Suppressor-Induced Structural Changes in a Missense L-Ribulokinase of *Escherichia coli*

RICHARD M. CRIBBS,* JAY C. LEONARD, AND ALEDA H. RICKELTON

*Department of Human Genetics, Virginia Commonwealth University, Richmond, Virginia 23298*

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A suppressor mutation specific for a missense codon in the L-ribulokinase structural gene of the L-arabinose operon of *Escherichia coli* B/r enhanced L-arabinose utilization by the strain containing the missense codon. Electrophoretic comparisons of the wild-type, missense, and suppressed missense L-ribulokinases indicated that the suppressor changed the structure of the missense kinase, thereby increasing its catalytic activity. Hyperinducibility imposed on an operator-distal gene by the missense codon was not affected by the suppressor mutation.

The L-arabinose gene-enzyme complex of *Escherichia coli* B/r (Fig. 1) consists of a regulatory gene, *araC*, and controlling sites, *araO* and *araI*, which govern the expression of the structural genes for L-ribulokinase (*araB*), L-arabinose isomerase (*araA*), and L-ribulose 5-phosphate 4-epimerase (*araD*) (11, 12, 19). Enzymatic analyses of structural gene mutations have shown that those occurring in the *araB* gene affect the level of isomerase and epimerase produced by the adjacent (and *araCOI-distal*) *araA* and *araD* genes (19, 20). The level of isomerase is greatly increased in L-arabinose-negative (Ara-) missense mutants (17). *araB* nonsense mutants, with one exception, have less than wild-type levels of isomerase (17). With regard to isomerase production, the former are classified as hyperinducible and the latter as polar mutants.

We previously reported the isolation and partial characterization of a unique revertant from an extremely polar *araB* gene mutant, *araB14* (2, 7). This revertant contained two mutations, a reverse mutation located at or near the *araB14* site and an extragenic suppressor mutation. Although the revertant is phenotypically Ara*, it has a slower growth rate in mineral salts-L-arabinose and a lower kinase activity than the wild type. When the suppressor mutation is removed by transduction, the resultant strain containing the reverse mutation still retains an Ara+ phenotype but grows slower and has less kinase activity than when the suppressor is present. Furthermore, the suppressor mutation shows allelic specificity since it neither affects L-arabinose utilization by the wild type nor suppresses the Ara- phenotype of a number of missense and nonsense *araB* mutants.

Data presented in this report show that the suppressor causes a structural change in the kinase of the strain carrying the reverse mutation, thus enhancing its activity, but, contrary to a previous report (2), does not alter the hyperinducible level of isomerase. Evidence is also presented that identifies the nonsense codon of *araB14* and the missense nature of the codon generated by the reverse mutation.

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MATERIALS AND METHODS

**Media.** Routine growth of bacteria was in L-broth (22). Tris-(hydroxymethyl)aminomethane (Tris)-glucose medium supplemented with agar (0.65 or 1.5%) for the growth of phage P1bt and for isolation of single plaques of T4 phages has been described (3). For preparation of extracts, cells were grown in a mineral salts-casein hydrolysate medium with or without L-arabinose (10).

**Bacterial and phage strains.** The bacterial strain *araB14r1sub* was derived from *araB14* and contains a reverse mutation, *r1*, to *araB14* and an extragenic suppressor mutation, *sub* (2). The strain designated *araB14r1* contains only the reverse mutation.

The growth and storage of phage P1bt, transduction using P1bt, and selection procedures have been previously described (3, 15). Single plaques of T4 strains (from I. R. Tessman and M. Abou-Sabé), containing either UGA, UAG, or UAA nonsense codons, were isolated from a lawn of *E. coli* CR63 Sul growing in Tris-glucose soft (0.65%) agar overlaid on...
Tris-glucose hard (1.5%) agar. Stocks of T4 phages were grown in L-broth cultures in E. coli CR63 Sul.

**Transduction and suppressor studies.** Since E. coli B/r is permissive for T4 phages containing nonsense codons in the rII region, the following strains were prepared and used in the transduction and suppressor studies. The ara' leucine-negative (leu') loci of B/r were transduced into E. coli K-12 KH600 (λ) Hfr P4x araC19 (from R. B. Helling), a strain restrictive for rII nonsense mutants. The newly prepared KH600 ara' leu' strain was then used as a recipient for phages carrying either the araB14 leu' loci or the rII mutation and leu' locus (see Table 1). Leu' transductants were selected initially. These were then grown on minimal agar with L-arabinose to determine their L-arabinose phenotype.

For suppressor tests a KH600 araB14 leu' transductant from the cross described above was purified and tested for lysisogeny. From this transductant 16 spontaneous Ara' revertants were independently isolated, purified, and tested for their ability to support the growth of T4 phages containing known nonsense mutations (17).

**Preparation of cell extracts.** Cell extracts were prepared by a method described previously (2). After the final centrifugation, the bacterial pellet was ground with alumina (Alcoa no. A-305), the resultant paste was taken up in 3 ml of suspension buffer (10⁻⁴ M glycylglycine, 10⁻⁸ M ethylenediaminetetraacetic acid [EDTA], 10⁻⁴ M dithiothreitol, pH 7.6) and centrifuged (48,000 x g) at 3 C for 45 min, and the supernatant fluid was stored at -20 C.

**L-Arabinose isomerase assay.** The method for the determination of isomerase activity has been described (8). To determine the period of maximum isomerase activity after induction, 10 ml of a casein hydrolysate culture (about 3 x 10⁸ cells/ml) was inoculated into 250 ml of prewarmed casein hydrolysate with a resultant optical density of 0.026 (420 nm, Spectronic 20). After 1 h of growth at 37 C with shaking, L-arabinose (final concentration, 0.4%) was added to the culture. Thirty-milliliter samples were removed at 10-min intervals for 80 min. Each sample was immediately chilled and centrifuged at 3 C for 30 min at 20,000 x g. The pellets were washed with 10⁻⁴ M EDTA, pH 7.4, resuspended in 1 ml of the same buffer, and stored at -20 C for 1 to 3 days before isomerase determinations. Isomerase activities were essentially unaffected by this short period of storage.

Whole cells were prepared for isomerase assays by the method described by Schleif (25). From 0.1 to 0.4 ml of the whole-cell preparation, a drop of toluene, and sufficient reaction mixture to give a total volume of 2.0 ml were added to the reaction tube, shaken vigorously, and incubated at 37 C. Samples (0.1 ml) were removed each minute after incubation. The specific activity was based on L-ribulose production during the first 6 min, the period of maximum isomerase activity. Under these conditions all three strains of E. coli B/r (wild type, araB14r1, and araB14r1sub) used in this study achieved maximum isomerase levels 60 min after induction with L-arabinose. Previously reported (2) isomerase activities of these strains were determined by using cell extracts. To validate comparisons between this and the previous report, isomerase assays were done on both whole cells and cell extracts prepared from the same culture 1 h after induction. Specific activities (micromoles of L-ribulose formed per hour per milligram of protein) from whole-cell preparations were 30.0, 56.5, and 63.5, whereas those for cell extracts were 30.0, 58.3, and 61.2 for the wild type and strains araB14r1 and araB14r1sub, respectively. In view of these results and since assays of whole cells involve less preparation, all isomerase activities in this report were determined from whole cells.

Protein content was determined by a modification of the Folin-Lowry method (24), using bovine serum albumin (Sigma Chemical Co.) as a standard.

**Partial purification of L-ribulokinase.** L-Ribulokinase from cell extracts of each strain was partially purified through the 50% ammonium sulfate fractionation step of the method described by Lee and Englesberg (21; with slight modifications suggested by N. Lee). An amount of MnCl₂ (1.0 M) equivalent to 5% of the total volume was added to the cell extract with stirring. Twenty minutes later the mixture was centrifuged for 1 h (34,850 x g) and the supernatant fluid was adjusted to pH 7.6 with 0.3% ammonia. Crystalline (NH₄)₂SO₄ was added over a period of 30 min to bring the supernatant to 40% saturation while maintaining the pH at 7.6. Thirty minutes later the mixture was centrifuged (1 h at 34,850 x g), and the pellet was discarded. The supernatant fluid was brought to 50% saturation with crystalline (NH₄)₂SO₄ over a period of 15 min. Forty-five minutes later the precipitate was collected by centrifugation (1 h at 34,850 x g), dissolved in 1 ml of phosphate buffer (10⁻⁴ M potassium phosphate, 10⁻⁴ M EDTA, pH 7.6), and dialyzed against 2 liters of the same buffer. After 12 h the dialysis fluid was changed, and dialysis was continued for an additional 2 h. The contents of the dialysis bag were removed and stored at -20 C.
L-Ribulokinase activity. Kinase activities were determined spectrophotometrically by measuring reduced nicotinamide adenine dinucleotide oxidation at 340 nm and 30 C as previously described (19). Where gel slices were assayed for kinase activity, d-ribulose (Sigma Chemical Co.) rather than L-ribulose was used as a substrate (18). Relative specific activities of all three partially purified kinases were not altered when d-ribulose was substituted for L-ribulose (prepared according to Tipson and Brady [27]) in the reaction mixture.

Electrophoresis. Vertical polyacrylamide gel (8%) electrophoresis (E. C. Apparatus Corp.) of the partially purified kinases and pure crystalline L-ribulokinase (from N. Lee) was carried out in 0.04 M Tris-glycine buffer, pH 8.3, at 14 C. A gradient of 18 V/cm was applied to the gel for 6 h. Gels were stained for 2 h in 0.25% Coomassie brilliant blue (Sigma Chemical Co.) in a methanol-water-glacial acetic acid (5:5:1) mixture at 25 C after fixing in 10% acetic acid for 1 h. The same methanol mixture without the dye was used to clarify the gels. The stained gels were scanned at 572 nm (E. C. Apparatus Corp., model 810 densitometer).

The migration of the proteins in each extract was compared by applying a sample of pure kinase (20 μg), each extract (200 μg), and a mixture of each extract and pure kinase to separate slots on the same gel. To localize the kinases on the gel, duplicate samples of each extract were applied to each half of the gel. After electrophoresis the gel was cut longitudinally. One half of the gel was stained and aligned with the other unstained half. Slices (7 mm), corresponding to the bands on the stained half of the gel, were cut from the unstained half, minced, and eluted in 0.5 ml of a 10⁻² M potassium phosphate buffer, pH 7.6, for 16 h at 4 C. As a control, gel slices were cut from areas of the gel that did not contain protein and treated similarly. The eluates were clarified through filter paper contained in a microsyringe. Kinase activities were determined on two sets of filtrates independently prepared from two different gels.

RESULTS

The original strain of araB14 has been shown to contain a nonsense codon (17). Since the araB14 stock used in this report was derived from the original strain a number of years ago, we confirmed the nature of the codon present in our araB14. By testing the ability of KH600 araB14 revertants to support the growth of T4 phages carrying the nonsense codons, we verified that our araB14 strain contains a UAG codon. It was also confirmed that B/r does not contain a UAG suppressor and the KH600 strain used in this study does not contain suppressors to either UAG, UGA, or UAA.

When P1 phages carrying the araB14 and leu⁺ loci were used to transduce KH600 ara⁺ leu⁻ recipients, 17% of the initially selected Leu⁺ transductants were Ara⁻ (Table 1). That is, araB14 and leu⁺ were cotransduced 17% of the time. The rI mutation and the leu⁺ locus should be cotransduced into the same recipient with roughly the same frequency since rI is located at or near the same site as araB14 (2). However, the results of the ara⁺ leu⁺ (recipient) x araB14rI leu⁺ (donor) cross in Table 1 show that all of the Leu⁺ transductants had an Ara⁺ phenotype, even though an estimated 17% contained the rI mutation. Failure to segregate Ara⁻ transductants in this same cross supports our previous conclusion that the rI mutation occurred at or near the same site as the original araB14 mutation.

These data and those showing an absence of nonsense suppressors in the KH600 strain indicate that the rI mutation most likely generates a missense codon that produces a functional kinase and enables L-arabinose utilization by the araB14rI strain.

Isomerase activity. Isomerase activity measurements of E. coli B/r wild type, strain araB14rI, and strain araB14rIsub were reproducible only when cells were harvested from dilute cultures in the exponential growth phase and when assays were done by measuring L-ribulose production at 1-min intervals for 6 min rather than at 3-min intervals for 15 min as formerly reported (2). With these modifications, the isomerase activities of the wild type, araB14rI, and araB14rIsub 60 min after induction were 31.2 ± 1.7, 64.1 ± 5.5, and 67.8 ± 10.0, respectively. These data show, unlike those reported previously (2), that araB14rI does not have a polar effect on the operator-distal araA gene product nor does the presence of suppressor mutation, sub, appreciably alter the level of isomerase of the araB14rI strain. A comparison of the rates of isomerase synthesis by the three

<table>
<thead>
<tr>
<th>Recipient KH600 strain</th>
<th>Donor B/r strain</th>
<th>Leu⁺ transductants tested</th>
<th>Ara⁺ phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara⁺ leu⁻</td>
<td>araB14 leu⁺</td>
<td>225</td>
<td>186 (83)</td>
</tr>
<tr>
<td>ara⁺ leu⁺</td>
<td>araB14rI leu⁺</td>
<td>215</td>
<td>215 (100)</td>
</tr>
</tbody>
</table>

*Abbreviation for strain E. coli K-12 KH600 (λ) Hfr P4x.

*Initial selection was for Leu⁺. The Leu⁺ transductants were then scored on mineral salts-L-arabinose agar to determine their Ara phenotype. The numbers in parentheses are the percentage of ara⁺ leu⁺/leu⁺ phenotype.
strains (Fig. 2) verifies these conclusions since araB14r1 and araB14r1sub had indistinguishable rates and both differed significantly from that of the wild type ($P < 0.01$).

**Electrophoresis of L-ribulokinase.** The densitometric tracings comparing the mobilities of proteins in the partially purified extracts are shown in Fig. 3. The migration distance of the pure kinase (indicated by an asterisk) was determined by electrophoresis of the kinase alone and mixed with each of the three partially purified kinase preparations. In each instance the pure kinase migrated to the same position, indicating that its mobility is unaffected by other proteins in the partially purified preparations. Neither araB14r1 nor araB14r1sub showed a distinct band corresponding to the wild-type kinase even though these extracts had 74 and 20% total activity, respectively, of the wild-type kinase. The kinases of each of the three strains (indicated by an arrow in Fig. 3) were located by assaying slices from separate slots on the same gel for activity. Initial assays of the gel from the entire slot localized the kinases of each strain in the vicinity of the pure kinase about one-third of the distance from the end of the gel. Subsequent assays on smaller gel slices from this region (see "schematic zymogram," Table 2) showed that the wild-type kinase migrated to the most anodal position, which corresponded to the pure kinase band, strain araB14r1sub kinase migrated to the most cathodal position, and the kinase of strain araB14r1 migrated to an intermediate position (Table 2).

**DISCUSSION**

The results of both the suppressor and transduction (Table 1) studies show that araB14 contains a UAG nonsense codon that is changed to a missense codon by the r1 mutation. The

![Fig. 2](http://jb.asm.org/)

**Fig. 2.** Kinetics of isomerase synthesis by E. coli wild type (○), araB14r1 (△), and araB14r1sub (●) after induction with L-araB14. Each point represents a mean specific activity (micromoles of ribulose formed per hour per milligram of protein) from either two (wild type) or three (araB14r1 and araB14r1sub) independent experiments. Before the means were determined, the data for replicate experiments were tested for homogeneity.

![Fig. 3](http://jb.asm.org/)

**Fig. 3.** Spectrophotometric scans of polyacrylamide gels of the partially purified extracts of E. coli B/r wild type (WT), araB14r1, and araB14r1sub. The asterisks indicate the location of the pure L-ribulokinase when electrophoresed alone or mixed with the extracts. The arrows indicate the respective locations of the WT, araB14r1, and araB14r1sub kinases. The direction of migration was from cathode to anode.
Table 2. L-Ribulokinase activity of polyacrylamide gel slices

<table>
<thead>
<tr>
<th>Strain</th>
<th>Kinase activity (%) of gel slice:*</th>
<th>Total activity A + B + C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>12 16 82</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>20 8 72</td>
<td>3.3</td>
</tr>
<tr>
<td>araBl4rl</td>
<td>18 41 41</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>8 86 6</td>
<td>7.0</td>
</tr>
<tr>
<td>araBl4rlsub</td>
<td>27 64 9</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>96 0 4</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Schematic zymogram

\[\text{ABC}\]

\[\text{→}\]

\[\text{→}\]

* Slices from two gels were assayed. Numbers in the top row of each strain were determined from one gel, those in the bottom row from a second gel.

* Total change in absorbancy (340 nm) \(\times 4 \times 10^{-5}/\text{min}\) for three gel slices from one slot.

* Migration was from cathode to anode. The vertical lines outline the areas (A, B, and C) of the gel slices assayed for activity.

nonsense-to-missense change has two noted phenotypic effects. First, a functional kinase with less activity than the wild-type kinase is produced by araBl4rl (2). The difference in the activity levels between the two kinases and, more importantly, their differential migration during electrophoresis illustrate the missense nature of the araBl4rl kinase. Second, the araBl44-imposed polarity on the products of the araCOI-distal araA, and presumably araD, genes is relieved and isomerase activity is increased to a level twice that found in the wild type.

When sub is in the genome of the araBl4rl strain, the isomerase activity remains at the same hyperinducible level. However, as noted previously (2), the specific activity of kinase (expressed as change in optical density \(\times 100\) per hour per milligram of extract protein) increases from 5 in araBl4rl to 12 in the presence of the sub mutation compared with 28 in the wild type. The electrophoretic data showing migration differences between all three kinases indicate that the extragenic sub mutation causes a qualitative change in the araBl4rl missense kinase. sub presumably effects replacement of the missense amino acid in araBl4rl kinase with a second missense amino acid, resulting in a kinase with a higher level of activity.

In the tryptophan synthetase system of E. coli, various missense mutants producing an inactive A protein are suppressed by substitution of the wild-type amino acid for the missense amino acid (5, 16). In these instances suppression is accomplished by a modification in one of the glycine-carrying transfer ribonucleic acids, enabling it to insert glycine in response to the missense codon. There are no a priori reasons why the suppressor must reinsert the wild-type amino acid at the site of the mutant codon. Any amino acid that enhances the catalytic properties of the mutant protein is potentially capable of suppression. This is illustrated by nonsense suppressors where different levels of suppression are achieved by the reinsertion of non-wild-type amino acids at the site of the nonsense codon (13, 14).

In the most extensively studied cases, transfer ribonucleic acid with altered coding properties have been shown to be responsible for the suppression of specific nonsense and missense codons (4, 5, 9, 16). The mechanism by which sub suppresses (or raises the activity of) the kinase present in the araBl4rl strain can also be explained on this same basis. However, our available data make such an explanation premature.

Based on extensive studies with missense and nonsense araB mutants, Katz and Englberg (17) have proposed a model of control to account for isomerase hyperinducibility of the araB missense mutants. According to their model, maximum expression of the araBAD genes in the fully induced wild-type strain is not achieved owing to repressors produced from L-arabinose metabolism. These "self-catabolite" repressors act, in a manner similar to glucose catabolite repressors, to lower the intracellular level of cyclic adenosine 3',5'-monophosphate (cAMP) (23). As with other inducible operons, cAMP is necessary to initiate transcription of the araBAD genes (6, 28). araB missense mutants that do not metabolize L-arabinose have sufficient cAMP (due to a lack of self-catabolite repressors) to achieve maximum (or hyperinducible) expression of the araA, and presumably araD, genes. As pointed out elsewhere (26), one prediction of this model is an inverse relationship between kinase and isomerase activities of araB mutants whose transcription/translation rate is unimpaired. The isomerase level of an araB mutant with an Ara* phenotype would be regulated by the degree of activity of the mutant kinase. araBl4rl does utilize L-arabinose and yet has a hyperinducible level of isomerase. Strain araBl4rlsub should have a higher level of self-catabolite repressors than araBl4rl since
the former has a faster growth rate in mineral salts-L-arabinose medium. Significantly, the isomerase activity of araB14rlsub remains at the same hyperinducible level. Our results also show that the rate of isomerase synthesis by araB14rl is not reduced by the suppressor mutation. Abou-Sabe' (1) has also shown a lack of correlation between the degree of kinase restoration and isomerase levels in a number of suppressed araB nonsense mutants. In view of these observations, hyperinducibility may be an all-or-none phenomenon; that is, the loss of hyperinducibility only occurs when the repressor concentration reaches some critical level in the cell. Our studies indicate that this critical level may only be achieved when the mutant kinase has some level of activity greater than 50% of the wild-type kinase.

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LITERATURE CITED