# PHENETHYL ALCOHOL RESISTANCE IN *ESCHERICHIA COLI*. II. REPLICATION OF F FACTOR IN THE RESISTANT STRAIN C600

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PHENETHYL alcohol (PEA) is known to inhibit nucleic acid and protein synthesis in bacteria (BERRAH and KONETZKA 1962; ROSENKRANZ, CARR and ROSE 1965; PREVOST and MOSES 1966). In *Escherichia coli*, PEA is believed to selectively inhibit the initiation of a new cycle of DNA replication without interfering with the completion of a cycle already initiated before addition of PEA to the medium (TREICK and KONETZKA 1964; LARK and LARK 1966). It also has been proposed that the primary action of PEA is at the level of the cell membrane (SILVER and WENDT 1967).

We have previously reported that strain C600 of *E. coli* K12 and its derivatives are resistant to PEA and carry a resistance gene (*pea*) located very close to the gene determining azide resistance (*azi*) (YURA and WADA 1968). Other evidence suggests that the *pea–azi* region of the *E. coli* chromosome is concerned with some essential function(s) related to the direct or indirect involvement of the cell membrane structure in the control of DNA replication and cell division (see also, VAN DE PUTTE, VAN DILLEWIJN and RÖRSCH 1964; NORMARK 1970; TAYLOR 1970).

The fertility (F) factor of *E. coli* is a typical episome present as either an autonomously replicating unit or an integrated part of the host chromosome (JACOB and WOLLMAN 1958). Although genetic evidence has suggested the participation of a specific protein in controlling the replication of the F′ factor (JACOB, BRENNER and CUZIN 1963), little is known about the nature and the mode of action of the cellular and episomal factors involved. During the course of an investigation of PEA resistance in *E. coli*, we noted that strain C600 carrying an autonomous F episome is rather unstable in that it segregates out $F^-$ cells at an unusually high frequency. Furthermore, segregation of $F^-$ clones was markedly accelerated by addition of sublethal concentrations of PEA to the culture medium. These observations prompted us to investigate the genetic and other factors affecting the stable maintenance of the autonomous F factor in this strain, and the results of some of the experiments will be reported in this paper.

Part of this work has appeared previously in abstract form (WADA and YURA 1965).

## MATERIALS AND METHODS

*Bacterial and phage strains:* All bacterial strains employed are derivatives of *Escherichia coli* K12 and are listed in Table 1. A number of strains were derived from strain C600.1 (GARTNER

TABLE 1

*Bacterial strains used and their known genetic characters**

| Strain | Sex | Chromosomal markers | Episome | Origin‡ |
|---|---|---|---|---|
| a. Strain C600 and its derivatives | | | | |
| C600 | F- | *thr, leu, lac, thi, tonA, pea, supE* | —— | A. CAMPBELL |
| C600.1 | F- | *lac, thi, tna, str, pea, supE, maf* | —— | M. RILEY |
| KY301 | F- | del(*trp-tonB*); other markers same as in C600.1 | —— | C600.1 |
| KY305 | F- | *his*; other markers same as in KY301 | —— | KY301 |
| KY306 | F- | *pro*; other markers same as in KY301 | —— | KY301 |
| KY6005 | F- | *gal*; other markers same as in KY306 | —— | KY306 |
| KY387 | Hfr | *str*+; other markers same as in KY301 | —— | CS101 × KY305 |
| KY344 | F+ | same as in KY301 | F+ | KY301 |
| KY311 | F′ | same as in KY301 | F*trp*+ | 5TD4 × KY301 |
| KY314 | F′ | same as in KY306 | F*trp*+ | 5TD4 × KY306 |
| KY327 | F′ | same as in KY301 (Mutant II)† | F*trp*+ | KY311 |
| KY334 | F′ | same as in KY301 (Mutant I)† | F*trp*+ | KY311 |
| KY2016 | F′ | *his*; other markers same as in KY334 | F*trp*+ | KY334 |
| KY312 | F′ | same as in KY301 | F*lac*+ | W3747 × KY301 |
| KY2142 | F′ | same as in KY301 | def-F*lac*+ | KY312 |
| KY6904 | F′ | same as in KY6005 | F*gal*+ | W4520 × KY6005 |
| b. Other K12 strains | | | | |
| KY131 | F- | *thr, leu, his, met, lac, gal, malA, xyl, mtl, ara, thi, tonA, azi, str* | —— | PA678 |
| 5TD4 | F′ | *trp, cysB, his, str* | F*trp*+ | P. FREDERICQ |
| KY113 | F′ | del(*trp-tonB*) (λ) | F*trp*+ | 5TD4 × KY120 |
| KY2054 | F′ | *trp*; other markers same as in KY131 | F*lac*+ | W3747 × KY2053 |
| W4520 | F′ | *met* (λ) | F*gal*+ | Y. HIROTA |
| KY2127 | F- | *his*+, *trp*; other markers same as in KY131 | — | KY387 × KY131 |

* Gene symbols: Genes determining biosynthesis; *cys*, cysteine; *his*, histidine; *trp*, tryptophan; *met*, methionine; *thr*, threonine; *leu*, leucine; *pro*, proline; *thi*, thiamine. Genes determining sugar utilization: *gal*, galactose; *lac*, lactose; *mal*, maltose; *xyl*, xylose; *mtl*, mannitol; *ara*, arabinose. Genes determining response to phages: *tonA*, T1 and T5; *tonB*, T1. Genes determining response to drugs: *str*, streptomycin; *azi*, azide; *pea*, phenethyl alcohol. *tna*, gene determining tryptophanase. *sup*, suppressor of amber mutations. *maf*, gene determining maintenance of autonomous F factors. Sexual crosses are represented as donor × recipient. del, deletion; def, defective.

† These strains carry a mutation giving rise to stable maintenance of F′ factors under certain conditions (see Table 6).

‡ Origin as indicated here does not necessarily represent an immediate origin.

and RILEY 1965), kindly supplied by M. RILEY, and will be referred to simply as strain C600 in this paper. Strain 5TD4 carrying F*trp* was obtained from P. FREDERICQ, and W3747 carrying F*lac* and W4520 carrying F*gal* from Y. HIROTA. Phage P1kc (LENNOX 1955) was employed in all transduction experiments. Male-specific RNA phage MS-2 was obtained from Y. KAWADE.

*Media:* Standard media used were minimal medium E (VOGEL and BONNER 1956) with glucose and other supplements, and a peptone-glucose (PG) medium containing 20 g polypeptone (Wako Drug Co.), 5 g NaCl and 2 g glucose per liter (pH 7.2). The determination of resistance or sensitivity to PEA was made using PEA-agar medium as described previously (YURA and WADA 1968). Segregation of F- clones from strains carrying F*trp* was scored on medium E supplemented with 0.2% glucose, 0.2% Difco Casamino acids, 1 μg/ml L-tryptophan, 2 μg/ml

thiamine, and 1.5% agar (Hakko Agar Co.). To score F⁻ segregants from strains carrying F*gal* or F*lac*, EMB agar (LEDERBERG 1950) with galactose or lactose was used, respectively.

*Measurement of frequency of segregation of F⁻ clones:* A PG medium was inoculated with $10^5$ to $10^6$ cells/ml of an F′ or F+ strain to be tested and was incubated at 37°C for 24 hr without aeration. In order to minimize the number of F⁻ cells that might initially be present, the F′ inoculum was grown in a selective medium which did not support the growth of F⁻ segregants. Samples from overnight cultures were diluted and plated on appropriate media (see above) and F⁻ segregants were scored after 24 hr incubation at 37°C. Cells carrying F*trp* formed normal size colonies, whereas F⁻ segregants, requiring tryptophan for growth, formed small flat colonies on the medium with limited tryptophan. Similarly, F⁻ segregants were easily detected among colonies of F′ strains harboring F*lac* or F*gal* by their inability to utilize lactose or galactose, respectively. In the case of the F+ strain, F⁻ segregants were scored by their resistance to male-specific phage MS-2.

## RESULTS

*Instability of the F factor in strain C600:* It was initially observed that strain C600 carrying an F factor segregates F⁻ cells at frequencies much higher than those commonly observed with other K12 strains. Five percent or more (up to 60%) of the cells from overnight cultures of strain C600 were F⁻ whereas less than 0.1% F⁻ segregants could be detected with other strains (Table 2). This was true whether the strain carried F+ or any one of the three F′ factors employed, although some quantitative differences were noted in the frequencies of segregation of F⁻ clones among the F′ strains. The presumptive F⁻ segregants, as judged by their nutritional or sugar utilization properties, were also found to be resistant to male-specific RNA phage MS-2. It thus became clear that normal replication or distribution of the F factor is somehow hampered with RILEY's strain of C600. However, this F factor instability was not observed with our stocks of the original strain of C600 isolated by APPLEYARD (1954).

*Effect of PEA on segregation of F⁻ clones:* Most *E. coli* K12 F+ and F⁻ deriva-

TABLE 2

*Segregation of F⁻ clones from various F+ or F′ strains in the presence or absence of PEA**

| Strain | Episome carried | Sensitivity to PEA | Percent segregation of F⁻ cells: Control | Plus PEA (0.2 percent) | Plus PEA (0.23 percent) |
|---|---|---|---|---|---|
| a. C600 and its derivatives | | | | | |
| KY344 | F+ | R | 30 ± 10 | —— | 95 ± 5 |
| KY311 | F*trp* | R | 40 ± 20 | 75 ± 5 | 95 ± 5 |
| KY312 | F*lac* | R | 10 ± 5 | —— | 95 ± 5 |
| KY2142 | F*lac* | R | 5 ± 2 | —— | 95 ± 5 |
| KY6904 | F*gal* | R | 40 ± 20 | —— | 90 ± 10 |
| b. Other K12 strains | | | | | |
| 5TD4 | F*trp* | S | 0.07 | < 0.1 | < 0.1 |
| KY113 | F*trp* | S | < 0.01 | < 0.1 | < 0.1 |
| KY2054 | F*lac* | S | < 0.1 | < 0.1 | —— |
| W4520 | F*gal* | S | < 0.1 | < 0.1 | —— |

* The frequency of segregation of F⁻ clones was determined as described in MATERIALS AND METHODS. At least 500 colonies were examined to calculate the frequency for each experiment, and the ranges of such frequencies from several experiments are presented.

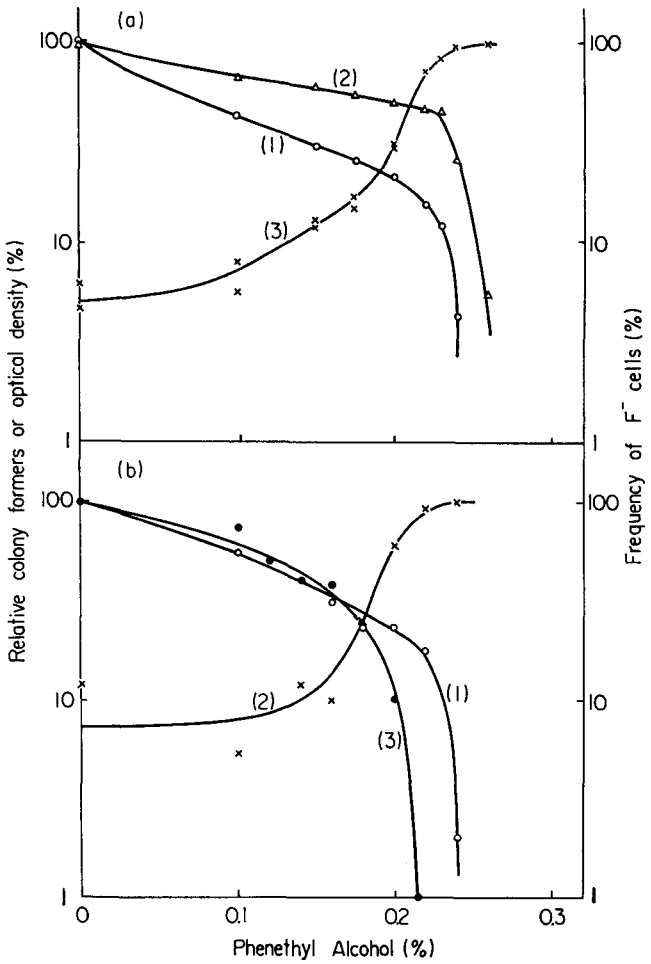


FIGURE 1.—Effect of PEA concentration on segregation of F⁻ clones from strain KY2142 carrying defective F*lac*. PG media containing various concentrations of PEA were inoculated with about $10^6$ cells/ml of strain KY2142 and were incubated at 37°C for 24 hr without aeration. Optical density was measured by a Klett-Summerson colorimeter with a No. 54 filter. Appropriate dilutions of each culture were plated on EMB-lactose agar to score Lac+ and Lac⁻ colonies after overnight incubation at 37°C. Experiment (a)—(1) Relative colony formers (○—○); (2) Relative optical density (△—△); (3) Frequency of F⁻ cells (x—x). Experiment (b)—(1) Relative colony formers (○—○); (2) Frequency of F⁻ cells (x—x); (3) Relative colony formers with a PEA-sensitive derivative of KY2142 (●—●). This strain was obtained by introducing *pea*+ (PEA-sensitivity gene) into strain KY2142 by P1 transduction.

tives show little or no growth in the presence of 0.23% PEA. At lower concentrations of PEA (0.18–0.2%), the cells grow at a reduced rate and they show no evidence of episome instability. In contrast, C600 F+ and F′ strains grow in 0.23% PEA, albeit at a reduced rate, and the majority of the cells become F⁻ after about 10 generations of growth at 37°C (Table 2).

Figure 1 shows the effect of PEA concentration on the segregation of F⁻ clones from an F′*lac* derivative of strain C600 (KY2142). This strain is defective with respect to episome transfer and was employed to minimize the possible re-infection of F⁻ segregant cells with the F′ factor. The effect of PEA can be seen at 0.15% or higher concentrations and is most striking at 0.23 to 0.24%, where the majority of cells became F⁻ after 7 to 8 generations of growth. Essentially similar results were obtained with a strain carrying F*trp*. The relatively high optical density observed as compared with colony formers at a given concentration of PEA (Figure 1a) may reflect, at least partly, the formation of non-viable filaments ("snakes") due to the inhibition of cell division by PEA. It may also be noted that the concentration of PEA required for episomal elimination corresponds to that required for growth inhibition of the PEA-sensitive derivative of strain KY2142 (Figure 1b). Since F⁻ segregants did not grow faster than the F′ strain, either in the presence or absence of PEA, the observed effect of PEA under our experimental conditions does not seem to be due to the selection of pre-existing F⁻ clones in the culture. This has been shown by reconstruction experiments as well as by kinetic experiments presented below. Table 3 gives a summary of the reconstruction experiments using an F′*trp* strain and an essentially isogenic F⁻ segregant strain. Similar results were also obtained with an F′*lac* strain.

*Kinetics of segregation of F⁻ clones:* The time course of the appearance of F⁻ segregant cells from strain KY2142 was followed in the presence or absence of PEA, and results obtained from several experiments are shown in Figure 2. A

TABLE 3

*Doubling time of F′ and F⁻ strains in pure and mixed cultures**

| Experiment number | Strain | Doubling time (minutes) | |
|---|---|---|---|
| | | F′ cell | F⁻ cell |
| (a) | Without PEA | | |
| 1 | F′ | 27 | — |
| 2 | F⁻ | — | 39 |
| 3 | F⁻(KY306) | — | 38 |
| 4 | F′ + F⁻(1:20) | 28 | 40 |
| 5 | F′ + F⁻(4 : 1) | 29 | 42 |
| (b) | With PEA (0.24 percent) | | |
| 1 | F′ | 130 | — |
| 2 | F⁻ | — | 180 |
| 3 | F′ + F⁻(1 : 24) | 140 | 180 |
| 4 | F′ + F⁻(4 : 1) | 130 | 180 |

* PG medium without (a) or with (b) 0.24% PEA was inoculated with 1 to 2 × $10^6$ cells/ml of F′ (KY2016), F⁻ (a spontaneous segregant of strain KY314, unless otherwise indicated) or a mixture of both in different proportions. Cultures were incubated at 37°C without shaking, aliquots were taken at intervals, diluted and plated on appropriate media to score the number of F′ and F⁻ colonies separately. Strain KY2016 was derived from strain KY311 by a chromosomal mutation that resulted in the stable maintenance of an F′ factor in the presence or absence of PEA (Mutant I in Table 6). The slower growth rate of the F⁻ strains observed is presumably due to the *trp-tonB* deletion present in the F⁻ segregants.

rapid and striking increase in the proportion of F⁻ cells occurred in the presence of PEA (0.24%), whereas only a slight increase was detected during this period in the absence of PEA. The initial lag observed in the PEA-stimulated segregation, although somewhat variable in different experiments, may represent the time required for the effect of PEA to reach its final site of action and/or for the completion of nuclear segregation, since the F′ cells may contain several episome copies per cell. The rapid rise in the rate of segregation of F⁻ clones after 2 to 3 generations may represent either a virtually complete inhibition of the replication or inhibition of the distribution of the F′ factor to daughter cells. The spontaneous rate of F⁻ segregation in the absence of PEA was estimated to be approximately $5 \times 10^{-3}$/cell/generation. The addition of PEA accelerated this rate about 200 fold, to almost unity, when calculated from the curve between the 3rd and the 7th generations of growth (Figure 2).

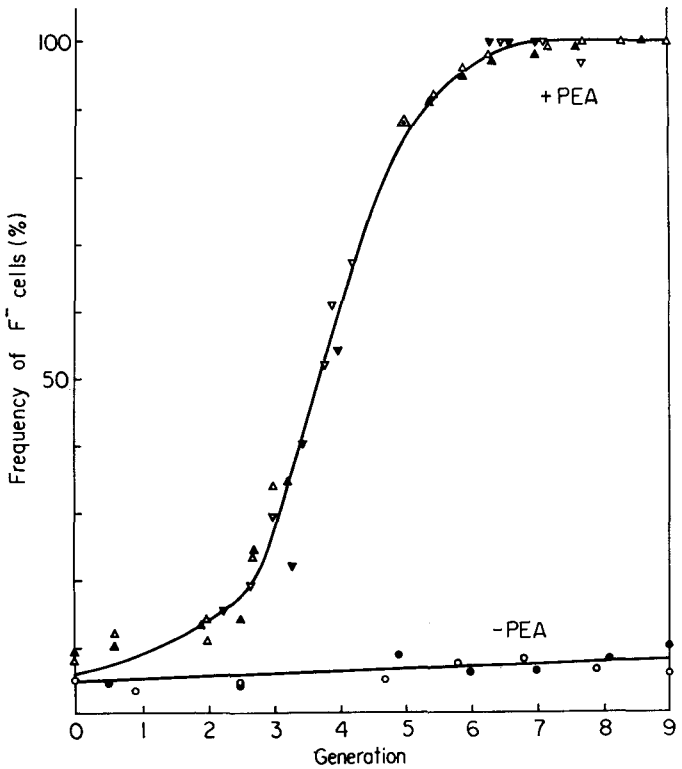


FIGURE 2.—Kinetics of segregation of F⁻ clones from strain KY2142 in the presence or absence of PEA. PG medium with or without PEA (0.24%) was inoculated by $2 \times 10^5$ cells/ml of strain KY2142 and was incubated at 37°C without aeration. Samples (0.1 ml) were taken at intervals, appropriate dilutions were plated on EMB-lactose agar, and F⁻ segregants (Lac⁻) as well as F′ colonies (Lac⁺) were scored after 24 hr incubation at 37°C. The results from two (—PEA; ○, ●) or four (+PEA; △, ▲, ▽, ▼) independent experiments are presented, and each point represents a frequency obtained by examining 200 to 1000 colonies. The doubling time under these conditions was 30 min in the absence of PEA and 135 min in the presence of PEA.

TABLE 4

*Effect of PEA on the transfer of Ftrp factor to F⁻ cells**

| F′ strain | Experiment number | Frequency of F′ transfer per donor cell | |
|---|---|---|---|
| | | Minus PEA | Plus PEA (0.24 percent) |
| KY314 | 1 | $2.5 \times 10^{-2}$ | $2.5 \times 10^{-3}$ |
| | 2 | $4.0 \times 10^{-3}$ | $< 5.0 \times 10^{-4}$ |
| | 3 | $5.3 \times 10^{-2}$ | $2.0 \times 10^{-3}$ |
| KY2016 | 2 | $7.0 \times 10^{-3}$ | $6.0 \times 10^{-3}$ |
| | 3 | $1.0 \times 10^{-1}$ | $5.0 \times 10^{-2}$ |

* Overnight cultures of F′ and F⁻ strains in medium E containing Casamino acids and thiamine (L-tryptophan also added for the F⁻ strain) were diluted 20 fold in PG medium and were grown to about $3 \times 10^8$ cells/ml. Cultures were centrifuged and resuspended in fresh PG medium. F′ cells ($10^8$/ml for Experiments 1 and 2; $10^6$/ml for Experiment 3) were mixed with a 10-fold excess of F⁻ cells (strain KY301) in PG medium with or without PEA (0.24%). Mixtures were incubated at 37°C for 30 min without shaking, samples (0.1 ml) were taken into 10 ml of saline and were sheared with a Flash Mixer. Appropriate dilutions were then plated on medium E with thiamine (2 μg/ml) to score F′ transfer, and on medium E with L-histidine (20 μg/ml) or L-proline (20 μg/ml) in addition to thiamine to determine viability of donor cells.

*Effect of PEA on the transfer of an F′ factor:* Since the transfer of F⁺ or F′ to F⁻ recipient cells may involve F-directed replication (JACOB, BRENNER and CUZIN 1963; OHKI and TOMIZAWA 1968), we determined whether F′ transfer was also affected by PEA. As shown in Table 4, the transfer of F*trp* from a C600 F′ strain (KY314) was inhibited by PEA (0.24%) to less than 10% of the control without PEA. There was no loss of viability of either donor or recipient strain during the 30 min incubation in the presence of PEA nor any appreciable increase in F⁻ segregants within the donor population during this short time period. In contrast, such a PEA effect was not observed with a mutant of KY311 (Mutant I in Table 6) in which F′ replication apparently became resistant to inhibition by PEA (see below). Thus, there seems to be a correlation between the PEA effect on F′ replication and that on F′ transfer. These results are also consistent with the notion that transfer of F factor involves its replication within the donor cell and that F-directed replication is inhibited by PEA.

*Location of the gene(s) responsible for high frequency segregation of F⁻ clones:* The results suggested that a gene (or genes) located on the chromosome of strain C600 might be responsible for the high frequency segregation of F⁻ clones from the F⁺ and F′ strains of C600. Furthermore, the same gene(s) might also be involved with the further PEA stimulation of F⁻ segregation from these strains. It was also of interest to see whether these characters are related to the PEA resistance of these strains. Thus a series of transduction experiments with phage P1 was performed to locate the gene(s) involved. As shown in Table 5a, a single gene tentatively designated as *maf* (maintenance of autonomous F) seems to be involved in the high frequency segregation character of strain C600, and either *maf* or *maf*⁺ was co-transduced with *ara*, *leu*, or *azi* at a frequency of 30 to 50% (Crosses 1–4). The examination of other unselected markers revealed that *maf* is distinct from *pea* or *azi* and that the gene order is probably *maf–leu–*(*pea*, *azi*)

TABLE 5

*Transductional mapping of the gene* (maf) *responsible for high frequency segregation of F⁻ clones from strain C600 carrying F*trp*

| Cross number | Parental strains | | | | | Donor marker selected | Number of transductants tested | Cotransduction of *maf*+ or *maf* | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Number | Frequency (percent) |
| (a) Cotransduction frequencies | | | | | | | | | |
| 1 | KY131 | *maf*+ | *azi* | *leu* | | *azi* | 22 | 7 | 32 |
| | KY311 | *maf* | *azi*+ | *leu*+ | | | | | |
| 2 | KY131 | *maf*+ | *azi* | *leu* | | *azi* | 11 | 2 | 18 |
| | KY301 | *maf* | *azi*+ | *leu*+ | | | | | |
| 3 | KY301 | *maf* | *azi*+ | *leu*+ | *ara*+ | *leu*+ | 29 | 11 | 37 |
| | KY2127 | *maf*+ | *azi* | *leu* | *ara* | *ara*+ | 15 | 7 | 47 |
| 4 | KY311 | *maf* | *azi*+ | *leu*+ | *pea* | *leu*+ | 50 | 21 | 42 |
| | KY2127 | *maf*+ | *azi* | *leu* | *pea*+ | | | | |
| 5 | KY334 | *maf* | *azi*+ | *leu*+ | | *leu*+ | 34 | 17 | 50 |
| | KY2127 | *maf*+ | *azi* | *leu* | | | | | |

(b) Distribution of other unselected markers (Cross #4 above)

| Selection | Unselected markers | | | Number of transductants obtained |
|---|---|---|---|---|
| | *maf* | *pea* | *azi* | |
| *leu*+ | 1† | 1 | 1 | 9 |
| | 1 | 0 | 0 | 11 |
| | 1 | 0 | 1 | 1 |
| | 0 | 1 | 1 | 15 |
| | 0 | 0 | 0 | 14 |
| | | | Total | 50 |

* In each cross, the first line represents a donor and the second, a recipient. Only relevant markers are indicated here. Strain KY334 was derived from KY311 as a mutant which can stably maintain the F′ factor (Mutant I in Table 6). To score the *maf* marker, transductants were purified and were cross streaked against cells of an F′*trp* strain (KY113) on appropriate medium for episomal infection. The resulting F′ strains were purified and duplicate colonies were tested for segregation of F⁻ clones as described in MATERIALS AND METHODS.

† 1 and 0 represent markers from donor and recipient strains, respectively.

(Table 5b). The location of *maf* relative to *thr* and *ara* could not be determined unambiguously for technical reasons.

In order to find out whether the same gene is responsible for the PEA-stimulated segregation of F⁻ clones, transductants that were PEA resistant (26 transductants altogether) were examined for F⁻ segregation in the presence of PEA. Thus the stimulatory effect of PEA on F′ elimination was demonstrated with all the transductants carrying *maf* as well as *pea* (20/26), whereas those carrying *maf*+ and *pea* (6/26) did not segregate F⁻ clones at high frequency regardless of the presence of PEA. The PEA-stimulated segregation, therefore, appears to be highly correlated with high spontaneous rate of segregation.

*Further mutations affecting stable maintenance of the autonomous F′ factor:* To further investigate the nature and the function of episomal and chromosomal factors controlling stable maintenance of the autonomous F episome, we attempted to isolate from an F′ strain mutants that had lost the characteristic high frequency segregation of F⁻ clones in the presence or absence of PEA. Such

mutants were easily selected by growing cells of strain C600 carrying F*trp* (e.g. KY311) in PG medium containing PEA (0.24%) and subsequently isolating F′ clones that could stably maintain F*trp* in the presence of PEA. A number of such mutants were isolated either spontaneously or after treatment with N-methyl-N′-nitro-N-nitrosoguanidine or 2-aminopurine. The mutants have been classified into three types with respect to the site of mutation and to the pattern of F⁻ segregation, as summarized in Table 6. To determine whether a mutation occurred on the chromosome or on the episome, segregation of F⁻ clones was examined with a pair of F′ strains constructed as follows: (1) An F′ factor of each mutant was transferred into the parental C600 F⁻ strain (KY301). (2) The parental F′ was transferred into an F⁻ strain that had been obtained by "curing" the F′ factor from each mutant.

Type I mutants stably maintained the F*trp* factor whether PEA was present or not in the medium. Type II mutants showed unchanged spontaneous instability of F*trp* but PEA-stimulated segregation was no longer observed. All the Type I and Type II mutations examined were found to be located on the host chromosome. On the other hand, Type III mutants carried the mutation in the genetic material of the episome, but these frequently reverted back to the parental type during serial transfer through several strains. Thus no stable Type III mutant was found despite extensive search for such mutants (but see DISCUSSION).

When representative strains of mutant Types I and II were infected with F*lac* or F*gal* instead of F*trp*, the same characteristic patterns of segregation were obtained, indicating that the effects of these chromosomal mutations are not restricted to F*trp*. Genetic analysis of one of the Type I mutants has shown that a mutation occurring outside of the *leu–maf* region is responsible for stabilization of the F′ factor in this strain (Cross #5 of Table 5a).

*Effect of penicillin on segregation of F⁻ clones:* When strain C600 carrying F*trp* was grown overnight in PG medium containing low concentrations of peni-

TABLE 6

*Mutants of C600 carrying* Ftrp *(KY311 and KY314) that are altered with respect to the pattern of segregation of F⁻ clones**

| Type | F′ strains tested (0) Minus PEA | (0) Plus PEA | (1) Minus PEA | (1) Plus PEA | (2) Minus PEA | (2) Plus PEA | Site of mutation | Number of mutants obtained Spont. | NG | AP |
|---|---|---|---|---|---|---|---|---|---|---|
| Parent | + | ++ | + | ++ | + | ++ | —— | — | — | — |
| Mutant I | — | — | + | ++ | — | — | Chromosome | 1 | 18 | 1 |
| Mutant II | + | + | + | ++ | + | + | Chromosome | 1 | 15 | — |
| Mutant III | — | — | — | — | + | ++ | F*trp* | 1 | 1 | — |

* Procedures for isolation of mutants and for construction of a pair of F′ strains (1) and (2) from each original F′ mutant strain (0) are described in the text. Segregation of F⁻ clones from these F′ strains was tested in PG medium in the presence or absence of PEA (0.24%) as described in MATERIALS AND METHODS. The frequencies of F⁻ segregation are presented semi-quantitatively as follows: +, 30 ± 10%; ++, 95 ± 5%; and —, < 0.1%. Spont. spontaneous; NG, N-methyl-N′-nitro-N-nitrosoguanidine; AP, 2-aminopurine.

TABLE 7

*Effect of penicillin on segregation of F⁻ clones from F′ strains carrying Ftrp**

| F′ strain used | Percent segregation of F⁻ cells | | |
|---|---|---|---|
| | Control | Plus Pen (40 units/ml) | Plus Pen (50 units/ml) |
| KY314 | 43 | 98 | > 99.8 |
| KY334 (Mutant I)† | < 0.3 | — | < 0.2 |
| KY327 (Mutant II)† | 51 | 97 | — |
| 5TD4 | < 0.4 | — | < 0.7 |
| KY113 | < 0.5 | — | < 0.3 |

* Frequency of segregation of F⁻ clones was determined as described in MATERIALS AND METHODS, except that penicillin G was added to the medium where indicated. Pen: penicillin.
† See Table 6 and text for properties of these strains.

cillin, the majority of cells became F⁻. Thus penicillin also affects replication or distribution of the F′ factor in the C600 subline, and, as in the case of PEA, stimulates segregation of F⁻ clones with strain KY314 but not with strains 5TD4 and KY113 (Table 7). The growth rate in the presence of penicillin (40–50 units/ml) as judged by number of colony formers did not vary appreciably among the strains tested. Again, as shown by reconstruction experiments, the observed effect of penicillin does not seem to be due to selection of pre-existing F⁻ cells in the F′ culture used nor does penicillin cause segregation of F⁻ clones from Type I mutants carrying F*trp*. The segregation of F⁻ cells from Type II mutants (KY327), however, seems to be accelerated by penicillin though not by PEA.

## DISCUSSION

We have previously shown that *E. coli* strain C600, as compared to other K12 strains, is resistant to PEA with regard to the initiation of the DNA replication cycle and to membrane permeability among other properties (YURA and WADA 1968). This strain's resistance to PEA is primarily determined by the gene *pea*, which is identical with, or adjacent to, the gene *azi*, determining azide resistance. These genes are presumably involved in the formation and/or activity of some unidentified membrane structure that might be related to the control of DNA replication and cell division (YURA and WADA 1968; TAYLOR 1970).

In view of these results, we first suspected that the observed instability of the F factor in strain C600 might be correlated with PEA resistance of these strains. This possibility became unlikely, however, when we found that our stocks of the original strain of C600 could stably maintain an F′ factor but are just as resistant to PEA as the RILEY's strain C600.1. The observed difference between the two C600 strains in F′ maintenance is presumably related to the origin of strain C600.1; it may be a recombinant between C600 and some other strain (GARTNER and RILEY 1965). The present results have shown that a gene or a group of genes that are closely linked to, but are distinct from, the gene *pea* is responsible for the high frequency segregation of F⁻ clones from strain C600 carrying an F′ or F⁺ factor. Not all PEA-resistant transductants showed the high

frequency segregation character, and conversely, some transductants that showed the high segregation were PEA sensitive. Thus PEA resistance appears to be neither sufficient nor necessary for the manifestation of spontaneous F′ instability caused by the *maf* mutation. On the other hand, some of the PEA-resistant mutants more recently isolated from a sensitive strain are incapable of maintaining an F′ factor with the usual stability (WADA and YURA, unpublished results). If single-gene mutations are responsible for both PEA resistance and F′ instability in these mutants, this finding suggests that mutation to PEA resistance can somehow hinder replication or distribution of F′ factors during the cell cycle of *E. coli*.

The precise mechanism leading to the high frequency segregation of F⁻ clones from C600 F′ strains, is not known at the present time. Such instability might be due to the presence of another plasmid in these strains. This possibility seems rather unlikely, however, since the mutant allele of the *maf* gene (causing instability) as well as the wild-type *maf*⁺ allele (causing stability) can be cotransduced with the specific chromosomal genes such as *leu* or *ara* (see Crosses #3 and #4 of Table 5a), unless the *maf* function is related to the incompatibility caused by a hypothetical plasmid. The results show that the *maf* gene is distinct from the mutants described by JACOB *et al.* (1963) which also affected replication of F′ factors, since the latter were mapped between *str* and *asp* (HIROTA, RYTER and JACOB 1968). *Maf* is also unrelated to the mutations which lead to the loss of the capacity to grow certain bacteriophages (DENHARDT, DRESSLER and HATHAWAY 1967; CALENDAR *et al.* 1970).

Strain C600 can regain the ability to stably maintain an F′ factor by mutation and, at least in the one case studied in detail, the second mutation occurred outside the *maf-leu* region. These results suggest that a number of chromosomal genes can affect the genetic stability of an F factor, either directly or indirectly, in *E. coli*. Mutation of the F′ factor itself which suppressed the high frequency segregation character of strain C600 could not be stably obtained by our present method of selection. However, some of the F′ mutants that acquired resistance to RNA phage MS-2 and are henceforth defective in their transfer to an F⁻ strain, can be stably maintained in strain C600 either in the presence or absence of PEA. These mutant F′ factors were cis-dominant; when strain C600 carries both the mutant and wild-type F′ factors in the same cell, only the mutant F′ is stably maintained and the wild-type F′ is lost at high frequency (WADA and YURA 1965).

Our results suggest that the mutant *maf* allele is also responsible for the PEA-stimulated (and presumably also penicillin-stimulated) segregation of F⁻ cells. Since both PEA and penicillin are known to act primarily on cell surface structure, the results suggest that the gene *maf* is involved in certain aspects of the formation or activity of the cell surface structure that is essential for stable maintenance of an autonomous F factor in *E. coli*. Furthermore, the occurrence of the cis-dominant F′ mutants that can replicate normally in strains carrying *maf* raises the interesting possibility that the formation or activity, of some structural component (possibly of the cell membrane) which interacts directly

with the DNA of the autonomous F factor, is altered in strain C600 as a result of the *maf* mutation.

The question of whether spontaneous and PEA- or penicillin-stimulated segregation of $F^-$ clones is primarily caused by inhibition of F replication or of distribution of newly-replicated F into daughter cells during cell division is not clear at present. However, it was noted that inhibition of F′ transfer by PEA can be observed only with F′ strains in which PEA- or penicillin-stimulated segregation of $F^-$ clones can be demonstrated (Table 4). DNA replication but not F factor distribution may be involved in the process of F′ transfer in *E. coli*. Since PEA inhibits the initiation of transfer–replication during mating (ROESER and KONETZKA 1964), these results suggest that the effect of PEA is exerted primarily on replication rather than F factor distribution. However, this point should be examined by more direct experiments. It is hoped that further genetic characterization of various mutants described in this paper, as well as the biochemical analysis of their membrane structure, will provide a useful system for obtaining a more concrete understanding of the control of DNA replication and cell division in bacteria.

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## SUMMARY

Replication of the F factor in a phenethyl alcohol resistant strain of *Escherichia coli* C600.1 (GARTNER and RILEY 1965) has been investigated. When cells of this strain carrying an $F^+$ or F′ factor are grown in broth overnight at 37°C, $F^-$ cells are segregated out at high frequency (5–50%). When phenethyl alcohol ($0.23 \pm 0.01\%$) or penicillin (40–50 units/ml) is added to the culture medium, the majority of cells become $F^-$ during overnight incubation. The inhibitory effect of phenethyl alcohol is virtually complete under certain conditions, and appears to be exerted primarily on replication itself rather than on the distribution of the F factor into daughter cells during cell division. Transduction analysis has shown that a gene designated as *maf*, located close to the *leu–ara* region but distinct from the gene *pea* (or *azi*), is responsible for the high spontaneous rate of segregation of $F^-$ clones and for its further stimulation by phenethyl alcohol. Several types of chromosomal and episomal mutations affecting F factor replication in the presence or absence of phenethyl alcohol were obtained. Some of them can stabilize the F factor both in the presence or absence of phenethyl alcohol, whereas others render the F factor resistant to inhibition by phenethyl alcohol while retaining the spontaneous instability of the F′ strains. Available evidence suggests that the *maf* gene is involved in the formation or activity of cell surface structure essential for stable maintenance of autonomous F factor in *Escherichia coli*.

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