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The lactose system of *Klebsiella aerogenes* V9A

5. Lac-permease defective mutants of two *Klebsiella* Lac plasmids and their apparent reversion to wild type

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SUMMARY

The Lac plasmids in two *Klebsiella* strains, V9A and RE1544, give characteristic Lac-permease defective mutations which produce a lactose-negative (ML$^-$) phenotype in the host strain, in spite of the presence of a *lac* operon on the chromosome. These ML$^-$ clones can revert to the wild-type (ML$^+$) phenotype, and the mechanism of this reversion is examined. In V9A carrying its own Lac plasmid (F_K*lac*), it is shown that reversion of ML$^-$ to ML$^+$ is usually the result of mutation to constitutivity of one of two galactoside permeases which are not induced by lactose but accumulate lactose when otherwise induced or made constitutive. However, in one out of the 51 ML$^+$ revertants tested the mechanism of reversion appeared to be a change back to wild type of the Lac plasmid's own permease gene. In V9A carrying the Lac plasmid (pRE6) from RE1544, successive changes of phenotype were obtained of ML$^+$ to ML$^-$ to ML$^+$ to ML$^-$ to ML$^+$; these were all found to be the result of changes in the plasmid permease gene, and could be simply explained if an IS sequence could insert and excise from this gene.

1. INTRODUCTION

Many *Klebsiella* strains exhibit a strong lactose-positive phenotype after incubation for 16–20 h on MacConkey Lactose Agar (ML agar). A number of such strains have been shown to contain a (Lac) plasmid which carries a *lac* operon, and also a chromosomal *lac* operon giving about 10 % as much β-galactosidase activity as the plasmid operon. In consequence, loss of the plasmid gives a host strain showing a weak lactose-positive phenotype on ML agar (Reeve & Braithwaite, 1973*b*).

A curious feature shown by V9A, one of the *Klebsiella* strains examined which carries a Lac plasmid, is that it gives colonies of three distinct phenotypes on ML agar. These are strong lactose-positive (ML+), weak positive (ML$^{-/+}$) and negative (ML$^-$), and the three phenotypes are consistently obtained and easily distinguished from each other on ML agar. ML$^-$ and ML$^{-/+}$ colonies are found occasionally when cells of the ML$^+$ type are plated out, and each breeds true except that ML$^-$ clones

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aged for a few days on ML agar always contain some cells of the other two types. No doubt this ageing process causes a severe depletion of nutrients other than lactose in the agar, and so results in strong selection favouring cells with an improved ability to utilize lactose. A number of tests have further suggested that it is possible to obtain each of the three ML phenotypes by occasional spontaneous reversion from the other two (Reeve & Braithwaite, 1972).

The Lac plasmid of V9A, F_K*lac*, is not able to promote its own transfer, but carries a gene which represses F transfer. This repressor gene is always transferred with F_K*lac* to *E. coli*, and it is present in V9A cells of all three ML phenotypes, so that at least that part of F_K*lac* carrying the repressor gene must be retained in cells of ML$^-$ and ML$^{-/+}$ phenotypes. ML$^{-/+}$ cells show about 10 % and ML$^-$ cells 100 % of the level of activity for β-galactosidase shown by the wild-type strain, so it appears that the plasmid *lac Y* gene is inactivated in ML$^-$ and the plasmid *lacZ* (and probably also *lac Y*) is inactivated in ML$^{-/+}$ cells. This conclusion is supported by the fact that conjugation with ML$^-$ cells carrying a suitable transfer factor can convert LacZ$^-$ but not LacY$^-$ *E. coli* to Lac$^+$, while conjugation with ML$^{-/+}$ cells cannot convert either type of recipient to Lac$^+$.

The occurrence of the three ML phenotypes and their apparent interconvertibility could be explained in several ways, e.g. (1) recombination between the Lac plasmid and the host chromosome could lead to inactivation of the *lac Y* gene to give ML$^-$, or of both the Y and Z genes to give ML$^{-/+}$, and further recombination could recreate the original plasmid or the third form of the plasmid; or (2) transposition of an IS fragment could produce the same effects without involving the chromosome; or (3) illegitimate recombination within the Lac plasmid could be responsible for the changes $+ \rightarrow -/+$ and $+ \rightarrow - \rightarrow -/+$, while the apparent reversions to the + state were the result of mutations to constitutivity of one of the two host galactoside permeases (other than that of the *lac* operon) able to accumulate lactose (Reeve & Braithwaite, 1973*a*). The results described below suggest that both mechanisms (1) or (2) and mechanism (3) are at work in different cases. We shall first describe tests on a second Lac plasmid, which has the advantage over F_K*lac* that it can mediate its own transfer.

2. MATERIALS AND METHODS

(i) *Bacteria and plasmids.* The *Klebsiella* strains used are listed in Table 1, the stock reference being RE with a four-figure number. When no such reference number is given it means that the strain was lost or discarded. Table 2 lists the strains of *E. coli* K12 used, my own stocks being coded RE with a three-figure number. Table 3 lists the plasmids assumed to be present in the various strains. *Klebsiella* plasmids are coded pRE1, 2, etc.

(ii) *Maintenance of stocks.* A number of bacterial stocks have survived for many years, without apparent change, on Dorset Egg slants (without Crystal Violet, purchased from Oxoid), but more recently our stocks have been kept as L-broth overnight cultures (plus glycerol 30 %) in a domestic deep-freeze.

(iii) *Media.* ML agar was Oxoid MacConkey Lactose agar No. 3. MacConkey raffinose agar was made to the same formula, using raffinose instead of lactose. L-broth and L-broth agar, LZ (lactose tetrazolium) agar, M9 minimal medium and agar were made up as described by Reeve & Braithwaite (1972, 1973*a*). EMB

Table 1. Klebsiella *strains*

Name	ML phenotype	Origin, characters, reference
RE1401	+	*Klebsiella* V9A (Reeve & Braithwaite, 1970)
RE1403	+	V9A Thy$^-$ (Reeve & Baithwaite, 1972)
RE1409	+	V9A Thy$^-$His$^-$ by NTG treatment of RE1403
RE1427	−	V9A Thy$^-$(ML$^-$18) variant of RE1403 (Reeve & Braithwaite, 1972)
RE1434	+	V9A Thy$^-$; variant of RE1427
RE1435	−/+	V9A Thy$^-$; variant of RE1427
RE1468	−/+	RE1409 (*FtraO304his*$^+$); see text
RE1469	−/+	RE1468 cured of F304; see text
RE1476	−/+	RE1469 Nal^r; spontaneous mutant
RE1483	+	RE1469 (pRE6$^+$); see text
RE1544	+	*Klebsiella* clinical isolate CI3 from S. B. Primrose
RE1701	−	Variant of RE1483
RE1702	−/+	Variant of RE1701
RE1703	+	Variant of RE1701
RE1706	−	Variant of RE1483
RE1707	−/+	Variant of RE1706
RE1708	+	Variant of RE1706
RE1712	−	Variant of RE1483
RE1713	+	Variant of RE1712
RE1714	−	Variant of RE1713
RE1716	+	Variant of RE1714
RE1715	−/+	Variant of RE1714
RE1754	+	RE1544(0) spontaneous capsule$^-$ mutant of RE1544
RE1755	−	Variant of RE1754, completely LacZ$^-$ (see text)
RE1758	−	Variant of RE1754 made Str^r
RE1759	−	Variant of RE1754
RE1762	−	Variant of RE1754
RE1764	−	RE1544 ML − 1; variant of RE1544
RE1765	−	RE1544 ML − 2; variant of RE1544
RE1767	+	RE1764 ML$^+$; variant of RE1764
RE1768	+	RE1765 ML$^+$; variant of RE1765
ML$^-$20	−	Variant of RE1403 (Reeve & Braithwaite, 1972), now lost
ML$^-$21	−	Variant of RE1403 (Reeve & Braithwaite, 1972), now lost

'Variant' in all cases means a clone showing a spontaneous change in ML phenotype (see text).

Galactose agar was made according to the Oxoid formula for Eosin Methylene Blue agar, using galactose instead of lactose. Antibiotics were added to a final 20 μg/ml when selecting for the presence or transfer of a resistance determinant. Streptomycin at 1 mg/ml or nalidixic acid at 20 μg/ml were used as counter-selective agents against the donor when plating for recombinants after matings.

(iv) *Mating.* To transfer plasmids from *Klebsiella* to *E. coli*, both strains were

grown up in L-broth, and 0·1 ml of each was spread together on a plate of L-broth agar and incubated overnight. Loopfuls of the mixture were then spread by successive cross-streaking with sterile loops over plates selective for the transferred plasmid. This method provides serial dilution on the plate so that even a very low frequency of transfer gives some colonies, and the transfer frequency can be judged

Table 2. *Strains of* E. coli *K12*

	Designation and genotype*	Origin†
RE1	F⁻ *proA lacY ton trp his strA* (*λ*)	J62 from W. Hayes (1962)
CA237	HfrH Δ (*lacZ*: M445) *thi*	From J. Scaife (1968)
RE253	F⁻ Δ (*lacZ*) *trp his strA*	From CA237 × RE1
RE254	F⁻Δ (*lacZ*) *trp his strA nalA*‡	*nalA* mutant of RE253
E7074	Δ (*lac pro*) *supE thi* (F¹*lacI proA*⁺, *B*⁺)	From J. D. Bond (1974)
RE296	F⁻Δ(*lac pro*) *supE thi*	E7074 cured of F¹*lac*
RE298	F⁻ *metB lamB nalA*	*nalA* mutant of W1485
RE299	F⁻ *metB lacZ*⁻*Y*⁺ *lamB nalA*§	*lacZ*⁻ mutant of RE298
RE300	F⁻ *metB lacZ*⁺*Y*⁻ *lamB nalA*§	*lacY*⁻ mutant of RE298
MS3	F⁻ *galE pyrD strA melA*	From J. Shapiro (1966)
RE303	F⁻ *lacZ*⁺*Y*⁻ *gal*⁻ *pyrD mel*⁺‖	From MS3
RE307	F⁻ *lacZ*⁻*Y*⁺ *gal*⁻ *pyrD mel*⁺‖	From MS3
ED2704	Δ(*lac*: X74) *trp his thy spcA*(F*traO304his*)	From D. J. Finnegan (1972)
JC6535	*trp his* (F57his⁺)	From N. S. Willetts (1973)

* Gene symbols follow Taylor & Trotter (1972).

† Date given indicates year stock was obtained.

‡ RE254 was found to be LacI⁻Z⁻Y⁻, when tested with F*lacI* and for its ability to grow on melibiose at 42 °C. CA237 and RE253 have not been tested in this way.

§ RE299 and 300 were selected after NTG mutagenesis and classified on the basis of their ability to synthesise *β*-galactosidase and to grow on melibiose at 42 °C.

‖ MS3 is galactose-sensitive. RE303 and 307 were obtained by first selecting Lac⁻ mutants on ML agar, then selecting Gal⁻ (instead of Gal-sensitive) mutants of these on EMB galactose agar, then transducing these to *melA*⁺ with P_1 grown on K12, followed by the classification steps under footnote §, above.

Table 3. *Plasmids*

	Designation and genes carried	
F_K*lac*	*lac* operon	Lac plasmid in V9A*
T_K	T determinant	T plasmid in V9A*
pRE5	T determinant, conjugative	T plasmid in RE1544†
pRE6	*lac* operon, conjugative	Lac plasmid in RE1544†
FR5	F*gal* linked to A, C, S, Su determinants*	
R1*drd*16	A, C, K, S, Su determinants. *o*ᶜ mutant of R1‡	
R1*drd*19	A, C, K, S, Su determinants. *i*⁻ mutant of R1‡	

* See Reeve & Braithwaite (1970), Reeve (1970).

† See text.

‡ See Meynell & Cooke (1969), Reeve & Braithwaite (1970).

qualitatively as 'high' or 'low'. Thus donors with a repressed sex factor normally give 10 to 100 colonies per plate. Colonies growing on the selective plates were routinely purified and checked for the characters of the recipient. Colonies of *Klebsiella* can easily be distinguished from those of K12 by their appearance on MacConkey agar, and by their ability to grow on citrate, raffinose or sucrose as carbon source.

(v) *Assays of β-galactosidase and α-galactosidase.* Assays of β-galactosidase in *Klebsiella* strains were normally made on cells growing in log phase on citrate as carbon source, with and without previous induction by 0·1 mM IPTG for 60 or 90 min, and using the method of Miller as modified by Reeve & Braithwaite (1974). A further modification was that uninduced cells of $ML^{-/+}$ strains were diluted 1/5 and not 1/20 into Z-buffer for assay. The enzyme activity was always converted to units per ml at OD_{550} 1·0, using the conversion factors of Reeve & Braithwaite (1974). In addition, 'live' assays (i.e. assays on cells not treated with SDS and chloroform) were made for both β-galactosidase (βG) and α-galactosidase (αG) as follows: bacteria were grown to OD_{550} 0·2–0·4 in a suitable minimal medium and then resuspended in the same medium without carbon source at about the same OD. The reaction mixture contained 2 ml this bacterial suspension, 1 ml ONPαG or ONPβG (0·9 mg/ml in M/4 phosphate buffer) and chloramphenicol at 20 μg/ml unless the bacteria were resistant to chloramphenicol, in a total vol. of 3 ml. αG was assayed at 35 °C, βG at 28 °C, and the reaction was stopped by adding 2 ml of 1 M-K_2CO_3 when colour had developed. The optical densities were then read at 420 and 550 nm, and enzyme activity was calculated as

$$\frac{OD_{420} - 1{\cdot}75\ OD_{550}}{RT \times \text{bacterial } OD_{550}} \times \frac{1}{0{\cdot}0075},$$

where RT is reaction time (min) and Bacterial OD_{550} is OD of bacterial suspension before it was added to reaction mixture.

(vi) *Abbreviations.* A, C, K, S, Su, R-factor determinants for resistance to ampicillin, chloramphenicol, kanamycin, streptomycin and sulfonamides, respectively. T, plasmid determinant for tetracycline resistance. Cm, chloram phenicol; Str, streptomycin. IPTG, Isopropyl-β-D-thiogalactoside. NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. ONPαG, *O*-nitrophenyl-α-D-galactoside; ONPβG, *O*-nitrophenyl-β-D-galactoside.

3. RESULTS

(i) *Lac plasmid in a clinical isolate of* Klebsiella, *strain RE1544*

RE1544 was obtained as one of a series of clinical isolates (no. CI 3), from a Warwick Hospital, and was supplied by S. B. Primrose. It is capsulated and gives thick mucoid colonies on ML agar with a typical ML^{+} phenotype, suggesting that it carries a Lac plasmid; and it is also resistant to ampicillin and tetracycline. RE1544 was infected with two conjugative plasmids, FR5 and R1*drd*16, and was then mated to a Lac^{-} strain of K12, with the results shown in Table 4. RE1544 on its own proved able to transfer both Lac^{+} and tetracycline resistance, separately, to K12, and the recipients were then able to transfer each of these characters to other K12 strains. FR5 converted RE1544 to a Lac^{-} phenotype but did not eliminate its tetracycline resistance, and the infected host was then able to transfer FR5 but not Lac^{+} to K12. R1*drd*16 did not affect the Lac phenotype of RE1544, but was not transferred from RE1544 to K12. This result could be explained by

either a mutation in R1 or an effect of the host genes on its ability to transfer itself, but has not been analysed further.

We conclude that RE1544 contains two plasmids, one carrying a T determinant and a transfer system (pRE5) and the other a *lac* operon and transfer genes (pRE6). K12(pRE6$^+$) is resistant to F-specific phage (f2), and becomes sensitive to f2 when infected with R1*drd*16 but not when infected with R1*drd*19 (the o^c and i^- mutants of R1). pRE6 was also not compatible with an F-prime or with FR5 in *E. coli* K12, so this plasmid is fi^+ and belongs to the FI compatibility group. In matings of RE1544 to K12, pRE5 is always transferred much more frequently than pRE6, and may be derepressed for transfer; it is compatible with F and R1.

Table 4. *Effect of mating RE1544 to RE254 (K12 Lac$^-$Str^r)*

Plasmid first transferred into RE1544	Resultant phenotype of RE1544		Transfers to K12†		
	Lac	Tc	Plasmid	Lac$^+$	Tc^r
None	+	R	−	+	++
FR5	−	R	+	−	NT
R1*drd*16	+	R	−	+	NT

Overnight matings were made on L-broth plates as described under Methods, and the mixture was then streaked on ML agar containing streptomycin to kill the donor and Cm or Tc to select for transfer of the plasmid (FR5 or R1) and Tc-resistance, respectively. Minimal lactose streptomycin agar was used to select for transfer of Lac$^+$.

* +, − indicate ML$^+$ and ML$^-$ phenotypes, R indicates resistance to tetracycline.

† − no transfer; +, ++, low- and high-frequenty transfer; NT not tested.

The fact that pRE6 can transfer itself makes it much easier than F_K*lac* to study in different genetic backgrounds; and tests were started after a non-mucoid mutant, RE1754, of the host *Klebsiella* had been selected. This mutant gives smooth but non-mucoid colonies on ML agar, and retains the ML$^+$ phenotype and the ability to transfer both pRE5 and pRE6 to K12.

To obtain derivatives of RE1754 with a changed Lac phenotype, colonies were kept on MacConkey Raffinose agar (to prevent selection on the plates favouring ML$^+$), and were grown up in L-broth and plated on LZ agar. About 1 in 8000 colonies so obtained were red (i.e. Lac$^-$) on the LZ agar, and looked ML$^-$ on ML agar, while retaining tetracycline resistance and other characters of the *Klebsiella* parent. Eleven such lines were assayed for β-galactosidase activity, and fell into the three classes represented by lines −1, −2 and −3 in Table 5, which gives the enzyme levels in uninduced and induced cells and also the effects of mating each line to strains of K12 which were, respectively, LacZ$^-$, LacY$^-$ and LacZ$^-$Y$^-$.

Lines −3 and −9 retained the same ability to produce β-galactosidase as the parent RE1754, and were able to convert LacZ$^-$ but not LacY$^-$ strains of K12 to Lac$^+$: they thus resemble the ML$^-$ strains of V9A, and must carry a mutant of pRE6 with a defective Lac permease. Lines −2 and −4 possessed about 10% as much enzyme activity as the parent strain, and could not transfer either a LacZ$^+$

or a LacY$^+$ gene to K12: they appear to have lost either the complete Lac plasmid or the LacZY part of it, but retain the chromosomal *lac* operon.

Line -1 (RE1755) was unique in that it possessed no β-galactosidase activity at all, and no further mutants of this type have been obtained in a study of some 200000 colonies plated from the parent strain. This clone must have resulted from a double event: loss of the plasmid and a mutation which inactivated at least the *lacZ* gene on the chromosome. The two events might, of course, have resulted from recombination between the plasmid and chromosomal *lac* operons, but large-scale selection has not converted this strain back to Lac$^+$, as might then be expected to occur.

Table 5. *Lac$^+$ transfers by mutants of RE1754 (RE1544 Caps$^-$1)*

Mutant and Lac phenotype		β-galactosidase activity*		Transfer of Lac$^+$ to K12 strains†			RE no.‡
		U	I	LacZ$^-$	LacY$^-$	LacI$^-$Z$^-$Y$^-$	
+	Lac$^+$	4·0	330	+	+	+	1754
−1	Lac$^-$	0·05	0·07	−	−	−	1755
−2	Lac$^-$	0·14	38	−	−	−	—
−3	Lac$^-$	4·1	340	+	−	−	1759
−4	Lac$^-$	0·11	34	−	−	−	1762
−9	Lac$^-$	3·2	170	+	−	−	—

* Assayed growing in log phase on citrate as carbon source. U, Uninduced; I, induced with 10^{-4} M IPTG for 60–90 min.

† Transfer obtained (+) or not obtained (−) in matings to strains of *E. coli* K12. The recipients were RE299 and RE307 (LacZ$^-$), RE300 and RE303 (LacY$^-$), RE254 (LacZ$^-$Y$^-$). Matings were made overnight on L-broth agar and loopfuls of the mixture were then streaked on selective plates.

‡ Numbers assigned indicate that the lines have been retained. Mutant −2 was lost after these tests were carried out, but a Str^r derivative (RE1758) has been kept.

One difference between this series of mutants and the parallel series in V9A is that the type represented by lines −3 and −9 in table 5 could not be distinguished from that represented by lines −2 and −4 by their phenotypes on ML agar, since the latter looked just as lactose-negative as the former. So it is not possible to test whether a clone such as line −3 can lose its plasmic *lacZ* gene, in contrast to the easily recognized change from ML$^-$ to ML$^{-/+}$ in V9A.

Revertants of the ML$^-$ strains of RE1754 to ML$^+$ were not easy to obtain, but at this point we discovered ML$^-$ variants of RE1544 (the mucoid parent of RE1754), during routine plating, and their study proved instructive. Three such clones were independently isolated, and all three gave strong lactose-positive revertants when purified from colonies aged on ML agar, thus resembling the behaviour of ML$^-$ strains of V9A. In mating tests, neither the three ML$^-$ variants of RE1544 nor a Lac$^+$ revertant of each was able to transfer either a *lacZ* or a *lacY* gene to K12, so that either the Lac plasmid had been lost or its transfer system had become defective in the ML$^-$ lines.

The enzyme assays summarized in Tables 6 and 7 enable us to explain both the ML$^-$ and the revertant ML$^+$ behaviour. Table 6 gives the levels of β-galactosidase

in uninduced and induced cells of the three ML⁻ lines and of an ML⁺ revertant of each. The three ML⁻ lines clearly have the low enzyme activity associated with loss of the plasmid *lac* operon (compare Table 5), and the three ML⁺ revertants have precisely the same low enzyme levels, so that the striking change in phenotype from ML⁻ to ML⁺ cannot be explained by a change in the plasmid. This left the possibility that a permease able to take up lactose (but not inducible by lactose) had become constitutive in the ML⁺ revertants. Assays of β-galactosidase and α-galactosidase were therefore made on live cells growing on citrate and on

Table 6. *β-Galactosidase activity in lines derived from RE1544*

	Enzyme			Enzyme	
Line	U	I	Line	U	I
ML − 1	0·09	31·8	ML − 1 → +	0·12	30·6
ML − 2	0·09	29·4	ML − 2 → +	0·12	30·5
ML − 3	0·08	30·6	ML − 3 → +	0·06	26·0

Assays as for Table 5. Lines ML − 1, − 2 are RE1764, 1765. Lines − 1 → +, − 2 → + are RE1767, 1768.

Table 7. *α-Galactosidase (αG) and β-galactosidase (βG) activity in lines derived from RE1544: 'live' assays*

Carbon source	Line ML − 1		Line ML − 1 → +	
	αG	βG	αG	βG
Citrate	0·29	0·05	19·6	0·28
Raffinose	6·08	0·07	6·5	0·07
	Line ML − 2		Line ML − 2 → +	
	αG	βG	αG	βG
Citrate	0·20	0·05	18·8	0·25
Raffinose	6·95	0·08	6·8	0·07

Log phase cultures, growing in minimal medium on the carbon sources shown, were centrifuged and resuspended in minimal medium containing chloramphenicol but no carbon source, and were then assayed as described in 'Methods'.

raffinose as carbon source, with the results shown in Table 7. Bearing in mind that, in *Klebsiella* the melibiose permease takes up lactose, while the melibiose operon is induced by raffinose but not by lactose or citrate (Reeve & Braithwaite, 1973*a*), Table 7 demonstrates that the two ML⁻ strains tested are inducible for the melibiose operon (α-galactosidase activity is 20 × higher in cells grown on raffinose than in cells grown on citrate). Both ML⁺ revertants, on the other hand, are clearly constitutive for the melibiose operon, since they show actually higher α-galactosidase activity on citrate than on raffinose. The lower level on raffinose in these strains is probably the result of catabolite repression exerted by raffinose or one of

its breakdown products. The β-galactosidase levels show that the *lac* operon had not become constitutive in the ML$^+$ lines.

These results show that even a *Klebsiella* strain which has lost its Lac plasmid genes and retains only a chromosomal *lac* operon of low activity can give rise to clones with a strong lactose-positive phenotype (i.e. ML$^+$) by mutations to constitutivity of the melibiose operon; and one may assume that ML$^-$ lines carrying a plasmid with a defective Lac permease can revert to ML$^+$ in the same way. Moreover, a third inducible galactoside permease (GP III) in *Klebsiella* V9A can accumulate both lactose and melibiose though it is not induced by either (Reeve & Braithwaite, 1973*a*), so that a mutation of this permease to constitutivity should also convert ML$^-$ and ML$^{-/+}$ strains to ML$^+$. It therefore becomes necessary to re-examine the ML$^+$ revertants of ML$^-$ lines of V9A. It had previously been thought, for reasons which will appear, that these were the result of changes in the Lac plasmid.

(ii) *The Lac plasmid in V9A*

It will be recalled that ML$^-$ mutants of V9A carry a permease-defective Lac plasmid, while the *lacZ* and *lacY* genes of the plasmid are both missing or inactive in ML$^{-/+}$ strains. An early mating experiment is summarized in Table 8, in which

Table 8. *Plasmids transferred by V9A(FR5$^+$) strains mated to* E. coli *RE1*

	Recipient of T_K plasmid		
Donor	No. Lac$^+$	No. Lac$^-$	% Lac$^+$
ML$^+$(FR5$^+$)	478	125	79
ML$^-$20(FR5$^+$)	0	138	0
ML$^-$20 → +(FR5$^+$)	0	92	0
ML$^-$21(FR5$^+$)	0	200	0
ML$^-$21 → +(FR5$^+$)	266	97	73

Overnight L-broth cultures of donor and recipient were diluted together 1/10 into fresh L broth and incubated 6 h at 37 °C. 0·1 ml samples were then plated on ML agar containing 20 μg/ml tetracycline and 1000 μg/ml streptomycin. Only RE1 cells which have received the T_K plasmid can grow on these plates. 0·33 ml was plated from each mating.

Donors: ML$^+$ is RE1403, ML$^-$20 and ML$^-$21 were derived from RE1403 (Reeve & Braithwaite, 1972) and a (+) derivative of each was selected on ML agar. The five strains were infected with FR5 shortly before the matings to RE1 were made. RE1 is LacY$^-$ and has not been found to revert to lac$^+$.

the wild-type strain RE1403, two ML$^-$ variants and an ML$^+$ revertant of each were infected with FR5 and shortly afterwards mated to a LacY$^-$ strain of K12 (RE1). At this time the nature of the ML$^-$ defect was not understood, and selection was only made for transfer of the (T_K) plasmid in V9A which codes for tetracycline resistance. Selection was on ML agar containing tetracycline (and streptomycin to eliminate the donor), so that transfer of the Lac plasmid could immediately be recognized. Table 8 shows that the original ML$^+$ strain and the + revertant of ML$^-$21 gave 70–80 % Lac$^+$ clones among the recipients of T_K, while ML$^-$21 and the two other donors gave no Lac$^+$ transfer, though all transferred T_K. A repe-

tition of these matings gave the same result. Strain ML⁻21 was typical of other ML⁻ strains in its general behaviour (Reeve & Braithwaite, 1972), and must have possessed a Lac plasmid with a defective permease, so it is difficult to avoid the conclusion that its reversion to + was the result of a change in the Lac plasmid.

Table 9 gives the result of a more recent experiment on some strains derived from V9A. RE1427 is a spontaneous ML⁻ variant, and RE1434 and 1435 are spontaneous revertants of RE1427 to + and −/+. Finally, one ML⁺ and two ML⁻ lines were selected from the ML⁻/⁺ line RE1435. The last three lines were

Table 9. *β-Galactosidase assays on V9A (ML⁻) and its derivatives*

Strain and derivation		Enzyme activity U	Enzyme activity I
RE1427	ML⁻	4·1	111
RE1434	ML⁻ → ML⁺	5·6	104
RE1435	ML⁻ → ML⁻/⁺	0·19	29
—	ML⁻ → ML⁻/⁺ → ML⁺	0·18	24
—	ML⁻ → ML⁻/⁺ → ML⁻	0·13	23
—	ML⁻ → ML⁻/⁺ → ML⁻	0·12	21

Cells were assayed in log phase growing on citrate with (I) or without (U) previous induction by IPTG. Strains without an RE number were not retained.

Table10. *α-Galactosidase activity in RE1427 and 1434*

Strain		Enzyme activity in cells grown on: Citrate	Raffinose
RE1427	ML⁻	0·44	12·0
RE1434	ML⁻ → ML⁺	28·7	22·9

Table 11. *Summary of tests on ML⁺ revertants of RE1427*

State of melibiose operon	No. of ML⁺ clones
Inducible	2
Constitutive	46
Total clones tested	48

obtained by an enrichment procedure using the fact that, of the three phenotypes, only ML⁻/⁺ is unable to grow for several hours after transfer from raffinose to lactose as carbon source (Reeve & Braithwaite, 1972). No mutagen was used in this process. Table 9 shows that RE1427 and RE1434 had the wild-type β-galactosidase activity, while the ML⁻/⁺ line and both + and − lines derived from it had the low enzyme activity characteristic of the chromosomal *lac* operon. Thus the ML phenotype can be dissociated from β-galactosidase activity in V9A, as in RE1544. As a further test, FR5 was mated into both RE1427 and 1434, and each was then mated to a LacY⁻ and a LacZ⁻ strain of K12. Both donors transferred FR5 to

both K12 strains, but only converted the LacZ$^-$ recipient to Lac$^+$, so the ML$^+$ phenotype of RE1434 is not attributable to a change in the Lac plasmid. Assays of α-galactosidase levels on RE1427 and 1434 when grown on citrate and on raffinose as carbon source (Table 10) finally made it clear that the reversion to ML$^+$ in RE1434 was the result of mutation to constitutivity of the melibiose operon.

The ML$^-$ strain RE1427 was then selected to obtain a number of independent ML$^+$ revertants, and 48 of these were assayed for the state of the melibiose operon: as Table 11 shows, all but two were constitutive, thus explaining the cause of the 46 ML$^+$ revertants. The two lines which were still inducible for the melibiose operon were infected with FR5 and mated to LacY$^-$ and LacZ$^-$ strains of K12, and each transferred FR5 to both K12 strains but converted only the LacZ$^-$ strain to Lac$^+$. We conclude, then, that the Lac plasmid had not reverted to LacY$^+$ in these two ML$^+$ lines, and it seems probable that the cause of the reversion was mutation to constitutivity of the third glactoside permease, GP III, referred to earlier.

(iii) *Behaviour of pRE6 in* Klebsiella *V9A* ($F_K$lac$^-$)

The evidence presented so far indicates that reversion of the ML$^-$ phenotype to ML$^+$ is usually the result of mutation to constitutivity of the *mel* operon, but may also occur through conversion of the plasmid Lac permease gene to an active

Table 12. *Enzyme activity in RE1483 and its derivatives*

Strain	Origin	ML phenotype	β-Galactosidase U	β-Galactosidase I	α-Galactosidase operon
RE1483	RE1469(pRE6$^+$)	+	4·4	202	Ind
RE1712	RE1483	−	3·6	178	Ind
RE1713	RE1712	+	3·5	129	Ind
RE1714	RE1713	−	3·8	165	Ind
RE1716	RE1714	+	3·7	161	Ind
RE1715	RE1714	−/+	0·13	13	Ind

All except the first strain arose as spontaneous variants of the strains listed in the 'Origin' column. β-Galactosidase activity assayed as in Table 4; I and U: with and without induction by IPTG. α-Galactosidase activity was compared by 'live' assays on cells grown on citrate and raffinose as carbon source; 'Ind' means that the melibiose operon was clearly inducible from this test.

state (in 1 out of the 51 revertants tested). Strong evidence that such plasmid changes do occur is provided by another set of experiments in which V9A was cured of its resident Lac plasmid and was then infected with pRE6, and ML$^+$ variants of the resulting strain were studied.

ML$^{-/+}$ variants of V9A can be assumed to have lost most if not all of the *lac* operon in F_K*lac*, but retain part of this plasmid since they are still able to repress F, a character always associated with the plasmid when it is transferred to another host. Curing of F_K*lac* from V9A cannot be achieved by the usual methods, since not only is the plasmid refractory to treatments such as acridine orange but cured cells would be expected to give clones of ML$^{-/+}$ phenotype. A number of

ML$^{-/+}$ clones which arose spontaneously at various times have been tested for their ability to repress F activity (when infected with FR5), and none had lost this ability.

Cure of F_K*lac* was in fact obtained by chance, in an unexpected way, when a His$^-$ mutant of V9A (RE1409) was infected with the *traO303* mutant of F*his* from *E. coli* strain ED2704. The resulting *Klebsiella* strain was ML$^+$, but was sensitive to F-specific phage and gave ML$^{-/+}$ variants such as RE1468 which proved to have lost the entire Lac plasmid. The evidence for this was that His$^-$ descendants of RE1468 such as RE1469, which had lost F*traO304his*, became sensitive to

Table 13. *Effect of mating RE1483 and its derivatives to* E. coli *K12 strains*

Donor	Origin	ML phenotype	Transfer of Lac$^+$ to K12 strains* Z$^-$Y$^+$	Z$^+$Y$^-$	Z$^-$Y$^-$
RE1483	—	+	+	+	+
1701	RE1483	−	+	−	−
1702	1701	−/+	−		−
1703	1701	+	+	+	+
1706	1483	−	+	−	−
1707	1706	−/+	−		−
1708	1706	+	+		+
1712	1483	−	+	−	−
1713	1712	+	−	−	−
1714	1713	−	+		−
1716	1714	+	+		+
1715	1714	−/+	−		−

Each *Klebsiella* strain except the first arose as a spontaneous variant of the strain in the 'Origin' column. Matings were made overnight on L-broth agar to one or both of the K12 strains of each of the following types: RE299 and RE307 (LacZ$^-$Y$^+$); RE300 and RE303 (LacZ$^+$Y$^-$) for those donors indicated; RE254 and RE296 (LacZ$^-$Y$^-$).

* +, Lac$^+$ transfer; −, no Lac$^+$ transfer; blank indicates mating not made.

F-specific phage f2 when infected with wild-type F*his* (from JC6535), or with FR5, showing that the sex-factor repressor gene as well as the *lac* operon of the plasmid had been lost.

The cured strain of V9A, RE1469, was infected with the Lac plasmid pRE6 to give the new strain RE1483 = RE1469(pRE6$^+$). This strain gives a strong ML$^+$ phenotype much like that of V9A and is able to transfer its Lac plasmid to K12, so it can be assumed to carry pRE6 (which alone is able to promote its own transfer). RE1483 gave rise spontaneously to occasional ML$^-$ and ML$^{-/+}$ clones, and the ML$^-$ clones were able to revert to +. Such ML$^+$ revertants were able to give ML$^-$ derivatives, and from these second-stage ML$^-$ lines both ML$^+$ and ML$^{-/+}$ clones could be obtained.

Assays of both β-galactosidase and α-galactosidase on one series of these strains are summarized in Table 12, where it is seen that RE1712 is an ML$^-$ variant of RE1483, 1713 is an ML$^+$ revertant of 1712, and so on. The β-galactosidase activity

of the successively derived ML$^-$ and ML$^+$ lines is the same as that of the parent strain, while the ML$^{-/+}$ derivative has the low enzyme activity attributable to the host's chromosomal *lac* operon. The melibiose operon was inducible in all the lines tested, so it cannot be responsible for the ML$^+$ reversions. These lines and others derived from RE1483 were then mated to K12 strains which were LacZ$^-$, LacY$^-$ and LacZ$^-$Y$^-$, respectively, with the results shown in Table 13.

We see that the three ML$^{-/+}$ Klebsiellas could not convert any of the K12 strains to Lac$^+$, the four ML$^-$ strains could convert LacZ$^-$ but not LacY$^-$ recipients to Lac$^+$, and three of the four ML$^+$ revertants behaved just like the parent + line: they could convert even the LacZ$^-$Y$^-$ strain of K12 to Lac$^+$. Strain RE1713 (ML$^+$) was not tested for mating ability until long after RE1714 had been derived from it, and was then unable to transfer a Lac plasmid, probably because the latter had meanwhile become defective for transfer. An additional point of interest shown by these matings is that both LacZ$^-$Y$^+$ strains used gave a much stronger Lac$^+$ phenotype on ML agar when carrying a Lac plasmid from an ML$^+$ strain than when carrying one from an ML$^-$ donor.

These results seem to demonstrate quite convincingly that the Lac plasmid pRE6, when transferred to a new host, V9A, which had lost its own Lac plasmid, was able to produce Lac-permease defective mutants and to revert back to the LacY$^+$ state again.

4. DISCUSSION

The two Lac plasmids, F$_K$*lac* and pRE6, studied in this paper, both give characteristic Lac-permease defective mutations which produce a Lactose-negative phenotype in the host *Klebsiella*. Thus F$_K$*lac* gives ML$^-$ variants in V9A which can only grow on high concentrations of lactose (Reeve & Braithwaite, 1972), while pRE6 in V9A gives ML$^-$ lines which are unable to grow on any concentration of lactose (unpublished observations). When either plasmid is lost from V9A, the *lac* operon of the chromosome gives the host a weak lactose-positive phenotype (ML$^{-/+}$) and enables it to grow almost normally on lactose as carbon source: so we must assume that the ML$^-$ lesion in the plasmid permease gene is not a complete deletion, but causes production of defective M-protein sub-units which interfere with (negatively complement) the chromosomal *lac* permease.

Such a characteristic effect would be most easily explained by a small deletion or insertion of DNA at a particular point in the permease gene, perhaps brought about by one of the IS sequences which have been found both in plasmids and in the *E. coli* chromosome (Saedler & Heiss, 1973; Ptashne & Cohen, 1975). If this is the case, a reverse process leading to recreation of the plasmid in its original form might be expected to occur. While reversion to the ML$^+$ phenotype is found frequently in ML$^-$ colonies aged for several days on ML agar, doubtless due to the strong selection for cells better able to utilize lactose on these plates, the search for plasmid reversion is made laborious by the fact that two host chromosomal galactoside permeases which can accumulate lactose but are not induced by lactose (the melibiose permease and GP III) can give the same phenotypic change by mutating to constitutivity – a phenomenon not observed in *E. coli*. Thus, of 51 ML$^+$

revertants of ML⁻ lines obtained from V9A (F_K*lac*⁺), only one appeared to be the result of reversion of the plasmid to wild-type. This occurred in the line ML⁻21, now lost, from which only one ML⁺ line was tested: so it is possible that the conditions for reversion of the plasmid itself were not present in the other ML⁻ lines so far tested.

When another Lac plasmid (pRE6), able to promote its own transfer, was put into V9A in place of F_K*lac*, the new host-plasmid complex was also able to give ML⁻ variants which were able to revert to the ML⁺ state, and from these a second generation of ML⁻ lines was obtained which could again revert to ML⁺. These phenotype changes appear to be attributable to reversible changes in the plasmid Lac-permease gene, since the ML⁻ lines could transfer a plasmid to K12 which would convert LacZ⁻ but not LacY⁻ strains to Lac⁺, while three of the four first and second generation ML⁺ revertants tested could convert LacZ⁻Y⁻ K12 strains to Lac⁺ (see Table 13), and must therefore carry a complete *lac* operon on the plasmid.

It seems clear from these results that the characteristic *Lac* permease defect which occurs in both plasmids F_K*lac* and pRE6 can be repaired, at least in the *Klebsiella* strain V9A. DNA heteroduplex analysis should throw further light on this problem.

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