INDUCTION BY MUTAGENS OF TANDEM GENE DUPLICATIONS IN THE *glyS* REGION OF THE *ESCHERICHIA COLI* CHROMOSOME

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ABSTRACT

Four mutagens (ultraviolet light, nitrous acid, the acridine half mustard ICR 372, and niridazole) have been found to increase the frequency of tandem gene duplications in the *glyS* region of the *Escherichia coli* chromosome. This result was obtained by quantitating the spontaneous and mutagen-induced reversion frequency of a glycyl-tRNA synthetase (*glyS*) mutant. Following mutagenesis, as many as 0.2% of the survivors were observed to contain duplications in the *glyS* region. In addition, several classes of stable revertants of the *glyS* mutant have been identified.

TANDEM gene duplications, like other chromosomal rearrangements in bacteria, are believed to arise from aberrant recombinational events (reviewed by FRANKLIN 1971). A number of different selective procedures have been used to isolate mutant strains containing tandem duplications, including selection for increased gene dosage (HORIUCHI, HORIUCHI and NOVICK 1963; MARGOLIN and BAUERLE 1966; GLANSDORFF and SAND 1968; FOLK and BERG 1971; JACKSON and YANOFSKY 1973) and selection for suppressors that have deleterious or lethal effects in the haploid state (HILL *et al.* 1969; SOLL and BERG 1969a,b; MILLER and ROTH 1971). Duplications have been found to occur at very high spontaneous frequencies ($\geqslant 10^{-5}$) in at least two regions of the *E. coli* chromosome (GLANSDORFF and SAND 1968; FOLK and BERG 1971).

Although some mutagens are known to induce deletions in bacteria (SCHWARTZ and BECKWITH 1969; HARTMAN *et al.* 1971; CORDARO and ROSEMAN 1972), little is known about the effect of mutagens on the frequency of other genetic events arising from aberrant recombination. In this study, the effect of four mutagens on the frequency of tandem duplications in the *glyS* region of the *E. coli* chromosome was evaluated, using a *glyS* mutant previously isolated and described by FOLK and BERG (1971). This strain contains a mutant glycyl-tRNA synthetase with a very poor affinity for glycine and therefore requires glycine for growth. Among the Gly^+ revertants obtained in this strain, many may be attributed to tandem duplications including the mutant *glyS* gene. Apparently duplication of the mutant *glyS* gene increases the level of mutant glycyl-tRNA synthetase sufficiently to permit growth on the endogenously synthesized glycine pool (FOLK and BERG 1971).

The present results indicate that the frequency of tandem duplications in the *glyS* region of the chromosome is elevated following treatment with all four mutagenic agents tested. Using a different selection procedure, HILL and coworkers have independently found that several mutagens stimulate the frequency of duplications in the *glyT* region of the *E. coli* chromosome (HILL and COMBRIATO 1973).

MATERIALS AND METHODS

Strains

Strain BF87 (*E. coli* K12, *glyS87 trpA36 su36+*) is a glycine-requiring glycyl-tRNA synthetase mutant described previously by FOLK and BERG (1971). This strain, obtained from P. BERG, has lost the *su36+* mutation and therefore requires tryptophan. Strains TS95 and TS96 are independent Gly+ revertants of strain BF87 obtained after mutagenesis with niridazole. Strain 157 (*xyl arg ilv met thi*) was obtained from E. C. C. LIN. To test for recovery of the *glyS87* mutation from revertants of BF87, P1 phage grown on each revertant were used to transduce strain 157 to growth on xylose medium. Transductions were performed as described by WALL and HARRIMAN (1974), using P1 *vir* phage (IKEDA and TOMIZAWA 1965).

Media

Minimal medium was medium E of VOGEL and BONNER (1956). Minimal glucose glycine medium contained medium E, supplemented with 0.5% glucose, 0.1 mM tryptophan, and 5 mg/ml glycine. The medium used in reversion tests of strain BF87 contained medium E, 1.5% agar, 2% glucose, and 0.1 mM tryptophan. Commercial preparations of agar (Difco-Bacto) were found to contain enough glycine to support a trace of growth by strain BF87. Therefore, agar for solid media used in reversion tests was prewashed three times with distilled water. Tryptone glucose extract agar medium was purchased from Difco.

Mutagenesis

Several independent cultures of strain BF87 were tested for Gly+ revertants. A culture having a low frequency of Gly+ cells was stored in 50% glycerol at −20° and used as the starting culture for all subsequent experiments.

Ultraviolet light irradiation was performed with a germicidal lamp at a dose rate of 21 ergs/mm²/sec. Stationary phase cells were diluted 1:2 into T2 buffer (HERSHEY and CHASE 1952), exposed for varying time intervals, resuspended in minimal glucose glycine medium, and grown to stationary phase at 37°.

Nitrous acid mutagenesis was performed by a modification of the procedure of SCHWARTZ and BECKWITH (1969). Stationary phase cells (5 ml) were centrifuged, washed once with sodium acetate buffer (pH 4.6), and resuspended in 0.3 ml of the same buffer plus 0.05 M sodium nitrite. Cells were shaken for 4 to 18 minutes at 33°, washed with 5 ml of minimal medium, resuspended in 10 ml of minimal glucose glycine medium, and grown for 24 hours at 37°.

Mutagenesis with ICR 372 was accomplished by diluting 10ml of stationary phase cells into 1 ml of minimal glucose glycine medium containing 5 μg of ICR 372, and shaking for 22 hours at 37°. Mutagenized cells were washed and plated directly on selective medium.

Mutagenesis with niridazole [1-(5-Nitro-2-thiazolyl)-2-imidazolidinone] was performed by diluting stationary phase cells 1:5 into minimal glucose glycine medium containing 5—50 μg/ml of niridazole and shaking at 28° for 30 minutes. Treated cells were centrifuged, resuspended in minimal glucose glycine medium and shaken for 24 hours at 28°.

Reversion of strain BF87

For quantitative reversion studies of strain BF87, approximately 2×10^4 to 2×10^5 cells were spread on solid minimal glucose medium. The plates were incubated for 44 hours at 37°, and Gly+ revertants were counted. It was necessary to strictly standardize the incubation time to obtain reproducible results, because more colonies tend to appear after further incubation.

Stability tests

Tests for segregation of Gly⁻ clones by revertants of strain BF87 were performed by plating cells on tryptone glucose extract medium following growth in minimal glucose glycine medium. Gly⁻ segregants can be easily identified as tiny smooth colonies on tryptone glucose extract medium after 16–20 hours at 37°, while colonies of *glyS* duplications are larger, and often rough in appearance. Strains were judged to be stable if they segregated Gly⁻ clones at a frequency less than 0.1% after growth from a single colony to stationary phase in 10 ml of minimal glucose glycine medium.

RESULTS AND DISCUSSION

Classification of Gly⁺ Revertants

The effect of mutagens on the frequency of tandem gene duplications in the *glyS* region of the *E. coli* chromosome has been determined by quantitating the spontaneous and mutagen-induced reversion frequency of strain BF87. The results are complicated by the observation that the Gly⁺ revertants of this strain may be divided into four classes, as shown in Figure 1. Interestingly, all 287 spontaneous and mutagen-induced revertants that were analyzed still contain the *glyS87* mutation; no reversion to wild type was observed.

1. *Tandem duplications:* Revertants containing tandem duplications of the mutant *glyS* gene have been described by FOLK and BERG (1971). Revertants of this type may be easily identified by their genetic instability. Cultures of the Gly⁺ duplication strains grown in medium containing glycine generally contain about 1–50% Gly⁻ cells. FOLK and BERG (1971) presented extensive biochemical and genetic evidence showing that the unstable Gly⁺ revertants contain tandem duplications of the mutant *glyS* gene and that the genetic instability of these revertants is due to *recA*-dependent recombinational loss of the duplication. The mutagen-induced and spontaneous unstable Gly⁺ revertants are similar in colony morphology, growth characteristics, and degree of instability. Therefore, it seems likely that they represent the same genetic event. In this study, genetic instability is used as the basis for classification of Gly⁺ revertants as duplications.

As reported by FOLK and BERG (1971), and confirmed by the results in Table 1, *glyS* duplications are the largest class of revertants obtained spontaneously. In the experiment shown in Table 1, of 29 spontaneous revertants of strain BF87 picked from 10 independent cultures, 27 contained *glyS* duplications.

2. *Glycine dissimilation mutants:* Since strain BF87 contains a mutant glycyl-tRNA synthetase with a poor affinity for glycine, it may be suppressed by muta-

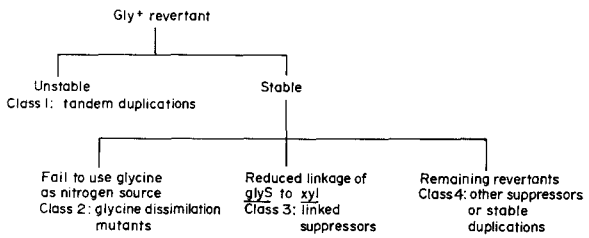

FIGURE 1.—Flow chart showing the procedure for classifying Gly+ revertants of strain BF87.

TABLE 1

Survival and reversion frequency of strain BF87 following mutagenesis

Gly+ revertants were picked and classified. The mutagen-induced increase of *glyS* duplications and glycine dissimilation mutants has been calculated in the last two columns.

Treatment	Percent survival	Gly+ revertants per 10^5 survivors	Classification of Gly+ revertants: Total Gly+ revertants analyzed	Tandem duplications	Glycine dissimilation mutants	Closely linked suppressors	Other suppressors or stable duplications	Mutagen-induced increase in frequency of revertants: Tandem duplications	Glycine dissimilation mutants
Spontaneous*	(100)	7 ± 5	29	27	2	0	0	(1x)	(1x)
	62	130	..	..	..	..	..	..	..
UV†	29	200	49	39	3	4	3	25x	25x
	7	300	.	..	..	..	..	..	..
	1	360	..	.	.	..	..	..	..
	70	13	..	..	..	..	..	..	..
	29	45	..	..	.	..	..	..	..
Nitrous acid‡	2	100	47	37	9	1	0	12x	40x
	0.1	150	..	..	..	..	..	..	..
	0.04	360	..	..	..	..	..	..	..
ICR 372§	..	200	76	25	51	0	0	10x	280x
	75	25	.	.	..	..	..	..	..
Niridazole¶	10	55	50	26	16	4	4	5x	37x
	5	98	..	..	..	..	..	..	..
	1	200	36	16	13	3	4	14x	150x

* The spontaneous reversion frequency of strain BF87 represents the average of ten experiments. The error of $\pm 5 \times 10^{-5}$ is the standard deviation. The spontaneous Gly+ revertants were picked from ten independent cultures.

† Ultraviolet doses ranged from 210 ergs/mm² (corresponding to 62% survival) to 840 ergs/mm² (1% survival).

‡ Mutagenesis was accomplished by treating cells for times ranging from 4 min (70% survival) to 18 min (0.04% survival).

§ Cells were grown in the presence of ICR 372 (5 μg/ml) for 22 hours at 37°.

¶ Niridazole concentrations ranged from 5 μg/ml (corresponding to 75% survival) to 50 μg/ml (1% survival).

tions that elevate the internal pool of glycine. A large number of stable Gly+ revertants of strain BF87 were found to have lost the ability to grow on glycine as the sole source of nitrogen. An extract of a typical revertant of this type was assayed for glycyl-tRNA synthetase activity by the method of FOLK and BERG (1971) and was found to contain, within an experimental error of 15%, the same activity as the parent Gly⁻ strain, BF87. Furthermore, when P1 phage grown on this strain were used to transduce an *xyl* recipient to growth on xylose, the mutant *glyS87* allele was recovered at the same frequency as from the parent strain (60–80%). It is concluded from these results that the *glyS87* mutation is indirectly suppressed in these strains by a second mutation blocking glycine dissimilation.

3. *Closely-linked suppressors:* A number of stable revertants of strain BF87 have been found in which the *glyS87* mutation can be recovered by P1 transductions with *xyl*, but at a greatly reduced co-transduction frequency. When P1 phage grown on these strains were used to transduce an *xyl* recipient to growth on xylose, Gly$^-$ transductants were recovered at frequencies of 0.3–4%, compared with 60–80% for the parent strain. The simplest explanation for these revertants is that they contain closely linked (perhaps intragenic) suppressor mutations. However, the possibility has not been precluded that some of the strains contain stable translocations of the *glyS* gene, in which the distance between *glyS* and *xyl* has actually been increased.

4. *Other suppressors or stable duplications:* Eleven of the revertants obtained after mutagenesis do not fall into any of the first three classes. They are all stable and utilize glycine as sole nitrogen source. The *glyS87* mutation is recovered from each of these strains by P1 transductions with *xyl* at the same high frequency as from the parent. This class has not been further analyzed, but presumably it contains other types of suppressors, and perhaps, stable duplications. In regard to the latter possibility, HILL and COMBRIATO (1973) have observed stable duplications of the *glyT* region of the *E. coli* chromosome.

Effect of mutagens on reversion of strain BF87

Four mutagens (UV, nitrous acid, the acridine half mustard ICR 372, and niridazole) have been found to increase the reversion frequency of strain BF87. Following each experiment, randomly picked Gly$^+$ revertants were classified as described above. By this procedure, it was possible to quantitate the increase in frequency of all types of revertants. The results (Table 1) indicated that all four mutagens increased the frequency of *glyS* duplications.

Reconstruction experiments were performed to determine whether the apparent induction of duplications by mutagens might be due to selection artifacts. In the first experiment untreated cells of strain BF87 were diluted 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} into minimal glucose glycine medium and grown to stationary phase. No significant increase in the frequency of Gly$^+$ revertants was observed with increasing dilution, indicating that Gly$^+$ revertants do not have a significant selective advantage in minimal glucose glycine medium.

The possibility still remained that the *glyS* duplication strains might be less sensitive to killing by mutagens than the parental haploid strain. If this were the case, the apparent induction of duplications by mutagens might actually result from higher survival or faster recovery of pre-existing spontaneous *glyS/glyS* merodiploids in the mutagen treated cultures. A reconstruction experiment was performed to test this possibility for one of the mutagens, niridazole.

Strains TS95 and TS96 are two independent unstable Gly$^+$ revertants recovered after treatment with niridazole. Strain TS96 is typical of the most unstable *glyS/glyS* merodiploids. Cultures of this strain, grown in minimal glucose glycine medium from single colonies as described in MATERIALS AND METHODS, contain approximately 42–49% haploid Gly$^-$ segregants (based on three determinations).

Strain TS95 is more stable, yielding 1–6% haploid Gly⁻ segregants under the same conditions.

Stationary phase cultures of these two strains were mixed in approximately 1:1 proportions with stationary phase cultures of strain BF87. The mixtures were treated with niridazole at several concentrations under exactly the conditions used in the experiments shown in Table 1. Following treatment and expression, the proportion of *glyS* and *glyS/glyS* cells was examined by plating the cultures on tryptone glucose extract medium. The results (Table 2) indicated that following mutagen treatment, strain TS95 had no pronounced selective advantage compared with the *glyS* haploid strain. The other duplication strain, TS96, was actually more sensitive to killing by niridazole than the *glyS* parental strain. These results suggest that the increased frequency of duplications observed following niridazole treatment (Table 1) could not result from selection for pre-existing spontaneous *glyS/glyS* duplications in the BF87 population.

Interpretation of the mutagenesis data

The mutagens employed in our study were selected because they induce different types of pre-mutational lesions in DNA. The mode of action on DNA of UV and nitrous acid has been extensively studied in many laboratories (reviewed by DRAKE 1970). ICR 372 is an acridine half mustard that induces frameshift mutations in bacteria (AMES and WHITFIELD 1966; CREECH *et al.* 1972). Niridazole is a nitrothiazole derivative which, in analogy with nitrofurans (MCCALLA, REUVERS and KAISER 1970; MCCALLA and VOUTSINOS 1974), may react with DNA following reduction to a nitroso or hydroxamino derivative. There have been no previous reports in the literature that this compound is mutagenic. However, YAMASAKI and AMES (personal communication) have found that it reverts one frameshift mutation (*hisD3052*) in the set described by AMES, LEE and

TABLE 2

Stationary phase cultures of strain BF87, and strain TS95 or TS96, were mixed in approximately equal proportions and treated with niridazole as described in MATERIALS AND METHODS

Following treatment and expression, the percentages of *glyS* haploid and *glyS/glyS* merodiploid cells in each culture were determined by plating on tryptone glucose extract agar and scoring colony types. The *glyS/glyS* merodiploid colonies were characteristically large and slightly rough; the *glyS* colonies were small and smooth.

Mixture	Niridazole concentration μg/ml	Composition of mixture after treatment and expression		
		Total colonies scored	Percent *glyS*	Percent *glyS/glyS*
BF87 + TS95	0 (Control)	794	43	57
	20	515	56	44
	40	950	36	64
	60	741	41	59
BF87 + TS96	0 (Control)	985	62	38
	20	984	90	10
	40	686	77	23
	60	561	95	5

DURSTON (1973), and we have found that it also reverts an ochre mutant, *hisC117*.

The observation that all four mutagens were active in inducing duplications suggests that the stimulation is due to some type of DNA damage (or DNA repair intermediate) common to a variety of mutagenic treatments, such as single-strand breaks and gaps. Such breaks could act as precursors for the "recombinational" intermediates involved in the formation of duplications. HILL and COMBRIATO (1973) have similarly found a lack of mutagen specificity in the induction of tandem duplications in *E. coli*. They found that UV, nitrous acid, ethyl methanesulfonate, and N-methyl-N'nitro-N-nitrosoguanidine all induce tandem duplications in the *glyT* region of the chromosome.

In the present study some mutagens appeared to give a much higher proportion of duplications, compared to stable suppressor mutations, than others. For example, under the conditions shown in Table 1, UV caused a 25-fold increase in both duplications and mutations in the glycine dissimilation pathway; while ICR 372 caused only a 10-fold increase in duplications but a 280-fold increase in glycine dissimilation mutants. This might reflect a different efficiency of the two mutagens in increasing the frequency of aberrant recombination *versus* point mutations. Further dose-response experiments must be performed, however, before rigorous conclusions can be drawn in this regard.

The genetic activity of niridazole is of interest because this compound is currently being used on an investigational basis for the treatment of schistosomiasis in humans (MOST 1972). In *E. coli*, at 1% survival, niridazole induces a 14-fold increase in tandem duplications in *glyS* and a 150-fold increase in forward mutations in the glycine dissimilation gene. The strong mutagenic activity of niridazole in bacteria suggests that it should be tested further for mutagenicity and carcinogenicity in higher organisms.

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