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Expression and Regulation of Lactose Genes Carried by Plasmids

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A number of plasmids carrying the lactose character have been studied. All of the plasmids examined so far code for proteins essential for lactose utilization, i.e., β-galactosidase and galactoside permease. None of them carries enzymatically or immunologically detectable thiogalactoside transacetylase. The expression of the two enzymes is both negatively and positively controlled: they are inducible by different galactosides and are sensitive to catabolite repression. Since the plasmid-coded lactose systems have many features in common with the *Escherichia coli* lactose operon, it is suggested that the plasmids could have acquired the lactose genes from an *E. coli* chromosome.

There are now many reports of transmissible extrachromosomal elements conferring the property of lactose utilization upon different species of *Enterobacteriaceae* normally and characteristically unable to ferment this sugar (3, 5-7, 9, 10, 14, 15, 18, 19, 21-23). Strains carrying such factors have been isolated from clinical specimens of organisms resistant to various antibiotics.

Numerous studies concerned with the nature of these lactose factors, their properties of transfer in other *Enterobacteriaceae*, their similarities with F *lac*$^+$ elements, their capacity to promote transfer of host chromosome, and their association with antibiotic resistance factors have been reported. However, very little is known about the expression and regulation of plasmid-carried lactose genes, their origin, or their possible relationship with the classical (chromosomal) "Lac" system of *Escherichia coli*. The studies reported here were aimed at answering some of these questions.

MATERIALS AND METHODS

Bacterial strains and media. Strains carrying different plasmids were generously given to us by L. Le Minor (Institut Pasteur) and J. Davies (University of Wisconsin). *E. coli* K-12 strain 2000X74 carrying a large deletion, including the entire lactose operon, and strain 2000X74/F *lac*$^+$ were from the collection in our laboratory. Some characteristics of these strains are given in Table 1. Minimal medium 63 [0.1 M KH_2PO_4, 0.02 M $(NH_4)_2SO_4$, 0.001 M $MgSO_4$, 0.001 mM $FeCl_3$; pH 7] supplemented with thiamine and a carbon source (generally 0.2% final concentration) was used. The cultures were grown at 37°C.

Transfer of plasmids. Plasmids carried by the strains listed in Table 1 were transferred into the *E. coli* Lac deletion strain 2000X74 by the method of Le Minor et al. (14).

Preparation of crude extracts. Overnight cultures were centrifuged, and the cells were suspended in buffer A [0.1 M Na_2HPO_4, 0.001 M $MgSO_4$, 10^{-4} M $MnSO_4$, 0.002 M (ethylene dinitrilo)tetraacetic acid, magnesium dipotassium salt; pH 7] and sonically treated. Generally, 1 g (wet weight) of bacteria was suspended in 2.5 ml of buffer A. The sonic extract was centrifuged at 15,000 rpm for 15 min, and the pellet was discarded.

Enzymatic assays. β-Galactosidase was assayed either in a toluene-treated bacterial suspension (to 2 ml of bacterial suspension, 50 μl of toluene and 50 μl of 1% sodium deoxycholate were added and agitated for 30 min at 37°C) or in crude extracts by the method of Pardee et al. (17). One unit is the amount of enzyme that hydrolyzes 1 nmol of *o*-nitrophenyl-β-D-galactoside (ONPG) per min at 28°C.

Galactoside permease was measured by the methyl-β-D-thiogalactoside (TMG) accumulation method of Rickenberg et al. (20). The results are expressed as nanomoles of intracellular TMG accumulated per milligram (dry weight) of bacteria.

Thiogalactoside transacetylase was assayed in crude extracts as described by Leive and Kollin (13) but with the following modifications. The final concentrations of the substrates acetyl coenzyme A and isopropyl-β-D-thiogalactoside (IPTG) were 0.01 and 1 M, respectively. Under these conditions, the assay is linear with respect to enzyme concentration provided that no more than 20% of the acetyl coenzyme A is used up.

Immunization procedures. Rabbits were immunized with wild-type *E. coli* β-galactosidase, which was purified to homogeneity by known procedures. A 500-μg amount of antigen in Freund adjuvant was injected in a footpad. Two months later, 1 mg of antigen in Freund adjuvant was injected intramuscularly. At varying intervals beginning 1 week after the booster, the rabbits were bled. The antiserum

TABLE 1. *Characteristics of strains used*

Strains	Lac⁺ character	Antibiotic resistance[a]	Origin
2000X74	–	None known	Institut Pasteur
2000X74/F *lac*⁺	+	None known	Institut Pasteur
Proteus mirabilis	+	Su	L. Le Minor
Klebsiella aerogenes	+	A, S, C, T, Su	J. Davies[b]
Salmonella typhimurium	+	A, S, Su	L. Le Minor
Enterobacter cloacae	+	A, S, C, T, Su	J. Davies[b]
Salmonella oranienburg	+	S, C, Su	L. Le Minor
Enterobacter hafniae	+	None known	L. Le Minor
Proteus morganii	+	None known	L. Le Minor
Enterobacter liquefaciens	+	None known	L. Le Minor

[a] Su, Sulfamides; A, ampicillin; S, streptomycin; C, chloramphenicol; T, tetracycline.
[b] Isolated in Peter Bent Brigham Hospital, Boston.

was kept at 4°C with 0.1% sodium azide.

Immunoprecipitation in liquid media. Precipitation in liquid media was used as a measure of the immunological relatedness of the different plasmid-coded enzymes (β-galactosidase) as well as for the detection of other cross-reacting material in the extracts.

(i) Antiserum obtained against *E. coli* β-galactosidase, at a given dilution, was incubated with increasing amounts of extracts containing either *E. coli* β-galactosidase or plasmid-carried β-galactosidase for 18 h at 4°C. The precipitate that formed, which contained the antibody-antigen complex, was separated from the free, soluble antigen by centrifugation. By assaying free antigen, i.e., β-galactosidase, in the supernatant, the amount of antigen precipitated by the antiserum could be calculated. The percentages of cross-reaction could be evaluated by comparing the amounts of different β-galactosidases precipitated by the same amount of antiserum.

(ii) To determine the presence of an antigen cross-reacting with anti-*E. coli* transacetylase antiserum, the following method was used. The antiserum was diluted so as to obtain 50% precipitation of the relevant antigen, i.e., *E. coli* transacetylase. This diluted antiserum was added to a mixture of two different extracts (at equal protein concentration), one containing *E. coli* transacetylase (1 volume) and the other containing the tentatively present cross-reacting material (20 volumes). The soluble-antigen assay was then performed as described above.

Sedimentation in sucrose density gradients. Sedimentation in sucrose density gradients was carried out in a Spinco SW56 rotor. A 0.1-ml volume of a crude extract containing 8 mg of protein per ml was layered on a linear sucrose gradient (5 to 20%) and centrifuged for 4 h at 56,000 rpm. The sedimentation coefficients were calculated assuming an *S* value of 6.3 for *E. coli* alkaline phosphatase.

RESULTS

For all the experimental work described here, plasmids were transferred in *E. coli* 2000X74, which carries a deletion of the entire lactose region. This was done to study the expression of the lactose system of different plasmids within the same genetic background.

General properties of Lac⁺ plasmids. *E. coli* 2000X74 carrying different plasmids does not contain significant levels of β-galactosidase when grown in minimal medium 63-thiamine (supplemented with glycerol). When IPTG was added to the medium or lactose was utilized as carbon source, high levels of β-galactosidase were observed (Table 2). As it can be seen from the results, the synthesis of β-galactosidase is inducible in all cases. The fact that lactose is able to induce β-galactosidase synthesis suggests the presence of a plasmid-coded galactoside permease. In the following sections, we shall consider successively the different elements of the lactose system.

β-Galactosidase. To characterize the different β-galactosidases, we have undertaken an extensive immunological, physicochemical, and kinetic study of this enzyme. A full account of these results will be presented separately (N. Guiso and A. Ullmann, manuscript in preparation). However, in the present paper, we should like to mention a few characteristics of these enzymes.

The fully induced levels of the different plasmid-determined β-galactosidases are quite different, ranging from 2,000 to 12,000 U/mg (Table 2). Some additional characteristics of these β-galactosidases are shown in Table 3. The kinetic constants were determined by measuring initial velocities as a function of substrate concentration in the absence or presence of added inhibitor (phenyl-ethyl-β-D-thiogalactoside [TPEG]). The Michaelis constants (K_m) for ONPG and inhibition constants (K_i) for TPEG were calculated from Lineweaver-Burk plots. These constants are quite similar to those found for the *E. coli* β-galactosidase. The sedimentation coefficients determined in sucrose density gradients are practically the same as those of the *E. coli* β-galactosidase, which is known to be a tetramer of 540,000 total molecular weight. The thermal stabilities of these β-galactosidases have been determined by measuring their inactivations at 52°C. The half-lives of inactivation can thus be calculated and they appear to be very different, giving evidence of some structural differences between these enzymes.

Antibodies against *E. coli* β-galactosidase recognize all plasmid enzymes, but the extent of cross-reactivity calculated as described in

TABLE 2. *Induced and uninduced levels of plasmid-determined β-galactosidases*

Plasmid origin[a]	β-Galactosidase sp act (U/mg [dry wt] of bacteria)		
	Glycerol[b]	Glycerol + IPTG[c]	Lactose[d]
E. coli F *lac*	36	20,000	8,400
P. mirabilis	41	12,000	2,300
K. aerogenes	57	8,000	1,800
S. typhimurium	14	5,000	3,000
E. cloacae	10	6,000	1,300
S. oranienburg	14	6,000	2,600
E. hafniae	70	12,000	3,400
P. morganii	11	2,000	1,300
E. liquefaciens	91	2,000	1,200

[a] All plasmids are carried by *E. coli* 2000X74.
[b] Final concentration, 0.2%.
[c] Final concentration, 5×10^{-4} M.
[d] Final concentration, 0.2%.

TABLE 3. *Properties of plasmid-determined β-galactosidases*

Plasmid origin	Kinetic constant (M)		Sedimentation constant	Thermal stability, half-life at 52°C (min)
	K_m	K_i		
E. coli F *lac*	2×10^{-4}	1.6×10^{-6}	15.5	1,920
P. mirabilis	2×10^{-4}	2.5×10^{-6}	15	1,620
K. aerogenes	2×10^{-4}	2.5×10^{-6}	15	1,320
S. typhimurium	2×10^{-4}	1.6×10^{-6}	15	220
E. cloacae	2×10^{-4}	3×10^{-6}	15.4	23
S. oranienburg	2×10^{-4}	1.6×10^{-6}	14.5	16
E. hafniae	2×10^{-4}	3×10^{-6}	15	7
P. morganii	2×10^{-4}	2.6×10^{-6}	15.2	5
E. liquefaciens	2×10^{-4}	1.6×10^{-6}	14.8	2

Materials and Methods is variable, ranging from 31 to 70%. At the present time, no definite conclusion can be drawn concerning the immunological relatedness of these enzymes, since their turnover numbers are not known.

Galactoside permease. The specificity and kinetic properties of the Lac permease system of *E. coli* were described by Rickenberg et al. (20). Based on these studies, and using the method of accumulation of a galactoside TMG against a concentration gradient, we determined some parameters of the plasmid-coded transport systems (Table 4). The concentration of external TMG, which corresponds to half-saturation of the system (K_m), is practically the same for each plasmid-carried permease and is quite similar to that found for the *E. coli* permease.The maximal capacities, i.e., the maximum internal concentrations of galactoside achieved when the system is saturated, vary slightly from one plasmid to the other. The β-galactosidase levels vary more widely (Table 2) than the permease levels, and for six plasmids the ratios of β-galactosidase/permease range from 10 to 50 and are quite similar to that obtained for *E. coli*. These results suggest that the expression of the "*z*" and "*y*" genes of the plasmids are coordinated, as in *E. coli*, and suggest further that the differences observed in absolute activities reflect differences in the levels of transcriptive activities of the genes, rather than differences in the specific activities of the proteins involved. However, the ratio for the *Enterobacter hafniae* plasmid (120) is significantly different. Whether this is due to an altered β-galactosidase or permease on this plasmid is not known since the molecular specific activities of the two proteins have not been determined.

Thiogalactoside transacetylase. The lactose operon of *E. coli* involves three structural genes coding, respectively, for β-galactosidase, galactoside permease, and thiogalactoside transacetylase. As it has been shown above, the first two enzymes are expressed by all plasmids.

To assay for transacetylase activity, extracts were prepared from fully induced cultures (Table 5). No significant acetylase activity could be detected. Since the acetylase assay method involves heating at 70°C (to inactivate acetyl coenzyme A deacetylase), the negative results could be due to inactivation of an eventually heat-labile transacetylase. Therefore, transacetylase activity was also measured, omitting the heating step (column 2 of Table 5). The results are not significantly different from those obtained after heating the extract, suggesting that thiogalactoside transacetylase is indeed absent in all these instances.

TABLE 4. *Properties of plasmid-determined galactoside permease*[a]

Plasmid origin	K_m for permease (M)	Maximal capacity for permease (nmol of TMG accumulated/mg [dry wt] of bacteria)
E. coli F *lac*	5.5×10^{-4}	280
P. mirabilis	5.5×10^{-4}	230
K. aerogenes	6×10^{-4}	260
S. typhimurium	5×10^{-4}	165
E. cloacae	5×10^{-4}	153
S. oranienburg	5.5×10^{-4}	285
E. hafniae	5.5×10^{-4}	100
P. morganii	5.5×10^{-4}	180
E. liquefaciens	5.5×10^{-4}	200

[a] Bacteria were grown in minimal medium in the presence of 0.2% glycerol and 2×10^{-4} M IPTG.

TABLE 5. *Thiogalactoside transacetylase of plasmids*

Plasmid origin	Transacetylase sp act (U/mg of protein)	
	After heating at 70°C	Without heating at 70°C
E. coli F *lac*	400	420
P. mirabilis	1	4
K. aerogenes	1.5	3
S. typhimurium	2	0.6
E. cloacae	1	4
S. oranienburg	5	8
E. hafniae	1	2
P. morganii	1.5	1.5
E. liquefaciens	1	1

To reinforce these observations, an immunological approach was used. Antibodies against *E. coli* transacetylase (a generous gift from I. Zabin and A. Fowler) were utilized to detect the eventual presence of an antigen. By using the method of precipitation in liquid media, as described in Materials and Methods, we tried to detect the presence of a cross-reacting material that would displace *E. coli* transacetylase from its antibody-antigen complex. None of the extracts prepared from strains carrying the different plasmids (even in 20-fold excess with respect to *E. coli* transacetylase containing extract) was able to displace *E. coli* transacetylase from the immunoprecipitate. It may be concluded that the plasmids do not code for a protein immunologically similar to thiogalactoside transacetylase.

Regulatory elements. Control of gene expression in the lactose operon of *E. coli* is known to be mediated by specific regulatory genes in the operon. It was of interest to know whether the regulation of plasmid-determined lactose genes can also be accounted for by the classical operon model of Jacob and Monod (4, 11). Negative control is mediated by the lactose repressor, which binds to the operator, thus preventing transcription of the structural genes. Coordinate transcription of the genes is initiated at the promoter. This genetic element contains not only recognition sites for ribonucleic acid polymerase but also for a protein exerting a positive control function (catabolite gene activator protein) in the regulation of catabolite repression (4).

Without performing binding measurements with purified systems, we were able to obtain information on the plasmid-determined repressors by studying the in vivo induction of β-galactosidase. It has been established (2) that the in vivo induction characteristics reflect adequately the repressor-inducer affinities measured by direct binding. Figures 1 and 2 show the differential rate of β-galactosidase synthesis as a function of inducer concentration. The inducer concentrations (TMG as well as IPTG) required for full induction are much lower in the plasmid-carrying strains than in *E. coli*. From these curves, one can see the inducer concentration necessary for half-maximal induction. Compared to that in *E. coli*, half-maximal induction in most plasmid-carrying strains can be achieved with 10 to 20 times lower IPTG or TMG concentrations. With all of the Lac plasmids, IPTG was a stronger inducer than TMG, showing a half-maximal induction concentration six to eight times lower for IPTG than for TMG.

To determine coordinate transcription of plasmid-carried lactose genes, we measure the differential rate of both β-galactosidase and galactoside permease synthesis at different levels of induction. For all plasmids examined, the expression of the two enzymes seems to be coordinated (Fig. 3).

The synthesis of all plasmid-carried β-galactosidases is sensitive to catabolite repression. The differential rate of enzyme synthesis is much higher in a medium containing glycerol or succinate as carbon source than it is in a medium containing glucose (Table 6). Moreover, this repression is reversed by addition of cyclic adenosine 3′,5′-monophosphate.

DISCUSSION

The main conclusion emerging from our studies is that the expression and regulation of plasmid-coded lactose genes resemble, in many respects, those of the lactose operon of *E. coli*. All plasmids carry genes for a functional β-galactosidase and galactoside permease. The synthesis of these proteins is both negatively and positively controlled. They are inducible by the inducers known to be active in the *E. coli* system. Moreover, they are sensitive to catabolite repression. Although we do not yet possess polar mutants, our results strongly suggest that the plasmid-carried genes involved in lactose utilization behave as a unit of coordinate expression, i.e., an operon.

It seems particularly significant that plasmid-coded β-galactosidases exhibit kinetic constants and sedimentation coefficients similar to those of *E. coli* enzyme. It is as yet difficult to evaluate the extent of their structural differences as revealed by different thermal inactivation properties and partial cross-reaction with anti-*E. coli* β-galactosidase antiserum. How-

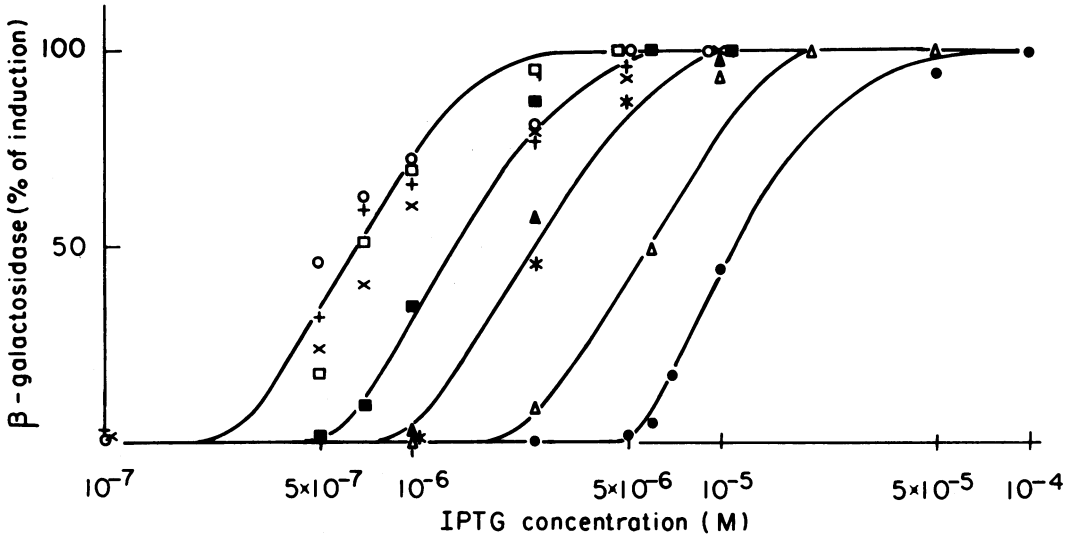

FIG. 1. *Differential rates of β-galactosidase synthesis as a function of IPTG concentration. Bacteria were grown for several generations in glycerol-minimal medium containing various concentrations of IPTG. The amount of β-galactosidase formed was then determined. 100% induction represents maximal induced enzyme levels. Symbols: ●, E. coli F lac⁺; ○, S. oranienburg plasmid; ■, S. typhimurium plasmid; □, K. aerogenes plasmid; ▲, P. mirabilis plasmid; △, P. morganii plasmid; ×, E. hafniae plasmid; +, E. liquefaciens plasmid; *, E. cloacae plasmid. All plasmids are carried by E. coli strain 2000X74.*

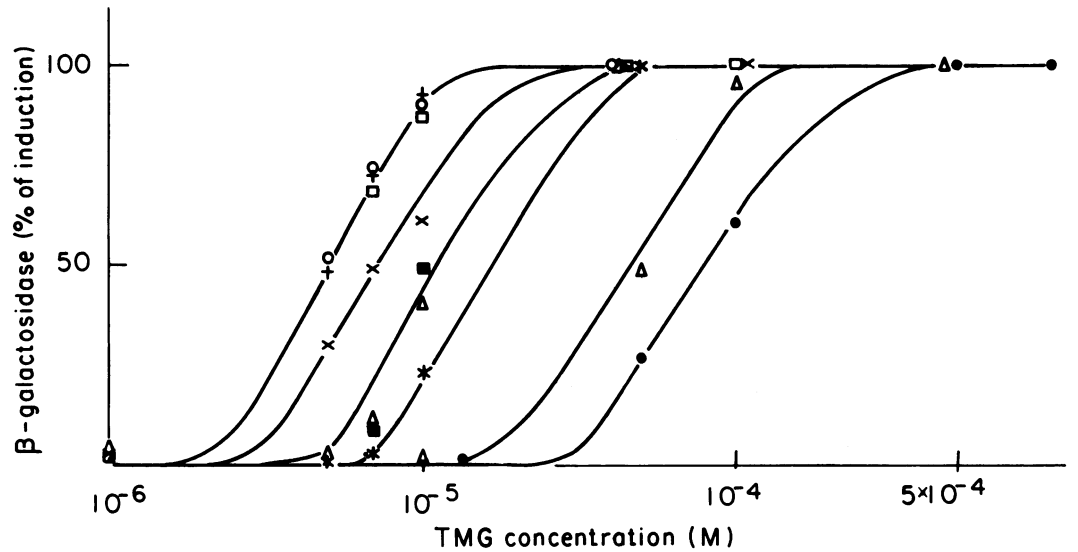

FIG. 2. *Differential rates of β-galactosidase synthesis as a function of TMG concentration. Conditions and symbols as in Fig. 1.*

ever, the fact that antiserum against the *E. coli* enzyme recognizes all of the enzymes is most significant, since the same antiserum does not react at all with the β-galactosidase produced by other *Enterobacteriaceae* such as *Klebsiella oxytoca*, *Enterobacter aerogenes*, and *Serratia rubidae* (data not shown).

Plasmid-coded galactoside permeases share most of the properties of the *E. coli* permease, i.e., the same affinity for substrates (Table 4) and the same inhibition characteristics by different galactosides (data not shown).

The regulatory elements carried by the plasmids are functionally similar to those of the *E.*

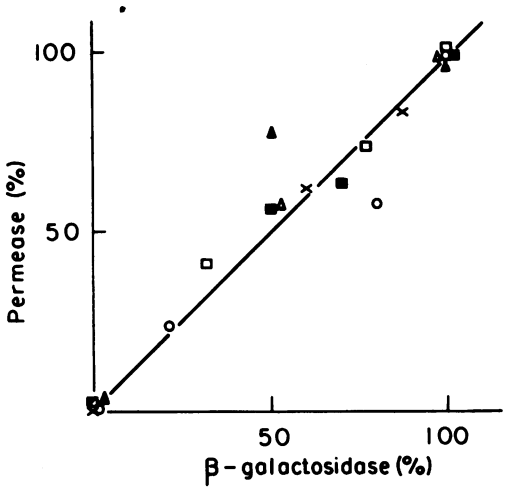

FIG. 3. *Coordination of β-galactosidase and galactoside permease synthesis. Conditions and symbols as in Fig. 1.*

coli lactose operon. The coordinate expression of the two genes (β-galactosidase and galactoside permease) and the sensitivity of enzyme synthesis to catabolite repression argue strongly in favor of the existence of a specific promoter site.

In the absence of inducer, the plasmid-coded lactose genes are barely expressed. Different galactosides known to induce enzyme synthesis or to be competitive inhibitors of induction in *E. coli* behave similarly in the plasmidic lactose systems. This strongly suggests the existence of an operator-repressor interaction similar to that described for the *E. coli* Lac operon.

The study of the in vivo induction characteristics revealed that half-maximal induction of all plasmidic enzymes can be achieved with 10 to 20 times lower inducer concentrations, as is the case in the *E. coli* system. This finding can be interpreted in two ways: the plasmidic repressors have either a lower affinity for the operator or a higher affinity for inducer. The first interpretation would imply high basal (uninduced) levels of enzymes—which is not the case. Therefore, we assume that lactose repressors carried by plasmids exhibit an increased affinity for inducer. Inducer affinity estimations based on in vivo measurements could be impaired by the presence of a functional permease. Therefore, we specifically blocked the permease by using thiodigalactoside (TDG) as described by Herzenberg (10). Under these conditions—where the permease is nonfunctional—the values for half-maximal induction of β-galactosidase changed significantly for *E. coli* (from 1.1×10^{-5} to 10^{-4} M) but only slightly for the *Salmonella oranienburg* plasmid (from 1.5×10^{-6} to 4×10^{-6} M). This suggests that the observed differences in affinity for the inducer cannot be accounted for by the functional properties of the permease itself. We conclude that plasmidic repressors compared with that of *E. coli* have much higher affinities for inducer. The *E. coli* Lac repressor has been studied extensively (for references see Müller-Hill [16]). A great number of mutants defective in inducer binding capacity have been isolated, but to our knowledge only one tight-binding repressor has been described (Gilbert and Müller-Hill [8]) exhibiting a binding constant increased by a factor of two compared with that of the wild-type repressor.

It is quite striking that, under experimental conditions of selection, tight-binding repressor mutants have not been found more frequently, whereas all of the plasmidic repressors examined so far seem to belong to this category. Whether the selection of highly inducible repressors on plasmids is correlated with their extrachromosomal localization remains an open question.

Since the expression and regulation of the lactose system coded by plasmids of various origins are strikingly similar to those of the *E. coli* Lac operon, it is tempting to suppose that plasmids acquired their lactose genes from the chromosome of *E. coli*. On the other hand, one of the most surprising results is that none of the plasmids examined so far codes for a thiogalactoside transacetylase. There are, however, two factors that might account for the absence of

TABLE 6. *Catabolite repression of plasmid-determined β-galactosidases*[a]

Plasmid origin	β-Galactosidase sp act (U/mg [dry wt] of bacteria)			
	Glucose[b]	Glucose + cAMP[c]	Glycerol[d]	Succinate[e]
E. coli F *lac*	6,800	14,000	20,000	24,000
P. mirabilis	6,000	9,100	12,000	24,000
K. aerogenes	2,100	5,800	8,700	15,000
S. typhimurium	1,000	2,600	3,000	6,500
E. cloacae	1,700	3,500	6,000	10,100
S. oranienburg	1,600	4,000	6,900	10,800
E. hafniae	5,500	8,800	12,000	14,000
P. morganii	540	1,200	2,000	3,500
E. liquefaciens	1,600	3,100	4,000	5,300

[a] Bacteria were grown in minimal medium in the presence of 5×10^{-4} M IPTG.

[b] Final concentration, 0.2%.

[c] Final concentration, 5×10^{-3} M. cAMP, Cyclic adenosine 3',5'-monophosphate.

[d] Final concentration, 0.2%.

[e] Final concentration, 0.1%.

this enzyme on plasmids: (i) the lactose genes could have been "picked up" from bacteria lacking this enzyme; (ii) the gene sequence coding for transacetylase could have been excised or inactivated during integration into the plasmid.

In conclusion, our work appears to furnish rather strong evidence that lactose enzymes coded by plasmids are of bacterial origin. In contrast, most of the other known enzyme systems coded by plasmids do not appear to have stemmed from bacteria but perhaps from fungi (1).

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