medium containing galactose, glucose, glycerol, lactose or raffinose at 0·2 % and incubated as for Fig. 1.

differences. Nevertheless, a diauxic growth test in which cells grown in glucose minimal medium were transferred to minimal medium containing 0·03 % glucose plus 0·2 % lactose and then incubated at 37 °C, brings out striking differences between the three phenotypes (Fig. 1). The three strains compared are ML⁻ 18 and a (−/+) and (+) revertant of this strain. The (+) revertant behaves like the original ML⁺ strain in every respect so far tested.

The three strains clearly use up the glucose first, all growing at the same rate with a doubling time of 60 min, and then take different times to switch over to lactose. The (+) strain takes less than 1 h and the (−/+) strain over 2 h for this adjustment, but both then grow on lactose at the same rate, with a doubling time of about 100 min. (−) cells take as long as (−/+) cells to adjust to lactose, but can then only grow very slowly with a doubling time of about 560 min – a rate which is maintained at least for several hours. Control cultures in which no lactose has been added (not shown in figure) gradually decline in optical density when the glucose has been exhausted. This and similar experiments make it clear that ML⁻ strains do succeed, after a long lag, in growing slowly on lactose. One possibility, that the eventual slow growth on lactose was the result of gradual replacement of (−) cells by (+) or (−/+) revertants, was ruled out by the fact that the proportion of such revertants was less than 1 % at the end of the test.

When ML⁻ cells are grown overnight on glucose and then transferred to another carbon source, they fail to grow only on lactose. Growth is normal after
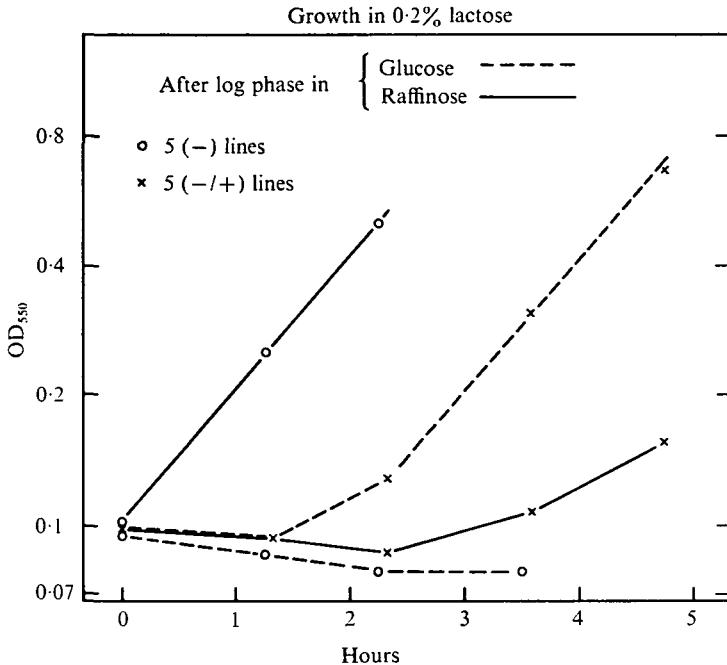
Growth in 0·2% lactose



Fig. 3. Effect of transferring ML⁻ and ML⁻/⁺ cells from minimal glucose or raffinose to lactose. Cells grown to log phase in minimal medium containing glucose or raffinose were centrifuged and resuspended in minimal medium containing 0·2% lactose. Incubation as for Fig. 1. Five ML⁻ lines (ML⁻ 1, 4, 11, 18, 20 and 21) and a (−/+) derivative of each were tested. The OD readings of each set of five lines were averaged to give the points plotted, since they all followed very similar growth trends.

a short lag on galactose, glycerol or raffinose (Fig. 2), making it difficult to accept catabolite repression as the cause of the lactose effect.

A strikingly different result is obtained when cells are transferred from raffinose to lactose, as shown in Fig. 3. For this test cells of five different ML⁻ lines and of a (−/+) revertant of each were grown to mid-log phase in both glucose and raffinose minimal medium, centrifuged and resuspended in minimal medium containing 0·2% lactose. All (−) lines showed very similar growth trends, as did all (−/+) lines, and the readings of the five lines of each type have therefore been averaged to give the points on the graph. We now see that the (−) lines, though unable to grow for 3 h after transfer from glucose, can grow rapidly on lactose without any lag after transfer from raffinose. In contrast, the (−/+) strains grow normally after a 2 h lag on transfer from glucose, but take much longer to adjust to lactose when transferred from raffinose.

This unexpected finding led us to examine the effect of transferring (−) and (−/+) strains from a variety of other sugars to lactose. Fig. 4 shows the effect of transferring ML⁻ 18 and its (−/+) derivative from stationary phase in glucose, glycerol, raffinose and sucrose to lactose. (−) cells again grew immediately on transfer from raffinose, but grew only slowly with a lag of about 4 h when trans-
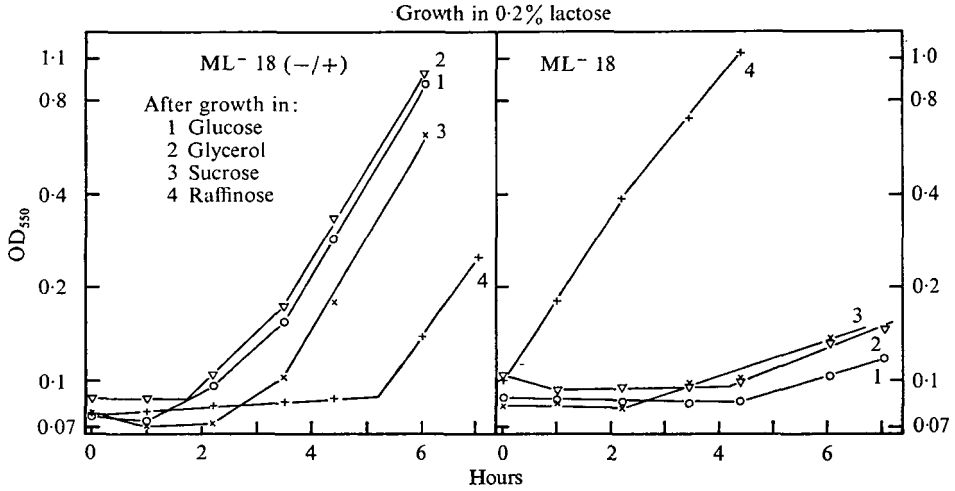
Fig. 4. Growth of ML⁻18 and a (−/+) derivative when transferred from stationary phase grown on various carbon sources to minimal lactose (0·2%). Incubation from time 0 in 500 ml flasks rotating in orbital incubator at 37 °C.
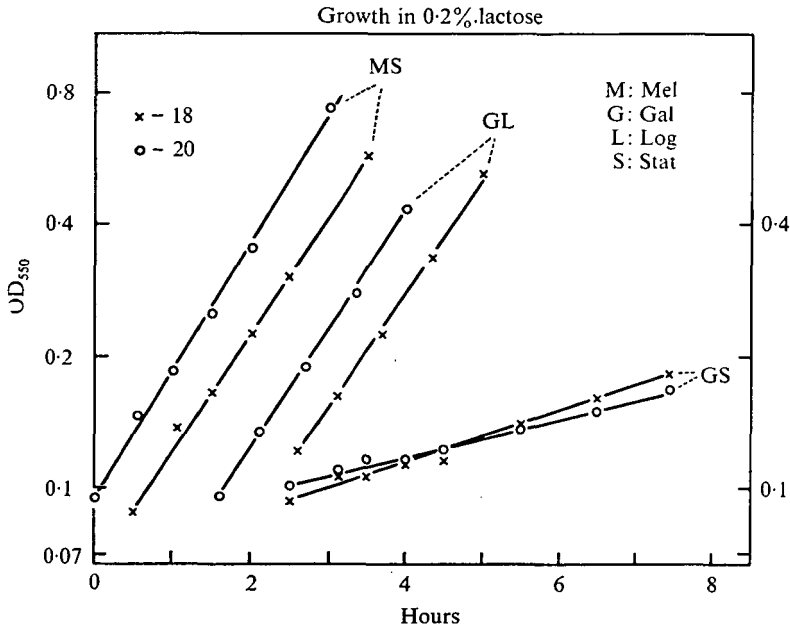


Fig. 5. Effect of transferring two ML⁻ strains, 18 and 20, from minimal melibiose or galactose to lactose. Cells grown to log or stationary phase in the first medium were centrifuged and resuspended in minimal lactose (0·2%). Incubation from time 0 in 500 ml flasks rotating in orbital incubator at 37 °C. M and G: previous growth on melibiose and galactose, respectively. L and S: transferred from log and stationary phase, respectively.
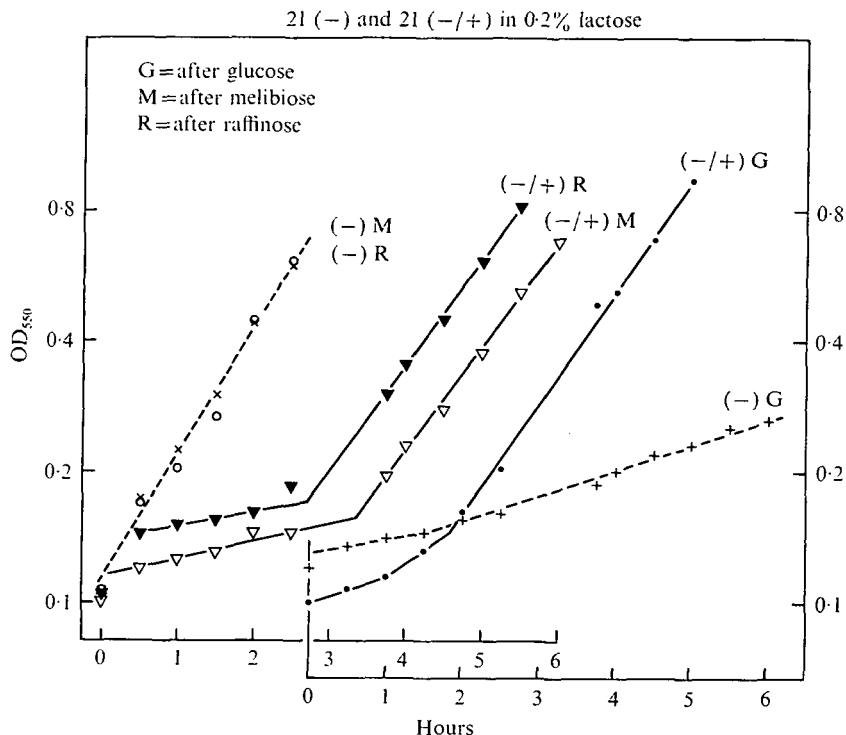
Fig. 6. Effect of transferring ML⁻ 21 and a $(-/+)$ derivative from stationary phase grown on various sugars to minimal lactose $(0.2\%)$. Methods as for Fig. 5.

ferred from glucose, glycerol or sucrose. $(-/+)$ cells grew rapidly after a lag of 2–3 h when transferred from glucose, glycerol or sucrose but needed 5 h before they could grow on lactose after transfer from raffinose. Transferring cells from log phase in these sugars to lactose has been found to give virtually the same result as transfer from stationary phase.

Fig. 5 shows the effect of growing cells of two $(-)$ strains, 18 and 20, to log phase on galactose and to stationary phase on melibiose or galactose and then transferring to lactose. This test brings out two new features: $(-)$ cells of both lines can grow rapidly without lag on lactose when transferred from stationary phase in melibiose (as in the case with raffinose), while transfer from log phase in galactose also gives rapid growth but transfer from stationary phase in galactose leads to the same lag followed by slow growth on lactose as is found on transfer from glucose.

Fig. 6 shows some results for a third $(-)$ strain, ML⁻ 21, and a $(-/+)$ line derived from it, transfer being from stationary phase on glucose, melibiose and raffinose to lactose. Again, $(-)$ cells grow rapidly without lag on transfer from melibiose or raffinose, but can only grow very slowly when transferred from glucose. $(-/+)$ cells show a shorter lag on transfer from glucose than from either melibiose or raffinose.

We thus see that several independently selected ML⁻ lines are able to grow rapidly on $0.2\%$ lactose, with little or no lag, after growth to log or stationary
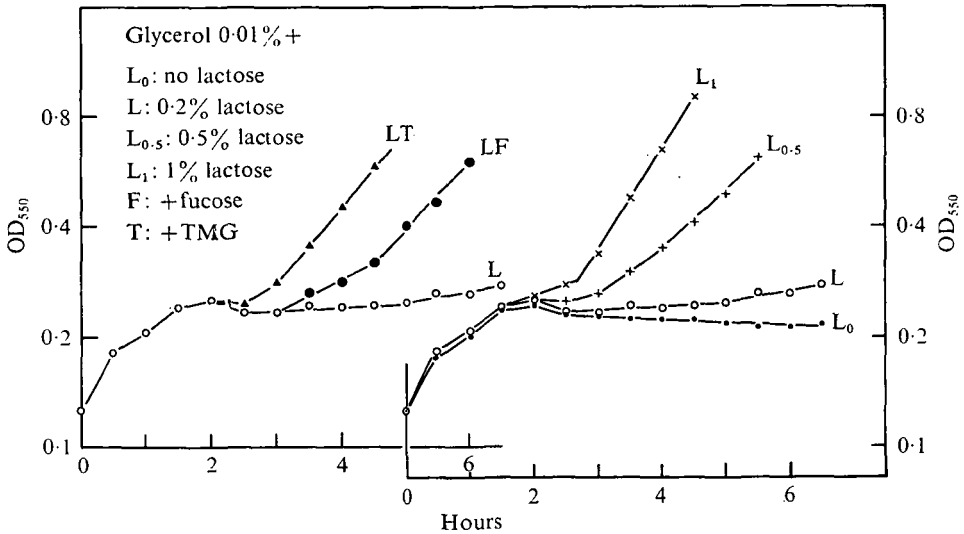
Fig. 7. Glycerol-lactose diauxie tests on ML⁻ 18. Cells grown up in minimal glycerol were centrifuged and transferred to minimal medium containing 0·01% glycerol and the following additions: $L_0$, no addition; L, 0·2% lactose; LT, 0·2% lactose + $10^{-3}$ M TMG; LF, 0·2% lactose + 0·05% D-fucose. $L_{0.5}$, 0·5% lactose; $L_1$, 1·0% lactose. Incubation from time 0 in 500 ml flasks rotating in orbital incubator at 37° C. All additions were made before incubation started.

phase on melibiose or raffinose or to log but not stationary phase on galactose, while transfer from stationary phase on galactose or from either phase on other carbon sources leads to a long lag followed by very slow growth. In the case of $(-/+)$ cells, normal growth on lactose was achieved after a substantial lag with all the carbon sources tested, but the lag was much greater on transfer from melibiose or raffinose than after growth on any other carbon source, including glucose. These results suggest that $(-)$ cells have a defect in the *lacY* permease gene which can be bypassed by previous growth on raffinose, melibiose or galactose but not by growth on other sugars. This defect has the peculiarity that it allows very slow growth on lactose. Some further change has occurred in $(-/+)$ cells which we have not yet been able to explain. This, in particular, dramatically affects their ability to grow on lactose after raffinose or melibiose.

The nature of the permease defect in $(-)$ cells is clarified by the diauxie tests shown in Fig. 7, in which cells grown on glycerol were transferred to a mixture of lactose and a small concentration of glycerol, to which other substances were added as inducers of the lactose system. The graphs on the left show that growth in 0·2% lactose alone after glycerol (line L) was very slow but either TMG or D-fucose added at the start of diauxic growth (lines LT and LF) led to rapid growth on the lactose after a short lag, once the glycerol had been exhausted. Neither TMG nor D-fucose can serve as a carbon source for *Klebsiella* V 9 A, so that both must act by inducing a permease which accumulates lactose efficiently but cannot be induced by lactose, at least at the concentration used (0·2%). The

graphs on the right show the effect of varying the lactose concentration without adding an inducer. We see first that 0·2 % lactose, by comparison with the $L_0$ culture containing no lactose, clearly does allow slow growth to occur, since the L and $L_0$ lines gradually diverge. More remarkably, the growth rate was increased by increasing the lactose concentration, since after a short lag it was very fast on 1 % lactose and intermediate in rate on 0·5 % lactose (lines $L_1$ and $L_{0·5}$).

The following two hypotheses could explain these results:

(1) The ML$^-$ mutation is in the *lacY* permease gene and results in the production of a defective lactose permease which can only accumulate lactose rapidly from a high external concentration. TMG and D-fucose induce another galactoside permease, which accumulates lactose efficiently but cannot be induced by lactose.

(2) Less plausibly, the mutation is in the *lac* repressor gene, and results in lactose becoming a very poor inducer of the lactose operon while TMG and D-fucose remain efficient inducers.

The simplest way of discriminating between these two hypotheses is to compare the behaviour of ML$^-$ cells growing fast on 1 % lactose and on TMG + 0·2 % lactose, when they are resuspended in minimal medium containing 0·2 % lactose alone. On the first hypothesis the TMG-induced cells should alone be able to grow fast on 0·2 % lactose, since no efficient accumulation system will have been induced by growth on 1 % lactose. The second hypothesis requires that both TMG and 1 % lactose are able to induce the lactose permease, so that both cultures will be able to grow fast on 0·2 % lactose.

These comparisons are made in Fig. 8. ML$^-$ 18 was first grown in glucose–lactose diauxie, as shown in the left-hand graphs. All cultures received 0·01 % glucose, which was exhausted before growth on lactose began. The four cultures *a–d* received, respectively, no lactose (*a*), 0·2 % lactose (*b*), 0·2 % lactose + 10$^{-3}$ M TMG (*c*), and 1 % lactose (*d*), and their behaviour confirms the results of the corresponding glycerol–lactose diauxie tests in Fig. 7. The two fast-growing cultures (*c*) and (*d*) were then centrifuged, washed in minimal medium without sugar, and resuspended in minimal lactose at lower OD so that their subsequent growth could be followed. Culture (*c*), growing rapidly on 0·2 % lactose + TMG, was able to grow rapidly on 0·2 % lactose alone when the TMG had been removed (culture *e*). However, culture (*d*) growing rapidly on 1 % lactose, grew slowly immediately it was transferred to 0·2 % lactose (culture *f*), though cells of (*d*) washed and resuspended in 1 % lactose were able to maintain their previous rapid growth rate (culture *g*). In fact, culture (*f*) could only grow at the same slow rate as cells growing on 0·2 % lactose after glucose (*b*). Evidently rapid growth on 1 % lactose does not induce the permease induced by TMG, ruling out the second hypothesis listed above.

Fig. 8 also shows that the effect of induction by TMG is temporary once the inducer has been removed. To test this point, cultures (*e*) and (*g*) were chilled and kept at about 4 °C overnight and reincubated next morning. After 90 min at 37 °C both cultures were diluted to low OD in the media they were growing in so that their growth rates could be measured accurately. Growth of these diluted cultures
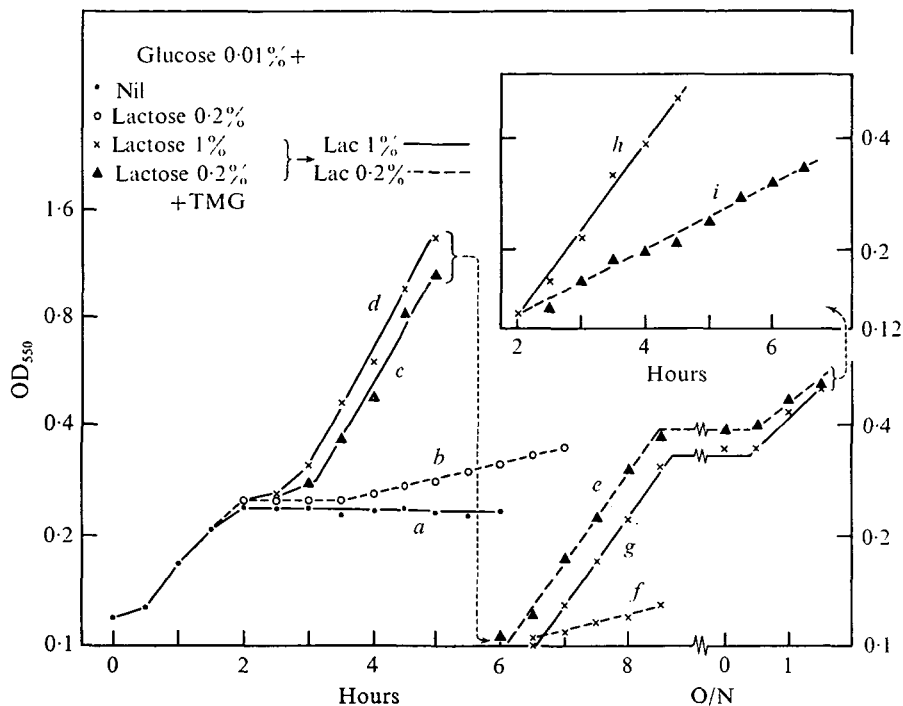
Fig. 8. Glucose–lactose diauxie test on ML⁻ 18: effect of changing lactose concentration or removing TMG. Cells grown up in minimal glucose were centrifuged and resuspended in minimal glucose (0·01 %) with the following additions, and then incubated from time 0 in 500 ml flasks rotated at 37 °C:

(a) No addition.　　　(c) 0·2 % lactose + 10⁻³ M TMG.
(b) 0·2 % lactose.　　　(d) 1·0 lactose,

At 5 h cultures (c) and (d) were centrifuged, washed once and resuspended to OD about 0·1 in minimal medium, then reincubated with carbon sources as follows:

(c) transferred to minimal lactose (0·2 %) without TMG becomes (e).
(d) part transferred to minimal lactose (0·2 %) becomes (f).
(d) part transferred to minimal lactose (1·0 %) becomes (g).

At 8½ h cultures (e) and (g) were chilled in ice and kept at about 4 °C overnight (O/N), then reincubated for 1½ h next morning. They were then diluted into medium with the same sugar concentration as before and incubation was continued (inset graphs)

(e) diluted into minimal lactose 0·2 % becomes (i).
(g) diluted into minimal lactose 1·0 % becomes (h).

is shown in the inset graph of Fig. 8. Culture (g) becomes (h), and shows that the strain continues indefinitely to grow rapidly on 1 % lactose. Culture (e) becomes (i), growing at a much reduced rate. Following the steps from (c) to (e) to (i), it is clear that cells induced by TMG (which is then removed) grow rapidly on 0·2 % lactose alone for about two cell generations (OD increase from 0·1 to 0·4), after which the growth rate falls to less than half its previous value. This may be assumed to be the result of the dilution out of the permease induced by the TMG.

Perhaps the most striking characteristic of ML⁻ 18 is its ability to grow only slowly on 0·2 % lactose and very rapidly on 1 % of the same sugar, and this pro-
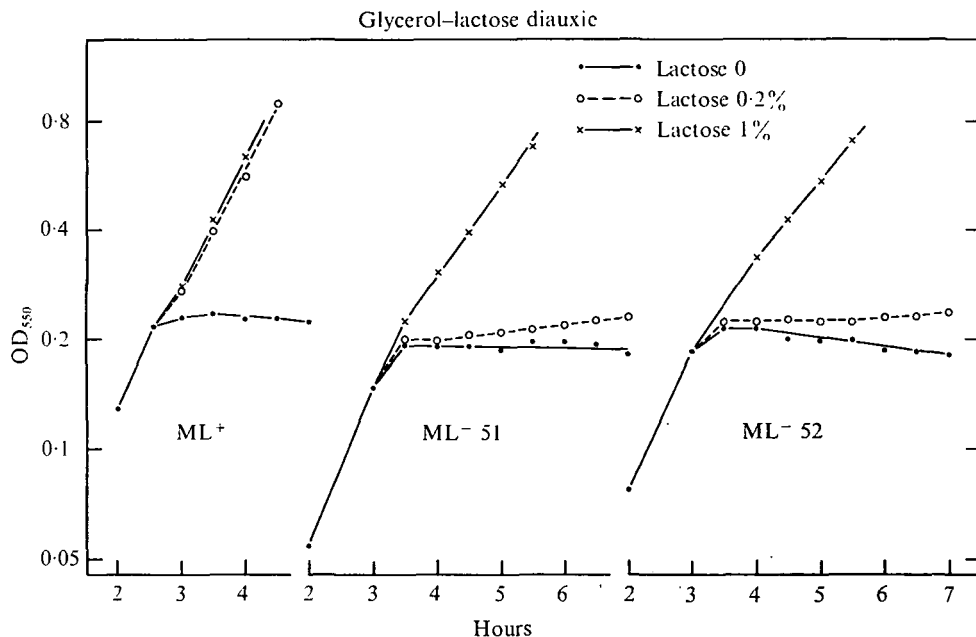
Fig. 9. Glycerol–lactose diauxie test on ML⁺ and two ML⁻ strains: effect of different lactose concentrations. Methods as for Fig. 7. Incubation was from time 0.

vides a very convincing test as to whether other ($-$) mutants have arisen by the same genetic change. Fig. 9 shows glycerol–lactose diauxie tests on strains ML⁺, ML⁻ 51 and ML⁻ 52, in which cells grown up in minimal glycerol medium were washed and transferred to minimal medium containing 0·01 % glycerol and lactose at either 0, 0·2 or 1 %. The original wild-type strain, ML⁺, grew almost as rapidly on 0·2 % as on 1 % lactose, but both ($-$) strains grew fast on 1 % but very slowly on 0·2 % lactose, giving precisely the same growth pattern as ML⁻ 18. The two ($-$) lines, ML⁻ 51 and ML⁻ 52, are recent ($-$) isolates from ML⁺. Exactly the same result has been obtained with ML⁻ 1, 4, 11, 20 and 21. Hence all eight ML⁻ lines tested show this same growth reaction to different concentrations of lactose. These lines were all derived directly as mutants of ML⁺, and we have not yet tested any ($-$) lines obtained from ($+$) or ($-/+$) revertants of primary ($-$) lines.

A striking characteristic of ($-/+$) lines is the increased lag before they can grow on lactose engendered by previous growth on raffinose. ($-/+$) revertants from six ML⁻ lines have been tested and all show this character, and in addition two ($-/+$) lines obtained directly from plating ML⁺ cells have now been tested and show the same behaviour. This, coupled with the similarities of phenotype and of enzyme induction pattern, strongly suggest that ($-/+$) strains derived from ($-$) and from ($+$) cells result from the same genetic change.

## 4. DISCUSSION

We have shown that *Klebsiella* strain V 9 A, the subject of this study, regularly produces lactose-defective mutants of a particular type, the ML⁻ mutants, and also mutants of an intermediate type (−/+) with reduced efficiency in adjusting to lactose utilization. Originally, (−) mutants were obtained from the parent (+) strain and gave rise to both (−/+) and (+) revertants, but (+) and (−/+) cell lines each appear to be able to mutate to both the other types.

The two mutant types are each readily identifiable by their appearance on MacConkey lactose agar and by their behaviour when transferred to liquid lactose minimal medium after growth on other carbon sources. Thus (−/+) cells take longer than (+) to adapt to lactose, with a particularly long lag when transferred from melibiose or raffinose, but they eventually grow normally on lactose 0·2 %. (−) cells can only grow very slowly on 0·2 % lactose unless previously grown on melibiose or raffinose or growing in log phase on galactose, or unless induced with TMG or D-fucose – all these treatments enable the cells to grow as rapidly on 0·2 % lactose as on other carbon sources. This ability to utilize lactose efficiently is then lost after a few cell generations. (−) cells can also grow rapidly without previous induction on 1 % lactose, but this does not enable them to grow rapidly on 0·2 % lactose.

Clearly the (−) mutant lines have a defect in the lactose permease with the unusual property that lactose can be taken up slowly from an external concentration of 0·2 %, and at an increasing rate as the external concentration is increased: 1 % concentration appears sufficient to allow maximum growth rate on lactose. Judged by enzyme assays, these (−) mutants possess a normal inducible β-galactosidase.

The fact that (−) mutants can grow rapidly in 0·2 % lactose after induction by TMG or D-fucose, or previous growth in melibiose, raffinose or galactose, means that at least one additional galactoside permease exists which can be induced by these compounds but not by lactose itself, and can then accumulate lactose efficiently. We have evidence that more than one such permease exists in V 9 A, but the problem is still under study.

It will be noted that the inducibility pattern and substrate specificities of the galactoside permeases in *Klebsiella* are different from those in *E. coli*. Thus Rotman, Ganesan & Guzman (1968) found evidence for four such permeases in *E. coli* K 12, of which only one, the lactose permease (TMG–1) can accumulate lactose. Moreover, melibiose induces both TMG–I and TMG–II in K 12, but we have found that melibiose and raffinose do not induce the lactose operon in V 9 A. More detailed studies on these permeases will be published separately.

In attempting to explain the origin of the two mutant phenotypes, (−) and (−/+), we must take into account the fact that *Klebsiella* strain V 9 A carries a Lac⁺ plasmid, F$_K$lac (Reeve & Braithwaite, 1970), which can be transferred to K 12 and is there found to contain an inducible *lac* operon, enabling the recipient cells (in which the complete *lac* operon is deleted) to grow normally on lactose. Mating tests (unpublished) indicate that ML⁻ mutants of V 9 A either fail to trans-

fer the Lac$^+$ plasmid to K 12, or transfer it at a very low frequency which could be the result of reversion of a small proportion of the ($-$) donors to ($+$) during the period of the mating. This suggests that the reversible mutation responsible for the change to the ML$^-$ phenotype occurs in the *lacY* (permease) gene of the plasmid, F$_K$*lac*.

Several possibilities then arise. If the *Klebsiella* chromosome also possesses an active *lacY* gene, then we must suppose that the products of the two $Y$ genes in the cell, one normal and one mutant, combine to form a modified M-protein (Fox, Carter & Kennedy, 1967) with the modified permease properties of ($-$) cells. This would require that the M-protein is made up of two or more subunits. Alternatively, the ($-$) mutation may completely (but reversibly) inactivate the plasmid $Y$ gene, the properties of the ($-$) cells depending on a defective chromosomal $Y$ gene. Another hypothesis could be based on the assumption that part of the F$_K$*lac* plasmid is lost in ($-$) cells and can then be recreated by duplication of chromosomal genes, the extra copy being transferred by recombination to the plasmid – as happens with the penicillinase genes and the $\pi$ plasmid in *Staphylococcus aureus* strain PS 80 (Asheshov, 1969). On this hypothesis a further assumption is needed to explain the peculiar nature of the lactose permease system in ($-$) cells. We have not yet succeeded in curing the host strain of its F$_K$*lac* plasmid, or in getting completely lactose-permease negative mutants, or in developing a transfer system for chromosomal genes in this strain; but it is hoped that work now in progress will lead to a much more specific explanation of a curious phenomenon.

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