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The Induction in a *Saccharomyces* sp. of the Gene-mutation controlling Utilization of Galactose by Exposure to Galactose

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SUMMARY: The Carbondale yeast stock contains cultures which are characterized by the delayed fermentation of galactose. Long-term adaptation to galactose results from gene-mutation followed by the selection of the mutant in the presence of galactose. The original non-fermenter and the derived fermenter mutant contain no galactozymase and possess no selective advantage over each other when grown in glucose. Three different methods (variance analysis, fluctuation-redistribution tests, and chemostat experiments) which have been used with success in the detection of spontaneous mutations showed that the mutation to ability to ferment galactose did not occur spontaneously in these stocks. Exposure of non-dividing non-fermenter cells of the long-term fermenter to galactose induced the mutation to the fermenter type in a small but relatively constant number of cells, while exposure to glucose, ribose or lactose was ineffective; dividing cells were not susceptible to the mutagenic action of galactose. Prolonging the exposure of the cells on galactose agar plates increased the number of mutations. It is inferred that the induction of the mutation results from the impingement of galactose on the gene-surface thus modelling the gene-surface into a mirror-image of the substrate.

The phenomenon of long-term adaptation to galactose in the Carbondale yeast stock was shown by Mundkur & Lindegren (1949) to result from gene-mutation, followed by selection of the mutant in the presence of galactose. Tetrad analysis of hybrids between haploid galactose-positive mutants originating from the long-term adapted cultures and galactose-negative cultures carrying several other markers proved that each rapid-fermenter culture possessed a gene for galactose fermentation which segregated regularly in the hybrid and, therefore, that gene mutation had occurred. Mundkur (1952) used the Luria-Delbrück (1943) method for distinguishing between spontaneous and induced mutations and found the variance approximately equal to the mean. He showed that the phenomenon of long-term adaptation in the Carbondale stocks is specifically different from long-term adaptation occurring in *Saccharomyces chevalieri* analysed by Winge & Roberts (1948), and that the generalizations of Spiegelman and his collaborators (1950, 1951) concerning *S. chevalieri* do not apply to the long-term fermenters in the Carbondale stocks.

It has been shown recently that the non-fermenters of the Carbondale stock are incapable of producing galactozymase. A series of experiments has revealed that many misconceptions concerning the phenomenon of the adaptation of rapid fermenters of galactose arose from the occurrence of impurities in the

galactose (Lindegren & Palleroni, 1952; Sheffner & Lindgren, 1952). Tests with pure galactose revealed that rapid fermenters of galactose are incapable of adapting to the utilization of galactose unless supplied with some other source of energy. The non-fermenters of the Carbondale stock are incapable of fermenting pure galactose, contain no detectable galactozymase, and do not (as Lindgren once proposed, 1949) possess a subthreshold capacity for galactozymase production, too small to be registered by the Durham tube test. These non-fermenters are the result of an intensive genetical selection which has produced a paired stock: one member incapable of fermentation, the other capable of vigorous fermentation (Lindegren, 1953).

Long-term adaptation in the Carbondale stocks involves two steps: (a) the mutation from the non-fermenter gene to the dominant allele, (b) the adaptation of the derived fermenter to galactose fermentation through the utilization of some source of energy other than galactose. In Mundkur's experiments these two phenomena were inextricably joined. This paper reports a series of experiments with the objective of separating the two events. They reveal that (a) the mutation cannot be demonstrated to occur spontaneously, but (b) the mutation can be induced by exposure to galactose but *not* by exposure to glucose, ribose or lactose (the latter two substrates are not utilizable by the cell).

METHODS AND MATERIALS

Cultures. Culture 5991 (*Saccharomyces* sp.) of the Carbondale stock is a long-term fermenter of galactose, closely related to culture 6166 analysed by Mundkur. When a number of different transfers from this culture are tested simultaneously in Durham tubes containing full nutrient medium and galactose, fermentation occurs at different times after prolonged delays. The variant cells from a positive tube on transfer to galactose medium cause fermentation to occur within 24 hr. In the present study, three rapid fermenters of galactose were derived from 5991 by plating cells on galactose plates and selecting three of the large colonies. Each is inferred to carry the mutant gene which controls the rapid fermentation of galactose. Since the fermentation of galactose is an adaptive process, the fermenter is enzymically similar to the non-fermenter when grown in glucose, for in 'short-term' adaptation the enzyme appears in the cells of fermenters only after from 2-8 hr. exposure to galactose. Neither the non-fermenter nor the fermenter contain enzyme during growth on glucose.

Culture 5991 is a fermenter of maltose, melibiose and sucrose, and does not grow in the absence of adenine, thiamine, pyridoxine or inositol. It is capable of utilizing lactate, acetate, pyruvate and formate.

Media

Purified galactose. Galactose was purified by treating a solution with yeast to metabolize the glucose and drying the resultant solution *in vacuo* to crystals. The crystals were dissolved in water and evaporated to a thick syrup which was refrigerated as the purified stock reagent.

Complete nutrient media. The complete nutrient medium contained: peptone, 3.5 g.; liquid yeast extract, 4 ml.; $(\text{NH}_4)_2\text{SO}_4$, 4 g.; MgSO_4 , 1 g.; KH_2PO_4 , 2 g.; glucose, 20 g.; water, 1 l. This medium was made into galactose complete agar by adding 22 g. Difco agar and substituting galactose for glucose.

Eosin methylene blue purified galactose agar. (EMB-PG) agar was prepared by adding to 1 l. of water the following materials: peptone, 3.5 g.; $(\text{NH}_4)_2\text{SO}_4$, 2 g.; MgSO_4 , 1 g.; KH_2PO_4 , 2 g.; Difco yeast extract, 2.5 g.; purified galactose, 20 g.; agar, 22 g.; eosin solution (2%), 20 ml.; methylene blue solution (0.5%), 5 ml. This medium gives the same results as that used by Spiegelman, Sussman & Pinska (1950), producing the characteristic colour reactions of non-fermenter and fermenter clones.

Chemically-defined media. Defined glucose medium was prepared by dissolving in 1 l. of water the following constituents: glucose, 20 g.; KH_2PO_4 , 1.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.33 g.; $(\text{NH}_4)_2\text{SO}_4$, 4 g.; KI, 2.5×10^{-5} g.; the metals B, Cu, Mo, Mn, 0.0025 p.p.m. each; Fe, 0.0013 p.p.m.; Zn, 0.0175 p.p.m. Thiamine, pyridoxine, niacin, pantothenate, 200 μg .; biotin, 2 μg .; inositol, 0.01 g.; adenine and methionine 2 mg. each. Defined galactose medium was prepared by the substitution of purified galactose for glucose in the above medium.

'Non-nutrient' agar. Agar medium designated as 'non-nutrient' contained 22 g. Difco agar/l. water. This medium was used to cover galactose nutrient agar; by itself it did not support any visible growth.

Experimental procedure

Variance analysis. In all the analyses reported here, non-fermenter cells of culture 5991 were grown from 24 to 48 hr. at 30° in full nutrient glucose broth in Erlenmeyer flasks on a shaker. A small sample of broth was centrifuged, the supernatant fluid discarded and the cells serially diluted in M/15-potassium dihydrogen phosphate to a concentration of less than 1000 cells/ml. Twenty-five to thirty 'growth tubes', containing 2 ml. each of glucose-defined medium, were inoculated with 0.1 ml. of diluted culture. Direct plate counts on glucose agar were made with at least two of the 'growth tubes' in each series to determine the number of cells in the inoculum. The 'growth tubes' were incubated for from 36 to 48 hr. and replicate haemocytometer counts were made on at least three tubes to determine the final population. The supernatant fluid was discarded after centrifugation, the cells resuspended in M/15-potassium dihydrogen phosphate, and the suspension used to inoculate galactose plates.

The inoculum was sufficiently small to ensure detection of fermenter mutants carried in it and to exclude the chance of seeding a population in equilibrium with respect to fermenters and non-fermenters. Centrifugation and dilution served to eliminate much of the original nutrient and to concentrate the cells previous to plating small volumes on EMB-PG agar. Pour plates were made by circulating molten galactose agar (39–43°) in Petri dishes containing equal sample volumes of washed cell suspensions. To prevent growth on the agar surface and to impose anaerobic conditions, each pour

plate was layered with approximately 12 ml. of 'non-nutrient' agar. In experiments employing EMB-PG agar, plates were rotated and cells spread evenly over the surface with a sterile, bent glass rod.

Stability of the derived mutant. The stability of the rapid fermenters of galactose derived from non-fermenter culture 5991 was investigated, since any considerable amount of reverse mutation could have serious effects on the analysis. A derived mutant which had been subcultured on glucose for more than 12 months yielded large galactose fermenter colonies on galactose plates and fermented galactose in fermentation tubes in from 18 to 24 hr. Mass reversion to inability to ferment galactose as a result of ultraviolet irradiation has not been detected in this culture. In this respect, it was quite unlike the galactose-fermenting yeast described by James (1952) in which as many as 10% of the surviving cells lost the ability to ferment galactose following irradiation. Fermenter cells from the derived culture were irradiated to 3% survival and plated on EMB-PG agar. The plates were covered with the typical red colonies characteristic of the rapid fermenter genotype; no pink colonies were detected. Plating irradiated cells on glucose agar before testing by Durham fermentation tubes gave similar results. The possibility that the non-fermenter cells produced by irradiation might have been incapable of surviving direct transfer to EMB-PG is not necessarily excluded, but the results are apparently different from those found by James.

RESULTS

Preliminary experiments

Growth of non-fermenters on galactose plates. The number of mutants appearing on galactose complete medium is not directly proportional to the number of cells seeded on the plate, although it may bear a relation to the final titre of cells on a plate. A direct microscopic examination of inoculated plates revealed that non-fermenter cells go through one to six divisions during the first 24 hr. after which the growth of clumps and single cells is greatly retarded. Attempts to prepare a medium which is completely incapable of supporting growth of non-fermenters but capable of supporting the growth of mutant fermenters were unsuccessful; even on defined medium with purified galactose from one to five divisions occurred, probably due to the presence of endogenous reserves and the failure to eliminate completely the nutrient after centrifuging the inoculum. Variation of the inoculum from 10^5 to 10^8 cells/plate gave no significant difference between the total number of mutants per plate.

Absence of competition between non-fermenter and mutant fermenter in glucose medium. A series of test-tubes containing 2 ml. of defined medium with glucose as sole added carbohydrate were inoculated with a mixed population consisting of approximately 250,000 non-fermenter and 350 fermenter cells. The fermenter cells were obtained from a 48 hr. complete nutrient glucose broth culture inoculated with cells from a rapid fermenter originally derived from culture 5991, but which had been carried for 12 months on glucose slopes. The tubes were incubated at 30° on a reciprocal shaker and were sampled for

total populations of fermenters and non-fermenters by the plate count technique (Fig. 1a). Samples were plated on galactose agar and glucose agar plates. The total count of the cells was obtained by colony counts on glucose agar;

Fig. 1. Population analyses of mixed cultures of fermenter (GA) and non-fermenter (ga) cells. Fig. 1a presents the results of growing populations in defined glucose medium; Fig. 1b depicts the growth curves of a mixed population grown on complete glucose medium. The parallel growth curves under both conditions indicate the absence of selective factors favouring either type.

counts on galactose agar gave the numbers of galactose fermenters. Three serial dilutions were made of each sample; each dilution was plated and read after incubation for 8 days. The numbers of galactose fermenter colonies appearing on the plates were proportional to the dilutions, showing that the growth curves (Fig. 1) are accurate reflexions of the population sampled and

not in error due to growth of non-fermenters on the plates. The difference between the two values gave the number of non-fermenters. The parallel growth curves of fermenter and non-fermenter populations indicate that the non-fermenter has no selective advantage over the fermenter and establish the absence of mass reversion of fermenter to non-fermenter.

A chemostat (Novick & Szilard, 1950) was set up containing complete medium with glucose as the carbohydrate source, and inoculated with *c.* 534 cells of the original non-fermenter culture 5991 and *c.* 200 cells of a galactose fermenter mutant derived from 5991. The experiment ran over 5 days (developing a population density of 6.9×10^7 cells/ml.). The parallel growth curves (Fig. 1*b*) indicate the absence of a selective advantage of either type over the other.

Variance analysis

The experiments described in Table 1 were undertaken for the purpose of detecting variations in the numbers of colonies appearing on galactose when pour plates were inoculated with samples from a single culture. In each experiment ten pour plates were made with 1 ml. of cells from a single tube. The readings were made on the fifth day, and the numbers of mutants appearing on the plates were recorded. The statistical analysis reveals that no significant fluctuation was encountered as a result of sampling error ($P=0.2-0.9$).

Table 1. *The numbers of fermenter mutants detected on replicate platings from the same culture*

Sample	No. of mutants detected			
	Exp. 11	Exp. 18 <i>a</i>	Exp. 18	Exp. 10 <i>a</i>
1	15	6	9	10
2	9	11	11	8
3	10	16	9	14
4	8	12	11	12
5	10	15	8	16
6	18	10	10	12
7	5	8	9	13
8	12	15	5	14
9	8	9	8	19
10	9	11	10	15
Average	10.4	12.5	9	13.3
Variance	14.0	12.4	3.1	9.5
χ^2	12.18	8.9	3.1	6.4
<i>P</i>	0.2	0.4	0.9	0.8

The data from a series of variance analyses following the procedure already described are given in Table 2. Exps. A and B present the results of fluctuation tests in which cells were plated on agar containing synthetic medium and purified galactose. The variance closely approximates the mean, but the average number of mutants/plate/day is slightly lower on defined medium than on complete medium (Exps. C-E, Table 2). This may be because yeasts generally adapt and grow slowly in defined medium.

Since as many as five divisions may occur when non-fermenter cells are plated on galactose agar (or on nutrient agar containing no added carbo-

hydrate), the mutants detected after incubation could be the result of mutations occurring during growth on glucose in the culture tube before plating the population on galactose and during residual growth on the agar plates after contact with galactose. If mutations do occur at both sites, magnifying the number of mutant cells present at either locality should, therefore, produce results differing from those obtained in routine variance analyses. In Exps. C and D an opportunity was afforded for the growth and selection of any fermenter mutants present in the inoculum. Glucose-grown non-fermenter cultures were centrifuged, the supernatant discarded, and the cells resuspended in $M/15$ -potassium dihydrogen phosphate with 0.1 ml. of 4 % galactose broth. The cultures were incubated for 7 hr., after which pour plates were made on galactose agar. By the aid of tables supplied by Lea & Coulson (1949), the mean number of mutations/culture was calculated to be 3.6 with a P of 0.224 that a single culture would be detected containing between nine and sixteen mutants. In considering the selective environment and the clumping nature of culture 5991 during rapid growth, an upper range of from twenty to eighty mutants would have been expected in some cultures. No large fluctuations in the numbers of mutants were found, however, after passing the cultures through the selective medium.

Cultures in routine fluctuation tests are derived from small inocula to decrease the chance of seeding mutants in the growth tubes. When a given mutation occurs with a certain frequency (e.g. 1×10^{-8}), it is unlikely that it will occur during the first few divisions of a small population. A large inoculum, however, should lead to large numbers of mutants since a large inoculum is more likely (*a*) to transfer randomly-distributed mutant cells which could multiply during the early divisions, and (*b*) to give rise to mutant cells during the first few divisions. With this in mind, fluctuation tests were made when using large inocula. The results in Exps. E and F (Table 2) show that the mean number of mutants/plate is low and essentially the same as for cultures derived from small inocula. The plates in Exp. F were scored after 6 days. The higher mean is not due to the delayed expression of mutants originally present, but due to prolonged incubation of the plates. This is proved by comparison with results of Exp. G in which a small inoculum of fifty cells was used and the plates were scored after 6 days incubation.

Fluctuation-redistribution experiments

Newcombe (1949) developed a modified fluctuation test which exploits the fact that mutant cells form colonies on solid medium. The cells in the colonies are redistributed over the agar after varied periods of incubation. He found that when mutation to virus resistance occurred before redistribution and exposure to phage, redistribution of the cells after incubation magnified the number of mutants by dispersing the cells of mutant clones.

When mutation to the form able to ferment galactose occurs before plating cultures, rapid fermenter variants which arose before sampling would be selected on the galactose plates and a larger number of fermenter mutant colonies would appear on the plates in which the clones had been redistributed.

Table 2. *The number of fermenter mutants detected after plating a series of similar cultures on galactose agar plates*

Exp.	...	A	B	C*	D*	E	F	G
No. of cultures	...	25	25	25	25	25	25	25
Vol. of cultures/ml.	...	5	5	2.1	2.1	2.1	2.1	2.1
Vol. of samples/ml.	...	1	1	1.0	1.0	1.0	1.0	1.0
Inoculum	...	50	50	15	15	1×10^6	1×10^6	50
Days of incubation	...	5	5	4	4	4	6	6

Culture no.	No. of fermenter mutants						
	8	15	8	7	10	19	28
1	8	15	8	7	10	19	28
2	5	7	6	9	7	18	18
3	5	5	12	6	7	12	14
4	4	10	9	9	10	17	12
5	7	9	12	7	5	16	12
6	6	5	8	13	7	19	14
7	4	3	12	6	11	21	19
8	6	7	7	10	10	19	19
9	5	8	10	15	8	18	20
10	7	15	12	10	6	20	25
11	4	4	15	13	7	12	20
12	6	4	8	9	6	21	24
13	7	7	14	9	10	14	21
14	8	12	8	13	11	20	23
15	7	6	14	12	7	14	24
16	4	4	15	9	9	17	23
17	6	5	11	9	11	15	14
18	10	8	9	8	8	19	21
19	5	5	11	11	7	18	13
20	15	5	10	13	4	16	12
21	12	6	6	13	13	15	18
22	11	6	9	10	8	15	15
23	3	9	11	6	12	18	21
24	5	6	7	8	5	21	12
25	7	7	10	10	13	20	21
No. cell/ml. $\times 10^7$	3.2	3.0	6.7	6.6	24	18	5.8
Average	6.68	7.12	10.16	10.12	8.48	17.4	18.52
Variance	7.89	9.94	7.06	9.11	6.34	7.24	19.68
χ^2	28.36	33.52	16.67	21.60	17.95	9.98	25.50
P	0.3	0.1	0.7	0.6	0.81	0.9	0.4

* Cultures passed through a selective medium by adding 0.1 ml. 4% galactose broth to washed cells suspended in 1 ml. of $M/15-KH_2PO_4$.

When mutation does not occur before plating, no significant difference is to be expected between redistributed ('R') and undisturbed ('U') plates. A series of tests (shown in table on p. 502) revealed that when the cells of deliberate mixtures were redistributed after short periods of incubation striking increases did occur in the numbers of fermenter colonies.

Duplicate EMB-PG agar plates were paired, and both members of each pair were inoculated with cells from the same tube. In all analyses cultures were first centrifuged, the supernatant poured off and the cells resuspended in half the original volume of $M/15-KH_2PO_4$. One-tenth ml. samples from each tube were spread evenly on the plates. The plates were incubated for limited periods

of time after which 0.1 ml. of sterile phosphate buffer was added to one plate (marked 'R') of each pair and the cells redistributed by spreading for approximately 30 sec.

Fermenter inoculum (no. of cells)	Fermenter colonies; Redistribution after			Inoculum mixed with 10 ⁴ -10 ⁷ Non-fermenter cells/plate		
	2 hr.	6 hr.	10 hr. No. of colonies	2 hr.	6 hr.	10 hr.
495	510	535	> 1000	426	711	> 1000
566	518	673	> 1000	486	598	> 1000
49	57	87	138	74	83	151
40	39	56	195	38	120	175
201		801			870	
104		1112			1170	

The length of the incubation period elapsing before redistribution should be sufficiently long to insure that a detectable increase in mutant colonies will result from the multiplication of mutant fermenters on the plate. Control experiments showed that a minimum of 6 hr. was sufficient to produce a significant difference between redistributed and undisturbed plates. The incubation period should not be so prolonged that the effect of the substrate, if any, might obscure the results. Since rapid fermenter variants become barely visible after approximately 3 days, the incubation time was set arbitrarily at a maximum of 10 hr.

The data shown in Table 3 reveal no significant difference between the average number of mutants detected on plates on which the cells had been redistributed or left undisturbed after incubation periods of 7 or 10 hr. In some pairs the redistributed plate showed a higher number of mutants than the undistributed control. In other pairs the number of mutants which appeared on the redistributed plates was smaller. It may be inferred that the frequency of mutation before redistribution is so small as to be undetectable, and hence that the mutations must have occurred on the plates. The fact that incubation periods of from 7 to 10 hr. did not change significantly the numbers of mutants strongly suggests that mutation did not occur until cell division had ceased or the rate had slowed down considerably. A comparison of Exps. 32, 38 and 41 (Table 3) reveals that the number of fermenter mutant colonies appearing on the galactose agar plates is relatively independent of the number of cells used to inoculate the plate. In these three experiments 3.9 , 4.5 and 8.8×10^6 cells were used respectively in each inoculation. No significant difference in the number of fermenter mutants occurred in the 150 cultures analyzed. The counts on these plates were made on the fifth day. Exp. 43 (Table 3) is a parallel experiment to 41, with the exception that the mutants were counted on the thirteenth day. The number of mutant colonies which appeared on the plates in Exp. 43 was almost double the number appearing on the plates counted on the fifth day, thus showing that the number of mutants increased as incubation progressed. The experiment on redistributing the cells shows that no mutants were present in the cells used as an inoculum, since, if that had been the case, the numbers of colonies in

some of the redistributed plates should have been larger than the numbers on the undisturbed plates. It may be inferred, therefore, that the mutations occur only on the plates. This view is further supported by the increase in the number of mutants with continued incubation as revealed by the higher counts observed after 18 days.

Table 8. *Numbers of galactose fermenter mutants appearing on paired plates (undisturbed 'U' and redistributed 'R') in a series of fluctuation-redistribution tests (Newcombe, 1952)*

Exp.	...	32	38	41	48				
No. of cultures	...	25	25	25	25				
Vol. of cultures (ml.)	...	2.1	2.1	2.1	2.1				
Inoculum	...	50	100	50	50				
No. of samples	...	0.1	0.1	0.1	0.1				
No. of cells/plate $\times 10^6$	...	3.9	4.5	8.8	8.8				
Incubation time/hr.	...	7	7	10	10				
		U	R	U	R	U	R	U	R
		No. of fermenter mutants							
Culture no.									
1		11	10	18	19	15	13	40	49
2		10	13	17	24	16	15	36	27
3		13	19	23	21	21	19	33	41
4		15	14	20	22	19	22	39	37
5		14	9	16	8	10	15	37	33
6		12	10	11	20	12	19	32	35
7		10	12	17	10	15	14	35	32
8		21	12	15	20	13	10	34	40
9		10	14	12	21	17	14	39	36
10		9	13	24	23	13	16	30	41
11		12	11	17	20	10	11	27	28
12		16	20	22	10	12	13	21	25
13		12	13	20	19	25	24	44	34
14		10	13	13	21	18	9	46	29
15		14	19	24	18	13	16	35	32
16		10	12	20	21	17	17	36	21
17		15	13	10	16	8	19	22	32
18		22	16	21	17	18	19	39	41
19		15	16	14	12	12	17	29	37
20		14	14	17	24	25	14	26	36
21		10	11	24	15	11	14	29	40
22		13	9	22	13	15	12	26	35
23		19	10	12	17	17	16	33	31
24		11	9	16	22	11	23	32	34
25		16	15	14	21	19	16	40	45
Average		13.8	13.1	17.5	18.2	15.3	15.9	34.6	34.8
Variance		12.2	9.0	18.5	20.4	19.5	14.6	39.5	39.9
χ^2		22.0	16.5	25.4	26.9	30.6	22.0	27.4	27.5
P		0.5	0.8	0.4	0.3	0.1	0.5	0.2	0.2

The mutability of non-fermenter cells grown under different conditions

The data presented above indicate that mutation from non-fermenter to fermenter does not occur during growth in glucose broth. Table 4 presents several series of variance analyses on culture 5991 in which the cells comprising the inoculum for the galactose pour plates were grown either on media of

different composition or under different conditions. Routinely, the growth tubes used to inoculate the galactose plates contained glucose-defined medium; this medium was used in Exp. 45 (Table 4). In Exp. 46 (Table 4) glucose complete medium was used. A parallel pair of experiments in which fructose replaced glucose was also performed. In all four experiments the cultures contained substantially the same number of cells and no significant differences were found between the numbers of mutants appearing on the plates, suggesting that these variations in the medium did not affect the mutability of the cells.

In Exp. 49*a* (Table 4), twenty EMB-PG plates were inoculated with 2×10^6 non-fermenter cells and the number of mutant colonies counted after 5 days. In Exp. 49*b* (Table 4) the inoculum was grown on an EMB plate containing no added carbohydrate, and after 6 days incubation, the cells were washed from the surface of each EMB plate and transferred to molten galactose defined agar. During the 6 days on the EMB plate, the population had increased from 2×10^6 to 3.1×10^8 . Washing removes some of the dye, thus giving the galactose plate a distinct pink tint. The fermenter colonies were also faintly coloured. Comparison of Exps. 49*a* and 49*b* shows little difference in the numbers of mutants on the EMB plates containing galactose (49*a*) and on the galactose plates (49*b*) inoculated with 3.1×10^8 cells which had been washed from the EMB plates lacking added carbohydrate.

In Exp. 50 (Table 4) glucose was added to the EMB plate, and the population of cells increased to 6.1×10^8 . Plating the cells on galactose agar yielded approximately the same number of mutants as in the two preceding experiments, indicating that neither exposure to EMB nor any of the other conditions imposed by growth on these plates had increased the number of mutants.

*Conditions controlling the variation in the numbers of mutants
which appear on galactose plates*

Luria & Delbrück (1943) in analysing the frequency of mutation of *Bacterium coli* from bacteriophage-sensitivity to bacteriophage-resistance, found that the distribution of bacteriophage-resistant mutants on different plates was characterized by an extremely high variance when plates flooded with bacteriophage were smeared with cells grown in different tubes. They concluded that the mutation occurred spontaneously in the absence of the bacteriophage; if the mutation occurred early in the growth tube, many mutants would be found, but if it occurred late only a few would appear on the plate. The extremely low variance of the distribution of galactose fermenters in experiments carried out according to the Luria-Delbrück procedure raises the question of whether the experiment can be carried out under conditions in which a high variance would be observed.

Non-fermenter cells of culture 5991 were inoculated into full nutrient medium and aerated for 24 hr. The cells were washed, resuspended in phosphate buffer and 1 ml. was added to each tube of two series: (*a*) thirteen tubes containing 6 ml. of 2% glucose in phosphate buffer, and (*b*) eighteen tubes containing 2% reagent galactose in phosphate buffer. The tubes were held for

Table 4. *The numbers of rapid fermenter colonies detected on galactose agar containing non-fermenter populations grown on different substrates*

Exp.	45	46	47	48	49 a	49 b	50
Inoculum	...	...	...	...	...	...	...
Vol. of cultures (ml.)	100	100	100	100	2 × 10 ⁶	2 × 10 ⁶	2 × 10 ⁶
Vol. of samples (ml.)	2	2	2	2	0.1	0.1	0.1
No. of cultures	1	1	1	1	1.0	1.0	1.0
No. of cells/plate	20	20	20	20	20 (rep)	20 (rep)	20 (rep)
Substrate (carbohydrate)	58 × 10 ⁷ Glucose defined medium	6.4 × 10 ⁷ Glucose complete medium	8.5 × 10 ⁷ Fructose defined medium	5.6 × 10 ⁷ Fructose complete medium	EMB (+ Gal.)	3.1 × 10 ⁸ (b) EMB (- Gal.) (- Glu.)	6.0 × 10 ⁸ (a) EMB (- Gal.) (+ Glu.)
Culture no.	No. of mutant colonies						
1	16	22	28	14	20	17	13
2	15	16	15	19	13	23	14
3	32	23	16	25	23	28	24
4	19	25	21	27	26	24	19
5	17	21	22	15	17	41	20
6	13	20	12	22	9	25	20
7	23	26	22	12	22	29	27
8	18	19	18	19	24	31	29
9	16	34	26	28	12	26	21
10	15	16	19	22	11	21	13
11	27	19	17	19	12	24	12
12	14	15	17	21	16	22	17
13	25	27	22	9	12	22	20
14	13	16	20	19	13	24	12
15	22	18	18	17	18	28	16
16	24	22	15	22	10	23	22
17	22	28	10	27	11	29	20
18	24	27	17	18	20	18	19
19	17	18	22	16	25	32	23
20	17	26	13	28	17	18	19
Average	19.45	21.90	18.50	19.95	16.55	25.25	19.00
Variance	26.78	24.66	20.86	23.51	29.62	31.46	22.65
χ ²	26.16	21.39	20.91	27.1	34.01	23.67	22.63
P	0.12	0.3	0.3	0.1	0.03	0.2	0.2

a, cells suspended in 0.2 % glucose complete broth before spreading on galactose agar plates. b, populations determined by assays of four different plates.

4 days at 30°, after which 1 ml. of water containing 2.5 mg. peptone and 0.1 ml. of liquid yeast extract was added to each tube. The population densities after 24 hr. incubation at 30° were found to be 2.4×10^6 in the glucose series and 9.6×10^5 in the galactose series. The complete culture from each tube was mixed with molten galactose agar in a Petri dish and the plates were incubated for 4 days at 30°. The following tabulation shows the number of fermenter colonies appearing on the plates:

Colonies per plate (4 days)	No. of plates	
	Glucose series	Galactose series
1-6	12	2
7-12	1	1
13-18	.	7
19-24	.	6
25-30	.	1
31-36	.	.
37-42	.	.
43-48	.	.
49-54	.	1

The glucose series shows the characteristic low variance which indicates that the mutations only occurred after removal from the glucose broth. The variance of the galactose series is in excess of that expected on random sampling of a homogeneous group, indicating that some mutations occurred in the growth tubes.

Absence of spontaneous mutation of non-fermenter to fermenter in glucose

If spontaneous mutation occurs in the chemostat, the number of mutants detectable on plating should increase linearly with time provided (a) that the mutant type is stable, (b) that the number of mutants forms a very small fraction of the total population, (c) that neither mutant nor non-mutant possesses a selective advantage. If the growth of the mutant type were suppressed by the parent strain, an initial rise in the number of mutant colonies would be followed by levelling off.

Experiments were performed with the chemostat in which populations of non-fermenter cells (Fig. 2) were grown over long periods of time under conditions which simulated those occurring (a) in growth tubes containing defined medium, (b) in growth tubes containing complete nutrient medium, and (c) on agar plates, where the rate of multiplication is greatly reduced after the first few divisions. Non-fermenter cells of culture 5991 were grown for 14 days at a generation time of 12 hr. (30°) on a defined medium with a low concentration of orthophosphate (10 mg. $\text{PO}_4(\text{L})$) at a population density of 2.7×10^7 cells/ml. Samples taken at regular intervals were centrifuged, the supernatant discarded and the cells resuspended to the original volume in sterile vials which were shaken by hand for at least 1 min. before sampling. Since population analyses (Fig. 1a, b) showed neither suppressive effects nor selective advantages for either fermenter or non-fermenter, it was concluded

that the curve (Fig. 2) which represents the numbers of colonies appearing on the galactose plates resulted from mutations occurring on the plates rather than mutation, followed by selection, in the chemostat, although the latter could yield similar results (Novick & Szilard, 1950).

Fig. 2. Experiments with the chemostat in which populations of non-fermenter cells were grown over long periods of time under conditions simulating those occurring: (a) in growth tubes containing defined medium, (b) in growth tubes containing complete nutrient medium, and (c) on agar plates, where the rate of multiplication is greatly diminished after the first few divisions. The results show no detectable increase in GA mutants over periods of continuous growth extending as long as 14 days.

The absence of a detectable spontaneous mutation rate to ability to ferment galactose could be ascribed to a two-step mutation resulting in a deficiency capable of preventing the expression of the mutant type unless a specific nutrient were supplied. Although the growth studies on defined medium (Fig. 1) strongly suggest that this does not occur, non-fermenter cells of culture 5991 were grown for long periods in the chemostat on complete medium and on defined medium. The results (Fig. 2) revealed no significant differences in the numbers of mutant colonies detected on galactose agar. These findings are in agreement with those obtained from variance and redistribution analyses, which show that the mutation to galactose fermentation does not occur while the cells are growing in glucose medium, and that the constant low numbers of mutants which appear on the galactose plates are not due to suppression of fermenters by non-fermenters.

Pretreatment of non-fermenter cells with galactose

Non-fermenter cells from culture 5991 were exposed to reagent galactose (either Merck or Pfanstiehl, without apparent difference) in phosphate buffer, and to phosphate buffer alone, in the absence of an external source of nitrogen. The cells were aerated in tubes for from 24 to 30 hr. on a reciprocal shaker at 30°. Ten different tubes of galactose-treated and of untreated cells were plated

on galactose agar plates. The large (fermenter) colonies appearing on the plates were counted on the fifth, sixth and seventh days; the results are presented in Fig. 3. After 5 days the ten plates inoculated with cells pretreated with galactose buffer contained a total of ninety-eight colonies, while the ten plates inoculated with cells pretreated with buffer alone contained a total of thirty-six colonies. The fact that the pretreatment was carried on in the absence of an external source of nitrogen excluded selection and growth as

Fig. 3. The total number of large (galactose-fermenter) colonies appearing on separate galactose test plates inoculated from two series of ten flasks in which non-fermenter cells were pretreated (a) with galactose in buffer, and (b) with buffer without sugar. The plates were incubated at 30° and counted after the first, fifth, sixth and seventh days.

complicating factors. The data show the characteristic low variance. The difference in the number of mutants obtained on the diagnostic plates following the different pretreatments cannot be ascribed to the difference in the number of cells plated. Plate counts of the viable cells, made on glucose medium as each sample was taken, revealed that both the plain buffer and the galactose buffer tubes contained 2×10^6 cells/ml. Remarkably few cells died during the treatments. Cells from both test and control were examined with a modified methylene blue dead cell stain (Townsend & Lindegren, 1953) with the following results: the percentages of dead cells in six glucose tubes were respectively, 2.9, 7.4, 8.1, 10.3, 1.9, 5.7; in seven galactose tubes, 10.7, 14.1, 11.8, 7.2, 11.5, 7.5, 8.9. Nine other experiments were run at different times, each involving approximately the same number of plates but using various concentrations of cells. Each showed a low variance and confirmed the findings described above.

Table 5 describes another experiment in which glucose- and galactose-treated cells were planted on galactose agar and the number of mutant colonies counted after 6, 8 and 10 days. Instead of using complete medium, the treated cells were mixed with molten galactose-defined medium agar. In this experiment the numbers of dead cells in both series approximated 10%. In spite of the fact that the defined medium, which tends to diminish the number of divisions occurring on the plate, was used, the same difference was found between plates seeded with glucose- and galactose-treated cells.

Table 5. *The total number of large (galactose-fermenter) colonies appearing on separate galactose test plates inoculated from twenty different tubes in which non-fermenter cells were kept for 48 hr. in (a) phosphate buffer with 2% galactose and (b) phosphate buffer with 2% glucose*

Solutions contained 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The plates were incubated at 30° and counted after the 1st, 6th, 8th and 10th days.

Pretreatment (48 hr.)	Sample	Days				Pretreatment (48 hr.)	Sample	Days			
		1	6	8	10			1	6	8	10
Galactose in phosphate buffer	1	0	5	8	11	Glucose in phosphate buffer	1	0	10	16	16
	2	0	4	6	6		2	0	4	8	9
	3	0	6	9	9		3	0	7	9	9
	4	0	3	5	7		4	0	9	9	10
	5	0	5	12	14		5	0	3	11	11
	6	0	5	20	21		6	0	8	11	11
	7	0	12	13	15		7	0	8	12	14
	8	0	3	4	14		8	0	12	13	13
	9	0	9	12	12		9	0	7	12	13
	10	0	11	13	13		10	0	11	20	21
Total		0	63	102	112			0	79	121	127

Mutability of the non-fermenter allele in dividing and non-dividing cells

Table 6 records the frequency distributions of galactose fermenter colonies on galactose plates seeded with similar cultures grown as follows: (a) full nutrient medium containing 2% glucose (Exp. 12a); (b) full nutrient medium containing 2% reagent galactose which has as impurities *c.* 0.3% glucose and 0.3% ethanol (Exp. 14b); and (c) Burkholder's (1943) medium containing 1% glucose + 1% galactose (Exp. 16a). In Exps. 14a and b the growth tubes were incubated for 5 days, while in Exp. 16a, the growth tubes were incubated for only 48 hr. The contents of each tube were mixed in a Petri dish with 15 ml. molten 2% galactose nutrient agar. The plates were counted after 5 days.

The number of large (galactose fermenter) colonies appearing on the galactose plates which had been seeded with the cells grown in the absence of galactose varied from 5 to 30, showing the characteristic low variance (Exp. 14a).

In Exp. 14b the glucose was all consumed long before the end of the incubation period, thus allowing several days' exposure to galactose in the absence of growth. The number of large colonies appearing on the galactose plates which were seeded with cultures that had been grown in the impure

galactose showed an exceedingly high variance. Two-thirds of the cultures produced plates containing more than 80 colonies/plate, showing that mutation had occurred in the growth tube before plating. The fact that it is possible to set up conditions under which a high variance is observed proves that the characteristic low variance is not due to the suppression or crowding of the mutant cells on the galactose plates.

The plates seeded with the cells grown in the tubes containing equal amounts of glucose and galactose showed a low variance. In this experiment the growth tubes were incubated for only 48 hr. before seeding the galactose agar plate, and the cells had just completed the logarithmic phase. The presence of galactose produced no effect because the cells were still dividing at the time of transfer to the galactose agar. Galactose apparently cannot exert its mutagenic action on actively growing cells.

Table 6. *The effect of the presence of galactose in the growth tube on frequency distributions of galactose fermenter colonies appearing on galactose agar plates*

Exp.	...	14a	16a	14b
No. of cultures	...	56	50	58
Vol. of cultures (ml.)...		5.5	2.1	5.5
Vol. of samples (ml.)...		5.0	2.0	5.0
		No. of cultures		
No. of GA colonies				
0	0	0	0	0
1	0	0	0	0
2	0	0	0	0
3	0	1	0	0
4	0	0	0	0
5	1	1	1	0
6-10	18	26	0	0
11-15	28	18	1	1
16-20	6	4	3	3
21-25	0	0	5	5
26-30	3	0	6	6
31-500	0	0	18	18
500	0	0	20	20
Mean	12.82	10.52	—	—
Variance	20.51	13.59	—	—

Appearance of fermenter on galactose agar

When a rapid fermenter mutant is planted on galactose agar, it forms a large colony in less than 3 days, whether it is planted together with large or small numbers of non-fermenter cells. When non-fermenter cells grown in glucose are planted on galactose agar, the first count of the fermenter colonies is made on the fifth day. On the third day distinct tiny fermenter colonies make their first appearance and become large and easily counted on the fifth day. The fact that fermenter cells form large colonies before the third day is another piece of evidence supporting the view that they are absent from glucose grown cultures of the non-fermenter.

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