PHENETHYL ALCOHOL RESISTANCE IN *ESCHERICHIA COLI* I. RESISTANCE OF STRAIN C600 AND ITS RELATION TO AZIDE RESISTANCE

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BERRAH and KONETZKA (1962) reported that phenethyl alcohol (PEA) selectively and reversibly inhibits DNA synthesis in *Escherichia coli*. It was subsequently shown that PEA inhibits the initiation of a new cycle of DNA replication in this organism but permits the completion of those cycles initiated before addition of PEA to the medium (TREICK and KONETZKA 1964). We reasoned therefore that genetic and biochemical characterization of strains resistant to PEA might give a clue to our understanding of the nature and function of factor(s) controlling the initiation of the DNA replication cycle in bacteria.

Examination of several strains of *E. coli* K-12 revealed that strain C600 is markedly resistant to PEA compared to other strains tested (WADA and YURA 1965). As will be shown below, strain C600 is resistant to PEA with respect to DNA, RNA, and protein synthesis, initiation of the DNA replication cycle, and membrane permeability to potassium ions. The PEA resistance of this strain is primarily determined by a single gene (*pea*) located between *leu* and *azi*, being extremely close to the latter. The mutational analysis of both the *pea* and *azi* genes revealed that the expression of these genes is closely interrelated so as to suggest that they are either identical or closely linked genes whose expression is coordinated perhaps by forming an operon. Evidence also suggests that both genes are involved in the formation, activity or maintenance of cell membrane structure in *E. coli*.

MATERIALS AND METHODS

Bacterial and phage strains: Bacterial strains used in this study are all derivatives of *Escherichia coli* K-12 and are listed in Table 1. Phage P1kc (LENNOX 1955) has been used in all transduction experiments.

Media: Standard media used are 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 l (pH 7.2).

For determination of the PEA phenotype, medium E was supplemented with 0.2% glucose, 0.2% Difco Casamino acids, 40 μg/ml DL-tryptophan, 2 μg/ml thiamine, and 1.5% agar (Hakko agar Co.). Redistilled PEA (Nakarai Chemical Co.) was added to the sterilized medium to a final concentration of 0.23% (PEA agar). The concentration of PEA was most critical and was carefully adjusted so as to give best discrimination between PEA-sensitive and resistant strains. Plates were preincubated for 24 hrs at 37°C before use. For determination of resistance vs. sensitivity to azide, PG medium containing 3.4 mM sodium azide was employed. Dihydro-

TABLE 1

*Bacterial strains used and their known genetic characters**

Strain no.	Genetic characters	Origin†
C600	F⁻ : *thr leu thi lac tonA*	A. CAMPBELL
KY109	F+ : *cys*	Y-mel
KY112	F+ : *trp tonB*	Y-mel
KY113	F′ : *trp tonB* / F*trp*+	5TD4 × KY120
KY120	F⁻ : *trp tonB*	KY112
KY131	F⁻ : *thr leu his met thi lac gal mal xyl mtl ara tonA azi str*	PA678
KY301	F⁻ : *trp thi lacY tna tonB pea str*	C600.1 (M. RILEY)
KY305	F⁻ : *trp his thi lacY tna tonB pea str*	KY301
KY311	F′ : *trp thi lacY tna tonB pea str* / F*trp*+	5TD4 × KY301
KY316	Hfr : *trp thi lacY tna tonB pea str*	KY301
KY387	Hfr : *trp thi pea*	CS101 × KY305
KY2120	F⁻ : *thr leu his met thi azi str*	KY387 × KY131
KY2121	F⁻ : *his met thi pea str*	KY387 × KY131
KY2151	Hfr : *trp thi pea str*	KY131 —× KY387
KY2192	F⁻ : *trp his met pea str*	KY387 × KY131
KY2670	F⁻ : *his thi*	KY316 × KY131
KY2671	F⁻ : *his thi pea*	KY316 × KY131
5TD4	F′ : *trp cysB his str* / F*trp*+	P. FREDERICQ
JC2207	F⁻ : *leu-2 his-1 arg-6 met-1 lac-1,4 str*	J. CLARK

* Gene symbols: Genes determining biosynthesis: *cys*, cysteine; *his*, histidine; *trp*, tryptophan; *thr*, threonine; *leu*, leucine; *met*, methionine; *thi*, thiamine. Genes determining sugar utilization: *gal*, galactose; *lac*, lactose; *mal*, maltose; *xyl*, xylose; *mtl*, mannitol; *ara*, arabinose. Genes determining response to drugs: *str*, streptomycin; *azi*, azide; *pea*, phenethyl alcohol. Genes determining response to phages: *tonA*, T1 and T5; *tonB*, T1. *tna*, genes determining tryptophanase. Sexual cross is represented as donor × recipient; transduction by phage P1*kc* as, donor —× recipient.

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

streptomycin sulfate (150 μg/ml unless otherwise indicated) was used to score or select for streptomycin-resistant clones.

Mating experiments: Matings were carried out in Difco Penassay broth for 2 hrs at 37°C and appropriate dilutions were plated on selective media. Recombinant colonies were picked and streaked with sterile toothpicks on the same selective medium. After incubation overnight at 37°C, the plates were replicated onto appropriate media to determine unselected markers (amino acid and sugar markers). To determine PEA and azide resistance, recombinants were grown in the same but liquid selective medium overnight at 37°C. Cell suspensions were streaked on agar medium and incubated for 1 or 2 days at 37°C to score azide or PEA resistance, respectively. Resistance to phage T5 was scored by cross streaking cell suspensions against T5 phage on PG medium.

Transduction experiments: Transduction with phage P1kc was performed essentially as described by LENNOX (1955). Phage lysates that have been grown on donor bacteria by two passages were employed. Determination of unselected markers was done as described above.

C^{14}-thymidine incorporation: Medium E supplemented with 0.2% glucose, 20 μg/ml each of twenty common L-amino acids, 2 μg/ml thiamine, 250 μg/ml deoxyadenosine and 8 μg/ml 2-C^{14}-thymidine (specific activity, 300 μc/mmole) was employed. Aliquots (0.3ml) were taken at various times and added to 3 ml of 5% cold trichloroacetic acid. After standing 30 to 60 min in the cold, cells were filtered through a membrane filter (Millipore, HA 0.45 μ), washed with cold 5% trichloroacetic acid and each filter was placed on a planchet. A few drops of 2 N NH_4OH

was added to the sample, dried, and the radioactivity counted by a Nuclear Chicago gas-flow counter.

K^{42} efflux and influx: A general procedure of SILVER and WENDT (1967) was followed with minor modifications. For efflux experiments, cells were grown in tryptone broth containing K^{42} (National Inst. of Atomic Energy, Japan) for several generations at 37°C. Radioactive cells were centrifuged at 20°C, washed and resuspended in nonradioactive broth at 3×10^8 cells/ml. The culture was quickly divided into two tubes, one of which contained PEA. Tubes were shaken at 30°C and 1 ml aliquots were taken at intervals and immediately filtered through Millipore filter, HA (0.45 μ). Both filters (cells) and 0.2 ml samples of filtrates were dried and counted in a gas-flow counter.

For influx experiments, cells grown in tryptone broth (3×10^8/ml) were placed in two tubes, with or without PEA; K^{42} was added, and 1 ml samples withdrawn at intervals during incubation at 30°C with shaking. Samples were immediately filtered, and filters (cells) were dried and counted as above.

RESULTS

Effect of PEA on growth: Among the strains of *E. coli* K-12 initially examined for their sensitivity to PEA on agar medium, strain C600 and its derivatives (e.g. KY301, 311) were found to be markedly resistant compared to all other strains tested. Thus, strain C600 showed heavy growth when cell suspensions were streaked on medium containing 0.23% PEA whereas most other strains failed to show any visible growth after 2 days incubation at 37°C. In liquid PG medium, PEA-resistant (Pea-R) and sensitive (Pea-S) strains are clearly distinguishable under PEA concentrations of around 0.25% (Figure 1). Similar

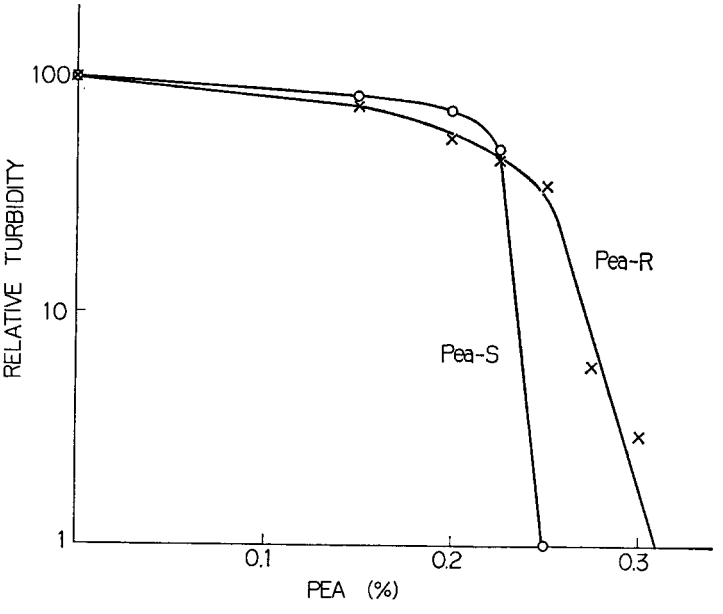

FIGURE 1.—Effect of PEA on growth of Pea-S (5TD4) and Pea-R (KY311) strains. Peptone-glucose media with various concentrations of PEA were inoculated with 10^6 cells/ml of each strain, and were incubated for 18 hrs at 37°C without aeration. Turbidity was measured in a Klett Summerson colorimeter with a No. 54 filter.

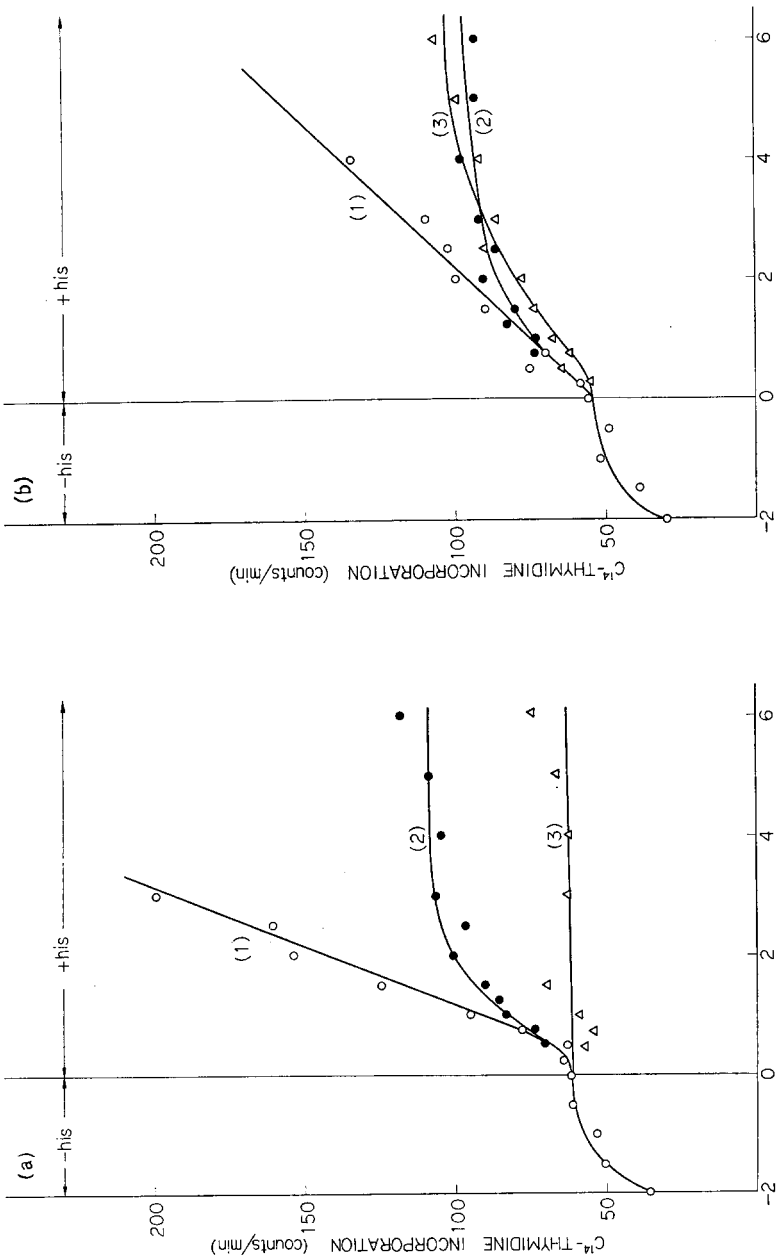

FIGURE 2.—Effect of PEA on initiation of DNA replication cycle in Pea-S (KY2670) and Pea-R (KY2671) strains. Overnight cultures in medium containing C^{14}-thymidine (see MATERIALS and METHODS) were diluted 15-fold with the same medium and were aerated to about 3×10^8 cells/ml. Cultures were filtered through a sterile Millipore filter HA 0.45 μ), washed and resuspended in prewarmed medium lacking histidine and were aerated for 2 hrs at 37°C. Histidine (20 μg/ml) was then added and cultures divided into three parts; (1) Control without PEA (-O-O-) (2) 0.2% PEA added after 30 min aeration at 37°C (-●-●-) (3) 0.2% PEA added at 0 time (-Δ-Δ-). Aeration was continued at 37°C and samples removed at appropriate intervals for measurements of radioactivity in acid-insoluble fraction. (a) KY2670 (Pea-S) (b) KY2671 (Pea-R).

TABLE 2

Effect of PEA on DNA, RNA, and protein synthesis in Pea-S (KY120) and Pea-R (KY301) strains of E. coli*

PEA (percent)	DNA		RNA		Protein	
	Pea-S	Pea-R	Pea-S	Pea-R	Pea-S	Pea-R
0	5.5	6.5	3.5	5.3	5.8	10.0
0.2	2.2	4.0	1.0	2.6	2.0	4.6
	(73)†	(45)	(100)	(63)	(79)	(60)
0.25	2.2	2.5	1.0	1.4	1.8	2.5
	(73)	(73)	(100)	(91)	(83)	(83)
0.3	2.0	2.0	1.0	1.2	1.4	2.6
	(78)	(82)	(100)	(95)	(92)	(82)

* Figures represent relative increase over the initial values (set as unity) during 120 min after addition of PEA to the PG medium.

† Values in parentheses indicate per cent inhibition by PEA. Chemical assays for DNA, RNA and protein were performed by colorimetric methods essentially as described by BERRAH and KONETZKA (1962).

results were also obtained with agar media. Hence, in all subsequent experiments, PEA concentrations of 0.23–0.24% (liquid medium) or 0.22–0.23% (agar medium) were employed to determine resistance vs. sensitivity to PEA.

Effect of PEA on macromolecular synthesis: Effect of PEA on the synthesis of DNA, RNA and protein in the Pea-S and Pea-R strains was studied and the results are summarized in Table 2. It can be seen that the Pea-S strain is more severely inhibited than the Pea-R strain with respect to all the macromolecular syntheses studied. The difference between the two strains is evident when 0.2% PEA is present in the medium and is particularly marked with respect to RNA synthesis. RNA synthesis was completely inhibited by 0.2% PEA in the Pea-S strain but was only partially inhibited in the Pea-R strain.

We then examined the effect of PEA on the initiation of DNA replication cycle with Pea-S and Pea-R strains. For this experiment, a pair of strains with similar growth rate carrying the same *his* marker was isolated as recombinants from a cross between strains KY316 and KY131. As can be seen in Figure 2, cells starved for histidine for 2 hrs underwent DNA replication in most of the bacteria to reach the point where no further DNA synthesis is observed. At this point, addition of histidine to the culture immediately allowed initiation of a new cycle of DNA replication. If PEA (0.2%) is added simultaneously with histidine, this initiation of DNA synthesis was completely inhibited in the case of the Pea-S strain (Figure 2a, curve 3), but hardly in the case of the Pea-R strain, although PEA prevented DNA synthesis beyond doubling of the DNA as judged by C^{14}-thymidine incorporation (Figure 2b, curve 3). The latter observation may be due either to the prolonged generation time under these conditions or to the increased PEA concentration caused by aeration of small volumes of media or both. It may also be added that the increase in optical density (not shown) was inhibited by PEA to 20% and 40% of the control without PEA for

Pea-S and Pea-R strains, respectively. Finally, PEA added at 30 min after addition of histidine allowed doubling of the DNA in both strains (Figure 2a,b, curve 2). These results clearly indicate that the Pea-R strain is resistant to PEA compared to the Pea-S strain with respect to the initiation of the DNA replication cycle as well as to the bulk of DNA, RNA and protein synthesis.

Effect of PEA on membrane permeability: Effect of PEA on membrane permeability was studied by examining K^{42} efflux from, and influx into, cells of Pea-S and Pea-R strains. As can be seen in Figure 3a, PEA (0.25%) accelerated loss of intracellular potassium, and the PEA-induced permeability change was significantly greater with Pea-S (KY2670) than with Pea-R (KY2671) strain (Figure 3b). Moreover, the Pea-R strain appears to be altered in its permeability properties in the absence of PEA as compared to the Pea-S strain. Similar results were also obtained with another pair of strains, KY387 (Pea-R) and KY131 (Pea-S).

Figure 4 shows the effect of PEA on K^{42} influx. It was found that after transient inhibition by PEA at the initial stage, PEA (0.25%) induced an appreciable increase of K^{42} influx in the Pea-S but not in the Pea-R strain. Essentially the same results were obtained with strains KY387 and KY131. It thus appears that the Pea-R strains employed are resistant with respect to PEA-induced alteration in membrane permeability as judged by both K^{42} efflux and influx under growing conditions.

Chromosomal location of a gene determining PEA resistance: In order to study the genetic control of PEA-resistance in strain C600, crosses were made between Pea-S and Pea-R strains. Preliminary experiments showed that a single gene located on the *thr-gal* region of the chromosome determines the resistance to PEA as scored on PEA-agar. A cross was then performed between strains KY387 (Hfr, Pea-R) and KY131 (F^-, Pea-S), and various unselected markers were examined among recombinants obtained by three different selections. The Hfr strain (KY387) had been shown to be similar to Hfr H as to the point of origin and to the direction of chromosomal transfer. As seen in Table 3, the results obtained from selections (1) and (2) indicate that a gene determining PEA resistance (designated as *pea*) is located between *leu* and *azi*, being rather close

TABLE 3

*Recombinants from a cross KY387 (Hfr, Pea-R) × KY131 (F^-, Pea-S)**

	Selection	Unselected marker from Hfr parent (percent)										Number of recombinants tested
		thr	*leu*	*pea*	*azi*	*tonA*	*gal*	*trp*	*his*	*met*	*mal*	
(1)	*thr*+*leu*+*str*	100	100	78	75	51	2	0	0	0	0	99
(2)	*gal*+*str*	99	88	82	81	69	100	5	7	0	..	93
(3)	*his*+*str*	94	59	29 (50)†	49	30	11	31	100	4	..	70

* Hfr : *thr*+ *leu*+ *pea* *azi*+ *tonA*+ *gal*+ *trp* *his*+ *met*+ *mal*+ *str*+
F^- : *thr* *leu* *pea*+ *azi* *tonA* *gal* *trp*+ *his* *met* *mal* *str*
† Scored on medium containing streptomycin as well as PEA.

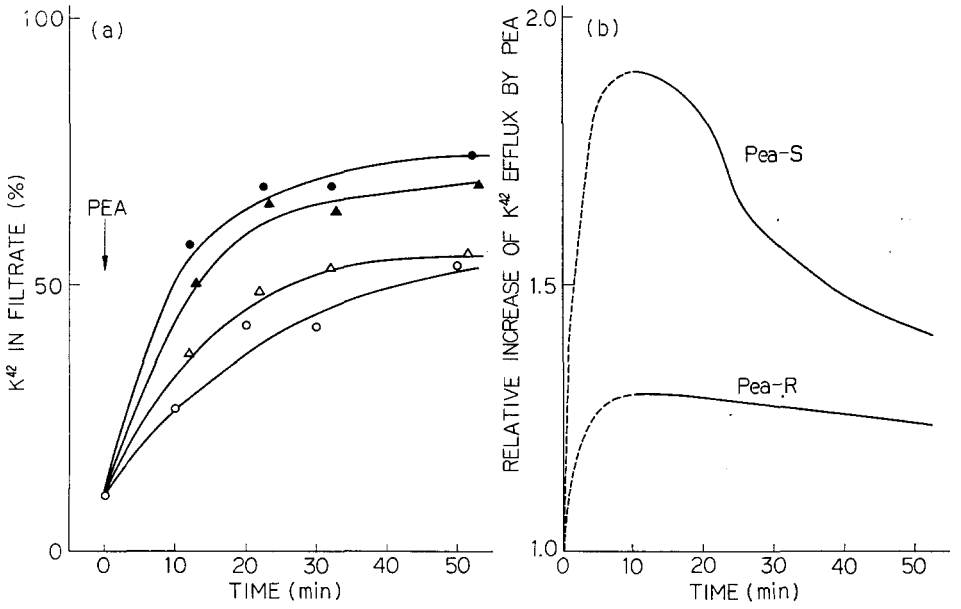

FIGURE 3.—Effect of PEA on K^{42} efflux with Pea-S (KY2670) and Pea-R (KY2671) strains. Ten ml of K^{42}-labeled cells (0.2 μc/ml) were suspended in prewarmed nonradioactive broth with or without PEA (0.25%) and 1 ml aliquots were taken at intervals and filtered. (a) Time course of K^{42} efflux at 30°C; KY2670 (Pea-S) with (-●-●-) or without (-O-O-) PEA, and KY2671 (Pea-R) with (-▲-▲-) or without (-△-△-) PEA. (b) PEA-induced K^{42} efflux; ratios of K^{42} in filtrate in the presence and absence of PEA were plotted from the data of (a).

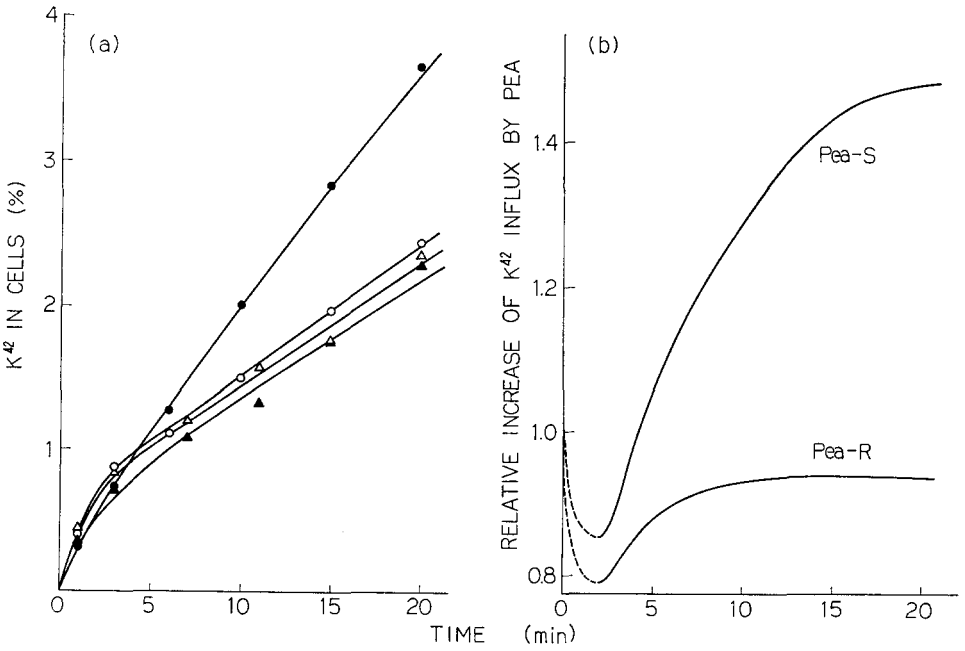

FIGURE 4.—Effect of PEA on K^{42} influx with Pea-S (KY2670) and Pea-R (KY2671) strains. PEA (0.25%) was added to the culture which was immediately followed by addition of K^{42} to 0.2 μc/ml. One ml aliquots were taken at intervals and filtered. (a) Time course of K^{42} influx; KY2670 (Pea-S) with (-●-●-) or without (-O-O-) PEA, and KY2671 (Pea-R) with (-▲-▲-) or without (-△-△) PEA. (b) PEA-induced K^{42} influx; ratios of K^{42} in cells in the presence and absence of PEA were plotted from the data of (a).

to the latter. In selection (3), however, the transfer of the *pea* gene from the Hfr parent was too low to be consistent with the results of selections (1) and (2). This has been satisfactorily accounted for by the finding that some of the apparently Pea-S recombinants show the Pea-R phenotype when tested on PEA medium containing streptomycin. As will be shown later, these recombinants that inherited the *pea* marker from the Hfr and the *str* marker from the F⁻ parent show the Pea-S phenotype in the absence of a certain suppressor gene, unless streptomycin is present in the medium. It may also be noted that in this cross the frequency distribution of unselected Hfr markers proximal to the selective one does not follow the usual pattern. The reason for this is not clear at the present time.

Further mapping of the *pea* gene was carried out by transduction experiments using phage P1kc and the results are shown in Table 4. In complete agreement with the data from the mating experiments, *pea* is mapped between *leu* and *azi* and is extremely close to the latter. In fact, the *pea* and *azi* genes are so close as to suggest that they might represent the same gene or genes adjacent to each other.

Interrelationships between pea *and* azi *genes:* In order to test the possibility that the *pea* and *azi* genes are interrelated functionally as well as structurally, a number of azide-resistant (Azi-R) mutants were isolated from strain KY387 which is azide sensitive (Azi-S) and PEA-resistant, and the possible simultaneous change in the PEA phenotype was examined. As shown in Table 5, most of the Azi-R mutants obtained from this strain turned out to be Pea-S unlike the parental strain. To test the generality of this finding, Azi-R mutants derived from three other strains were similarly examined. From the results presented in Table 5, it seems clear that simultaneous changes in phenotype of genes *azi* and *pea* by single-step mutations are rather common, since they were found with three of the four strains tested. In some cases, the coordinate changes were re-

TABLE 4

Transduction of the pea *gene by phage P1kc*

Experiment	Selective marker	Unselected marker				Number of transductants
		leu	*pea*	*azi*		
I*	*leu*+	1†	1	1		176
		1	1	0		13
		1	0	1		1
		1	0	0		209
					Total	399
II	*azi*	1	1	1		510
		0	1	1		259
		1	0	1		1
		0	0	1		10
					Total	780

* Experiment I : KY2121 (donor) —× KY2120 (recipient)
Experiment II : KY2120 (donor) —× KY2121 (recipient)
KY2121 : *leu*+ *pea azi*+; KY2120 : *leu pea*+ *azi*
† 1 and 0 represent markers from donor and recipient strains, respectively.

TABLE 5

*Simultaneous changes in resistance to PEA among spontaneous mutants selected for azide resistance**

Parental Azi-S strain				Azi-R derivatives			
	Azi	Pea	Str	Azi	Pea	Pea(+Str)†	Numbers obtained
KY387	S	R	S	R	S	..	62
					R	..	8
KY301	S	R	R	R	R	R	36
					R	S	10
					S	S	3
KY112	S	S	S	R	S	..	30
JC2207	S	S	R	R	S	R	6
					S	S	17

* S or R represent sensitive or resistant phenotype, respectively.
† PEA phenotype in the presence of streptomycin (400 μg/ml) in the medium.

vealed only when streptomycin was present in PEA-agar medium. That such changes in PEA phenotype are correlated with an alteration in membrane permeability was shown by the finding that one of the Azi-R mutants with Pea-S phenotype obtained from strain KY387 shows a similar response to PEA in K^{42} efflux and influx as other Pea-S strains studied (cf. Figures 3 and 4).

To find out whether these mutations that simultaneously affect azide and PEA resistance occurred at the *pea-azi* region of the chromosome, several such mutants obtained from strain KY387 were crossed with strain KY131, and the *thr*$^+$*leu*$^+$ *str* recombinants were scored for their sensitivity to resistance to azide and PEA in the presence or absence of streptomycin. Since very few, if any, recombinants with Azi-S or Pea-R phenotype (with or without streptomycin) were found from any of these crosses, it is tentatively concluded that these mutations occurred at or around the *azi-pea* region, although the possibility that they occurred at suppressor genes located proximal to *azi* has not been excluded. These results suggest that *azi* and *pea* are either identical or closely linked genes whose expression is correlated perhaps by forming an operon.

Temperature-sensitive azide-resistant mutants: In an attempt to gain some insight into the mechanism of action of the *azi* as well as the *pea* gene, temperature-sensitive Azi-R mutations in strain KY387 were studied. In particular, temperature-sensitive mutants that grow only at some restricted temperature range in PG medium (without PEA or azide) were looked for among mutants that have been selected by virtue of their resistance to azide at a certain temperature. Such mutants, if isolated, would be most useful in functional analysis of genes *azi* and *pea*. In fact, several mutants that can grow only at low (25°C) or high (42°C) temperature have been obtained as shown in Table 6 (type 6–8). Besides, many mutants whose phenotype with respect to PEA and/or azide resistance is temperature dependent have been isolated (Table 6, type 1–5).

When representative mutants of type 6 and 7 were crossed with strain KY131

TABLE 6

*Temperature-sensitive Azi-R mutants obtained from strain KY387**

Type	Growth at 42°C PG	42°C +AZI	42°C +PEA	Growth at 25°C PG	25°C +AZI	25°C +PEA	Number obtained: Spontaneous	Number obtained: Nitrosoguanidine
Parental	+	—	+	+	—	+	..	..
1	+	+	—	+	+	+	19	24
2	+	+	+	+	+	—	0	2
3	+	+	—	+	—	—	3	8
4	+	—	—	+	+	—	1	1
5	+	+	+	+	—	+	0	2
6	+	+	—	—	—	—	0	3
7	—	—	—	+	+	—	0	2
8	+	+	—	—	+	—	0	1

* Growth was determined by streaking cell suspensions onto PG medium or PG medium containing sodium azide (3.4 mM) or PEA (0.23%) and incubating for 1 or 2 days at 42°C or 25°C, respectively.

(F^-, *azi*), the *thr*$^+$*leu*$^+$*str* recombinants obtained were all azide resistant and many of them inherited the temperature sensitivity of each Hfr parental mutant used. It appears therefore that these mutations occurred at the *azi-pea* region of the chromosome and that the *azi* gene (or genes) plays a certain essential role in the growth of *E. coli*. Furthermore, this function seems to be somehow related to cell division, since all these mutants were found to elongate markedly to form "snakes" upon transfer to the nonpermissive temperature.

Effect of suppressors on Pea-R phenotype: It was noted in the crossing experiment of Table 3 that some of the *his*$^+$*str* recombinants that presumably received *pea* as well as *azi* from the Hfr parent did not show the Pea-R phenotype unless streptomycin is added to the PEA medium. By examining the unselected markers of these recombinants, it was noted that most of them (15/17) inherited the *trp* as well as the *his* region from the Hfr parent. In contrast, most of the recombinants (22/26) carrying the *trp* region of the F^- parent showed the Pea-R phenotype. Moreover, the *gal*$^+$*str* recombinants (selection 2 in Table 3) to which the *azi* and *trp* but not the *his* regions have been transmitted from the Hfr parent were all Pea-R. These results suggested that at least two genes each located near *trp* or *his* in addition to *str* are involved in determining the phenotype of PEA resistance vs. sensitivity. The effect of these genes in turn appeared to be affected by streptomycin in some cases.

To further test this possibility, several transduction experiments were performed and the results are presented in Table 7. It was first shown that transduction of *str* from strain KY131 to KY387 altered the PEA phenotype from resistance to sensitivity in most of the transductants, but all of them exhibited resistance when scored in the presence of streptomycin (Experiment 1). In Experiment 2, the *his* region of KY387 was transduced to KY2192 carrying the *trp* region of KY387 and the *his* and *str* regions of KY131. About 20 percent of the

TABLE 7

Transduction experiments showing the effects of str *and other suppressor genes on Pea-R phenotype*

Experiment	Parental strains					Donor marker selected	Number of transductants tested	Streptomycin in the medium	Number of transductants obtained	
									Pea-S	Pea-R
1	KY131*	*trp*+	*his*	*str*	*pea*+	*str*	50	—	42	8
	KY387	*trp*	*his*+	*str*+	*pea*			+	0	50
2	KY387	*trp*	*his*+	*str*+	*pea*	*his*+	100	—	19	81
	KY2192	*trp*	*his*	*str*	*pea*			+	7	93
3	KY131	*trp*+	*his*	*str*	*pea*+	*trp*+	30	—	7	23
	KY2151	*trp*	*his*+	*str*	*pea*			+	0	30
4	KY113	F*trp*+	*his*+	*str*+	*pea*+	*trp*+	100	—	53	47
	KY2151	*trp*	*his*+	*str*	*pea*			+	6	94

* In each experiment, the first line represents a donor and the second, a recipient.

transductants tested became Pea-S indicating that a gene closely linked to *his* is responsible for the alteration from the Pea-R to the Pea-S phenotype. Similarly, when the *trp* region of KY131 was introduced into strain KY2151 carrying the *trp* and *his* regions of KY387 and exhibiting the Pea-S phenotype, many of the transductants became Pea-R, suggesting that a gene closely linked to *trp* is responsible for this change (Experiment 3). Finally, the result of Experiment 4 suggests that a gene with a similar effect is present on the F*trp* episome (FREDERICQ 1964) and that it is dominant over the corresponding allele on the chromosome of KY2151. Table 8 summarizes the effects of various genes on the phenotypic expression of the *pea* (resistant) gene indicating the origin of the four relevant regions of the chromosome for several strains used. It seems apparent

TABLE 8

Summary of effects of various genes on phenotypic expression of the pea *(resistant) gene**

	Chromosomal region				PEA phenotype	
	pea	*trp*	*his*	*str*	−STR	+STR
KY387	1(R)†	1	1	1(S)	R	. .
KY131	0(S)	0	0	0(R)	S	S
Recombinant I‡	1	1	1	0	S	R
Recombinant II	1	0	1	0	R	R
Recombinant III	1	1	0	0	R	R
Recombinant IV	1	0	0	0	R	R
KY2151	1	1	1	0	S	R
KY2192	1	1	0	0	R	R

* Data were collected from experiments shown in Tables 3 and 7.

† 1 and 0 represent markers derived from strain KY387 and KY131, respectively. R or S in parenthesis indicate, respectively, the resistant or sensitive allele.

‡ Recombinants obtained from a cross KY387 (Hfr) × KY131 (F^-). Strains KY2151 and KY2192 are the representatives of type I and III recombinants, respectively.

that at least two suppressor genes, one located near *trp* and the other near *his*, as well as the *str* region affect the PEA phenotype under these conditions.

DISCUSSION

Although BERRAH and KONETZKA (1962) first reported that PEA acts by selectively inhibiting DNA synthesis in *E. coli*, more recent experiments in several laboratories failed to find conditions for such selective inhibition of DNA synthesis (e.g., ROSENKRANZ, CARR and ROSE 1965; PREVOST and MOSES 1966). The results reported in the present paper also indicate that synthesis of RNA rather than of DNA is inhibited most severely by relatively low concentrations of PEA in the Pea-S strain of *E. coli* employed. It seems worth recalling, however, that PEA inhibits the initiation of a new cycle of DNA replication as far as DNA synthesis is concerned (TREICK and KONETZKA 1964; LARK and LARK 1966). Recent work with *Neurospora crassa* (LESTER 1965) and with *E. coli* (SILVER and WENDT 1967) revealed that PEA alters cellular permeability characteristics and led to the proposal that the primary action of PEA is at the level of the cell membrane with resultant breakdown of the permeability barriers. The inhibition of DNA synthesis would then be caused by a coupling of the initiation of DNA replication to the membrane as suggested by TREICK and KONETZKA (1964) or by an indirect effect of the initial change in membrane permeability.

The present experiments showed that strain C600 and its derivatives are more resistant to PEA than other strains of *E. coli* tested with respect to the synthesis of DNA, RNA and protein, and in particular, to the initiation of the DNA replication cycle. It was further found that the membrane structure of the Pea-R strains is characterized by its resistance to the PEA-induced permeability change as revealed by K^{42} efflux and influx experiments. These results are therefore in line with the above proposal that the primary site of action of PEA is the cell membrane (LESTER 1965; SILVER and WENDT 1967) and suggest that it is an alteration of the membrane structure which is primarily responsible for the differences observed between Pea-R and Pea-S strains. LARK and LARK (1964; 1966) have found that the initiation of chromosome replication involves two types of protein, and the synthesis or the activity of one of them, presumably the structural protein, is sensitive to PEA. Whether the membrane alteration observed with the Pea-R strain affects in some way the latter protein that could correspond to the hypothetical replicator (JACOB, BRENNER and CUZIN 1963) or the proreplicator (LARK 1966) is not known at present.

Genetic data from both conjugation and transduction studies established that the PEA resistance in strain C600 is determined by a single gene located very close to *azi* on the *E. coli* chromosome. All the differences found between Pea-R and Pea-S strains are presumably due to different allelic states of the *pea* gene, although this point has not been rigorously tested by employing isogenic strains. Moreover, mutations to azide resistance are often accompanied by alterations in PEA resistance suggesting that *azi* and *pea* are either identical or closely linked genes whose expression is coordinated, perhaps by forming an operon. In this

connection, it is particularly interesting that the function of the *azi* gene(s), and possibly the *pea* gene as well, appears to be related somehow to the process of cell division and/or DNA replication cycle.

Studies of DNA synthesis with temperature-sensitive Azi-R mutants obtained may give further insight into the nature and function of the *pea-azi* region. Thus, with one such mutant (type 7, Table 6) examined, DNA was found to be selectively degraded after about doubling when temperature was shifted from 25°C to 42°C (WADA and YURA, unpublished result). A possible relevance of this finding to the control of DNA replication is under investigation.

Analyses of the effect of suppressor genes on PEA resistance phenotype (Tables 7 and 8) revealed that the chromosome of strain KY131 as well as the F*trp* episome carries the active form (*sup*$^+$) of at least one suppressor, closely linked to *trp* genes, that might be identical with one of the known amber or ochre suppressors (see review by GORINI and BECKWITH 1966). In fact, more recent experiments show that the expression of the PEA phenotype in strains carrying the *pea* allele is affected by *supI*$^+$, *supII*$^+$ or *supIII*$^+$ (WADA and YURA, unpublished results). It may be concluded then that the *pea* allele of strain KY387 and other derivatives of strain C600 is a nonsense mutant carrying presumably an internal nonsense (amber) codon. It seems as though the nonsense codon(s) must be translated, at least part of the time, into certain amino acid(s) to exhibit the Pea-R phenotype, although it remains to be seen whether the *pea* allele gives rise to Pea-R, Pea-S or possibly a lethal phenotype in the absence of any amber suppressors.

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SUMMARY

Escherichia coli K-12, strain C600, is resistant to phenethyl alcohol (PEA) as compared to other strains tested. The PEA resistance was observed with respect to the synthesis of DNA, RNA and protein, the initiation of the DNA replication cycle, and to the permeability barrier of the cell membrane. The PEA resistance of strain C600 is primarily determined by a gene (designated as *pea*) located between *leu* and *azi*, being extremely close to the latter. Mutational analysis revealed that the *pea* gene is also functionally related to the *azi* gene which in turn was found to play a certain essential role in the growth of *E. coli*. Phenotypic expression of the *pea* gene is affected by at least two suppressor genes, the *str* gene, and by streptomycin. It is postulated that the *pea-azi* region of the chromosome participates in the formation, activity and/or maintenance of some unidentified membrane structure directly or indirectly involved in the control of DNA replication and cell division.

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