GENETIC CONTROL OF RAFFINOSE UTILIZATION IN *ESCHERICHIA COLI*[1]

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The genetic and physiological control of β-galactosidase in *Escherichia coli* has been the subject of extensive investigations. It has been clearly shown that the presence of this inducible enzyme is dependent on the genic constitution of the cell (Lederberg *et al.*, 1951). In many cases gene mutation has led to a complete loss of ability by the cell to produce the enzyme; in other instances a decrease in the amount of enzyme produced was noted. With some lactose mutants a lack of ability of certain inducers to promote the formation of β-galactosidase was observed, and in one instance (Rickenberg, 1954) was shown to be associated with a decreased permeability to lactose. However, no clear evidence demonstrating a genic control over inducer specificity rather than innate ability to form enzyme has been presented.

The formation of β-galactosidase in *E. coli* strain K-12 can be induced by galactose and many β-galactosides (Monod *et al.*, 1951; Lester and Bonner, 1952). Neolactose (altrose-β-D-galactoside) is an exceptional substrate, for it is not an inducer of the enzyme; neolactose utilizing strains have been shown to be constitutive β-galactosidase producers (Lederberg, 1951). Some α-galactosides, such as melibiose, are also effective inducers of β-galactosidase, but raffinose, although containing a melibiose moiety, is ineffective as an inducer and as a carbon source (Monod *et al.*, 1951; Lester and Bonner, 1952). α-Galactosidase has been shown to be induced by galactose and some α-galactosides, but the ability of β-galactosides to induce α-galactosidase appears to depend on the strain of *E. coli* used. Thus, Porter *et al.* (1953) report that, with *E. coli* strain B, lactose can serve as an inducer of α-galactosidase, whereas, in our experience, lactose is ineffective as an inducer in K-12. Such variations in inducer specificity are suggestive of a genic control over the sensitivity of the cell to various inducers.

The work to be described is concerned with the utilization of raffinose by mutants of *E. coli* strain K-12. This organism is capable of utilizing melibiose (glucosyl-α-galactoside) with concomitant production of α-galactosidase, but it is unable to utilize raffinose (sucrosyl-α-galactoside) as a sole carbon source. Mutants have been obtained which can utilize raffinose, and show α-galactosidase activity when raffinose is used as a carbon source. Evidence will be presented which indicates that the ability of raffinose to act as an inducer of α-galactosidase is under genic control. The phenotypic expression of the raffinose gene will be shown to depend on the presence of genes for both constitutive and induced β-galactosidase formation.

MATERIALS AND METHODS

The organisms used were *E. coli* strain K-12 and five strains derived from K-12: W-1485, a non-lysogenic strain; W-1317, a constitutive β-galactosidase producer; Y-10, which requires threonine, leucine, and thiamin; H-34, which requires histidine; and 58-161, which requires methionine and biotin. A defined salts medium (Rickenberg *et al.*, 1953) was used for liquid culture, and for the plates used in recombination experiments; the salts medium was supplemented with the appropriate vitamins and amino acids for the culture of auxotrophic strains. Washed agar was used in the preparation of solid media. The sugars and the salts medium were autoclaved separately. All cultures were incubated at 37 C, the liquid cultures with agitation.

Quantitative determinations of β-galactosidase were made with the chromogenic substrate *o*-nitrophenyl-β-D-galactoside (β-ONPG) (Lederberg, 1950; Rickenberg *et al.*, 1953). To distinguish between adaptive and constitutive β-galactosidase producers, the following test was employed. Cells were grown in 1.0 ml of medium

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containing 0.1 to 0.2 per cent glycerol for 24 to 48 hr. Two drops of toluene were added and the cultures were shaken for 30 min at 37 C. Then 0.5 ml of 1×10^{-3} M β-ONPG in 0.1 M sodium phosphate buffer, pH 7.0, was added. Cultures of constitutive β-galactosidase producers showed a strong yellow color within 5 min, while adaptive strains showed no appreciable color on incubation for 30 min at room temperature.

α-Galactosidase was determined using a similar qualitative method employing *o*-nitrophenyl-α-D-galactoside (α-ONPG) as the substrate (Porter *et al.*, 1953). To 1.0 ml of culture was added 1.0 ml of 3×10^{-3} M α-ONPG in 0.1 M phosphate buffer, pH 7.0, and incubated for 1 hr at 37 C. Cells grown on glycerol plus galactose, methyl- or thiomethyl-β-D-galactoside, showed very weak color after incubation for 1 hr with α-ONPG, and cultures grown on lactose or on glycerol alone showed no appreciable color. Only melibiose-grown cells showed a strong yellow color.

Turbidity measurements were made with a Klett-Summerson colorimeter using the 660 (red) filter. Protein determinations were made on whole cells by the method of Lowry *et al.* (1951) using, in essence, their procedure for insoluble proteins. Values for protein determined in this fashion were higher than those obtained from extracts of cells prepared by sonic disintegration.

The procedure for crosses was as follows. Cultures of the two strains to be crossed were grown in 5 ml of nutrient broth in 50-ml centrifuge tubes. After incubation for 16 to 18 hr, 10 ml of fresh nutrient broth was added and the cultures were incubated for an additional 2 to 3 hr. The cultures were then centrifuged and resuspended in 20 ml of saline and recentrifuged. After three washings with 20-ml portions of saline, the cells were resuspended in 15 ml of minimal medium without any carbon source. The suspensions were mixed, and 0.05- or 0.1-ml aliquots were spread on minimal agar plates containing 1 per cent glycerol. For a control, aliquots taken from the cultures before mixing were also spread on minimal plates containing 1 per cent glycerol; the same amounts of cells were used in the control plates as were used in the mixtures. In no case did colonies appear in the control plates. After incubation for 72 hr colonies were picked from the recombination plates and streaked in a single straight line on fresh glycerol plates. The growth which appeared was picked into tubes containing 1.0 ml of 0.15 per cent glycerol minimal, and incubated for 18 to 24 hr. A scant 3-mm loopful of the glycerol culture was used to inoculate tubes containing 1.0 ml of 0.15 per cent glycerol and 1.0 ml of 0.6 per cent raffinose minimal medium. The tubes were incubated for 48 hr. The raffinose tubes were examined for growth, and the glycerol cultures were tested for constitutive β-galactosidase formation.

RESULTS

E. coli strain K-12 is unable to utilize raffinose as a carbon source, but grows well on melibiose. Two possibilities were considered which might have accounted for this anomaly: (a) the cells were impermeable to raffinose, and (b) raffinose did not induce the formation of α-galactosidase. These possibilities were tested by examining the ability of cells to utilize raffinose while α-galactosidase was being induced by melibiose. Basal salts media were prepared containing melibiose, raffinose, melibiose + raffinose, or sucrose. Ten ml of each medium in a 125-ml erlenmeyer flask were inoculated with a drop of a glycerol grown culture of strain K-12 or strain W-1485. The flasks were incubated for 48 hr and the turbidity determined. The results are given in table 1. It can be seen that no growth was observed on either raffinose or sucrose alone, whereas on a mixture of raffinose and melibiose more growth was obtained than could be accounted for by melibiose alone. The additional growth corresponds to the galactose moiety of raffinose. These results indicate that hypothesis (a) is unlikely.

A search for mutants of strains Y-10 and H-34 capable of utilizing raffinose as a sole carbon source was instituted to determine whether such strains were constitutive α-galactosidase producers. The plating of large numbers of cells on raffinose agar yielded no raffinose utilizers. If

TABLE 1

Growth of Escherichia coli strains K-12 and W-1485 on melibiose and raffinose

Sugar	Relative Turbidity	
	K12	W-1485
0.05% Melibiose	68	69
0.15% Raffinose	2	2
0.05% Sucrose	2	2
0.05% Melibiose + 0.15% raffinose	118	122

raffinose utilizers were constitutive α-galactosidase producers, such cells might have a shorter lag on melibiose. Then, alternate culture in glucose and melibiose media might increase the proportion of raffinose utilizers (Cohen-Bazire and Jolit, 1953). However, this procedure also proved unsuccessful. Finally, a washed suspension of strain H-34 was treated with ultraviolet light. About 99.9 per cent kill was observed; the viable population was approximately 2×10^6 cells per ml. Most of the irradiated suspension was spread on several plates, and 1.0 ml was added to a tube containing 10 ml of 0.3 per cent raffinose medium. The cultures were incubated at 37 C and observed daily. At 7 days no growth was observed in either the plates or the tube, and the plates were discarded. At 10 days there appeared to be an increase in turbidity in the tube, and by 11 days the turbidity was that of a fully grown culture. Aliquots of the tube were streaked on 1.5 per cent raffinose agar, and at 72 hr some of the colonies which appeared were picked and restreaked. The isolates were capable of growth in both solid and liquid media with raffinose as the sole carbon source, and showed a requirement for histidine. One isolate was kept for further investigation.

A similar procedure was carried out with strain Y-10, and again no growth was observed on plates. Seven tubes of 0.3 per cent raffinose were inoculated with the irradiated suspension, about 5×10^6 viable cells per tube. At 4 to 5 days the turbidity began to increase, and the cultures were fully grown on the following day. Raffinose utilizers were isolated, and all showed the nutritional requirements of the parent strain. Subsequently, five more raffinose utilizers were isolated using strain W-1485 as the parent strain. The isolates were further characterized with respect to valine inhibition and lysogenicity (strains H-34 and Y-10) or sensitivity to phage (strain W-1485). Only two isolates were used extensively in subsequent work; they are designated as strains H-34R and Y-10R.

The raffinose-utilizing strains showed slower growth on raffinose than on other carbon sources, and no growth on sucrose. To determine the extent of raffinose utilization 10 ml of basal medium containing either 0.1 per cent galactose or 0.3 per cent raffinose (equivalent to 0.1 per cent galactose) was inoculated with one drop of a glycerol grown culture of strain H-34R, incubated for 40 hr, and the turbidity was measured.

TABLE 2

Growth of Escherichia coli strain H-34R on galactose and raffinose

Sugar	Relative Turbidity
0.1% Galactose	140, 137
0.3% Raffinose	142, 138

TABLE 3

β-Galactosidase activity of Escherichia coli strains Y-10 and Y-10R grown on various carbon sources

Carbon Source	Specific Activity*	
	Y-10	Y-10R
Glycerol	0.09	52
Glucose	0.05	13
Lactose	15	51
Melibiose	18	63
Raffinose		46

* mμmoles β-ONPG hydrolyzed per μg protein per 10 min at 37 C.

The results shown in table 2 indicate that only the galactose moiety was utilized suggesting that α-galactosidase activity was present.

When the raffinose utilizing cultures were tested for α-galactosidase using α-ONPG as substrate, only cultures grown on melibiose and raffinose showed high activity. On glycerol, glucose, maltose and lactose the activity was nil. Such results indicated that α-galactosidase was not a constitutive enzyme, and that raffinose was now serving as an inducer of α-galactosidase. Raffinose utilizing strains were tested for the production of β-galactosidase after growth on various sugars. The results obtained with strain Y-10R are shown in table 3, and these are typical of the results obtained with the other seven strains of raffinose utilizers. All the raffinose utilizing strains form β-galactosidase constitutively.

The simultaneous appearance of characters for constitutive β-galactosidase production and raffinose utilization might appear to be the result of a single mutational event. Yet, the inability to obtain raffinose utilizers upon plating large numbers of cells (even after ultraviolet irradiation) on raffinose plates would indicate that if a single mutation is implicated its occurrence must be rare indeed. The time period required for the appearance of raffinose utilizers in liquid

TABLE 4

Spontaneous mutation of various strains of Escherichia coli to raffinose utilization

Strain	No. Plated on Raffinose	No. of Colonies	Frequency (No. per 10^8)
W-1485....	5.4×10^8	0	
W-1485....	5.0×10^9	0	
K-12.....	5.2×10^{10}	0	
H-34.....	5.4×10^{10}	0	
W-1317....	4.5×10^7	3	6.7
W-1317....	3.9×10^7	3	7.7
W-1317....	3.0×10^9	40	1.3
W-1317....	3.4×10^{10}	485	1.4
#48*.....	1.15×10^{10}	1034	9.0

* #48 is a β-galactosidase constitutive strain obtained from one of the crosses to be described.

cultures suggests that mutations for β-galactosidase constitutiveness and raffinose utilization might have occurred in different cells. Perhaps upon recombination of these two mutant types the double character of the raffinose utilizers would emerge.

Several quantitative experiments were run to determine the frequency of mutation to raffinose utilization in both adaptive and constitutive strains. The cells were grown on 0.12 per cent glycerol agar, and the numbers of raffinose utilizers were ascertained by plating aliquots of the cultures in 1.5 per cent raffinose agar. The plates were incubated for 4 days at 37 C. The results are summarized in table 4. Among the adaptive strains (W-1485, K-12, H-34) no colonies appeared on the raffinose plates, although the numbers of cells plated exceeded 10^{11}. However, when constitutive strains (W-1317, #48) were examined, colonies did appear on the raffinose plates. About 50 colonies were picked at random; all were capable of utilizing raffinose as a sole carbon source and showed constitutive β-galactosidase production. These observations, and that of the β-galactosidase constitutive character of all raffinose utilizers so far obtained, are in accordance with a hypothesis that the phenotypic expression of a mutation to raffinose utilization is dependent on the concomitant presence of a β-galactosidase constitutive character.

A test of this hypothesis can be made by observing the raffinose (R) and β-galactosidase constitutive (C) characteristics of the progeny of a cross between a raffinose utilizer R^+C^+ with a raffinose negative strain R^-C^-. If raffinose and β-galactosidase constitutiveness are one gene, then only the parental types, R^+C^+ and R^-C^-, should be found among the progeny of the cross. On the other hand, if these characteristics are due to separable genes, four classes are possible among the progeny, R^+C^+, R^-C^-, R^-C^+, and R^+C^-. However, if the phenotypic expression of the raffinose gene is dependent on the presence of the gene for β-galactosidase constitutiveness, R^+C^- would not be distinguishable from the R^-C^- class.

TABLE 5

Segregation of β-galactosidase constitutiveness from raffinose utilization

Cross (Strains of *Escherichia coli*)	No. of Prototrophs Examined	Classes Recovered			
		R^+C^+	R^-C^-	R^-C^+	R^+C^-
		%	%	%	%
58-161 × Y-10R.....	108	67	30	3	0
H-34 × Y-10R	197	54	43	3	0
H-34R × Y-10	210	13	53	34	0
H-34R × Y-10	148	9	64	27	0

Four such experiments were run using the raffinose utilizing strains H-34R and Y-10R. The results are summarized in table 5. The data obtained for both crosses involving strain Y-10R are very similar, as are those for the duplicate crosses involving strain H-34R. The occurrence of the class R^-C^+ shows that these two characters are separable. Since R^-C^+ occurs, then the phenotype R^+C^- should be expected. Its absence supports the hypothesis that the expression of R^+ is dependent on C^+.

However, it is also possible to explain these results by assuming that (a) there is a very high frequency of mutation of R^+ to R^-, or (b) the combination R^+C^- is lethal. The first assumption was largely negated by examining the frequency of mutation of strain H-34R to R^-C^+. Strain H-34R was grown in nutrient broth, diluted in saline, and spread on glycerol minimal agar plates. The colonies were picked into 0.15 per cent glycerol broth, and also streaked on raffinose agar plates. The glycerol broth cultures were used to inoculate raffinose and glycerol media. The raffinose cultures were examined for growth and the glycerol cultures were tested for β-galactosidase activities. All of the 205 isolates examined showed growth on raffinose and constitutive β-galactosidase formation. Thus, the

occurrence of R^-C^+ in a cross of R^+C^+ by R^-C^- is probably not due to a spontaneous loss of the raffinose character.

The second possibility, that of the lethality of the R^+C^- combination, was also examined. If the genotype R^+C^- was not lethal, such strains would have the phenotype R^-C^-. Then amongst the progeny of a cross having the phenotype R^-C^-, there would be expected to be some with the genotype R^+C^-. Such a situation would be amenable to examination by crossing the phenotype R^-C^- recombinants to a R^-C^+ strain. However, inserting markers suitable for crossing experiments in these strains was not technically feasible. On the other hand, an examination of the frequency of spontaneous mutation to raffinose utilization by R^-C^- phenotypes might yield the desired information. Since it has already been shown that the spontaneous occurrence of raffinose utilizers in wild type (R^-C^-) strains is extremely rare, it might be assumed that those members of the phenotypic class R^-C^- which bear the same genotype would also show no obvious spontaneous mutation to raffinose utilization. On the other hand, members of the class R^-C^- which are genotypically R^+C^- should, upon mutation to C^+, manifest the raffinose utilizing character.

The phenotypically R^-C^- prototrophs obtained in the first cross shown in table 5 were examined for spontaneous appearance of raffinose utilizers in two ways. First, each of the R^-C^- strains were inoculated into tubes containing 1.0 ml of 0.01 per cent glycerol medium and incubated for 20 hr, giving a population of about 2×10^8 per tube. Each culture was then plated in 1.5 per cent raffinose agar and incubated for 4 days. Secondly, each R^-C^- strain was inoculated into 5.0 ml of minimal broth containing 0.01 per cent glycerol + 0.6 per cent raffinose; the glycerol would give a total population of about 1×10^9 cells per tube (light growth). The tubes were incubated and examined for 4 days for heavy growth, which would indicate utilization of raffinose. Out of 32 phenotypically C^-R^- prototrophs so examined, 29 showed growth in either raffinose plates or tubes. Thus, it appears likely that the class R^+C^- is not lethal and gives the phenotype R^-C^-.

The evidence so far presented has shown that the R^+ genotype is not expressed unless the genome is also C^+. However, it might be that the C^+ gene does not exert its primary control on β-galactosidase, but determines the formation of an internal β-galactosidase inducer, or otherwise modifies the adaptive system normally present under the control of another gene(s). Then it may be asked whether the manifestation of R^+ is dependent solely on the presence of the gene C^+ or on the constitutive system created by C^+ acting in concert with a gene (L) which is directly concerned with β-galactosidase formation If the above assumption is correct it should be possible to obtain from an R^+C^+ strain a mutant incapable of producing β-galactosidase, but still carrying the genes R^+ and C^+. With such a mutant it should be possible to determine whether phenotypic expression of R^+ is dependent on C^+ alone, or requires both C^+ and L^+. Incidentally, it would then be possible to ascertain whether C^+ is a modification of a gene controlling β-galactosidase, i. e., an allele of L, or whether C^+ is another gene which plays a supporting role to L.

A search for a β-galactosidase negative mutant was instituted using strains Y-10R as the parent strain. Strain Y-10R was irradiated with ultraviolet to give a kill of 99.9 per cent, and the irradiated suspension was spread on plates of EMS agar (Lederberg, 1949) containing 1.0 per cent lactose. Colonies not showing a typical lactose fermentation reaction were picked and streaked on EMS lactose plates. Potential lactose negative isolates were picked and tested for growth on various carbon sources, and for β-galactosidase production. One isolate grew normally on the several carbon sources tested, except lactose and raffinose, on which no growth was observed. No β-galactosidase could be found in this strain even when it was grown on melibiose, which is normally an excellent inducer of β-galactosidase formation. Furthermore, the basal level of β-galactosidase activity, observed in adaptive strains grown on non-inducing carbon sources, was also absent. The mutant showed the nutritional requirements of the parent strain, valine inhibition, and production of a phage which lysed the sensitive strain W-1485. The mutant was designated strain Y-10RL-4.

Mutant strain Y-10RL-4, in addition to being unable to produce β-galactosidase, also showed an inability to utilize raffinose as a carbon source. Before examining the genetic constitution of strain Y-10RL-4, the presence of the R^+ gene

was first established in a preliminary fashion by examining revertants for lactose utilization. Revertants of strain Y-10RL-4 were obtained from platings in lactose agar at a frequency of 1 per 2.3×10^7 cells. Several such colonies were isolated and tested for β-galactosidase production and raffinose utilization; every isolate grew on raffinose and showed constitutive β-galactosidase formation. Thus, it would appear that the R^+ gene was present in strain Y-10RL-4.

To ascertain the nature of C, i. e., whether C is an allele of L or an accessory to L, as well as to establish the relationship of R to C and L, strain Y-10RL-4 was crossed to strain H-34. If strain H-34 is denoted as $R^-C^-L^+$ and strain Y-10RL-4 as $R^+C^+L^-$, then an examination of raffinose utilization and β-galactosidase formation in the prototrophs obtained should yield the following classes or genotypes and phenotypes, assuming that L and C are separate genes, and that R^+ is expressed only in the presence of C^+L^+:

Genotype	*Phenotype*	*Description*
$L^+C^-R^-$, $L^+C^-R^+$	$L^+C^-R^-$	Adaptive β-galactosidase, raffinose negative
$L^-C^+R^+$, $L^-C^-R^-$, $L^-C^+R^-$, $L^-C^-R^+$	$L^-C^-R^-$	β-Galactosidase negative, raffinose negative
$L^+C^+R^-$	$L^+C^+R^-$	β-Galactosidase constitutive, raffinose negative
$L^+C^+R^+$	$L^+C^+R^+$	β-Galactosidase constitutive, raffinose positive

If, however, R^+ expression is dependent only on C^+ then the class $L^-C^+R^+$ would appear phenotypically as $L^-C^-R^+$. If L and C are alleles then neither constitutive β-galactosidase producers nor raffinose utilizers should be obtained. The prototrophs of a cross between strains H-34 and Y-10RL-4 were tested for constitutive β-galactosidase formation and raffinose utilization as described previously. The type L^+C^- was distinguished by a positive reaction of a streak on EMS-lactose and the lack of β-galactosidase when grown in a glycerol medium. The results are given in table 6. It can be seen that the classes obtained are consistent with the first hypothesis suggested above, which proposes that L and C are not allelic, and that the phenotypic expression of R^+ is dependent on the concomitant presence of C^+ and L^+ in the genome.

TABLE 6

Characteristics of prototrophs from a cross between H-34 and Y-10RL-4 strains of Escherichia coli

Phenotypic Class	No.	Per Cent
$L^-C^-R^-$	124	38
$L^+C^-R^-$	178	54.6
$L^+C^+R^-$	23	6.8
$L^+C^+R^+$	2	0.6

Although the utilization of raffinose as sole carbon source appears to be under genic control, its expression seems to depend on a functioning β-galactosidase synthesizing system. This relationship between raffinose utilization and β-galactosidase might raise the question of whether raffinose can be attacked by β-galactosidase. However, the cleavage of raffinose is not mediated by β-galactosidase, prepared from either constitutive, adaptive, or raffinose utilizing strains. From the results shown in table 1, and the fact that raffinose grown cells contain α-galactosidase activity, it appears that the utilization of raffinose is mediated, initially, by an α-galactosidase. Yet, the strains employed are capable of forming α-galactosidase when grown on melibiose; the same observation was made with strain Y-10RL-4, which is unable to produce β-galactosidase. The main difference between parental and raffinose utilizing strains seems to be that raffinose does not induce α-galactosidase activity in the former, but does so in the latter. It is possible to conceive of a different α-galactosidase in each case with unique specificities for melibiose and raffinose. If so, the α-galactosidase induced by melibiose should have no action on raffinose. The experiment described in table 1 would suggest that this is not the case. It was necessary to ascertain further the identity of the α-galactosidase produced by R^+ and R^- strains.

The α-galactosidase of *E. coli* strain K-12 (and *E. coli* B, Porter *et al.*, 1953) has not, to date, been obtained in cell extracts. Thus, the usual characterization of an enzymatic activity could not be made, and the problem of the identity of the α-galactosidase of R^+ and R^- strains was attacked in a different fashion. If it is as-

TABLE 7

Induction of α- and β-galactosidase activities in various genotypes

Carbon Source	Galactosidase Activity*									
	R^-C^-		R^-C^+		R^+C^+		$R^+C^+ L^-$		$R^+C^+ M^-$	
	α	β	α	β	α	β	α	β	α	β
Glycerol	−	−	−	+	−	+	−	−	−	+
Lactose	−	+	−	+	−	+	0	0	−	+
Melibiose	+	+	+	+	+	+	+	−	0	0
Raffinose	0	0	0	0	+	+	0	0	0	0

* + or − indicates presence or absence of activity well above any basal level; 0 indicates inability to utilize carbon source.

sumed that the same α-galactosidase is induced by both melibiose and raffinose, mutants selected for their inability to utilize melibiose and showing an inability to form α-galactosidase should also be unable to utilize raffinose. If, however, two distinct α-galactosidases are involved, then such a mutant should utilize raffinose.

A search for a melibiose negative mutant was made using H-34R as the parental strain. Strain H-34R was irradiated with ultraviolet light and spread on EMB melibiose agar. Colorless colonies were picked and screened for melibiose utilization. Two isolates were found which did not grow on melibiose (M^-). The M^- strains grew well on several carbon sources, but no α-galactosidase activity could be detected, even in cells grown on galactose which is an inducer of α-galactosidase activity in the parent strain. The M^- strains remained constitutive β-galactosidase producers. The two isolates selected for their inability to grow on melibiose also showed no growth on raffinose. Thus, the loss of ability to utilize melibiose and to form α-galactosidase was accompanied by a simultaneous loss of ability to utilize raffinose. Revertants of these mutants which could utilize melibiose also could utilize raffinose.

For recapitulation and orientation, table 7 shows the α- and β-galactosidase activities of the genotypes which have been examined.

DISCUSSION

The genetic control of enzyme formation has been well documented during the past several years. However, no concrete evidence for a genic control of enzyme formation with respect to inducer specificity has appeared. In yeast it has been shown (Hestrin and Lindegren, 1952) that maltose and α-methyl-glucoside are capable of inducing α-glucosidase activity, and that the utilization of each inducer is under separate genic control. However, it was found that these substrates induced the formation of specific and distinct α-glucosidases, indicating that the genic action was concerned with enzyme formation rather than with inducer specificity. In *E. coli*, mutants incapable of utilizing lactose have been found which are able to synthesize β-galactosidase when presented with inducers other than lactose (Lederberg *et al.*, 1951). A strain of this sort has been extensively examined by Rickenberg (1954), who showed that the inability of this strain to utilize lactose was primarily due to the impermeability of the cell to lactose rather than to a lack of inducer activity by lactose.

The work described in this paper suggests that the inability of the K-12 strains to use raffinose cannot be explained by (a) the impermeability of the cell to raffinose, or (b) the inability of the cell to form a raffinose specific α-galactosidase. The fact that cells induced to form α-galactosidase by melibiose are capable of utilizing raffinose indicates that the transport of raffinose into the cell does occur, and argues against a permeability hypothesis. However, the possibility that inducible concentration systems (Monod, 1956) may be involved is not entirely excluded. The evidence demonstrating the concomitant establishment of a genetically controlled, constitutive β-galactosidase system, and a genic change uniquely concerned with raffinose utilization, suggests that the utilization of raffinose depends on internal systems, rather than the character of the cell surface.

The possibility that the utilization of raffinose is dependent on the presence of a raffinose specific α-galactosidase does not seem likely. Such a hypothesis would require the induction, by melibiose, of two α-galactosidases since, as has been pointed out, cells induced by melibiose are capable of utilizing raffinose. While such a situation is not unlikely (melibiose induces both α- and β-galactosidase activity) it is not very tenable in view of the results obtained with mutants of strain H-34R which are unable to use melibiose. Thus, it is reasonable to assume

that the same α-galactosidase is involved in the utilization of both melibiose and raffinose.

The presence of a functional α-galactosidase forming system in the parent strains, as evidenced by the induction of this enzyme by galactose or melibiose, would argue that the mutation from raffinose negative to raffinose positive is not primarily related to an entirely new α-galactosidase forming system. Rather, the mutation of the parental strain to raffinose utilization indicates a change in susceptibility of the α-galactosidase forming system to induction by raffinose. Thus, the mutation from raffinose negative to raffinose positive appears to demonstrate a genic control of inducer specificity.

The genetic factors which permit the phenotypic expression of the raffinose gene indicate a complex interrelationship between α- and β-galactosidase formation. It would appear that for the utilization of raffinose as sole carbon source at least four conditions must be realized; (1) a constitutive β-galactosidase forming system, (2) β-galactosidase formation, (3) α-galactosidase formation, and (4) raffinose induction of α-galactosidase formation. Each condition is under the control of a separate gene.

The complex genic interrelationships which have been described would suggest, at first glance, that a single specific function cannot be readily assigned to a single locus. It appears that the gene for β-galactosidase formation and the gene for constitutiveness have two functions, one in respect to β-galactosidase formation and another in permitting raffinose to induce α-galactosidase. The observation that one gene affects more than one phenotypic character may be considered an example of pleiotropy in *E. coli*. A similar situation might exist in those pleiotropic mutants of *E. coli* where selection for a strain unable to use a single sugar afforded strains unable to use several sugars (Lederberg *et al.*, 1951). The question arises as to whether the pleiotropic effects observed can be explained on the basis of a polyfunctional gene, or whether they represent secondary effects of a single genetic activity (Goldschmidt, 1955). Although it is not possible to decide between these alternatives (or others) at present, the evidence presented would suggest that, if the gene is not polyfunctional, its unifunctional activity can have quite diverse effects.

SUMMARY

Strains able to utilize raffinose as a sole carbon source have been obtained from auxotrophs of *Escherichia coli* strain K-12 which do not utilize raffinose. These strains probably utilize raffinose via the action of an α-galactosidase which can be induced by melibiose in both parent and raffinose utilizing strains. The phenotypic expression of raffinose utilization is dependent on the simultaneous presence of a gene for constitutive β-galactosidase. A genetic analysis has shown that these characteristics are under separate genic control. In addition it has been demonstrated that the presence of other genes for α- and β-galactosidase formation are also prerequisites for the expression of the raffinose gene. These results have been interpreted as signifying a genetic control of inducer specificity. The multigenic system observed has been discussed with respect to certain aspects of gene action.

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