

REVERSIONS OF THE L-RIBULOKINASE STRUCTURAL GENE OF *ESCHERICHIA COLI*¹

RICHARD M. CRIBBS

Department of Biology and Genetics, Medical College of Virginia, Richmond

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MUTATIONS in the *araB* gene, the structural gene for L-ribulokinase, of the arabinose gene-complex in *Escherichia coli* B/r (Figure 1) produce a two-fold effect (ENGLESBERG 1961; LEE and ENGLESBERG 1962, 1963): they are deficient in L-ribulokinase, and they differ in their level of L-arabinose isomerase and L-ribulose-5-phosphate-4-epimerase, the products of the adjacent structural *araA* and *araD* genes. Certain *araB* mutants show a higher inducible level of isomerase and epimerase than the wild type while others show less. In addition, the levels of the isomerase and epimerase enzymes of each *araB* mutant are coordinated. The L-arabinose isomerase produced by the *araB* mutants is indistinguishable from the isomerase produced by the wild-type strain of *E. coli*. A genetic map of the *araB* gene fails to show any relationship between the relative location of the mutant sites and their isomerase activity (CRIBBS and ENGLESBERG 1964).

In this study, the *araB* mutants were further examined to determine if the mutants which produced approximately the same levels of isomerase were results of the same type of genetic alteration. Since certain chemical mutagens, particularly base analogues, have been considered to induce specific types of nucleotide

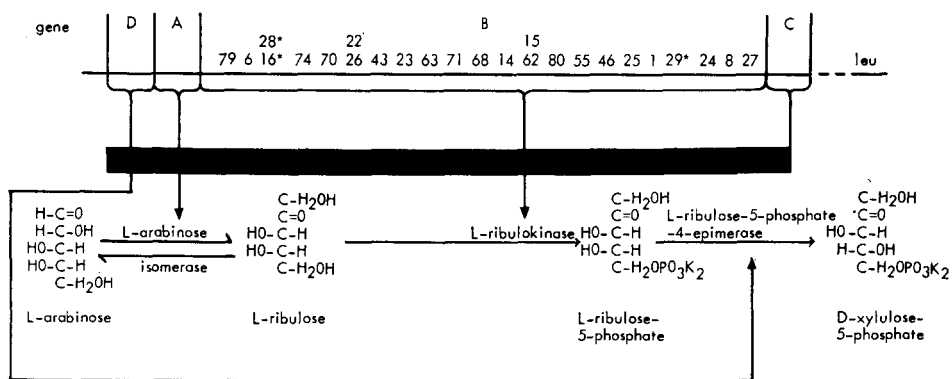


FIGURE 1.—The L-arabinose gene-enzyme complex. The numbers in the *B*-gene represent mutant sites. Two numbers at one site are "identical site" mutants. Mutant sites which cannot be ordered unambiguously are starred.

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changes in phage (FREESE 1959; CHAMPE and BENZER 1962) and bacteria (RUDNER 1961a, b), the reversion response of each *araB* mutant to four chemical mutagens was determined and compared to the level of isomerase in each of the mutants. A brief summary of this work has been published (CRIBBS 1964).

MATERIALS AND METHODS

Bacterial strains: The isolation, enzymatic characterization, transduction procedures, and genetic order of the *araB* L-arabinose negative (*ara*) mutants of *E. coli* B/r have been reported elsewhere (GROSS and ENGLEBERG 1959; ENGLEBERG 1961; CRIBBS and ENGLEBERG 1964). Two of the *araB* mutants, *ara-79* and *ara-80* were induced by X rays, the remainder were induced by ultraviolet light (UV).

Induced reversions: The procedures for testing the response of mutants to various chemical mutagens have been described (IYER and SZYBALSKY 1958; RUDNER 1961a; ALLEN and YANOFSKY 1963). The *ara* mutants were grown 18 hours in tubes containing 5 ml of L-broth (LENNOX 1955) with shaking at 37°C. The cultures were diluted with an equal volume of sterile saline, and 0.1 ml of the cells placed into 3.0 ml of warm 0.65% minimal agar enriched with 0.02% nutrient broth and L-arabinose (2 mg/ml). The warm agar containing the cells was poured onto a 1.5% minimal agar base (ALLEN and YANOFSKY 1963) containing the same enrichments. After the soft agar layer hardened, a sterile filter paper disc was placed in the center of the agar, and the mutagens, diethyl sulfate, bromodeoxyuridine, beta-propiolactone, were added directly to the disc. The 2-aminopurine was spread over a small area of the soft agar (EISENSTARK and ROSNER 1964). Sterile water was added to the disc on the control plates. The plates were incubated at 37°C for 3 to 4 days. Duplicate plates were used for each mutant tested. When the results were not clear, the tests were repeated.

Revertant analysis: Most of the reversion colonies on any given plate were uniform in size. A few reversion plates contained, in addition to wild-type size colonies, noticeably smaller colonies. A number of small colonies were picked, purified, and their growth rate in liquid minimal arabinose compared to the growth of the wild type and the original arabinose negative mutant. To test these revertants for a mutation at a second site, phage P1bt was grown on the purified revertants and adsorbed to *ara⁺ leu* bacteria for 30 minutes (GROSS and ENGLEBERG 1959) (*leu* = leucine dependent). Aliquots of the transduction mixture were then plated on minimal glucose plates (selection for *leu⁺*). The remaining transduction mixture was washed and resuspended in minimal arabinose medium containing penicillin (200 units/ml). After 18 hours incubation, the mixture was washed, resuspended, and plated on minimal glucose agar. The *leu⁺* transductants from both the plain and penicillin selected mixtures were then transferred to minimal arabinose plates. Failure of the *leu⁺* transductants to grow on the arabinose agar would indicate the original *ara* marker had been recovered from the revertant.

Mutagens: The volume of each mutagen placed on the agar was 0.05 ml. Undiluted diethyl sulfate (DES) was added, and β -propiolactone (BPL) was diluted 1:10 before use. Solutions of 2-aminopurine (AP, heated to dissolve 40 mg/ml) and 5-bromodeoxyuridine (BDU, 16 mg/ml), both from Sigma Chemical Company, were made prior to use and sterilized by filtration. The base layer of the plates on which BDU was used contained 0.05 mg/ml of aminopterin (Sigma). The aminopterin was sterilized by filtration and added to the agar medium prior to pouring the plates.

RESULTS

Reversions: Twenty-five *araB* mutants were tested for their reversion response to the mutagens AP, BDU, BPL, and DES. Twenty-three of the mutants were induced to revert at a rate higher than the spontaneous frequency by at least one of the mutagens. *ara-27* did not revert either spontaneously or when exposed to the mutagens and is assumed to be a deletion, although small, since it recom-

bines to prototrophy with adjacent mutant sites (CRIBBS and ENGLSBERG 1964). *ara-1* spontaneously reverted to prototrophy, but the rate of reversion was not increased by the mutagens. The mutants are listed in Table 1 according to their levels of L-arabinose isomerase (CRIBBS and ENGLSBERG 1964) and their reversion response to each mutagen.

The difficulty in classifying mutants according to either their spontaneous or induced frequency is the occurrence of "false revertants," i.e., reversions due to base rearrangement other than restoration of the wild-type base sequence. The "false revertants" tend to obscure the true back-mutation frequency. Because of the large number of revertants obtained in this study, it was not practical to test each one to determine whether it was a true back mutation. Instead, the smaller colonies, which could have been due to either a reversion that occurred late in the incubation period or to a second-site mutation that restored at least partial

TABLE 1

Reversion response of mutants in the L-ribulokinase structural gene, araB

ara mutant	L-arabinose isomerase level*	Total cells plated ($\times 10^8$)	Total spontaneous reversions	Frequency of reversion† <i>ara</i> → <i>ara</i> ⁺ due to:			
				AP	BDU	BPL	DES
14	3	0.8	5	5	1	3	94
27	5	9.4	0				
25	7	8.4	5	1	1	1	4
55	8	4.8	2	8	1	4	100
29	14	1.0	1	1	1	1	15
68	16	4.4	9	3	2	4	83
1	25	9.4	16	1	1	1	1
80	26	0.9	26	1	1	1	17
74	47	0.1	2	1	1	1	3
6	51	1.9	18	3	1	4	2
8	75	5.2	22	2	1	1	16
28	89	3.8	30	10	1	10	10
46	127	0.3	2	5	4	1	100
16	156	0.4	3	1	1	1	4
63	157	1.6	12	2	1	2	41
70	160	2.1	5	1	1	1	100
22	171	0.7	1	17	20	4	100
43	175	2.1	41	1	1	2	11
23	211	4.3	1	1	1	100	100
24	217	5.0	5	3	1	4	23
15	218	4.3	4	1	1	2	100
26	230	12.1	1	16	7	15	100
79	235	0.02	7	1	6	1	61
62	249	1.9	2	1	1	1	100
71	300	0.9	4	4	3	3	39

* μ moles of ribulose produced per hr per mg protein (CRIBBS and ENGLSBERG 1964).

† Times the total number of spontaneous revertants on the control plates. AP=2-aminopurine; BDU=5-bromo-deoxyuridine; BPL= β -propiolactone; DES=diethyl sulfate.

enzyme activity, were tested for second-site mutations. Of these small colony revertants from ten different *ara* mutants, 120 were purified and their growth rates compared to the wild-type cells. The following fractions indicate the numbers of slow-growing (partial) revertants over the total number of colonies tested: *ara*-68, 3/12; *ara*-71, 1/12; *ara*-63, 0/14; *ara*-79, 2/13; *ara*-43, 2/13; *ara*-26, 0/11; *ara*-15, 0/8; *ara*-8, 0/14; *ara*-14, 9/13; *ara*-24, 5/10. Twenty-two (18%) of the colonies tested showed a growth rate less than the prototroph. The remaining colonies were apparently due to late reversion, since they grew at the same rate as the wild-type cells. Growth rate alone, however, is not sufficient to classify a revertant as a true back mutation, since it has been shown that (1) a mutation at a second, extragenic site, or (2) a mutation in the same coding triplet causing an amino acid replacement other than the amino acid in the wild-type enzyme, may result in a revertant having the same growth rate as the wild-type strain (GAREN and SIDDIQI 1962; HENNING and YANOFSKY 1962).

To further test the two classes of revertants, phage was grown on 17 full revertants (growth rate equal to the wild type) and 17 partial revertants, which were used as donors in transduction experiments with *ara*⁺ *leu* recipient bacteria. The *leu*⁺ transductants (from 100 to 400 colonies in each case) obtained from all 17 transductions using phage grown on full revertants were *ara*⁺, and on the basis of colony size were indistinguishable from wild-type cells. *leu*⁺ transductants resulting from phage grown on partial revertants showed three classes of response when scored on minimal arabinose agar (Table 2). In the first class, all the *leu*⁺ colonies tested were *ara*⁺, with a colony size similar to wild type (Revertant numbers 1, 3, 4, 5, 7, 8, and 17, Table 2). In the second class, a proportion of the *leu*⁺ transductants were clearly arabinose negative and the remainder grew to wild-type size (Revertant numbers 2, 6, 10, 14, 15, and 16, Table 2). In the third class, the *leu*⁺ transductants, when scored on arabinose agar, were either the same size as wild type or noticeably smaller (Revertant numbers 9, 11, 12, and 13, Table 2). The response of the second class of partial revertants indicates that the second-site mutation was loosely linked to the *ara* region, since it was not cotransduced with the *ara leu* region of the chromosome. The response of the third class, i.e. partial growth of approximately one half of the *leu*⁺ transductants on the arabinose plates and the absence of strict *ara* colonies, indicates a very close linkage between the original *ara* site and the second-site mutation. The response of the first class of partial revertants cannot be accounted for by either of the above explanations, since only *ara*⁺ recombinants were recovered among the *leu*⁺ transductants. Failure to recover *ara* or partial *ara*⁺ colonies from the latter class of partial revertants implies repair of the original mutant site. Concurrent damage to another, distant gene whose product is involved in arabinose utilization could cause the observed partial growth of the revertant. Mechanisms that could account for the nature of these partial revertants are being investigated.

Twelve of the mutants listed in Table 1 (*ara*-14, 55, 68, 6, 8, 28, 46, 63, 22, 24, 26, and 71) showed increased frequency of reversion in response to AP at frequencies from 2 to 17 times that of the spontaneous rate. The mutants responding

TABLE 2

Genetic analysis of small colony revertants of araB mutants

Revertant No.	Original arabinose negative mutant	Reversion induced by†	<i>ara/leu</i> ⁺ transductants‡	
			penicillin nonselected	penicillin selected
1	71	AP	0/588	0/228
2	68	BDU	32/74	41/105
3	..	BPL	0/300
4	..	DES	0/399
5	43	SPON	0/161	0/361
6	79	SPON	2/34	1/10
7	..	BPL	0/23	0/13
8	14	SPON	0/140	0/168
9	..	AP	95/225*	82/149*
10	..	DES	24/67	59/123
11	..	BPL	38/111*	35/143*
12	..	BDU	41/102*	89/140*
13	24	SPON	52/112*	103/203*
14	..	DES-5	0/150	5/75
15	..	DES-2	72/194	47/77
16	..	AP2	1/229	0/177
17	..	AP3	0/213	0/187

* Partial growth of *ara*⁺ colonies.† AP=2-aminopurine; BDU=5-bromodeoxyuridine; BPL= β -propiolactone; DES=diethyl sulfate; SPON=spontaneous reversion.‡ In each case the *leu*⁺ transductants were obtained by transductions of *ara*⁺ *leu* bacteria by phage grown on small colony *ara*⁺ revertants (*leu*⁺).

to AP are randomly distributed among the low (1 to 100), medium (100 to 200) and high (200 to 300) isomerase producers (*L*-arabinose isomerase units in μ moles of ribulose produced per hour per mg protein of the cell free extract). Six of the mutants (*ara*-68, 46, 22, 26, 79, and 71) show an increase in their reversion frequency in response to BDU. All of these except *ara*-79 also responded to AP. Three of the mutants (*ara*-26, 79, and 71) are located among the high, two (*ara*-46 and *ara*-22) in the medium, and one (*ara*-68) in the low isomerase producers. Thirteen of the mutants (*ara*-14, 55, 68, 6, 28, 63, 22, 43, 23, 24, 15, 26, and 71) show an increase in reversion frequency in response to the alkylating agent BPL, and are randomly distributed among both the low and high isomerase producers. The frequency of reversion of only two of the mutants (*ara*-27 and *ara*-1) did not increase in the presence of DES. The effect of DES on the reversion frequency, as a rule, was much more pronounced than the other three mutagens; and those mutants that had a weak response to DES responded little, if any, to the other three mutagens. The effectiveness of DES as a mutagen has also been demonstrated with leucine (MARGOLIN and MUKAI 1961), tryptophan (BALBINDER 1962), and cysteine (EISENSTARK and ROSNER 1964) mutants of *Salmonella typhimurium*.

"Identical site mutants": Transduction results of CRIBBS and ENGLSBERG (1964) show that *ara*-28 and *ara*-16 are located at "identical sites" in the *araB* gene, i.e. recombination to prototrophy occurs at a rate equal to or less than the

TABLE 3
Comparison of "identical site" *araB* Mutants

<i>araB</i> mutant	Reversion response to:*				CRM production†	Isomerase level‡	Negative interference§
	AP	BDU	BPL	DES			
28	++	—	++	++	no	89	yes
16	—	—	—	+	no	156	yes
22	++	++	+	+++	no	171	no
26	++	++	++	+++	yes	230	no
15	—	—	+	+++	no	218	no
62	—	—	—	+++	no	249	yes

* +=2 to 5 times; ++=6 to 49 times; +++=greater than 50 times; —=no increase in spontaneous reversion frequency.

† CRM="cross reacting material," identified by LEE and ENGLSBERG 1963.

‡ μ moles of ribulose produced per hr per mg protein.

§ Previously reported (CRIBBS and ENGLSBERG 1964).

spontaneous reversion frequency of the recipient bacteria. *ara-22* and *ara-26* are also located at "identical sites," as are *ara-15* and *ara-62*. The various properties of these "identical site" point mutations (all six spontaneously revert to prototrophy) are shown in Table 3. *ara-22* and *ara-26* differ, since *ara-26* has been shown to produce a protein antigenically similar to L-ribulokinase while *ara-22* does not produce such a protein (LEE and ENGLSBERG 1963), although they have a similar response to the mutagens employed in this study. Previous transduction studies (CRIBBS and ENGLSBERG 1964) show that *ara-62* and *ara-15* differ widely in their recombination frequencies when crossed with adjacent *ara* markers. Differences in recombination behavior of mutants located at the same site could be attributed to qualitative differences in their mutant base substitution (BALBINDER 1962). *ara-28* and *ara-16*, although similar in three of the properties compared, can be distinguished by their response to the mutagens, since *ara-28* responds strongly to AP and BPL while *ara-16* does not. Each mutant differs from its "identical site" partner by at least one of the properties examined and is presumed to differ in its substituted mutant base. Yet none of the three mutants produce levels of isomerase significantly different from their "identical site" partners. These data also emphasize the need of examining a number of properties before considering two mutants at the same site as being similar in their base rearrangement.

DISCUSSION

Thirteen of the L-arabinose negative mutants of the *araB* gene did not respond to AP and are nontransition type mutations (FREESE 1963). The nontransition mutations are scattered throughout the *araB* gene and include both high and low L-arabinose isomerase producers. The reversion frequency of the 12 remaining mutants was increased, compared to their spontaneous rate, by AP, and could be considered transition ($A_T \leftrightarrow G_C$) mutations (FREESE 1963). Only six of the mutants responded to BDU, and of these, five also responded to AP. These mutants that respond to either or both analogues are also randomly located in the *araB*

gene and have widely different levels of isomerase. CHAMPE and BENZER (1962) concluded from their reversion studies with bacteriophage T4 that a mutant resulting from an $\frac{A}{T} \rightarrow \frac{G}{C}$ transition responds to both AP and BDU while a $\frac{G}{C} \rightarrow \frac{A}{T}$ transition responds to AP only. In this study, "false revertants" were shown to occur both spontaneously and in response to all of the chemical mutagens employed. It could be, then, that some and possibly all of the AP and/or BDU revertants of any particular mutant were due to suppression of nontransition mutants rather than to true back mutation of transition type mutants. The number of *araB* revertants due to suppression, by the criteria used in this study, is small; nonetheless, without an extensive analysis of each revertant, the classification of the *araB* mutants as transition mutations of either type is open to question.

A similar response of two mutants to the chemical mutagens does not provide irrefutable evidence on the similar nature of the mutants. This is apparent from *ara-22* and *ara-26*, two mutants that show a similar response to the four mutagens, yet differ in another property indicating they are not the same in their type of base rearrangement. The same is true of *ara-15* and *ara-62*. Differences in the reversion response of two mutants could be due to a number of causes; different base substitution at each mutant site, the influence of neighboring nucleotides on reversion frequency, or the frequency with which second-site mutations occur resulting in suppression of a particular mutant.

On the basis of these results, the reversion response (whether due to a true back mutation or a suppressor mutation) of 25 arabinose negative mutants of the *araB* gene shows no correlation in the amount of L-arabinose isomerase produced by a mutant and its reversion response to the four chemical mutagens employed in this study. Although the number of mutants tested was small, UV induces approximately 50% nontransition type mutations.

Genetic tests of revertants that exhibit partial growth indicate, in one instance, a loosely linked suppressor site which segregated from the original arabinose deficiency. In the second instance, the original and suppressor site segregate together. In the latter case, either these two sites are closely linked or the partial revertant resulted from a base change in the original coding triplet causing replacement of an amino acid in the L-ribulokinase enzyme. The new amino acid reduces, but does not destroy, the activity of the enzyme (HENNING and YANOF-SKY 1962). In the third instance, the partial revertant does not appear to contain the original *ara* site but rather a deficiency in a gene distal to the *ara leu* region of the chromosome. It may be that this distal gene (or genes) plays a role in the translation or regulation of a genetic message necessary for the efficient use of arabinose.

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SUMMARY

Twenty-five L-arabinose negative mutants of the *araB* gene of *Escherichia coli*

B/r have been examined with regard to their reversion response to four chemical mutagens. There appears to be no relationship between the reversion response and the characteristic level of L-arabinose isomerase, the product of the adjacent structural *araA* gene, produced by each mutant. A small proportion of the revertants were tested genetically and their reversion attributed to a mutation at a second site. The reversion response was also used to determine whether mutations, which map at the same site in the *araB* gene, differ qualitatively.

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