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The lactose system in *Klebsiella aerogenes* V9A

6. Lactose transport

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SUMMARY

Klebsiella aerogenes V9A carrying a *lac* plasmid in addition to its chromosomal operon showed strongly positive fermentation of lactose on MacConkey lactose agar plates, and was found to transport the lactose analogue thiomethyl-β-galactoside (TMG) at a rapid rate. The strain that had been freed of the plasmid showed moderate transport due to the chromosomal *lac* operon. When a plasmid bearing a mutation in *lacY* was inserted into a strain with a normal *Y* gene, the resulting diploid became lactose-negative in phenotype. The presence of *E. coli* F′*lac* factors that carried *lacY* mutations, whether deletions or missense or nonsense mutations, also rendered *lacY*$^+$ *Klebsiella* lactose-negative. Such diploids, after growth in 1% lactose, transported TMG at a much lower rate than the corresponding plasmid-free *lacY*$^+$ *Klebsiella*. However, this interference by *lacY*$^-$ plasmids with the expression of the chromosomal *lacY* gene was not seen when cells were induced with IPTG or when the chromosomal and plasmid *lac* operons were both constitutive. It was found that this effect of the plasmids was dependent on their possessing an intact *lacZ* gene.

1. INTRODUCTION

A number of *Klebsiella* strains possess a chromosomal *lac* operon of low specific activity and, in addition, a plasmid *lac* operon of much greater activity (Reeve & Braithwaite, 1970; Reeve, 1970; Brenchley & Magasanik, 1972; Reeve & Braithwaite, 1973*b*). Studies with *Klebsiella* V9A have indicated that, when both operons are present, there is efficient fermentation of lactose, as shown by a strongly positive phenotype (ML$^+$) on MacConkey lactose agar (ML). Loss of the plasmid results in a weakly positive phenotype (ML$^{-/+}$) which can be induced to give only 10% as much β-galactosidase as ML$^+$ cells. When ML$^+$ cells are spread on ML plates spontaneous mutants arise which fail to ferment lactose and are designated ML$^-$. These cells have been shown to possess a plasmid carrying a normal *Z* gene but with a *Y* gene mutation (Reeve, 1976). Although the host cell retains a normal

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chromosomal operon, lactose fermentation is inhibited when the mutant plasmid is present.

The present study is a further exploration of this phenomenon in *Klebsiella*. The insertion of $lacZ^{+}Y^{-}$ genes via the *Klebsiella* plasmid or an *E. coli* F-factor into an episome-free *Klebsiella* ($ML^{-/+}$) uniformly results in the inhibition of expression of the chromosomal *Y* gene. Several hypotheses that might account for this phenomenon have been tested, and it appears that the presence of a *Z* gene on the episome is the necessary factor involved.

2. MATERIALS AND METHODS

(i) *Bacteria and plasmids*

Table 1 lists the K12 and the *Klebsiella* strains, and Table 2 lists the plasmids used.

Reeve & Braithwaite (1973*a*) deduced that *Klebsiella* V9A possesses an extra galactoside permease, GPIII, which can accumulate lactose and melibiose after induction by IPTG or TMG. However, studies on the lactose transport system of 1740 have failed to confirm the existence of this permease, and the 'GPIII-minus' mutants described by Reeve & Braithwaite appear to be the result of loss of the chromosomal *lacY* gene. We therefore assume that the transport systems examined in this paper depend entirely on the lactose operon(s) present in the cell.

(ii) *Media*

For most transport experiments cells were grown in medium 9 (Miller, 1972), containing 1 % lactose (ML^{-} strains would grow well in 1 % lactose but only very slowly in 0·2 %); in a few experiments citrate 0·4 % was the carbon source. Thymine was added when appropriate.

Broth was made as described by Reeve & Braithwaite (1972). ML agar was BBL MacConkey agar (Becton, Dickinson & Co.). MTS was ML agar supplemented with tetracycline at 20 μg/ml and streptomycin at 1000 μg/ml. The K12 deletion mutants were grown on the minimal agar of Miller (1972, p. 432) except that trisodium citrate was included at 1 g/l. For recombination tests, thiamine, proline and tryptophan were added, and lactose was used as carbon source. Antibiotics, where required, were added at 20 μg/ml except for streptomycin (at 1000 μg/ml).

(iii) *Construction of conjugative plasmids carrying the V9A Lac plasmid and a resistance determinant from R1 as a marker*

The Lac plasmid of V9A is non-conjugative, and to create a suitably marked conjugative plasmid carrying the *lac* operon (either from the wild-type, $F_{K}lac$, or from the ML^{-} mutant pRE3), we used the fact that the Lac plasmid of V9A is compatible with FR5 in V9A but not in *E. coli*. V9A carrying $F_{K}lac$ or pRE3 was infected with FR5, and then mated to a Lac^{-} strain of K12, using resistance to streptomycin or nalidixic acid to counterselect against the donor. Matings were made overnight at 37 °C on broth agar, and aliquots were then spread on ML agar

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containing chloramphenicol 20 μg/ml together with streptomycin 1 mg/ml or nalidixic acid 20 μg/ml according to the recipient strain used (RE254 when the donor carried F_K*lac*, RE299 for pRE3). All recipient clones had received FR5, and

Table 1. *Bacterial strains*

Klebsiella V9A strains	Relevant characters	Origin or reference
RE1403	V9A (F_K*lac*, T_K)	Reeve (1976)
RE1427	V9A (pRE3)	ML⁻18 (Reeve, 1976)
RE1469	V9A *his* (T_K)	RE1403 cured of F_K*lac*
RE1740	V9A (T_K)	Spontaneous His$^+$ revertant of RE1469
RE1746	V9A *thy* (pRE12, T_K)	ML⁻20 (Reeve, 1976)
RE1838	RE1740 *proAB*	This paper
RE1849	V9A *thy* (pRE10, T_K)	ML⁻4 (Reeve & Braithwaite, 1972)
RE1850	V9A *thy* (pRE11, T_K)	ML⁻11 (Reeve & Braithwaite, 1972)
RE1851	V9A *thy* (pRE13, T_K)	ML⁻21 (Reeve, 1976)
RE1852	RE1838 partially *lac* constitutive	This paper
E. coli K12 strains		
A324.5	$lacI^-Z^+Y^+/F'lacI^-Z^+Y^+$	E. P. Kennedy
CSH18	Δ $lac/F'lacI^-Z^-Y^+proAB^+$	Cold Spring Harbor strains (Miller, 1972)
CSH20	Δ $lac/F'lacI^-Z^-Y^+proAB^+$	Cold Spring Harbor strains (Miller, 1972)
CSH21	Δ $lac/F'lacI^+Z^-Y^+proAB^+$	Cold Spring Harbor strains (Miller, 1972)
CSH22	Δ $lac/F'lacI^+Z^-Y^+proAB^+$	Cold Spring Harbor strains (Miller, 1972)
DP90CNal	Δ (*lac-pro*) *thi nalA*	Hobson *et al.* (1977)
E5014.1	Δ (*lac proAB*)/$F'lac^+proAB^+$	R. Sanders from J. Beckwith
E7150	Δ $lac/F'I^+Z^+Y^{am}proAB^+$	J. Beckwith
RE254	$F^-\Delta$ (*lacZ*) *trp his strA nalA*	Reeve (1976)
RE299	$F^-lacZ^-Y^+$ *metB lamB nalA*	Reeve (1976)
RE307	$F^-lacZ^-Y^+$ *gal pyrD strA*	Reeve (1976)
RV/316	Δ $lac/F'I^-Z^+Y^-A^-proAB^+$	This paper
RV/MS1019	Δ $lac/F'I^+Z^+Y^{del}proAB^+$	Constructed from Y^{del} strain of Malamy (1966)
RV/MS1038	Δ $lac/F'I^+Z^+Y^{del}proAB^+$	Constructed from Y^{del} strain of Malamy (1966)
RV/MS1054	Δ $lac/F'I^+Z^+Y^{del}proAB^+$	Constructed from Y^{del} strain of Malamy (1966)
W4680	Δ $lac/F'I^+Z^{del}Y^+$	J. Beckwith
5	Δ $lac/F'lacI^-Z^+Y^-proAB^+$	This paper
15-1-C	Δ $lac/F'lacI^-Z^+Y^-proAB^+$	This paper
30-9-2	Δ $lac/F'lacI^-Z^-Y^+proAB^+$	This paper

a small proportion were Lac$^+$, showing that they had also received the Lac plasmid. Several such clones were purified, and proved to carry F′*gal* and the A, C, S, Su, markers of FR5 and also the complete *lac* operon and the *fi*$^+$ gene of the V9A plasmid. These genes all behaved as if in a single plasmid on transfer, but

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the two original plasmids would separate, with loss of one of them, at an appreciable frequency. The combination FR5-F_K*lac* is pRE7 referred to in Table 3.

To obtain a more stable combination, these plasmids were transferred back and forth, by conjugation, between two different *lacZ*$^-$*Y*$^+$ K12 strains, selecting for chloramphenicol-resistance and Lac$^+$, and very stable plasmids were finally obtained in which the *gal* operon of FR5 and the *fi*$^+$ gene of the Lac plasmid had been lost. These plasmids, pRE9 from pRE7 and pRE8 from the FR5-pRE3

Table 2. *Plasmids*

Name	Relevant genes	Whether conjugative	Origin or reference
pRE1	T_K = Tet plasmid in V9A (RE1401)	No	1
pRE2	F_K*lac* = Lac plasmid in V9A (RE1401)	No	2, 4
pRE3	Lac plasmid in V9A:ML$^-$18 (RE1427)	No	3
FR5	F*gal* linked to A, C, S, Su determinants	Yes	1
pRE7	pRE2 joined to FR5	Yes	This paper
pRE8	pRE3-FR5 complex: *Gal*, A, S, Su lost	Yes	This paper
pRE9	pRE2-FR5 complex: *Gal* lost	Yes	This paper
pRE10	Lac plasmid in V9A:ML$^-$4 (RE1849)	No	3
pRE11	Lac plasmid in V9A:ML$^-$11 (RE1850)	No	3
pRE12	Lac plasmid in V9A:ML$^-$20 (RE1746)	No	3, 5
pRE13	Lac plasmid in V9A:ML$^-$21 (RE1851)	No	3, 5
pRE17	pRE10-FR5 complex	Yes	This paper

(1) Reeve (1970); (2) Reeve & Braithwaite (1970); (3) Reeve & Braithwaite (1972); (4) Reeve & Braithwaite (1974); (5) Reeve (1976).

hybrid, carried the *lac* genes of the *Klebsiella* plasmid, F and resistance genes from FR5, and promoted efficient transfer and sensitivity to F-specific phage. pRE8 retained only the C resistance determinant while pRE9 carried A, C, S and Su.

The same method was used to make a composite conjugative plasmid out of pRE10 and FR5, but only a rather unstable hybrid plasmid, pRE17, which probably carried all the genes of both pRE10 and FR5, was obtained. When transferred by conjugation with selection for chloramphenicol resistance, 25% of the recipients received the whole pRE17 plasmid, the rest not inheriting the *lac* genes; but this was sufficient to enable us to test the ability of pRE10 to give *lacY*$^+$ recombinants with the *Y*$^-$ missense mutants MAA6, MAB6 and MAA38. For this test, DP90CNal carrying each F′*proAB*$^+$*lacY*$^-$ missense mutant was grown 2 days in L-Broth to obtain F$^-$ phenocopy cells, and was then mated with log phase cells of RE307/pRE17 for 3 h on L-Broth agar containing chloramphenicol 20 μg/ml. The surface growth was then washed off in buffer and plated on M9 glucose plates containing thiamine, chloramphenicol and nalidixic acid. Only recombinants between F′ *pro lac* and pRE17 retaining pro$^+$ and chloramphenicol

Abbreviations: IPTG, isopropyl-β-D-thiogalactoside; TMG, thiomethyl-β-D-galactoside; ONPG, *o*-nitrophenyl-β-D-galactoside; A, C, S, Su, T, plasmid determinants for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracyclines; XG, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

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resistance, respectively, from the two plasmids, in DP90CNal could grow on these plates. Colonies were purified on ML agar containing chloramphenicol and nalidixic acid and scored for Lac$^+$ recombinants.

(iv) *Transfer of Lac plasmids from ML^- strains into K12 tester strains where their presence could not be directly recognized*

The *lac* plasmids in five ML$^-$ strains have been tested: those in RE1427, RE1849, RE1850, RE1746 and RE1851. The presence of each of these plasmids gives a Lac$^+$ phenotype in a $lacZ^-Y^+$ but not in a $lacZ^+Y^-$ strain of K12. Rather than go through the long process of making a conjugative plasmid like pRE8 from each, we made use of a curious mating result. When V9A carrying its two plasmids, $F_K lac$ and T_K, and also FR5, is mated to *E. coli* and selection is made for transfer of T_K, nearly all recipients of T_K also receive the Lac plasmid but not FR5. To test for recombination between a Y^- plasmid and a K12 tester strain carrying a partial deletion of *lacY*, we mate the ML$^-$ strain carrying T_K and FR5 to the K12 strain, select about 20 clones on MTS agar, and look for subsequent Lac$^+$ recombinants when these clones, after 2–3 days at 37 °C, are streaked on the same medium or on minimal lactose–citrate agar. The Lac plasmid is of course cryptic in the K12 tester strain, but when the latter carries a deletion running from the left and thus deleting *lacZ*, assay for β-galactosidase in clones grown in minimal glycerol medium + IPTG makes it clear which clones contain the plasmid (since this carries an active *Z* gene). These clones were always in the majority.

(v) *Mapping the plasmid* lacY$^-$ *mutations*

Hobson, Gho & Müller-Hill (1977) have created a series of *E. coli* K12 strains with deletions running from either end of the *lac* operon into the *Y* gene, which divide this gene into 36 consecutive segments. These deletions make it possible to map the approximation position in the gene of Y^- mutations in any plasmid which can be transferred into the K12 strains, provided that the two *lac* operons can recombine. The Lac plasmid of V9A can recombine with the K12 *lac* operon (Reeve, unpublished), and the mutations responsible for the ML$^-$ phenotypes were mapped to their approximate positions.

Table 4 gives the results of the deletion mapping. pRE8 received a more detailed analysis than the other plasmids, since it is conjugative and marked by the C determinant.

(vi) *Construction of* Z$^-$Y$^-$ *F-factor*

A Y^- mutation was inserted into W4680 (Δ *lac*/F′ *lac* $I^+Z^{del}Y^+$) via P_1 transducing phage carrying the *lac* operon with a chloramphenicol resistance gene inserted into the *Y* gene. A P_1 lysate carrying an insertion of the chloramphenicol resistance translocon, Tn9, in the *lacY* gene obtained from strain MGB75 (MacHattie & Shapiro) was very kindly provided by Howard Shuman. Following the incubation of the P_1 lysate carrying chloramphenicol-resistance Y^- with W4680, cells were spread on plates containing glucose, XG, B_1, and chloramphenicol. White clones (Δ *lac*/F′ *lac* $I^+Z^{del}Y^-$::Tn9) and blue clones (Δ *lac*/F′ *lac* $I^+Z^+Y^-$::TN9) were picked and purified. The identity was confirmed by determination of transport

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pure β-galactosidase. These F-factors were then transferred into *Klebsiella* RE1838 selecting for chloramphenicol resistance on citrate plates (*Klebsiella* grows on citrate while *E. coli* does not). The same procedure was carried out with two additional strains, CSH21 and CSH22.

(vii) *Isolation of* proA$^-$ *mutant of RE1740*

Mutagenized cells of RE1740 were washed and grown in minimal medium plus radioactive glycerol (0·5 mg/ml, 1 mCi/ml). After three doublings cells were placed in the refrigerator for 3 days. Cells which possessed no amino acid requirements grew and incorporated radioactive products of [^{14}C]glycerol into cell constituents. 95 % killing was observed after 3 days at 4 °C. The survivors were grown overnight in minimal medium plus glycerol and proline. About 200 cells per plate were then spread on agar containing minimal medium, glycerol and proline. Two days later replicates were made onto similar agar plates with and without proline. Several proline-negative strains were isolated. Identification of the particular proline gene defect was accomplished by mating to cells containing F'*proC*$^+$ or F'*proAB*$^+$. Growth of the resultant strain in minimal medium without proline indicated which proline gene had been affected. It is not known whether *Klebsiella* has separate *proA* and *proB* genes.

(viii) *Transport assay*

Transport was determined by the accumulation of radioactive TMG, a non-metabolizable analogue of lactose. After 3 divisions growing in medium 9 on lactose or citrate, cells were washed twice with medium 9 containing chloramphenicol (50 μg/ml) and dithiothreitol (1 mM). The cells were resuspended in the same medium to a concentration of 0·82 mg wet wt./ml [100 units with a Klett–Summerson colorimeter (No. 42 filter)], and the reaction was started by the addition of [^{14}C]TMG to give a final concentration of 0·1 mM (0·25 μC/ml). After incubation at 25 °C for various time intervals 0·2 ml samples of the cell suspension were placed on pre-wet millipore filters (0·65 pore size), filtered and washed once with 5 ml of medium 9. The filter was placed in a counting vial with 15 ml Bray's solution (Bray, 1960) and counted in a liquid scintillation counter. The data are expressed as the ratio of the intracellular concentration of TMG divided by the external concentration.

The intracellular water was determined by exposing cells to tritiated water, which rapidly penetrates the cell water space, and [^{14}C]inulin which is excluded from the cell and is used as a measure of extracellular water trapped in the pellet. After incubation with the tritiated water for 10 min at room temperature the [^{14}C]inulin was added. Following a further 20 s incubation 1 ml of cells (6 mg wet wt.) was centrifuged for 4 min at 10000 *g* through 0·5 ml silicone oil in a 1·5 ml Microfuge tube (Beckman Instruments Co.), by a procedure described previously (Flagg & Wilson, 1977). The silicone fluid consisted of 60 % Corning 550 and 40 % Corning 510 (50 cs. viscosity). The intracellular water was found to be 0·58 μl/ml cells OD 100 Klett units (i.e. 71 % of the wet wt. of the cells).

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(ix) *β-Galactosidase assay*

The rate of *o*-nitrophenol production from *o*-nitrophenyl-*β*-galactoside (ONPG) was used as the assay for *β*-galactosidase. To 1 ml of cells was added 0·05 ml of toluene and 0·05 ml of 0·1 % sodium deoxycholate. Cells were vigorously vortexed for 30 s and allowed to stand at 25 °C for 30–60 min. Various dilutions were exposed to 5 mM-ONPG (final volume of 2 ml) at 25 °C and the reaction stopped by the addition of 6 ml of 0·6 M-Na_2CO_3. The yellow colour was read in a Klett colorimeter with a purple filter (No. 42). Units of *β*-galactosidase are given as *μ*mol *o*-nitrophenol produced/min/g wet wt. of cells (assuming 1 ml cells of optical density 100 contains 0·82 mg wet wt.).

(x) *Chemicals*

o-Nitrophenyl-*β*-D-galactopyranoside (ONPG), and isopropyl-*β*-D-thiogalactopyranoside (IPTG) were from Schwarz–Mann. [^{14}C]Thiomethyl-*β*-galactoside was obtained from New England Nuclear. *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine was from Aldrich. 5-Bromo-4-chloro-3-indolyl-*β*-D-galactoside (XG) was obtained from Cyclo Chemical Co.

3. RESULTS

(i) *Effect of* Z^+Y^- *plasmid on chromosomal* Y *gene expression*

In the first series of experiments a comparison was made of the TMG transport in three different cell types: (1) the wild-type *Klebsiella*, RE1403, which possesses both chromosomal and plasmid *lac* operons; (2) RE1740, a strain cured of its plasmid and containing only a chromosomal *lac* operon; and (3) a series of 5 mutants (derived from RE1403) with a defect in the *Y* gene of the plasmid but retaining a normal chromosomal *Y* gene. The first of these (RE1403) ferments lactose rapidly and gives bright red colonies (ML^+) on MacConkey lactose agar. The cell with only a chromosomal *lac* operon gives colonies with red centres, while the remaining five give white colonies. The latter (ML^-) cells grow poorly in 0·2 % lactose liquid minimal medium, but grow as rapidly as the parent strain when the concentration of lactose is increased to 1 %, the concentration used in the following experiments.

Table 3 shows that the TMG accumulation ratio in 20 s is 16·9 in the ML^+ cell, 5·4 in the $ML^{-/+}$ and 1·9–3·0 in the ML^- strains. Thus the values for transport of this non-metabolizable lactose analog follow the same pattern as the fermentation data obtained on indicator plates.

To confirm the fact that the effect on transport was indeed due to the presence of the plasmid in an otherwise normal $ML^{-/+}$ *Klebsiella*, similar strains were constructed by starting with RE1740, the plasmid-free strain, and inserting into it either the conjugable plasmid containing the normal *Y* gene (pRE7) or the plasmid bearing one of the mutant *Y* genes (pRE8). These reconstituted strains were tested in the same manner as before, and the same three lactose fermentation phenotypes were observed. RE1740/pRE7 was ML^+, RE1740 was $ML^{-/+}$ and RE1740/pRE8 was ML^-. Table 3 shows that the cell possessing a normal *Y* gene on the plasmid

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accumulated TMG 16·3-fold in 20 s, strain RE1740 accumulated 5·4-fold while the cell with a defective *Y* gene on the plasmid accumulated 1·7-fold.

The location of the defect in the plasmid *Y* gene of the five ML⁻ strains was mapped and the data are given in Table 4. Mutations in several different regions of the *Y* gene on the plasmid result in the ML⁻ phenotype.

Table 3. Klebsiella *containing Lac plasmid**

Strain	Genotype: Chromosome	Genotype: Episome	Lactose phenotype	β-Galactosidase†	TMG accumulation ratio at 20 s‡ (conc. in/conc. out)
RE1740	$I^+Z^+Y^+$	None	$ML^{-/+}$	42	5·4
RE1403	$I^+Z^+Y^+$	$F_KI^+Z^+Y^+$	ML^+	132	16·9
RE1427	$I^+Z^+Y^+$	$F_KI^+Z^+Y^-$	ML^-	248	1·9
RE1746	$I^+Z^+Y^+$	$F_KI^+Z^+Y^-$	ML^-	374	2·9
RE1849	$I^+Z^+Y^+$	$F_KI^+Z^+Y^-$	ML^-	381	3·0
RE1850	$I^+Z^+Y^+$	$F_KI^+Z^+Y^-$	ML^-	261	2·9
RE1851	$I^+Z^+Y^+$	$F_KI^+Z^+Y^-$	ML^-	375	2·6
RE1740/pRE7	$I^+Z^+Y^+$	$F_KI^+Z^+Y^+$	ML^+	128	16·3
RE1740/pRE8	$I^+Z^+Y^+$	$F_KI^+Z^+Y^-$	ML^-	277	1·7

* All cells were grown in medium 9 plus 1 % lactose.
† μmol *o*-nitrophenol produced/min/g wet weight cells. Mean value for three experiments.
‡ Mean values for at least three determinations.

(ii) *Effect of inserting an* E. coli *F′*lacZ⁺Y⁻ *on* Klebsiella *chromosomal* Y *gene expression*

In order to study this phenomenon in more detail a series of *E. coli* F′*lac* episomes bearing *Y* gene mutations was inserted into *Klebsiella* RE1838. The transfer was accomplished by the introduction of F′*lac*⁺*proAB*⁺ into this *proA*⁻ mutant of RE1740. The first series of *Y*⁻ mutants was isolated from an inducible F′*lac*⁺-*proAB*⁺ in *E. coli* (strain E5014.1), and the F-factors carrying these mutations were labelled the F′T series. When an F′*lac* containing a *Y*⁻ mutation was inserted into RE1838, no lactose fermentation was observed on MacConkey plates (ML⁻). Table 5 shows that *Y*⁻ *E. coli* factors in *Klebsiella* greatly decreased the TMG accumulation compared with RE1838. The presence of an *E. coli lac* operon in *Klebsiella* (RE1838/E5014.1) resulted in elevated β-galactosidase which is consistent with the ML⁺ phenotype. However, the low level of transport is not yet understood. A second series of missence *Y*⁻ mutants kindly provided by Hobson, Gho & Müller-Hill (1977), was inserted into RE1838. In every case the diploid cell showed an ML⁻ phenotype and the TMG transport was reduced compared with RE1838 (Table 5). These missense mutations were located in widely separated regions of the *Y* gene.

At this stage of the investigation a possible explanation for these results was the process of negative complementation. This hypothesis implies that an abnormal *Y* gene product of the episome combines with the normal *Y* gene product of the chromosome to give a defective hybrid dimeric or polymeric membrane transport system. However, the subsequent finding that both early nonsense mutations

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(E7150) and deletion mutants (MS1054) caused the same effect (Table 5) led us to discard this hypothesis as little or no defective gene product would be expected to accumulate in these cases.

Table 4. *Mapping of plasmid* lacY *mutations by recombination tests with K12* lacY *deletion strains*

K12 tester strain*		Plasmid (origin), recombination results and deduced map position of *Y* lesion				
Ref.	Y segments deleted	pRE8 (RE 1427)	pRE10 (RE 1849)	pRE11 (RE1850)	pRE12 (RE1746)	pRE13 (RE1851)
63b	1	+	+	+	+	+
118e	1, 2	+	−	+	+	+
21*a*	1–3	+	−	−	+	+
123b	1–4	+	−	−	−	+
116a	1–10	+	−	−	−	+
175a	1–12	+	−	−	−	−
189a	1–13	−	NT	NT	NT	NT
66X	12–36	−	−	+	+	−
75X	17–36	+	+	+	+	+
42X	21–36	+	+	+	+	+
Location in segment(s)†		13	2, 12	3	4	12

The plasmids were transferred to each K12 strain and tested as described under Methods. +, Many Lac$^+$ recombinants; −, no Lac$^+$ recombinants; NT, combination not tested.

* These strains are described by Hobson *et al.* (1977), and divide the *Y* gene into 36 segments, numbered consecutively from left to right.

† Assuming the plasmids contain only *Y* point mutations. The lesions could be large deletions (e.g. extending over segments 3–11 in pRE11 and 4–11 in pRE12), but pRE13 must carry a point mutation, since the plasmid itself reverts to Y^+. Mapping with missense mutations from the Hobson, Gho & Müller-Hill collection has shown that pRE8 is either a point mutation in segment 13 or a small deletion in 13 or 13–14; and that pRE10 contains two distinct point mutations, or deletions which do not cover segments 7–9.

(iii) *Insertion of F′*lacI$^-$

Since all of the episomes introduced so far bore the *lacI*$^+$ gene, there was a possibility that the repressor produced by the *E. coli* F-factor (or by the *Klebsiella* plasmid) might differ sufficiently from the *Klebsiella* repressor that the chromosomal expression would be inhibited. However, the insertion of a constitutive *lac* operon with a Y^- mutation gave cells with the same low transport (see Table 6) found in *Klebsiella* containing F-factors with inducible *lac* operons, indicating that the repressor produced by the F-factor is not necessary for the phenomenon. An additional point of interest is that F′316 is an A^- mutant, suggesting that the thiogalactoside transacetylase is not involved in this effect.

(iv) *Insertion of short episomal* Y *gene product into RE1838*

Another hypothesis considered was that a short N-terminal peptide coded for by the initial DNA sequence of the *Y* gene might prevent the normal activity of the chromosomal *Y* gene, perhaps by competing for a limited number of membrane

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receptor sites. To test this possibility three pairs of strains were constructed. In each case the starting strain was a Z^-Y^+ strain showing good transport activity. A Y gene split into two segments by the insertion of a chloramphenicol-resistance gene (Tn9) was transferred into each of the three Z^-Y^+ strains (W4680, CSH21,

Table 5. *Properties of* Klebsiella *containing* E. coli *F lac carrying* Y⁻ *mutation**

Strain	*Y* mutation	Lac phenotype	β-Galactosidase†	TMG accumulation ratio at 20 s (conc. in/conc. out)
RE1838	—	$ML^{-/+}$	43 (17)	5·2 (17)
RE1838/E5014.1	None	ML^+	229 (4)	5·3 (4)
RE1838/T series‡	Unknown	ML^-	354 (15)	1·2 (15)
RE1838/HGM series§	Missense	ML^-	216 (18)	2·0 (18)
RE1838/MAA23	Late nonsense	ML^-	200 (3)	1·4 (3)
RE1838/E7150	Early nonsense	ML^-	256 (2)	1·6 (2)
RE1838/MS1054	Deletion	ML^-	304 (3)	1·9 (3)
RE1838/MS1019	Deletion	ML^-	NT	NT
RE1838/MS1038	Deletion	ML^-	NT	NT

* All cells were grown in medium 9 plus 1% lactose. NT, Not tested.

† μmol *o*-nitrophenol produced/min/g wet weight cells. Numbers in parentheses indicate the number of experiments performed.

‡ Five different $I^+Z^+Y^-$ mutants (derived from E5014.1) tested three times each and the mean value for all experiments given.

§ Six different missense $I^qZ^+Y^-$ mutants (MAA38, MAA6, MAB6, MAA32, MAA2, MAA37) from Hobson, Gho & Müller-Hill were tested. Each mutant in *Klebsiella* was tested three times and the mean value is given for all mutants.

Table 6. *Properties of* Klebsiella *containing constitutive* F′ lac*

Strain	Genotype: Chromosome	Genotype: Episome	β-Galactosidase†	TMG accumulation ratio at 20 s (conc. in/conc. out)
RE1838	$I^+Z^+Y^+$	None	43 (17)	5·2 (17)
RE1838/316	$I^+Z^+Y^+$	$I^-Z^+Y^-$	1760 (3)	1·5 (3)
RE1838/15-1-C	$I^+Z^+Y^+$	$I^-Z^+Y^-$	1530 (2)	1·2 (2)

* All cells were grown in medium 9 plus 1% lactose.

† μmol *o*-nitrophenol produced/min/g wet weight cells. Numbers in parentheses indicate the number of experiments performed.

CSH22) by P_1 transduction. During this process Z^+Y^-::Tn9 recombinants were also isolated and these were tested in the same manner. Figure 1 shows that the Z^-Y^-::Tn9 mutant derived from W4680 showed no effect at all on TMG transport when inserted into *Klebsiella*, whereas the corresponding Z^+Y^-::Tn9 mutant showed the usual low level of accumulation. In addition the RE1838/F′$Z^{del}Y^-$::Tn9 showed the same lactose fermentation as RE1838 ($ML^{-/+}$) while the RE1838/F′Z^+Y^-::Tn9 gave an ML^- phenotype. Additional strains of this type (constructed from CSH21 and CSH22) gave similar fermentation results to those obtained with the strains constructed from W4680.

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The data thus far presented have indicated that the reduction in transport activity of *Klebsiella* containing an *Flac* with *Y* mutation is not due to the episomal *Y* gene or *I* gene products. On the other hand the comparison between RE1838 carrying F-factors with Z^-Y^- or Z^+Y^- suggests that it is the presence of the *Z* gene which is responsible for the reduction in chromosomal *lac* expression.

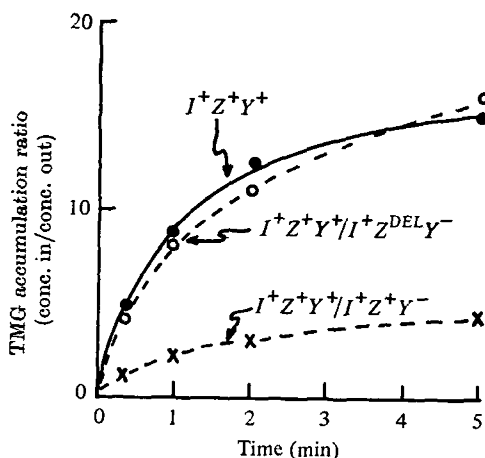

Fig. 1. Effect of episome carrying Z^{del} and Y^- mutations. The chloramphenicol resistant translocon, Tn9, inserted in the *Y* gene was introduced into W4680 (Δ *lac*/ $F'I^+Z^{del}Y^+$) by P_1 transduction. By this process two strains were constructed, Δ *lac*/ $F'I^+Z^{del}Y^-$: :Tn9 and Δ *lac*/$F'I^+Z^+Y^-$: :Tn9. The F-factors were then introduced into *Klebsiella* RE1838, selecting for chloramphenicol resistance on citrate plates (see Methods). The curve for RE1838 represents the mean values in seven experiments; the curves for the cells containing F-factors represent values for three experiments. Cells were grown in medium 9 + 1 % lactose + proline (100 μg/ml). Medium for the mated cells also contained 20 μg/ml chloramphenicol.

(v) *The role of the* Z *gene on the F-factor*

The next series of experiments was designed to evaluate the chromosomal *lac* operon expression when cells contained an F-factor which produced no β-galactosidase. A series of inducible and constitutive Z^- mutations on episomes was inserted into *Klebsiella*. When the $I^+Z^-Y^+$ episomes were inserted into *Klebsiella* and the cells grown on lactose, no reduction in chromosomal *Z* gene expression was observed (Table 7). In the case of constitutive F-factors carrying Z^- mutations, only a relatively small reduction in chromosomal *lac* expression was observed. In all cases the transport level was high as expected from a cell possessing two separate *Y* genes.

Another type of experiment confirmed the view that the *Z* gene on the F-factor was important for this phenomenon. Three different *Klebsiella*, each containing an F-factor with different *Y* deletions (MS1054, MS1019 and MS1038), were placed on 0·2 % lactose minimal agar plates. These cells failed to grow under these conditions. However, when 10^8 cells were spread on the plate, an occasional large clone grew after a 2- to 3-day period. One third of the 'revertants' were found to contain a Z^- mutation on the F-factor. The remaining two-thirds of the mutants proved to be

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constitutive for the melibiose operon which contains a transport system capable of lactose transport (Reeve & Braithwaite, 1973*a*). Under these latter conditions the cell is simply providing an alternative route for lactose entry into the cell. These studies strongly suggest that the presence of the *Z* gene on the F-factor is essential for the inhibitory phenomenon.

Table 7. *Properties of* Klebsiella *containing F′* lac *with Z⁻ mutation**

Strain	Genotype: Chromosome	Genotype: Episome	β-Galactosidase (% of RE1838)	TMG accumulation ratio at 20 s (conc. in/conc. out) (% of RE1838)
RE1838/T_1	$I^+Z^+Y^+$	$I^+Z^-Y^+$	100 (6)	150 (4)
RE1838/CSH22	$I^+Z^+Y^+$	$I^+Z^-Y^+$	118 (3)	174 (3)
RE1838/30-9-2	$I^+Z^+Y^+$	$I^-Z^-Y^+$	70 (3)	130 (3)
RE1838/CSH18	$I^+Z^+Y^+$	$I^-Z^-Y^+$	80 (2)	150 (2)
RE1838/CSH20	$I^+Z^+Y^+$	$I^-Z^-Y^+$	80 (2)	150 (2)

* All cells were grown in medium 9 plus 1% lactose.
The data are expressed as the percentage of the value obtained with RE1838 measured in the same experiment. Figures in parentheses represent the number of experiments performed.

(vi) Y *gene expression in IPTG induced cells*

A series of experiments was carried out on cells grown in citrate or glycerol in the presence of the gratuitous inducer IPTG. Table 8 shows that a *Klebsiella* strain carrying a Y^- mutation in the plasmid (RE1427) shows low levels of chromosomal *Y* gene expression (as measured by accumulation of TMG) when grown on lactose but relatively high levels when grown in the presence of IPTG. Under these latter conditions there was no reduction in TMG accumulation compared with the plasmid-free strain.

In a second experiment cells of *Klebsiella* with or without an *E. coli* F-factor carrying a Y^- mutation were grown in the presence of citrate and lactose or citrate and IPTG. Fig. 2 shows that when cells are grown in citrate and lactose, the presence of an F-factor carrying a *Y* deletion results in extremely low chromosomal *Y* gene expression. On the other hand, when these two cells were grown in citrate and IPTG, the transport levels were very similar. Two additional experiments gave the same results. Thus growth in the presence of IPTG completely abolishes the differences in *Y* gene expression seen in the *Klebsiella* containing a plasmid or F-factor bearing a Y^- mutation.

(vii) *Constitutive* Klebsiella *containing F-factors*

A partially constitutive *Klebsiella* was isolated and utilized for the next experiment. Table 9 shows that the constitutive level of chromosomal *Y* expression (3-fold TMG accumulation) is not affected by the presence of the *E. coli* $F'I^-Z^+Y^-$ mutations when cells are grown in lactose. This experiment indicates that it is the process of induction of the chromosome that is affected by the presence of an F*lac*.

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Table 8. *Effect of growth with IPTG or lactose on TMG transport*

Strain	Genotype: Chromosome	Genotype: Episome	TMG accumulation ratio in 20 s (conc. in/conc. out): Growth with lactose	TMG accumulation ratio in 20 s (conc. in/conc. out): Growth with IPTG
RE1838	$I^+Z^+Y^+$	None	5·2 (17)	9·5 (8)
RE1427	$I^+Z^+Y^+$	$I^+Z^+Y^-$	1·9 (3)	9·3 (3)
RE1403	$I^+Z^+Y^+$	$I^+Z^+Y^+$	17 (3)	33 (3)

Table 9. *Properties of a constitutive* Klebsiella *containing a constitutive F′*-lac*

Strain	Genotype	Carbon source	β-Galactosidase†	TMG accumulation ratio at 20 s (conc. in/conc. out)
RE1852	$I^-Z^+Y^+$	Lactose	35	4·0
RE1852	$I^-Z^+Y^+$	Citrate	18	3·0
RE1852/316	$I^-Z^+Y^+/I^-Z^+Y^-$	Lactose	359	3·4
RE1852/5	$I^-Z^+Y^+/I^-Z^+Y^-$	Lactose	692	2·7
RE1852/30-9-2	$I^-Z^+Y^+/I^-Z^-Y^+$	Lactose	28	8·6

* Mean values of three experiments.
† μmol *o*-nitrophenol produced/min/g wet weight.

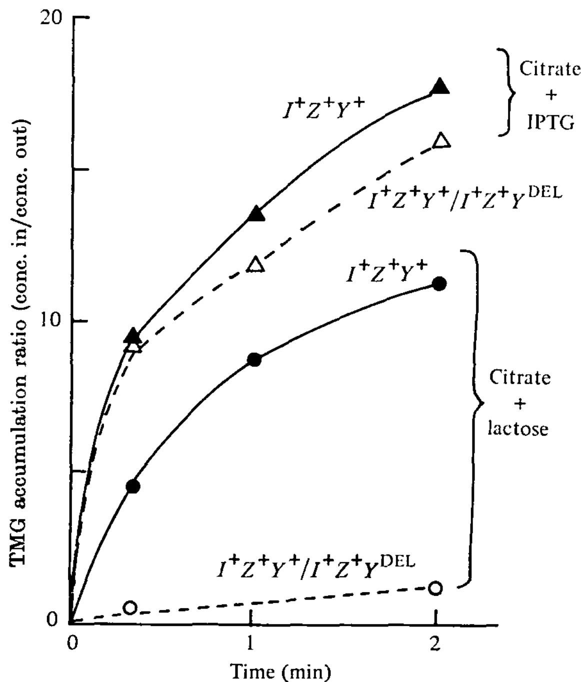

Fig. 2. Comparison of IPTG with lactose as inducers of RE1838 and RE1838/MS1054. Cells were grown in 0·4 % citrate plus 1 mM-IPTG or in 0·4 % citrate plus 0·4 % lactose. Washed cells were exposed to [^{14}C]TMG (0·1 mM) and cellular accumulation measured (see Methods).

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4. DISCUSSION

Klebsiella strains which carry an episome containing a *lacY* mutation show reduced levels of chromosomal *Y* gene expression compared with the parental strain of *Klebsiella* free of episome. This phenomenon cannot be a result of negative complementation, since both early nonsense mutations and *Y* deletions on the F-factor give rise to the same effect, although under these conditions little or no episomal *Y* gene product would accumulate in the cell. Another possibility considered was that a small N-terminal peptide coded by a short initial segment of the episomal *Y*-gene might prevent membrane insertion of the normal chromosomal *Y* gene product. No support was found for this hypothesis (Fig. 1). It was therefore concluded that the loss of chromosomal *Y* gene expression was a reflexion of lower activity of the entire *lac* operon on the chromosome. Unfortunately, because of the much higher levels of β-galactosidase produced by the *Z* gene of the episome as compared with the *Z* gene of the chromosome, it was not possible in these particular experiments to determine the extent of chromosomal *Z* gene expression.

Lowered expression of the chromosomal *Y* gene was still obtained when I^- mutants of the F-factor were inserted into *Klebsiella*. This finding eliminated the possibility that the *E. coli* repressor might cause the inhibition. Furthermore, the effect occurred in the absence of thiogalactoside transacetylase, showing that this enzyme was not involved.

The data indicate that the *Z* gene of the F-factor is essential for the phenomenon. When the F-factor contains a *Z* mutation, no reduction in chromosomal expression is observed. Under these conditions the chromosomal β-galactosidase can be measured, and the value is the same as or approaching that of the episome-free strain, whether the F-factor is Y^- or Y^+. In the presence of a Z^-Y^- F-factor the chromosomal *Y* is expressed to the same extent as in the strain bearing no episome, whereas the same F-factor carrying a normal *Z* gene shows inhibition of chromosomal *Y* expression. A further implication of the episomal *Z* gene in this process is the finding that revertants to growth on 0·2% lactose, obtained from *Klebsiella* with a *Y* deletion on the episome, show significant numbers which are now Z^- on the F-factor. This suggests that the loss of β-galactosidase produced by the F′*lacZ* relieves the inhibitory effect on the chromosomal expression.

It appears that the inhibitory phenomenon occurs only with inducible cells grown in lactose, and only when the episome carries a *Z* gene. One possible explanation for this effect is that the presence of unusually large amounts of β-galactosidase in a cell with only a very moderate level of transport could upset the normal process of production of the inducer, allolactose, from lactose. In the uninduced cell, the large quantity of extra basal β-galactosidase would rapidly hydrolyse the lactose entering the cell, thus keeping the disaccharide concentration in the cell at a very low level. Huber, Kurz & Wallenfels (1976) have shown that the proportion of allolactose to glucose, as a product of the action of an *E. coli* β-galactosidase preparation, is sharply decreased with decreased lactose concentration. In the present study this could mean that although the same amount of lactose enters the cell

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as in the episome-free strain, the more rapid hydrolysis of the sugar would result in the production of less allolactose. The hypothesis suggests that the level of allolactose reached in the cell is insufficient for the induction of either operon. However, when the cells are suspended in a liquid minimal medium containing 1 % lactose there is sufficient added entry of this sugar by diffusion to allow partial induction and growth of the cells.

Mieschendahl, Triesch & Müller-Hill (personal communication) have studied a similar situation in an *E. coli* system. They placed various F-*lac* episomes into an *E. coli* possessing a chromosomal *lac* promoter mutation (8 % expression). When F-factors bearing *Y* mutations were inserted, the cell failed to grow on lactose minimal plates in contrast to the strain free of episome, which showed growth. They, too have found that a Z^- F-factor does not cause the inhibition and have related this to their previous unpublished observations that Z^- strains of *E. coli* had become partially constitutive. They propose that an endogenously produced galactoside acts as an inducer and is responsible for the basal level of expression of the *lac* operon. This galactoside can be destroyed by the action of β-galactosidase, and the addition of large amounts of extra β-galactosidase via the F-factor would completely remove this endogenous inducer. No basal level of transport protein would be produced, and lactose therefore would be unable to enter the cell at all.

This hypothesis is consistent with their *E. coli* data as well as with some of the observations presented in this paper. However, an additional hypothesis would be necessary to explain the results obtained with *Klebsiella* grown in 1 % lactose minimal medium. Under these conditions lactose clearly enters the cell and yet the chromosomal *Y* gene is only partially expressed when the Z^+Y^- episome is present.

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