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Nitrosoguanidine Assay of Episomal Integration in *Escherichia coli*

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Mutations affecting utilization of lactose and resistance to the male-specific phages f1, f2, and Qβ tend to occur simultaneously more often than expected by chance in Hfr strains whose origin of transfer is close to the genes for lactose utilization, but not in F+ strains. Strains derived from the Hfr, but exhibiting poor ability to transfer early chromosomal genes, may or may not show this comutation phenomenon. These results support the concept that the F factor is integrated into the Hfr chromosome during vegetative growth, but is autonomous in the F+ strains and could serve as an assay for episomal localization.

N-methyl-*N'*-nitro-*N*-nitrosoguanidine is an effective mutagen in *Escherichia coli* (2, 11) and in many other organisms. The inactivation of the F factor by nitrosoguanidine, as we will abbreviate its name, has been described (13), and many mutations induced by this agent have been used in the genetic analysis of F functions (review in reference 1). Although all genes are susceptible to mutation by nitrosoguanidine, the mutations in any particular cell are not randomly distributed over the chromosome but crowded together in small regions, extending over less than 2 min of the *E. coli* chromosome (6), and placed at the positions occupied by the replication points at the time of the treatment (5). Genetic sites closer than 2 min exhibit comutation, that is, they tend to mutate simultaneously more often than expected by chance. Comutation may be a useful tool in some problems of genetic mapping, such as the recognition of the integrated and autonomous states of episomes. We have used the F factor as a model system, defining it as an episome conferring sensitivity to male-specific phages, such as the ribonucleic acid (RNA) phages Qβ and f2 and the deoxyribonucleic acid (DNA) phage f1.

MATERIALS AND METHODS

Bacteria and phages. χ15 is a prototrophic F+ strain of *Escherichia coli*, derived from the F+ strain W1485; χ493 is a prototrophic Hfr strain, derived from χ15 and transferring its chromosome in the order *O-proB-proA-leu-thr-···-lac-F*. Both were received from R. Curtiss (3). DF73 is an F− strain carrying the markers *strA*, *thyA*, *thr*, *leu*, *thi*, *pro*, *arg*, *lac*, *gal*, *ara*, *xyl*, *mtl*, derived from the strain AB1157 and received from K. Brooks.

The wild-type RNA phages Qβ and f2 were received from A. J. Clark, and the wild-type DNA phage f1 was from N. D. Zinder.

Culture conditions. Bacteria were grown at 37 C in tris(hydroxymethyl)aminomethane (Tris)-salts minimal medium (10), with D-glucose at 2 g/liter as carbon and energy source. Solid media were prepared in the same way with 25 g of agar (Difco) per liter. When appropriate, amino acids and thymine were added at 20 μg/ml, thiamine at 1 μg/ml, and streptomycin at 100 μg/ml, and β-lactose was substituted for glucose at 2 g/liter.

Nitrosoguanidine treatment. Cells from exponentially growing cultures were treated with 100 μg of nitrosoguanidine (Serva, Heidelberg, Germany) per ml of Tris-maleate buffer, pH 7.5, at 37 C for 30 min (4); they were washed twice by centrifugation both before and after the treatment.

Test for phage resistance. Phage stocks were produced in broth-grown *E. coli* strain χ493. A drop of phage stock, containing over 10^9 plaque-forming units of the indicated phage or phage mixture, was extended in a diametrical band on an agar plate with a wire loop. Bacteria from single colonies were streaked across the phage-containing band with a sterile toothpick. A colony was considered resistant when bacterial growth was the same on both sides of the phage-containing band, as observed after overnight incubation at 37 C.

RESULTS

New strains. Two Lac− derivatives of the Hfr strain χ493, unable to utilize lactose, were isolated after nitrosoguanidine treatment and called SE12 and SE13. To this end, exponentially-growing cells of strain χ493 were treated with nitrosoguanidine, allowed to grow in glucose liquid medium for 3 h, and plated on glucose solid medium; individual colonies were then tested for growth on lactose solid medium. The conjugational ability and the order of gene

transfer were checked in these strains by interrupted mating (9) with DF73, and they were found to be the same as in the original strain.

Two Lac$^-$ derivatives of the F$^+$ strain χ15 were isolated by the same procedure as for the Hfr, and called SE20 and SE21. They were checked to be still F$^+$ by observing the appearance of resistance to the male-specific phages f1, f2, and Qβ after prolonged incubation with acridine orange (7).

Strains exhibiting poor conjugational ability were isolated from the Lac$^-$ Hfr. Bacteria from SE12 colonies were spotted onto plates covered with some 10^9 cells of strain DF73 and containing adequate supplements for the selection of Leu$^+$ Thr$^+$ Str$^+$ recombinants (that is, containing streptomycin, thiamine, thymine, proline, and arginine). In most cases a thick growth of recombinants appeared, but some colonies, constituting about 1% of the total, did not produce any recombinants. After reisolation and retesting, four of them were called SE18, SE19, SE22, and SE23. The same procedure was applied to the strain SE13, and strains SE15, SE16, and SE17 were isolated. These strains were all found to be sensitive to male-specific phages.

Comutation. The *lac* mutations in strains SE12, SE13, SE20, and SE21 were obtained independently. The survival of these strains after nitrosoguanidine treatment and the frequencies of Lac$^+$ revertants are indicated in Table 1, which gives the results of typical experiments in which bacterial samples, taken just before or just after the treatment with nitrosoguanidine, were plated on glucose and lactose media. Higher survival rates can be obtained by decreasing the pH at the time of the treatment to 5.5. It can be noted that nitrosoguanidine usually increases the frequency of revertants more than 1,000-fold. The strains derived from SE12 and SE13 behave like their parents in these respects.

When untreated samples were plated on either glucose or lactose, the resulting colonies were always phage sensitive. Not a single phage-resistant colony was found after testing a total of 8,000 colonies belonging to all the strains obtained in the previous section.

After the mutagenic treatment, a sizable proportion of phage-resistant colonies was found both among the general population (glucose plates) and among the Lac$^+$ revertants (lactose plates). Table 2 gives the incidence of resistance to phages Qβ and f2, and Table 3 gives the incidence of resistance to phage f1. The colonies resistant to f1 were found to be resistant to Qβ and f2, but the reverse was often untrue. As a consequence, the proportions in Table 2 tend to be higher than those in Table 3.

TABLE 1. *Survival and reversion to Lac$^+$ after nitrosoguanidine treatment*

Strain	Surviving fraction	Frequency of Lac$^+$ revertants (per colony-forming unit)	
		Before the treatment	After the treatment
SE12 (Hfr)	0.15	2.2×10^{-6}	2.7×10^{-3}
SE13 (Hfr)	0.050	7.5×10^{-7}	9.4×10^{-4}
SE20 (F$^+$)	0.096	$<10^{-8}$	4.1×10^{-4}
SE21 (F$^+$)	0.072	$<10^{-8}$	2.8×10^{-6}

In the Hfr strains the mutation to resistance to male-specific phages was always more frequent among the Lac$^+$ revertants than among the general population; thus, both kinds of mutations tended to occur simultaneously in the same cells. No such association was found in the F$^+$ strains, where both kinds of mutations occurred as independent events. One of the seven strains isolated from the Hfr because of their poor ability to transfer early chromosomal genes showed comutation between phage resistance and Lac$^+$ reversion, but the others did not.

DISCUSSION

The two kinds of mutations investigated here were chosen because one is presumed to depend on chromosomal genes and the other is presumed to depend on F-factor genes. The experimental result is that in the Hfr the two kinds of mutations occur simultaneously more often than expected by chance after nitrosoguanidine treatment, whereas this does not happen in F$^+$ strains. This result indicates that the genes responsible for the two mutations replicate at about the same time in the Hfr but not in the F$^+$. This is in excellent agreement with the hypothesis (8) that the F factor of the Hfr is integrated with the chromosome and replicates as a part of it, even during vegetative growth unrelated to conjugation.

The strains isolated from the Hfr because of their poor ability to transfer early chromosomal genes may be expected a priori to fall into two groups, one lacking comutation between episomal and chromosomal genes (presumably due to detachment from the chromosome of an F factor that does not carry the relevant chromosomal genes) and another exhibiting comutation (detachment of an F factor carrying the relevant chromosomal genes, or mutational loss of conjugational ability without detachment or acquisition of resistance to male-specific phages). Six strains of the first group and one of the second group were isolated.

Guerola et al. (6) defined the comutation index as the frequency of double mutants rela-

TABLE 2. *Resistance to phages Qβ and f2 after nitrosoguanidine treatment*

Strain	Plated on glucose		Plated on lactose		Statistical significance[a]
	Colonies tested	Percent resistant	Colonies tested	Percent resistant	
Hfr strains					
SE12	800	4.6	800	9.2	$P < 0.001$
SE13	1825	4.8	1825	7.9	$P < 0.001$
F^+ strains					
SE20	700	5.0	800	5.1	NS
SE21	400	4.7	300	5.3	NS
Conjugation-defective strains derived from Hfr					
SE15	400	3.5	400	12.5	$P < 0.001$
SE16	400	4.7	400	5.0	NS
SE17	400	5.2	400	6.7	NS
SE18	400	4.0	400	4.0	NS
SE19	400	5.0	400	7.0	NS
SE22	500	5.0	500	5.6	NS
SE23	400	5.0	400	5.4	NS

[a] The probability of a chance deviation equal to or larger than the experimental one was calculated after the arcsin transformation and the *t* test (12). All "not significant" (NS) cases gave $P > 0.2$.

TABLE 3. *Resistance to phage f1 after nitrosoguanidine treatment*

Strain	Plated on glucose		Plated on lactose		Statistical significance
	Colonies tested	Percent resistant	Colonies tested	Percent resistant	
Hfr strains					
SE12	500	3.2	500	8.6	$P < 0.001$
SE13	375	2.1	375	7.2	$P < 0.001$
F^+ strains					
SE21	500	2.6	400	3.0	NS
Conjugation-defective strains derived from Hfr					
SE17	500	2.4	500	2.0	NS
SE18	500	1.4	500	1.4	NS

tive to the product of the frequencies of the single mutants; they found an index of 15 for genes placed 1 min away in the chromosome map. The comutation index for Lac^+ reversion and f1 resistance is about 3 in the Hfr strains. By comparison with Guerola's results, the relevant genes may be placed some 1.5 min away in the Hfr chromosome.

Comutation is clearer and the index is slightly higher when f1 is used to assay for resistance than when Qβ and f2 are used. On the other hand, bacteria resistant to f1 are usually resistant to Qβ and f2, but not vice versa. This may be due to mutations conferring resistance to Qβ and f2, but not f1, located in chromosomal genes far from *lac* and the F factor, and is probably related to the different participation of bacterial genes in the development of the two types of phage.

Comutation studies fashioned in different ways may be used to test for the integration of an episome into a chromosome. Such a test is independent of whether the episome confers conjugational ability, of whether a genetic system has been developed in the bacterial species under study, and of whether the episome carries any indispensable genes (and thus cannot be lost upon incubation with acridine dyes). For example, a mutational change in the episome would be selected, either in the forward or

reverse direction, after treatment with nitrosoguanidine, and the resulting mutants would be assayed for the presence of chromosomal mutations. Auxotrophic mutations are convenient to assay and are usually spread over the chromosome. If the episomal mutation is frequently associated with a particular growth requirement, the implication would be that the episome is close to the genes responsible for that kind of auxotrophy.

In fact comutation only means that the two genetic structures replicate simultaneously. Comutation might be found between genes in two independent replicons if there is a precise synchronization in their replication. Autonomous episomes are known to replicate at certain times of the cell cycle (14, 15), but there is no indication that the synchronization is good enough to show appreciable comutation with chromosomal genes.

Reversion to wild-type phenotype may sometimes be due more to intergenic suppression than to intragenic reversion and the comutation results would then be misinterpreted. In our experience such high relative incidence of intergenic suppression is uncommon and errors can be prevented by the study of different original mutations of the same type.

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LITERATURE CITED

1. Achtman, M. 1973. Genetics of the F sex factor in Enterobacteriaceae. Curr. Top. Microbiol. Immunol. **60**:79–123.
2. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
3. Berg, C. M., and R. Curtiss. 1967. Transposition derivatives of an Hfr strain of *Escherichia coli* K-12. Genetics **56**:503–525.
4. Cerdá-Olmedo, E., and P. C. Hanawalt. 1968. Diazomethane as the active agent in nitrosoguanidine mutagenesis and lethality. Mol. Gen. Genet. **101**:191–202.
5. Cerdá-Olmedo, E., P. C. Hanawalt, and N. Guerola. 1968. Mutagenesis of the replication point by nitrosoguanidine: Map and pattern of replication of the *Escherichia coli* chromosome. J. Mol. Biol. **33**:705–719.
6. Guerola, N., J. L. Ingraham, and E. Cerdá-Olmedo. 1971. Induction of closely linked multiple mutations by nitrosoguanidine. Nature (London) **230**:122–125.
7. Hirota, Y. 1960. The effect of acridine dyes on mating type factors in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **46**:57–64.
8. Jacob, F., and E. L. Wollman. 1957. Analyse des groupes de liaison génétique de différentes souches donatrices d'*Escherichia coli* K12. C. R. Acad. Sci. **245**:1840–1843.
9. Low, B., and T. H. Wood. 1965. A quick and efficient method for interruption of bacterial conjugation. Genet. Res. **6**:300–303.
10. Maaløe, O., and P. C. Hanawalt. 1961. Thymine deficiency and the normal DNA replication cycle. J. Mol. Biol. **3**:144–155.
11. Mandell, J. D., and J. Greenberg. 1960. A new chemical mutagen for bacteria: 1-methyl-3-nitro-1-nitrosoguanidine. Biochem. Biophys. Res. Commun. **3**:575–578.
12. Sokal, R. R., and F. J. Rohlf. 1969. Biometry, p. 607. W. H. Freeman and Co, San Francisco.
13. Takahashi, I., and R. A. Barnard. 1967. Effect of N-methyl-N′-nitro-N-nitrosoguanidine on the F factor of *Escherichia coli*. Mutat. Res. **4**:111–117.
14. Yabe, Y., and S. Mitsuhashi. 1971. Replication and transfer of the R factor in a synchronized culture of *Escherichia coli*. Jap. J. Microbiol. **15**:21–27.
15. Zeuthen, J., and M. L. Pato. 1971. Replication of the F'lac sex factor in the cell cycle of *Escherichia coli*. Mol. Gen. Genet. **111**:242–255.