SUPPRESSION OF A MISSENSE MUTATION IN THE L-RIBULOKINASE GENE OF ESCHERICHIA COLI

ROBERT G. BOST AND RICHARD M. CRIBBS

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

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The L-arabinose gene-enzyme complex, except for the unlinked L-arabinose permease gene, *araE*, is shown in Figure 1. *araB* and *araA* are the structural genes for the L-ribulokinase and L-arabinose isomerase, respectively; while *araD* is probably the structural gene for L-ribulose-5-phosphate-4-epimerase (Englesberg 1961; Englesberg et al. 1962; Lee and Englesberg 1962, 1963). The *araC* gene is a regulatory gene, and its product regulates, in a positive manner, the expression of the *araD*, *araA*, *araB* and *araE* structural genes (Englesberg, IRR, Power, and Lee 1965; Sheppard and Englesberg 1967). Transcription of the three adjacent structural genes begins at an initiator region located between the *araB* and *araC* genes (Englesberg, IRR, Power, and Lee 1965). Thus, the order of transcription, as well as translation, of these linked structural genes is presumably *araB*, *araA* and *araD*.

In a previous study (Cribbs 1965) a number of *araB* revertants were isolated that utilized L-arabinose at a slower rate than the wild type. Transduction analyses of these partial revertants indicated the presence of both the original *araB* mutation and a suppressor mutation. These suppressor mutations were either closely linked, or were not co-transducible (i.e. unlinked) with, the *araB* gene. When the suppressor was not present in the genome, the *araB* mutation prevented production of a functional L-ribulokinase.

However, a suppressed *araB* revertant has been isolated that can use L-arabinose
in the absence of its suppressor, although utilization is enhanced when the suppressor is present. In this report we show that the suppressor is unlinked to the \textit{araB} gene. In addition, it not only increases L-ribulokinase activity, but also has a polar effect on L-arabinose isomerase, produced by the adjacent \textit{araA} gene.

\textbf{MATERIALS AND METHODS}

The isolation, media, transduction procedures, and the biochemical and genetic properties of the L-arabinose negative mutants (\textit{ara}) have been described previously (Gross and Englesberg 1959; Englesberg 1961; Boyer, Englesberg, and Weinberg 1962; Cribbs and Englesberg 1964).

The suppressed mutant used in this study was a spontaneous revertant of \textit{araB14}, a mutant that exhibits extreme polarity; that is, it has very low levels of L-arabinose isomerase activity as well as L-ribulokinase activity (Englesberg 1961). This spontaneous revertant was obtained in the following manner. \textit{araB14} was cultured in 5 ml of L-broth (Lennox 1955) for 18 hr in a shaking incubator. The culture was diluted with an equal volume of saline and 0.1 ml aliquots were spread on a mineral agar base containing L-arabinose as the sole carbohydrate. The plates were incubated for 2 to 3 days. Spontaneous revertants were picked and purified on L-arabinose mineral agar. The suppressed mutant isolated in this manner, was designated \textit{araB14R1SuB\textsuperscript{R}}, the \textit{R1} denoting the reverted site at or near \textit{araB14}, and the \textit{Su\textsuperscript{R}}, the unlinked site that enhances L-arabinose utilization by \textit{araB14R1}. An \textit{araB14R1} strain was constructed from \textit{araB14R1SuB\textsuperscript{R}} by transduction with bacteriophage P1bt. For transduction studies the \textit{leu\textsuperscript{+}} (ability to synthesize leucine) marker was transduced to \textit{leu\textsuperscript{-}} (inability to synthesize leucine) so that each strain was available with either the \textit{leu\textsuperscript{+}} or the \textit{leu\textsuperscript{-}} marker. To obtain an \textit{araB14R1SuB\textsuperscript{R} leu\textsuperscript{-}} from \textit{araB14R1SuB\textsuperscript{R} leu\textsuperscript{+}}, phage grown on \textit{araB14 leu\textsuperscript{-}} was used to infect \textit{araB14R1SuB\textsuperscript{R} leu\textsuperscript{+}} bacteria. The transduction mixture was centrifuged and resuspended in saline to reduce lysogeny among the transductants. Aliquots of the saline suspension were then grown in liquid mineral L-arabinose containing penicillin (1000 units per ml). After growth in penicillin (usually 3 to 5 hr) the cells were washed with saline and plated on mineral agar containing L-arabinose and leucine. Final concentrations of L-arabinose and leucine in all solid and liquid media were 0.2 and 0.004%, respectively. These colonies were then scored onto L-arabinose mineral agar to determine their ability to synthesize leucine. A \textit{leu} colony was picked, purified, tested for lysogeny, and its growth rate compared to the original recipient, \textit{araB14R1SuB\textsuperscript{R} leu\textsuperscript{+}}. This insured the presence of the \textit{araB14R1} and \textit{Su\textsuperscript{R}} loci in the \textit{leu} transductant.

Bacterial growth was measured by growing the bacteria in L-broth to a concentration of approximately $5 \times 10^8$ cells per ml. Aliquots of 0.1 ml of this culture were added to sterile Klett tubes containing 4.9 ml of a mineral base and the appropriate carbohydrate and amino acid. The cells were incubated at 37°C in an incubator modified for shaking cultures and the turbidity was measured at twenty minute intervals by a Klett-Summerson colorimeter using a #42 filter. The Klett readings were converted to optical density and growth was recorded as increase in optical density per unit time.

Cell free extracts were prepared as described by Englesberg (1961) and Cribbs and Englesberg (1964). An overnight 5.0 ml mineral casein hydrolysate (1.0%) culture (Gross and Englesberg 1959) was added to 250 ml mineral casein hydrolysate contained in 1 liter Erlenmeyer flasks. The flasks were incubated at 37°C with shaking until a Klett reading (#42 filter) of 150 was reached. L-arabinose was then added (final concentration, 0.4%) to induce the enzymes. At a Klett reading of 300, usually about 2 hr after addition of L-arabinose, the cells were spun down in the cold. The pellet was washed with 5.0 ml 10^{-3} \text{ M} ethylenediamine tetraacetic acid (EDTA), pH 7.4, and resuspended in 50 ml EDTA and respun. The cells were finally resuspended in 5.0 ml of 10^{-3} \text{ M} EDTA, 10^{-4} \text{ M} glutathione, at pH 7.4. The cell suspension was then added to a cold mortar containing aluminum powder (Alcoa # A-305) and the cells were ruptured by grinding with a pestle. This mixture was spun in the cold and the supernatant assayed for enzyme activities.

The protein content of the cell-free extracts was determined by a modified Folin-Lowry
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L-arabinose isomerase activity was assayed by the method of CRIBBS and ENGLESBERG (1964). L-ribulokinase activity was assayed by a modified method of ANDERSON and WOOD (1961). The reaction mixture contained, in a total volume of 3.0 ml: tris (hydroxymethyl) aminomethane (pH 7.5), 100 μmoles; MgCl₂, 10 μmoles; adenosine triphosphate (ATP), 5 μmoles; phosphoenolpyruvate, 5 μmoles; glutathione, 15 μmoles; reduced nicotinamide adenine dinucleotide (NADH), 0.5 μmoles; L-ribulose (generously supplied by Drs. ELLIS ENGLESBERG and GILBERT ASHWELL), 15 μmoles; rabbit muscle lactic dehydrogenase (LDH) with pyruvate kinase, 0.005 ml; bacterial extract, 0.1 ml. (The ATP, NADH and LDH were obtained from the Sigma Chemical Co.) The oxidation of NADH at 25°C was followed at 340 nm using a Beckman DU spectrophotometer. L-ribulokinase activity of the extract was determined by subtracting NADH oxidation in the absence of L-ribulose.

As a control, each extract was assayed for NADH oxidase by measuring the decrease in NADH concentration using a Zeiss PMQ11 spectrophotometer at 341 nm at 30°C. The reaction mixture contained in 3.0 ml: 2.98 ml of 0.1 M KH₂PO₄-K₂HPO₄ buffer (pH 7.5) containing 0.3 mg NADH and 0.02 ml of the cell-free extract. All enzyme assays were performed on two independently prepared extracts.

RESULTS

Bacteriophage PIbt grown on the original leu+ partial revertant of araB14 (araB14R1Su8) served as a donor for araB14 leu bacteria. Initial selection for ara+ leu+ restricted recombination to the ara leu region of the chromosome. The ara+ leu+ transductants were homogeneous in size on the agar plates. A number of them were picked, purified, and their growth in liquid mineral L-arabinose was compared to the original revertant, the wild type, and araB14 (Figure 2). Student's t-test and analysis of variance showed that the growth rate of the ara+ leu+ transductants (P < 0.01) was significantly less than the wild type and the original partial revertant, but greater than that of araB14. The growth rates of these same strains were indistinguishable in liquid mineral glucose (Figure 3). The differences in colony size of these strains were easily recognizable when streaked on L-arabinose mineral agar. However, in this and subsequent transductions, a number of colonies were always picked, suspended in saline, and streaked onto L-arabinose agar to insure that our initial judgement of colony size was correct.

The results of the above transduction suggested that the original partial revertant contained two mutations, one linked and one unlinked to the ara leu region of the chromosome. The linked mutation, R1, enabled the strain to utilize L-arabinose at a slow rate, and the unlinked mutation, Su8, acted to increase this rate. This meant that either a simultaneous double mutation had taken place in araB14, which is unlikely, or that the Su8 mutation was already present in araB14 and a single mutation of araB14 to araB14R1 had occurred. To test these alternatives, phages from the original partial revertant were used to infect araB14 leu+ bacteria. Selection was carried out on mineral L-arabinose agar. Ten of the ara+ transductants were picked, purified, and their growth rates in liquid mineral glucose tested and found to be the same as the other strains shown in Figure 3. How-
Figures 2 and 3.—Growth of the wild type and the L-arabinose mutants of *Escherichia coli* B/r in mineral base supplemented with L-arabinose (Figure 2) and glucose (Figure 3). O—wild type; ●—the L-arabinose negative mutant, *araB14*; △—the suppressed missense mutant, *araB14R1SuB*; ▲—the missense mutant, *araB14R1*.

However, in liquid mineral arabinose their growth rates were the same as the original partial revertant, *araB14R1SuB*, shown in Figure 2. Since the *SuB* locus is not linked to the *ara leu* region, the growth characteristics of these phenotypically *araB* transductants, carrying the *R1* site, show that the *SuB* locus was present in the *araB14 leu*+ stock culture.

The location of the *R1* site is shown by the results of Table 1. When phage grown on *araB14R1SuB* *leu*+ bacteria were used to transduce the *araB* mutants

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Total <em>leu</em>+ <em>ara</em>+ transductants</th>
<th>Colony size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>small</td>
</tr>
<tr>
<td><em>araB14</em></td>
<td><em>araB14R1SuB</em></td>
<td>10,090</td>
<td>10,090</td>
</tr>
<tr>
<td><em>araB62</em></td>
<td><em>araB14R1SuB</em></td>
<td>1,869</td>
<td>1,853</td>
</tr>
<tr>
<td><em>araB68</em></td>
<td><em>araB14R1SuB</em></td>
<td>3,933</td>
<td>3,848</td>
</tr>
</tbody>
</table>

* The recipient bacteria were *leu*.
† The donor phage were *leu*+.
‡ Initial selection was for *ara*+ *leu*+ transductants. All but a few transductants were easily classified, on the transduction plate, as either the same size as *araB14R1* (i.e. small) or larger wild-type size colonies (i.e. large). All the intermediate colonies and a representative number of both large and small colonies were picked and restreaked on L-arabinose mineral agar and their size compared to *araB14R1*, *araB14R1SuB* and wild type before classification.
TABLE 2

Specificity of the suppressor locus (SuB)

<table>
<thead>
<tr>
<th>Recipient*</th>
<th>Donor†</th>
<th>leu+ transductants tested</th>
<th>ara+ leu+‡ leu+</th>
</tr>
</thead>
<tbody>
<tr>
<td>araB14R1SuB</td>
<td>wild type</td>
<td>163</td>
<td>163/163</td>
</tr>
<tr>
<td>araB14R1SuB</td>
<td>araB14</td>
<td>122</td>
<td>73/122</td>
</tr>
<tr>
<td>araB14R1SuB</td>
<td>araB16</td>
<td>184</td>
<td>125/184</td>
</tr>
<tr>
<td>araB14R1SuB</td>
<td>araB1</td>
<td>172</td>
<td>67/172</td>
</tr>
<tr>
<td>araB14R1SuB</td>
<td>araB25</td>
<td>147</td>
<td>44/147</td>
</tr>
</tbody>
</table>

* The recipient bacteria were leu.
† The donor phage was leu+.
‡ Initial selection was for leu+. The leu+ transductants were then scored on mineral L-arabinose agar to determine their arabinose genotype.

on either side of araB14, i.e., araB62 and araB68; (see Figure 1) two sizes of L-arabinose utilizing colonies were observed among the ara+ leu+ transductants. The larger colonies were the same size as the wild type and the others, noticeably smaller. However, when araB14 was transduced with phage grown on araB14R1SuB, smaller, but not wild-type size colonies were noted among the leu+ transductants tested. The linkage of the R1 site to the ara leu region together with the lack of wild-type recombinants in transductions with araB14 and R1, indicates R1 is at or near the same site as araB14.

Next, phage grown on leu+ araB14, araB16, araB1 and araB25 were used to infect the araB14R1SuB leu. A proportion of the initially selected leu+ transductants were scored on L-arabinose agar and found to be ara (Table 2). Since initial selection for the leu+ marker restricted recombination to this area, all recipient bacteria contained the unlinked SuB locus. Thus, the finding of ara transductants eliminates the SuB locus as a general suppressor of araB mutants. In addition, the SuB locus does not affect L-arabinose utilization by the wild type, since all leu+ transductants were phenotypically ara+ when araB14R1SuB was transduced with phage grown on wild-type cells (Table 2).

A comparison of the enzyme activities of the wild type, araB14, araB14R1, and araB14R1SuB strains is shown in Table 3. araB14R1 had a level of L-ribulokinase activity much below that of both the wild type and the araB14R1SuB strains but significantly (Student's t-tests, P < 0.01) higher than that of araB14. The R1 site in the araB gene also had a polar effect as evidenced by the reduced level of L-arabinose isomerase; the product of the adjacent araA gene. The presence of the SuB locus in the araB14R1 genome increased the kinase activity to roughly 50% of the wild-type activity. Even more striking was its effect on the isomerase level which was elevated to the wild-type level. araB14, as reported previously (Cribbs and Englesberg 1964) was a low isomerase producer and showed only a trace of kinase. The significance of these differences in L-ribulokinase and L-arabinose isomerase levels is reinforced by the very small variation in NADH oxidase activities of the four strains.
TABLE 3

**Enzymatic characterization of the suppressed and nonsuppressed strains**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>L-arabinose isomerase*</th>
<th>L-ribulokinase†</th>
<th>NADH oxidase‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>Average</td>
</tr>
<tr>
<td>wild type</td>
<td>42.6</td>
<td>65.8</td>
<td>54</td>
</tr>
<tr>
<td>araB14R1SuB</td>
<td>48.6</td>
<td>60.5</td>
<td>55</td>
</tr>
<tr>
<td>araB14R1</td>
<td>33.1</td>
<td>28.2</td>
<td>31</td>
</tr>
<tr>
<td>araB14</td>
<td>7.5</td>
<td>3.1</td>
<td>5</td>
</tr>
</tbody>
</table>

Columns 1 and 2 represent assays that were performed on separately prepared extracts.

* Isomerase activity is expressed as μmoles of ribulose formed/hr/mg protein of the cell extract.
† L-ribulokinase and NADH oxidase activities are expressed as the change in optical density × 100/hr/mg protein of the cell extract.

DISCUSSION

araB14 is a nonsense mutant (Ellis Englesberg, personal communication) and has been shown to be the result of a transition type mutation (Cribbs 1965). The spontaneous revertant of araB14 contains two altered sites. One site, R1, is linked to the ara leu region of the chromosome and probably at the same site as araB14. The second site, SuB, already present in araB14, is outside the araB gene and not co-transducible with the ara leu loci. It is likely that the intragenic reversion is the result of a nonsense (araB14) to missense (R1) mutation. The missense mutation has two effects. First, it enables the araB gene to be translated, resulting in a functional L-ribulokinase albeit a less efficient (missense) kinase than that of the wild type. Second, L-arabinose isomerase is produced in a much greater amount than that produced by araB14, but still less than the wild type. This is as expected, since R1 now enables translation to proceed and araA is more distal to the proposed initiation point of translation than is araB. The polar effect on isomerase production noted with R1 has also been reported for other araB gene missense mutants (Lee and Englesberg 1962). In those cases, however, the araB missense mutants produced a kinase that was enzymatically inactive, but immunologically crossreacting. Lee and Englesberg (1962) have also shown that mutations in the araB gene affect the rate of synthesis of isomerase but the isomerase produced by the araB mutants is indistinguishable from that produced by the wild type. For this reason, we believe that the isomerase produced by araB14R1, araB14R1SuB, and the wild type is the same. The differences of isomerase levels noted in Table 3, then, are due to different rates of synthesis of isomerase by these strains.

The unlinked SuB locus is specific for the R1 site and, most likely, any codon similar to it, since it does not affect the wild type or any of the araB mutants tested. The SuB locus increases the level of both kinase and isomerase; the former to a level below, and the latter to the same level as the wild type. In this study it is likely that the action of SuB involves a particular transfer RNA species as reported in cases of both nonsense (Capecchi and Gussin 1965; Engelhardt et al. 1965) and missense (Carbon, Berg, and Yanofsky 1966; Gupta and
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KHORANA 1966) suppression. SuB could act to alter the structure of the araB14R1 kinase by substituting an amino acid at the R1 site that differs from both the wild type and R1 amino acids. Since the anticodon and the recognition site for the aminoacyl-transfer RNA synthetase seem to be mutually exclusive (SCHULMAN and CHAMBERS 1968; SUNDHARADAS et al. 1968); the altered transfer RNA could be aminoacylated with a different amino acid and still bind to the R1 containing codon on the araB messenger RNA. This, in effect, would change the R1 missense kinase to a more efficient second type of missense kinase and would account for the difference in activities of araB14R1 and araB14R1SuB. If this is the case, the aminoacylated-transfer RNA of SuB must, in addition to carrying a different amino acid, be more readily available than the corresponding transfer RNA in the R1 strain. The increased quantity of SuB transfer RNA would permit faster translation of the araB14R1 codon and consequently araA, thus accounting for the increased isomerase activity noted when SuB is present in the genome.

Alternatively, the transfer RNA specific for the R1 containing codon in the araB gene may have an altered aminoacylation rate (CARBON and CURRY 1968). SuB would alter either the transfer RNA or the corresponding aminoacyl-transfer RNA synthetase in such a way that the aminoacylation rate for the same amino acid would be increased without altering the anticodon. Using the isomerase level (Table 3) as an indication of the translation rate, the increased availability of the aminoacylated-transfer RNA increases translation of the araB containing R1 and araA genes to the same rate as the wild type. In contrast to the former explanation, the missense kinase produced by araB14R1SuB would be identical to that of araB14R1 since they each contain the same missense amino acid. Even though the quantity of missense kinase, produced by araB14R1SuB, may be the same as the wild type, its catalytic activity is reduced because of the missense amino acid it contains. This would explain araB14R1SuB having both a lower kinase activity and a lower growth rate in mineral L-arabinose than the wild type. Since all the strains grow equally well in mineral glucose, it is likely that the transfer RNA involved does not have a major regulatory function in the cell.

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SUMMARY

A partial revertant of an L-ribulokinase structural gene (araB) nonsense mutant has been characterized. The revertant contains a nonsense to missense mutation in the araB gene and a suppressor locus that is unlinked to the araB gene. The missense mutation enables production of a partially functional L-ribulokinase and exhibits a polar effect on L-arabinose isomerase, produced by the adjacent structural araA gene. The presence of the suppressor locus has two effects on the strain containing the araB missense mutation. First, it increases the level of L-ribulokinase activity and, second, it relieves the polar effect on isomerase production. The suppressor mutation probably modifies a particular transfer RNA
species which, in turn, alters the quality and/or quantity of the missense kinase and increases the translation rate of the araA gene.

LITERATURE CITED


