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Long-Term Experimental Evolution in *Escherichia coli*. IX. Characterization of Insertion Sequence-Mediated Mutations and Rearrangements

Dominique Schneider,* Esther Duperchy,* Evelyne Coursange,* Richard E. Lenski† and Michel Blot*

**Laboratoire Plasticité et Expression des Génomes Microbiens, Université Joseph Fourier, 38041 Grenoble Cedex 9, France and †Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824*

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ABSTRACT

As part of a long-term evolution experiment, two populations of *Escherichia coli* B adapted to a glucose minimal medium for 10,000 generations. In both populations, multiple IS-associated mutations arose that then went to fixation. We identify the affected genetic loci and characterize the molecular events that produced nine of these mutations. All nine were IS-mediated events, including simple insertions as well as recombination between homologous elements that generated inversions and deletions. Sequencing DNA adjacent to the insertions indicates that the affected genes are involved in central metabolism (knockouts of *pykF* and *nadR*), cell wall synthesis (adjacent to the promoter of *pbpA-rodA*), and ill-defined functions (knockouts of *hokB-sokB* and *yfcU*). These genes are candidates for manipulation and competition experiments to determine whether the mutations were beneficial or merely hitchhiked to fixation.

INSERTION sequence (IS) elements are small DNA sequences that carry genetic information related to their transposition and its regulation (Mahillon and Chandler 1998). They are ubiquitous in bacterial genomes and contribute significantly to spontaneous mutagenesis in bacteria (Lieb 1981; Rodriguez *et al.* 1992; Kitamura *et al.* 1995; Hall 1999). Insertion of an IS element often leads to gene inactivation and strong polar effects (Jordan *et al.* 1968; Saedler *et al.* 1974). However, certain IS elements carry promoters or other protein-binding sequences, such that an IS insertion can also activate cryptic genes or alter the expression of adjacent genes (Saedler *et al.* 1980; Reynolds *et al.* 1981; Ciampi *et al.* 1982; Mahillon and Chandler 1998). Furthermore, IS elements act as substrates for recombination pathways leading to chromosomal rearrangements, such as inversions and deletions (Reif and Saedler 1975; Louarn *et al.* 1985; Deonier 1996).

IS and other transposable elements are presumably of considerable importance for the evolution of bacteria and other organisms that carry them, given the high rate and diversity of mutations they cause (McClintock 1965; Federoff 1983; Hartl *et al.* 1986; Blot 1994). However, there is some debate about whether transposable elements are an important source of variation for adaptive evolution or, alternatively, genomic parasites whose primary effect is to generate deleterious mutations (for reviews, see Blot 1994; Charlesworth *et al.* 1994). Several evolution experiments have shown that some IS-mediated mutations are beneficial (*e.g.*, Treves *et al.* 1998), but there has been no systematic study to identify the full range of IS-associated substitutions in an evolving population and then determine which ones were beneficial and which were deleterious mutations that presumably hitchhiked with some beneficial mutation. The findings that we present here represent an intermediate, but essential, step in such a study. Our analysis is based on two populations of *Escherichia coli* B that evolved in the laboratory for 10,000 generations (Lenski *et al.* 1991, 1998; Lenski and Travisano 1994). Papadopoulos *et al.* (1999) performed restriction fragment length polymorphism (RFLP) analyses using seven IS elements as probes with genomic DNA from 170 clones isolated at eight time points from these two populations. They found that several IS-associated mutations arose and were fixed in each population, all involving either IS*1* or IS*150*. In this study, we identify the affected loci and characterize the molecular events that led to many of these mutations. In the future, we intend to reconstruct isogenic genotypes, with and without these mutations, and perform competitions to determine whether particular mutations were beneficial or, alternatively, hitchhiked to fixation.

MATERIALS AND METHODS

Strains and plasmids: Twelve populations of *E. coli* B were founded from two variants of a common ancestor and propagated for 10,000 generations in glucose minimal medium (Lenski and Travisano 1994). Samples from each population were stored at −80° every 500 generations. The ancestor contains neither plasmids nor active prophages. One ancestral

Corresponding author: Dominique Schneider, CNRS EP2029, CEA LRC12, Université Joseph Fourier, Laboratoire Plasticité et Expression des Génomes Microbiens, CERMO, BP 53, 38041 Grenoble Cedex 9, France. E-mail: dominique.schneider@ujf-grenoble.fr

variant can grow on arabinose, whereas the other cannot; they differ by a single mutation that is neutral under the conditions used in the evolution experiment and serves as a genetic marker (Lenski *et al.* 1991). In this study, we used two populations, designated Ara − 1 and Ara + 1, which were arbitrarily chosen as foci for certain aspects of our research on these populations, including molecular genetic work. Numerous clones were isolated from the stored samples (Papadopoulos *et al.* 1999) and provide the genetic material used in this study.

The strain used in cloning experiments was Epicurian Coli XL10-Gold (Stratagene, La Jolla, CA). The plasmid used as the cloning vector was PCR-Script (Stratagene).

Media and growth conditions: Experimental conditions used for the evolving populations were described previously (Lenski *et al.* 1991). For routine work, we used Luria-Bertani broth (LB) and LB containing 12 g/liter agar as growth media (Sambrook *et al.* 1989). Media were supplemented with 30 μg/ml chloramphenicol (Cam), 40 μg/ml Xgal, or 50 μM IPTG as required. Strains were grown at 37° with shaking (200 rpm).

DNA handling and hybridizations: Genomic DNA (from 3-ml cultures) and plasmid DNA (from 1.5-ml cultures) were extracted following Sambrook *et al.* (1989). For sequencing, plasmid DNA was prepared with the QIAGEN (Chatsworth, CA) plasmid purification midi kit. Restricted DNA fragments were separated on 0.8% agarose gels and transferred to nylon membranes (Boehringer Mannheim, Indianapolis) as described by Southern (1975). Hybridizations were performed at high stringency (68°) with cold-labeled DNA probes using the Boehringer Mannheim DIG system.

Characterization of sequences adjacent to IS elements by inverse PCR: We refer to the left and right sides of an IS element according to the direction of transcription of its transposase gene. Papadopoulos *et al.* (1999) analyzed clones from populations Ara − 1 and Ara + 1 by RFLP experiments using *Eco*RV-digested genomic DNA. They used as probes internal IS fragments lacking *Eco*RV restriction sites to detect mutations associated with IS*1* and IS*150*. However, *Eco*RV cuts once near the right extremity of IS*150*, and the IS*150*-specific probe used by Papadopoulos *et al.* (1999) corresponds to the left part of the IS. Therefore, *Eco*RV fragments allow cloning of only the left IS*150*-adjacent sequence, whereas both IS*1*-adjacent sequences can be cloned using *Eco*RV because it does not cut within IS*1*. We reexamined the clones studied by Papadopoulos *et al.* (1999), using two other restriction enzymes, *Hinc*II and *Pvu*II, that do not cut within the IS*150* elements (D. Schneider, unpublished data). We detected no additional IS-associated mutations except for one IS*150*-mediated deletion recently discovered by Cooper (2000) that was fixed in both focal populations. This deletion was missed by Papadopoulos *et al.* (1999) because it occurred immediately adjacent to the right IS*150* extremity. Our new RFLP data also confirmed all the mutations previously detected with *Eco*RV digestions.

Genomic DNA of a clone was digested with *Eco*RV, and fragments were separated onto a 0.8% agarose gel with *Pst*I- and *Hin*dIII-digested lambda DNA as size markers. Gel fractions containing IS fragments were cut and DNA purified using a modification of the BIO 101 (Vista, CA) Geneclean kit (Boyle and Lew 1995). These fragments were self-ligated with T4 DNA ligase (Boehringer Mannheim) at 5–10 μg/ml, and the ligated mixtures were used as templates in PCR experiments. Primers used for inverse PCR to amplify sequences adjacent to IS*1* were G3, 5′-GTCATCGGGCATTATC TGAAC-3′; and G4, 5′-AGAAGCCACTGGAGCACC-3′. They are near the IS*1* extremities and directed outward. The IS*150* left-adjacent sequence was amplified using G5, 5′-GATCCTG TAACCATCATCAG-3′; and G21, 5′-CATCCTGTTCTGCACT CTGA-3′. G5 is near the left extremity of IS*150*, G21 is near the IS*150 Eco*RV site, and both are directed outward. All PCR reactions were performed using Pfu DNA polymerase (Stratagene) according to the manufacturer's recommendations. The PCR products containing IS*1*- and IS*150*-adjacent sequences were cloned using the PCR-Script Cam cloning kit (Stratagene). The plasmid content of white colonies was digested with *Eco*RV, allowing the isolation of two DNA fragments. For IS*1*, each fragment contained either the left- or right-adjacent sequence; for IS*150*, one fragment contained the left-adjacent sequence and the other fragment carried a small piece of the IS element. To obtain the right-adjacent fragments for IS*150*, additional RFLP experiments were performed using IS*150* as a probe, but genomic DNA was digested with either *Hinc*II or *Pvu*II, which do not cut within IS*150*; two restriction enzymes were needed because of comigrating fragments of similar size. The left-adjacent sequence of each IS*150* was then used as a probe with *Hinc*II or *Pvu*II reference membranes, allowing identification of fragments that contained the IS*150* element. These fragments were self-ligated and used in inverse PCR with primers G5 (see above) and G6, 5′-CTGAAGGATGCTGTTACGG-3′. G6 lies near the right extremity of IS*150* and is directed outward. This procedure allowed the characterization of both adjacent sequences of IS*150*.

All adjacent sequences were used as DIG-labeled probes with reference membranes (see below) to confirm that predicted IS-containing fragments hybridized. Sequencing of adjacent DNA was done by the dideoxy chain termination method (Sanger *et al.* 1977), using the same primers as in the PCR experiments. Sequences were compared to databases using BLAST (Altschul *et al.* 1997). We give chromosomal locations based on *E. coli* K-12 (Blattner *et al.* 1997).

Reference membranes: Sequences adjacent to IS elements were used to probe reference membranes that had been previously probed with the IS elements themselves. These membranes carry *Eco*RV-, *Hinc*II-, and *Pvu*II-digested genomic DNA from the ancestor and diverse evolved clones, including those from which the adjacent sequences were obtained. The membranes were probed with the IS elements, stripped, and reprobed with adjacent sequences to show that the correct sequences were cloned. By comparing clones with and without a particular IS element, we could identify its adjacent sequences and characterize the mutational event.

Nomenclature for IS elements: To denote a particular IS element present in the ancestral strain, the element's name is followed by an identifying numeral: *e.g.*, IS*150*-1 is one of five IS*150* elements present in the ancestor. To denote a particular element that was detected in a new restriction fragment from the genome of an evolved clone, the element's name is followed by an identifying letter: *e.g.*, IS*1*-A is an element in a fragment that was not present in the ancestor.

RESULTS

We characterized three mutations that arose and were fixed in population Ara − 1 and six that were fixed in Ara + 1 (Table 1). All nine mutations were IS-mediated events, including six simple insertions and three complex rearrangements. All six insertions involved IS*150* elements, including five such events in Ara + 1 (Table 1), which experienced a "burst" of IS*150* activity in which its copy number tripled (Papadopoulos *et al.* 1999). Each population exhibited a large inversion that occurred via homologous recombination between IS*1*

TABLE 1

IS-mediated mutations that arose and were fixed in two evolving populations of *E. coli*

Mutational event	Mutation described in	Mutation first detected at (generations)[a]	Mutation became fixed by (generations)[a]
Population Ara − 1			
Deletion of 6934 bp from IS*150* located upstream of *rbs* operon into *yieO*	Cooper (2000)	500	500
Insertion of IS*150* into *pykF*	Figure 1 (this study)	2000	5000
Inversion involving existing IS*1* elements located in *gatZ* and between *citC* and *dpiB*	Figure 2 (this study)	5000	5000
Deletion from IS*1* located in *nmpC* to *E. coli* B-specific sequence (within 0.7 min)	Figure 3 (this study)	5000	5000
Population Ara + 1			
Deletion of 6138 bp from IS*150* located upstream of *rbs* operon into *yieO*	Cooper (2000)	500	500
Insertion of IS*150* into *nadR*	Figure 4 (this study)	1000	2000[b]
Insertion of IS*150* into *hokB-sokB*	Figure 5 (this study)	1000	2000[b]
Inversion involving new IS*150* element in *nadR* and existing IS*150* in *hokX-sokX*	Figure 4 (this study)	2000	2000[b]
Insertion of IS*150* upstream of *pbpA-rodA* operon	Figure 5 (this study)	2000	2000[b]
Insertion of IS*150* into *yfcU*	results (this study)	5000	8000
Insertion of IS*150* into intergenic region between *glcB* and *yghK*	results (this study)	5000	8000

[a] Time in generations. At least seven clones from each population were analyzed at generations 500, 1000, 1500, 2000, 5000, 8000, and 10,000.

[b] These mutations were absent from 1 of 10 clones sampled at generation 2000, but were present in all 31 later clones.

elements in one population and IS*150* elements in the other (Table 1). Ara − 1 also underwent a deletion involving an IS*1* element (Table 1). In addition to the nine IS-mediated mutations identified and characterized in this study, Cooper (2000) found that both populations fixed deletions involving an IS*150* element located immediately upstream of the *rbs* operon; the proximal deletion endpoints were identical, although the distal endpoints were not. Aside from these parallel deletions, none of the other IS-mediated mutations involved the same genes in the focal populations (Table 1). In the paragraphs below, we briefly summarize the molecular evidence used to characterize the nine mutations discovered in this study.

Insertion of IS*150* into *pykF* in population Ara − 1: Figure 1 shows the essential features used to identify this mutation. A 6.4-kb *Eco*RV fragment containing the left-adjacent sequence of IS*150*-D was detected in 1 of 8 clones sampled at generation 2000 and in all 34 clones from later generations; the fragment was absent from 45 clones sampled before generation 2000. The left-adjacent sequence was cloned and used to probe *Hinc*II and *Pvu*II reference membranes, which confirmed the 1.4-kb size increase predicted for an IS*150* insertion. The 2.9-kb *Hinc*II fragment was self-ligated and used with inverse PCR to clone the sequences adjacent to IS*150*-D. Sequencing showed the insertion was in the *pykF* gene, 682 bp downstream from the ATG start codon; this insertion also led to a 3-bp duplication of the target site. The *pykF* gene encodes pyruvate kinase I, one of two *E. coli* pyruvate kinases (Bledig *et al.* 1996), and is located at 37.8 min on the K-12 chromosome. The insertion almost certainly inactivated *pykF*.

Inversion between two IS*1* in Ara − 1: The features used to characterize this chromosomal rearrangement are illustrated in Figure 2. The 6.2- and 2.3-kb *Eco*RV fragments containing IS*1*-A and IS*1*-C, respectively, were detected in all 34 clones from generation 5000 onward (Figure 2B), but not in any of 53 clones from earlier samples. The gains of IS*1*-A and IS*1*-C could have been generated by two different insertions, in which case (i) the IS*1*-A probe should hybridize with a 5.5-kb *Eco*RV fragment (6.2 kb for IS*1*-A reduced by 0.7 kb for IS*1*) in the ancestor and early clones; and (ii) the IS*1*-C probe should hybridize with a 1.6-kb *Eco*RV fragment (2.3 kb for IS*1*-C less 0.7 kb for IS*1*) in these same clones. However, the actual hybridization results were more complex. The left- and right-adjacent sequences of IS*1*-A and -C hybridized only with IS*1*-containing fragments. In the ancestor and early clones, hybridization showed two IS*1*-containing *Eco*RV fragments, one containing IS*1*-15 and the other IS*1*-6 (Figure 2A); both these fragments were absent in clones isolated from generation 5000 onward.

The IS*1*-C left-adjacent sequence and the IS*1*-A right-adjacent sequence both hybridized with the fragment containing IS*1*-6, and the IS*1*-C right-adjacent sequence and the IS*1*-A left-adjacent sequence both hybridized

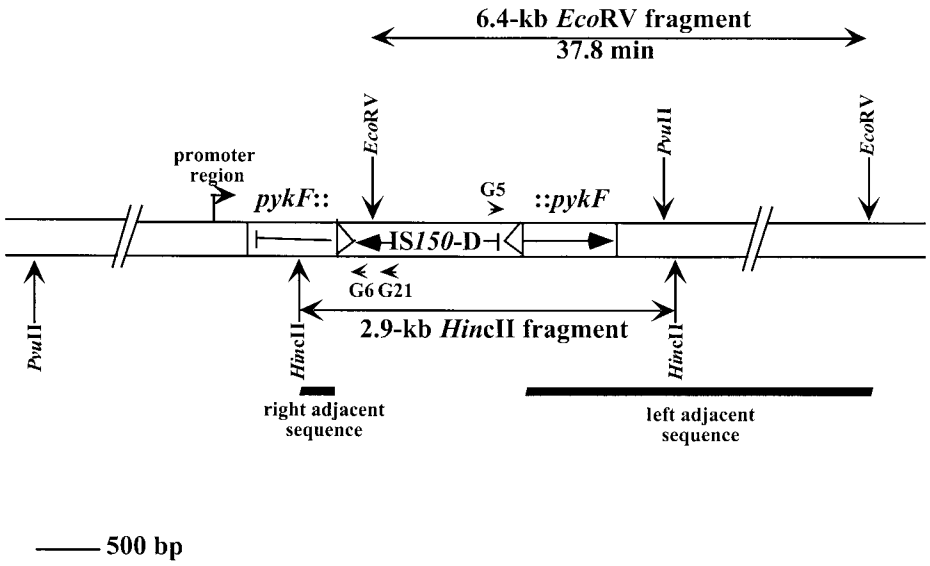

FIGURE 1.—Insertion of an IS*150* element into *pykF* in population Ara − 1. The gene *pykF* encodes pyruvate kinase I and is located at 37.8 min. The directions of transcription of the IS*150* transposase and *pykF* genes are shown by arrows. The 6.4-kb *Eco*RV fragment allowed cloning of the IS*150*-D left-adjacent sequence after self-ligation and inverse PCR using primers G5 and G21 (arrowheads). The 2.9-kb *Hinc*II fragment was identified using the left-adjacent sequence to probe reference membranes. The IS*150*-D right-adjacent sequence was cloned from this fragment by inverse PCR using primers G5 and G6 (arrowheads). Only relevant restriction sites are shown. IS*150* terminal inverted repeats are indicated by triangles. The *pykF* promoter region is shown as a bent arrow. Only *pykF* and IS*150*-D are drawn to scale.

with the IS*1*-15 fragment. Complementary results were seen using sequences adjacent to IS*1*-15 and IS*1*-6 as probes. These results imply that an inversion between ancestral elements IS*1*-15 and IS*1*-6 produced the new elements IS*1*-A and IS*1*-C. This inversion was confirmed by sequencing DNA adjacent to all four fragments, which also identified the endpoints of the inversion (Figure 2). One endpoint, corresponding to IS*1*-6 in the ancestor, was located in the *gatZ* gene at 46.9 min on the K-12 chromosome. The other inversion endpoint, corresponding to IS*1*-15, was in an intergenic region between *citCD* and *dpiBA* at 14.1 min. It is unlikely that this inversion would directly affect the expression of the genes at either endpoint.

Deletion associated with IS*1* in Ara − 1: Figure 3 summarizes information used to identify this mutation. A 1.7-kb *Eco*RV fragment containing IS*1*-B was detected in all 34 clones from generation 5000 onward (Figure 3B), but was absent from the ancestor and all 53 earlier clones. The two sequences adjacent to IS*1*-B were used to probe reference membranes of the ancestor and early clones. The right-adjacent sequence hybridized a 3.2-kb *Eco*RV fragment that carried the ancestral IS*1*-7 element, which disappeared from later clones, and the left-adjacent sequence hybridized with a 1.8-kb *Eco*RV fragment that did not contain any IS*1* in the ancestor and early clones. These results suggested a rearrangement that might be associated with the IS*1* element.

Sequencing on both sides of IS*1*-7 showed that this ancestral element was in *nmpC*, which encodes an outer membrane porin carried by a defective prophage; this gene is located at 12.4 min on the K-12 chromosome. This insertion was previously reported for another *E. coli* B derivative and leads to inactivation of *nmpC* (PRILIPOV *et al.* 1998). Sequencing around IS*1*-B revealed the same right-adjacent sequence as for IS*1*-7, *i.e.*, the 3′ end of *nmpC*. However, the left-adjacent sequence of IS*1*-B did not correspond to the 5′ end of this gene; in fact, it did not show homology with any sequence in the databases. The inverse PCR was repeated twice to clone and sequence the genes adjacent to IS*1*-B, with identical results. This left-adjacent sequence was used to probe *Eco*RV-digested genomic DNA of the *E. coli* B ancestor and various K-12 strains; hybridization occurred only with the ancestor, indicating a B-specific sequence. This novel sequence, whose function (if any) is unknown, was deposited in GenBank (accession no. AJ271006).

Replicative transposition of IS*1* often generates inversion or deletion of the sequence between IS*1* and its target (TURLAN and CHANDLER 1995); such an event might have produced IS*1*-B, with the target being the *E. coli* B-specific sequence. If the mutation were an inversion, then an additional IS*1*-containing fragment would have been detected. However, apart from the fragments already accounted for, no new IS*1*-containing fragment was seen, indicating a deletion event. An alternative explanation, which we cannot exclude, is that transposition and deletion events occurred in different generations. In either case, given the orientation of IS*1* elements in *nmpC* (IS*1*-B; Figure 3) and near *citC* (IS*1*-15 and IS*1*-C; Figure 2), the B-specific sequence must lie between 12.4 and 14.1 min. Moreover, three different IS elements located together at 13.1 min in the ancestor were all still present in evolved clones with the deletion. Therefore, the maximum extent of the deletion is from 12.4 to 13.1 min.

Insertion of IS*150* into *nadR* and a subsequent inversion involving that element in Ara + 1: Figure 4 shows the genetic features used to identify two mutations that were fixed in Ara + 1, the first being a simple insertion and the second a chromosomal inversion involving that new insertion. A 2.5-kb *Eco*RV fragment containing

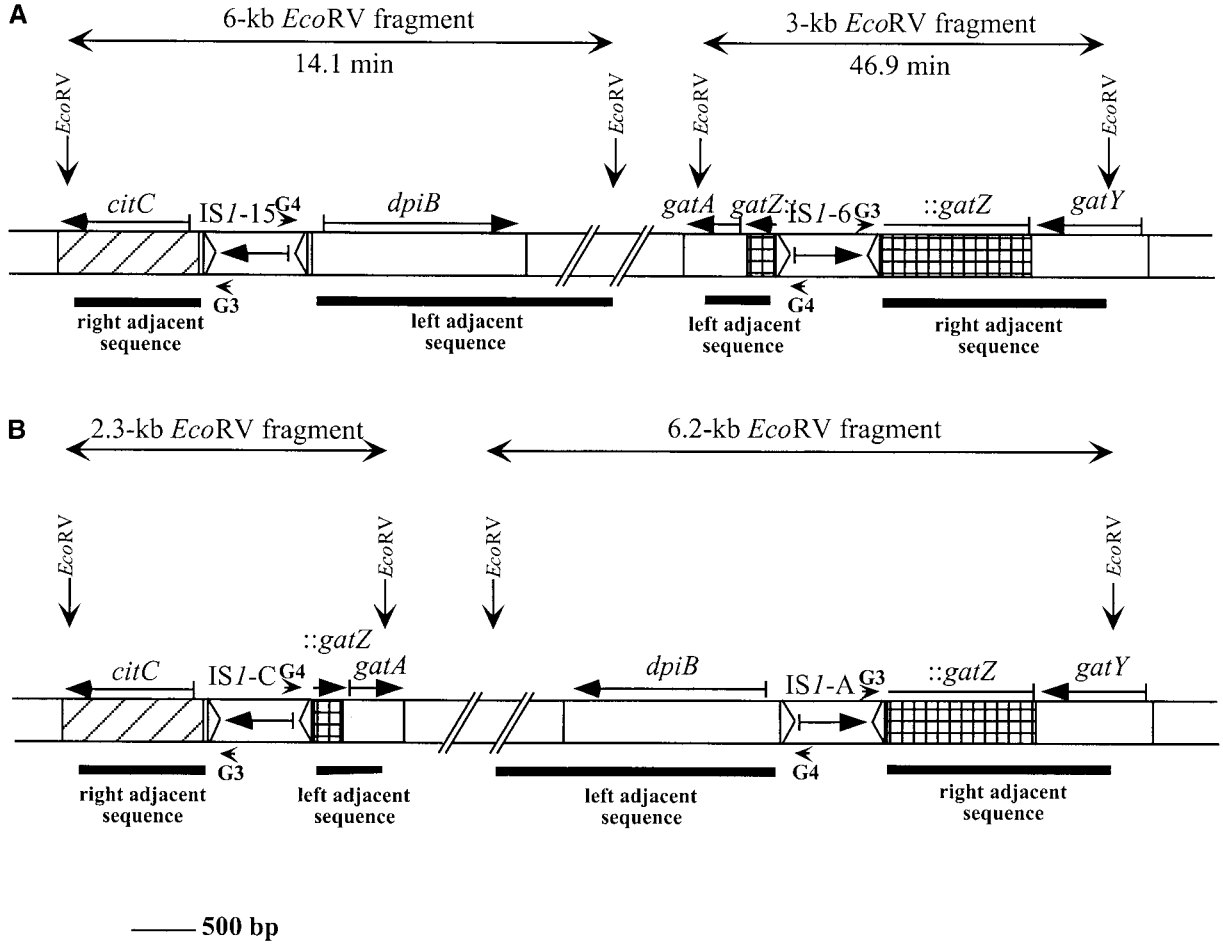

FIGURE 2.—Inversion between two IS*1* elements in population Ara − 1. Two new fragments, designated IS*1*-A and IS*1*-C, resulted from homologous recombination between two preexisting elements, IS*1*-6 and IS*1*-15; this event should lead to the inversion of the intervening region. Arrows indicate the directions of transcription of genes. (A) The chromosomal arrangement in the ancestor and clones isolated through generation 2000. (B) The rearrangement in clones isolated from generation 5000 onward. The adjacent sequences of the IS*1* elements are indicated; they were cloned from *Eco*RV-cut DNA, following self-ligation and inverse PCR using primers G3 and G4 (arrowheads). Only genes and IS*1* elements are drawn to scale, and only relevant restriction sites are shown. Open triangles represent IS*1* terminal inverted repeats. *citC*, similar to citrate lyase ligase gene in *Klebsiella pneumoniae* (BOTT and DIMROTH 1994); *dpiB*, gene encoding putative histidine kinase of a two-component signal transduction system involved in plasmid inheritance (INGMER *et al.* 1998); *gatY*, D-tagatose-1,6-bisphosphate aldolase gene involved in galactitol utilization; *gatZ*, gene of unknown function involved in galactitol utilization; *gatA*, gene encoding galactitol-specific enzyme IIA of phosphotransferase system (NOBELMANN and LENGELER 1996).

IS*150*-E was detected in 5 of 33 clones from generations 1000 and 1500, and in 40 of 41 clones from generation 2000 onward. The left-adjacent sequence of IS*150*-E was used to probe reference membranes and revealed a 3-kb *Hin*cII fragment and a 4.9-kb *Pvu*II fragment in the ancestor. In evolved clones from generations 1000 and 1500 that carried IS*150*-E, we saw a 4.5-kb *Hin*cII fragment and a 6.4-kb *Pvu*II fragment (Figure 4A), in accord with a new insertion of IS*150*. However, in clones from generation 2000 onward, the same probe hybridized with a 5.3-kb *Hin*cII fragment and a 5.5-kb *Pvu*II fragment (Figure 4B), indicating a further change. Both fragments from later clones contained IS*150*, which suggested a rearrangement involving the new element.

The 4.5-kb *Hin*cII fragment containing IS*150*-E prior to the putative rearrangement was used to clone the adjacent regions, and sequencing showed that IS*150*-E had inserted in the *nadR* gene, 168 bp downstream of its start codon (Figure 4A); the insertion also produced a 4-bp duplication of the target site. The *nadR* gene encodes a repressor of the nicotinamide adenine dinucleotide (NAD) biosynthesis genes (ZHU *et al.* 1991) and is located at 99.7 min in K-12. The IS*150*-E insertion almost certainly inactivated *nadR*. The 5.3-kb *Hin*cII fragment from later clones was used to characterize the subsequent rearrangement; the left-adjacent sequence of IS*150* was unchanged and corresponded to the 5′ end of *nadR*. However, the right-adjacent sequence was homologous to the *hokX-sokX* locus downstream of *cysJIH* at 62.2 min (PEDERSEN and GERDES 1999), where IS*150*-1 was inserted in the ancestor. IS*150*-E in *nadR* and IS*150*-1 in *hokX-sokX* have opposite orientation,

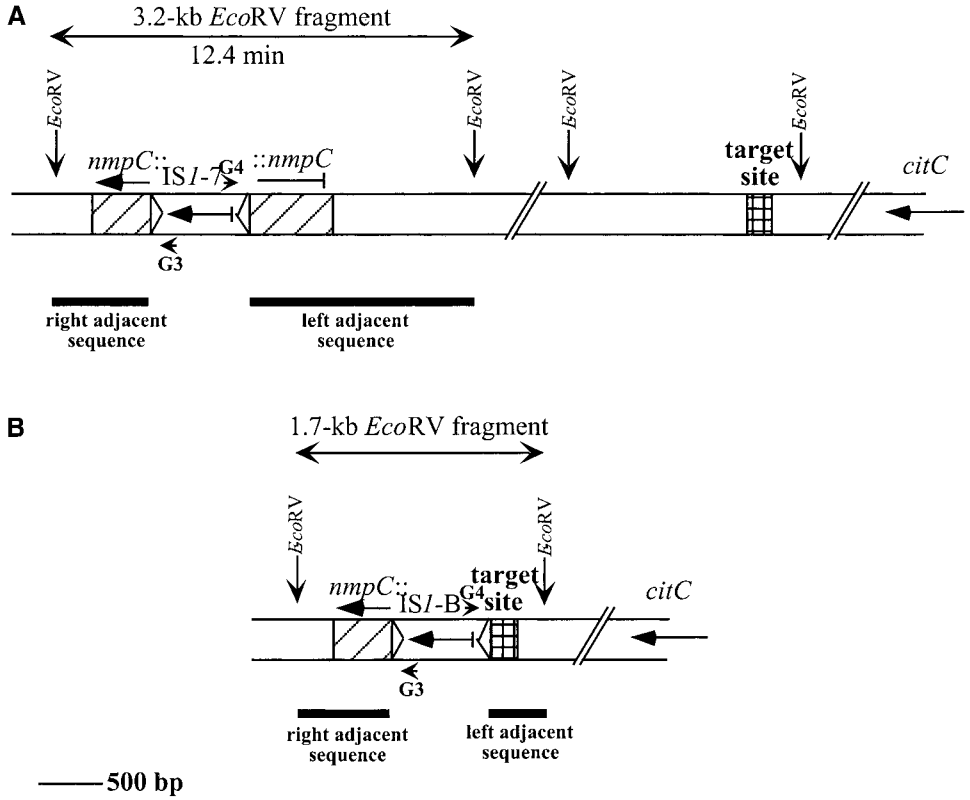

FIGURE 3.—Deletion associated with an IS*1* element in population Ara − 1. This mutation suggests a replicative transposition and associated recombination event, leading to deletion of the region between IS*1*-7 at 12.4 min and the target site (<0.7 min away). An alternative explanation is that the transposition preceded the deletion. Transcriptional directions are shown by arrows. (A) The arrangement seen in the ancestor and clones isolated through generation 2000. (B) The rearrangement in clones isolated from generation 5000 onward. Left- and right-adjacent sequences were cloned from *Eco*RV fragments, following self-ligation and inverse PCR with primers G3 and G4 (arrowheads). The target site of the transposition is shown as a cross-hatched box; it is a novel *E. coli* B-specific sequence. Only genes and IS*1* elements are drawn to scale. The precise distance between *nmpC* and the target site is unknown, but the persistence of other IS elements indicates that the target site is not beyond 13.1 min. Open triangles show IS*1* terminal inverted repeats. *citC*, similar to citrate lyase ligase gene (BOTT and DIMROTH 1994); *nmpC*, inactivated gene whose inferred product was a porin (PRILIPOV *et al.* 1998).

which suggested that recombination between them caused an inversion of the intervening sequence (Figure 4B). Amplification by PCR using primers at the 3′ end of *cysH* and overlapping the *Hin*cII site within *nadR* confirmed the inversion. Both *nadR* and *hokX-sokX* remained disrupted after this event.

Insertion of IS*150* upstream of *pbpA-rodA* in Ara + 1: Figure 5A shows the essential features of this mutation. A 2.7-kb *Eco*RV fragment containing IS*150*-F was seen in all 41 clones, except 1, from generation 2000 onward. Its left-adjacent sequence was used to probe reference membranes. In the ancestor, the probes gave *Hin*cII and *Pvu*II fragments of 1.7 kb and 4.3 kb, respectively; in clones containing IS*150*-F, both fragments were 1.4 kb larger (the size of IS*150*), which indicated an insertion. Sequencing the adjacent regions showed that IS*150*-E had inserted only 11 bp upstream of the putative promoter of an operon that contains *pbpA* and *rodA* (ASOH *et al.* 1986), located at 14.4 min; the insertion produced a 4-bp duplication of the target site. The *pbpA* gene encodes penicillin-binding protein 2, while *rodA* is involved in determining cell shape; the operon also contains two small open reading frames (ORFs) of unknown function (Figure 5A). Given its location so near the promoter, IS*150*-F is likely to influence the regulation or expression of this operon.

Insertion of IS*150* in *hokB-sokB* in Ara + 1: The important features of this mutation are summarized in Figure 5B. A 4-kb *Eco*RV fragment containing IS*150*-G was present in 5 of 33 clones sampled at generations 1000 and 1500, and in all except 1 of 41 clones sampled later. Using the left-adjacent sequence of IS*150*-G as a probe on reference membranes, we saw a 3.4-kb *Hin*cII fragment in the ancestor and a 4.9-kb *Hin*cII fragment in clones carrying IS*150*-G, indicating an insertion. Sequencing adjacent regions revealed that the insertion site was within the *hokB-sokB* locus (PEDERSEN and GERDES 1999) at 32.1 min, whose genes are homologous to plasmid-stabilization systems that operate by postsegregational killing. The IS*150* interrupts the stable mRNA and presumably prevents expression of the toxin. Five homologous loci are present in *E. coli* K-12 and a sixth was found in *E. coli* B (PEDERSEN and GERDES 1999). Only one, *hokB-sokB*, appears to be active in K-12, and that one was disrupted by this mutation in B.

Insertion of IS*150* in *yfcU* in Ara + 1: An *Eco*RV fragment carrying IS*150*-H was present in 5 of 7 clones from generation 5000 and in all 24 clones sampled later, but it was absent from the ancestor and all 51 clones sampled earlier. Sequencing around IS*150*-H indicated a new insertion in *yfcU*, an ORF of unknown function located at 52.9 min in K-12. The insertion probably inactivated this gene.

Insertion of IS*150* between *glcB* and *yghK* in Ara + 1: IS*150*-I was present in the same clones from generation 5000 onward that carried IS*150*-H. The sequences adjacent to IS*150*-I showed that this new element was inserted in the intergenic region between *glcB*, which en-

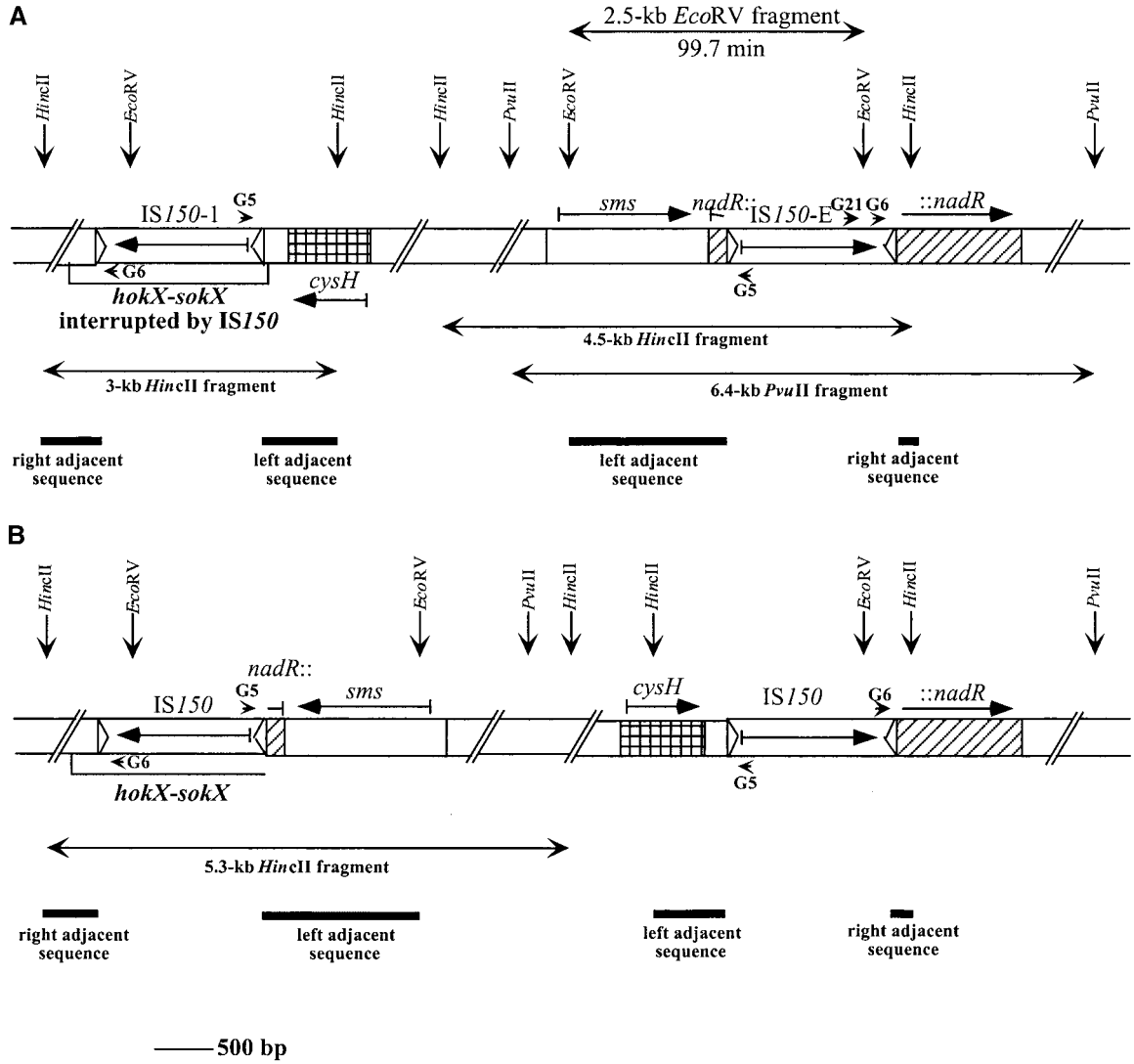

FIGURE 4.—Insertion of an IS*150* element into *nadR* in population Ara + 1, and a subsequent inversion involving that new element. The *nadR* gene encodes the repressor of NAD synthetic genes and is located at 99.7 min; this new IS*150*-E later undergoes homologous recombination with IS*150*-1, located at 62.2 min, producing an inversion. Arrows indicate directions of transcription. (A) The chromosomal arrangement in some clones at generations 1000 and 1500. The ancestral IS*150*-1 disrupts the *hokX-sokX* locus. The IS*150*-E insertion in *nadR* was detected in generation 1000. The 2.5-kb *Eco*RV fragment allowed cloning of the IS*150*-E left-adjacent sequence by inverse PCR using primers G5 and G21 (arrowheads). This left-adjacent sequence was used as a probe to identify the 6.4-kb *Pvu*II and 4.5-kb *Hinc*II fragments; the latter allowed cloning of the right-adjacent sequence using primers G5 and G6. The IS*150*-1 adjacent sequences were cloned from the 3-kb *Hinc*II fragment by inverse PCR using primers G5 and G6. (B) Rearrangement seen in clones isolated from generation 2000 onward. The 5.3-kb *Hinc*II fragment allowed cloning of IS*150* adjacent sequences by inverse PCR using primers G5 and G6. PCR amplification using a primer at the 3′ end of *cysH* and another overlapping the *Hinc*II site in *nadR* confirmed this rearrangement. Open triangles show IS*150* terminal inverted repeats. Only genes and IS*150* elements are drawn to scale. *cysH*, adenylylsulfate reductase gene; *hokX-sokX*, homologous to plasmid stabilization systems (PEDERSEN and GERDES 1999); *sms* (*radA*), gene involved in radiation sensitivity.

codes malate synthase G, and an uncharacterized ORF, *yghK*, at 67.2 min. Sitting 39 bp downstream of *glcB* and 387 bp upstream of *yghK*, IS*150*-I seems unlikely to affect expression of these genes.

DISCUSSION

LENSKI and TRAVISANO (1994) demonstrated that evolving populations of *E. coli* greatly improved their performance and had significant changes in their cell morphology during 10,000 generations in a glucose minimal medium. By performing RFLP analyses with IS elements as probes, PAPADOPOULOS *et al.* (1999) showed that these populations also underwent substantial genomic evolution. In each of two focal populations, they observed several mutations that were shared by all the descendants in a population, as well as many other mutations that were not fixed. They noted that the mutations that were fixed were the best candidates for having beneficial effects. PAPADOPOULOS *et al.* (1999) also suggested,

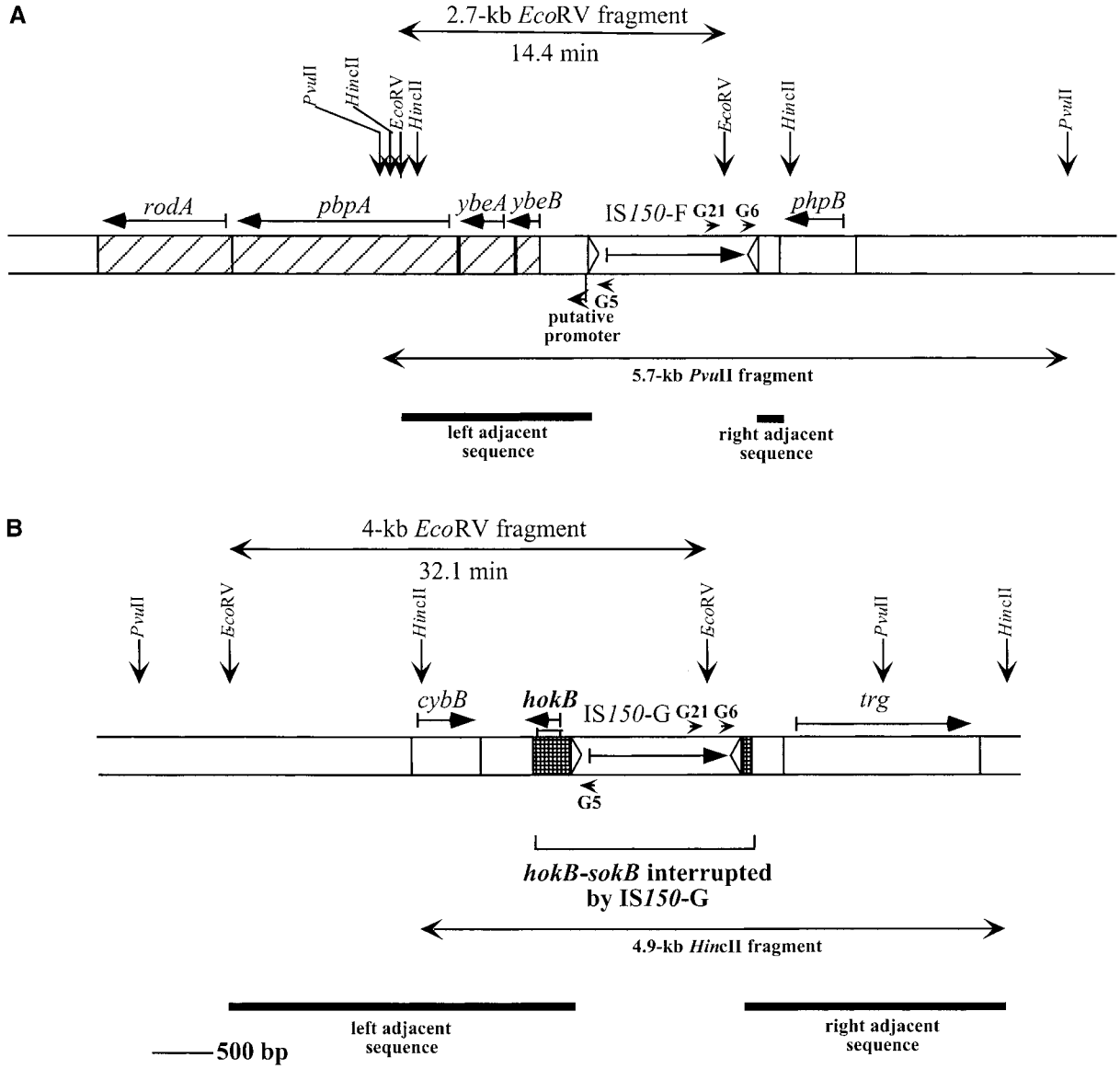

FIGURE 5.—Two insertions of IS*150* elements, upstream of *pbpA-rodA* and into *hokB-sokB*, in population Ara + 1. (A) IS*150*-F is a new insertion upstream of the *pbpA-rodA* operon at 14.4 min. The bent arrow indicates the putative promoter (ASOH *et al.* 1986). The 2.7-kb *Eco*RV fragment allowed cloning of the left-adjacent sequence by inverse PCR using primers G5 and G21 (arrowheads). This sequence was used as a probe to identify the 5.7-kb *Pvu*II fragment, which allowed cloning of the right-adjacent sequence using primers G5 and G6. *pbpA*, penicillin-binding protein 2 gene; *phpB*, gene for putative phosphohistidine protein; *rodA*, gene involved in determining cell shape; *ybeA* and *ybeB*, genes of unknown function. (B) IS*150*-G is a new insertion in the *hokB-sokB* locus near 32.1 min. The 4-kb *Eco*RV fragment allowed cloning of the left-adjacent sequence using primers G5 and G21. The left-adjacent sequence was used as a probe to identify the 4.9-kb *Hinc*II fragment, which allowed cloning of the right-adjacent sequence with primers G5 and G6. The disrupted *hokB-sokB* locus is shown by cross-hatching. *cybB*, cytochrome b561 gene; *hokB-sokB*, homologous to plasmid stabilization systems (PEDERSEN and GERDES 1999); *trg*, encodes protein III used in chemotaxis to ribose and galactose. Open triangles are IS*150* terminal inverted repeats. Only genes and IS*150* elements are drawn to scale.

based on indirect evidence, that the genomic changes they detected were caused by IS transpositions or rearrangements, as opposed to mutations in restriction sites. However, they did not characterize the underlying molecular events.

In this study, we identified the precise genetic bases of several mutations that were fixed in each focal population (Table 1). We showed that all these mutations arose either by transposition or homologous recombination between IS elements. The nine mutations characterized in this study involved different genes and were unique to one population or the other. However, COOPER (2000) found parallel losses of ribose catabolic function, which were used to identify similar but not identical IS*150*-mediated deletions of the *rbs* operon in all 12 replicate populations. Moreover, because the deletions produced

a phenotype that could be readily scored, Cooper (2000) isolated several spontaneous mutants (without resorting to genetic reconstruction) and then assessed their effects on fitness in the glucose medium. All of these IS-mediated *rbs* deletion mutations were beneficial, with selection coefficients of ~1.4%.

At present, it is unclear whether any of the nine mutations that we have identified gave a selective advantage in their respective populations. According to one hypothesis, IS elements are genomic parasites that persist owing to high transposition rates, especially when there is sufficient horizontal transfer to offset their harmful effects (Charlesworth *et al.* 1994). Alternatively, enough IS insertions have beneficial effects to offset their harmful effects, such that they persist by their overall positive contribution to bacterial fitness (Blot 1994). To address this issue, it will be necessary to construct genotypes with and without each of these mutations, but otherwise isogenic, and then perform competition experiments to test whether a mutation is beneficial or not.

Insertion events: Only one of the mutations characterized in population Ara − 1 was a simple transposition (Figure 1), and this was first detected at generation 2000. In particular, IS*150*-D inserted into the middle of the *pykF* gene, which encodes pyruvate kinase I, one of two glycolytic isoenzymes that catalyze the conversion of phosphoenolpyruvate (PEP) and ADP into pyruvate and ATP. Ponce *et al.* (1995) reported that a mutation in *pykF* impaired growth on glucose, albeit in a different strain and other conditions. This finding certainly suggests that the *pykF*::IS*150* mutation that was fixed in Ara − 1 will prove to be deleterious, although there are also physiological grounds for proposing that this mutation may be advantageous. In particular, PEP is the proximate source of phosphate for enzyme I in the phosphotransferase system (PTS), by which glucose and certain other substrates are transported across the inner membrane of *E. coli* (Saier and Reizer 1992). Working with the long-term lines, Travisano and Lenski (1996) presented evidence from competitions performed on various substrates that the PTS was an important target of selection. Viewed in this light, the *pykF*::IS*150* insertion may provide a benefit by slowing the conversion of PEP to pyruvate, such that more PEP is available to drive the PTS. We emphasize that this putative benefit is a hypothesis that will require testing, as discussed above.

Five of the six mutations characterized in Ara + 1 were new IS*150* insertions. Three of these were nearly fixed by generation 2000, whereas the others were polymorphic at generation 5000 and became fixed only later (Table 1). These late insertions are less promising candidates for having beneficial effects because they arose when fitness improvement was much slower and because they were associated with the burst of IS*150* hyperactivity in this population. Also, neither one suggests a particularly compelling basis for having a beneficial effect: one inserted into an ORF of unknown function and the other into an intergenic region that seems unlikely to affect gene expression. Nonetheless, it should be possible to measure their fitness effects using isogenic constructs, as discussed above.

The first three insertions in Ara + 1 are much more promising from the standpoint of adaptive evolution because they were substituted early and, moreover, they suggest physiological effects that might be beneficial. These insertions were IS*150*-E into *nadR* (Figure 4); IS*150*-G in the *hokB-sokB* locus (Figure 5B); and IS*150*-F near the promoter of the *pbpA-rodA* operon (Figure 5A). The *nadR* gene encodes the repressor of NAD biosynthetic genes (Zhu *et al.* 1991); *hokB-sokB* is homologous to a plasmid stabilization system (Pedersen and Gerdes 1999); and *pbpA-rodA* encodes proteins controlling cell growth and shape (Matsuzawa *et al.* 1989; Vinella *et al.* 1992; Begg and Donachie 1998).

NAD is involved in nearly all of the metabolic pathways within the cell. Two genes, *nadA* and *nadB*, of the biosynthetic pathway and one, *pncB*, of a recycling pathway are under negative transcriptional control by the product of *nadR*; disruption of *nadR* should therefore cause their constitutive expression and increase the intracellular level of NAD (Foster *et al.* 1990; Penfound and Foster 1996). Besides its regulatory role, NadR is a bifunctional protein involved in transport of nicotinamide mononucleotide (NMN) across the cell membrane. The regulatory activities of NadR depend on cell physiology and particularly on levels of NAD (Foster *et al.* 1990; Zhu *et al.* 1991; Penfound and Foster 1999). The NadR protein switches between a form that induces NMN transport (but does not repress the biosynthetic genes) at low NAD levels and a form that represses the biosynthetic genes (but does not induce NMN uptake) at high NAD levels. The new IS*150*-E lies at the 5′ end of the gene (Figure 4), which encodes the protein's N terminus responsible for its repressor function (Foster and Penfound 1993). Expression of the NadR domain involved in transport and located in the protein's C terminus might still occur if the IS*150* element provides the necessary transcription and translation signals. Whether the *nadR*::IS*150* mutation is beneficial or harmful to *E. coli* under the conditions of the experimental evolution is difficult to say, but it seems unlikely that such a mutation would be neutral, given the central role of NAD in metabolism. The evolved lines have higher maximum growth rates than their ancestor (Vasi *et al.* 1994), and these fast-growing cells may need more NAD than do cells growing slowly, suggesting a possible advantage for this mutation. The evolving populations also spend >16 hr each day in stationary phase, but have shorter lag times upon renewal of the medium (Vasi *et al.* 1994). High $NAD^+/NADP^+$ levels may promote repair of oxidized proteins in starving cells (Antelmann *et al.* 1997), which may give an advantage when growth resumes.

Another mutation fixed early in population Ara + 1

was the insertion of IS*150*-G in the *hokB-sokB* locus. The *hok-sok* locus of plasmid R1 is responsible for killing plasmid-free segregants, a function that seems more beneficial to a plasmid than to its bacterial host. The *hok* gene encodes a toxin, and *sok* encodes an antisense RNA that prevents translation of the toxin. Five *hok-sok* loci are present in *E. coli* K-12 and four—all except the *hokB-sokB* locus—have been functionally inactivated, in most cases by IS elements (Pedersen and Gerdes 1999). Repeated inactivation of these loci suggests that their function is deleterious to the host, at least in the absence of plasmids. If so, then the IS*150*-G mutation would have been beneficial under the plasmid-free conditions of the evolution experiment. Alternatively, this mutation may simply indicate a preferential insertion site because only IS*150* and IS*186* are found in the *hok-sok* loci (Pedersen and Gerdes 1999).

Another early substitution in Ara + 1 was the IS*150*-F insertion only 11 bp from the putative promoter of the *pbpA-rodA* operon (Asoh *et al.* 1986), which encodes two essential cell cycle proteins (Begg and Donachie 1998). The *pbpA* gene encodes penicillin-binding protein 2, which is involved in peptidoglycan synthesis and elongation of the cell wall; the characteristic rod shape of *E. coli* cells also depends on the *rodA* gene (Matsuzawa *et al.* 1989). Peptidoglycan is a dynamic structure that shows a balance between hydrolysis and synthesis (Pisabarro *et al.* 1985; Tuomanen and Cozens 1987; Huisman *et al.* 1996), and the insertion in the regulatory region of the *pbpA-rodA* operon may alter this balance. It is interesting that the evolving populations studied here underwent large increases in cell volume and commensurate increases in surface area (Lenski and Mongold 2000), which may require more peptidoglycan synthesis. It is also noteworthy that a non-IS-related mutation in the upstream regulatory region of another operon involved in peptidoglycan synthesis, *glmUS*, was fixed early in the evolution of the Ara − 1 population (M. Stanek, personal communication). These findings suggest that the populations may have achieved parallel physiological adaptations by different genetic solutions, which can be tested by reconstructing the relevant genotypes and testing their fitness effects.

IS-mediated rearrangements: During 10,000 generations, each focal population underwent two or more chromosomal rearrangements that were mediated by IS elements (Table 1). These rearrangements included a very large inversion in each population that involved different IS elements and affected different regions of the chromosome; parallel deletions of the *rbs* operon discovered by Cooper (2000); and another deletion present only in Ara − 1. The parallel deletions of *rbs* involved an IS*150* element located immediately upstream of this operon, and they were substituted in the first 500 generations in both focal populations. The deletions appear to have involved transposition of a new IS*150* element into (or downstream of) the operon coupled with homologous recombination between the upstream and new elements, leading to deletion of the intervening region. As noted above, these *rbs* deletions were shown to be beneficial (Cooper 2000). The additional deletion in Ara − 1 may have occurred by a similar mechanism, but with IS*1*; it was not seen until generation 5000. In this case, there was evidently recombination between an ancestral IS*1* element in *nmpC* at 12.4 min and a new copy in a sequence specific to *E. coli* B (Figure 3). Alternatively, the transposition and deletion events might have occurred at different times. In either case, the net result was deletion of the region between the IS*1* element at 12.4 min and the nearby B-specific sequence. The precise location of this B-specific sequence is unknown, but it must lie between IS*1*-7 at 12.4 min and three different IS elements located at 13.1 min that were still present in evolved clones carrying the deletion. It may be possible to test the effect of this deletion on fitness by constructing an ancestral strain that carries the deletion.

The inversion in Ara − 1 was generated by homologous recombination between two IS*1*'s, one located in *gatZ* at 14.1 min and the other between *citC* and *dpiB* at 46.9 min (Figure 2). The inversion was not detected at generation 2000 but it had become fixed by generation 5000. In Ara + 1, the inversion occurred between two IS*150* elements, one in *hokX-sokX* at 62.2 min and the other in the *nadR* gene at 99.7 min (Figure 4). The latter IS*150* element was a new insertion first seen in several clones at generation 1000; the inversion was first detected at generation 2000 and was subsequently fixed in the population. Both inversions involve about one-third of the chromosome, based on the K-12 map. In terms of potential fitness effects, some inversions affect expression of genes at their boundaries, but this seems unlikely here because both involved IS elements already there when the inversions occurred. Inversions may change the direction of transcription relative to replication of genes within the inverted region, and they may alter the effective copy number of genes during growth. With one-third of the chromosome involved in each inversion, these effects could produce subtle but widespread differences in gene expression.

Large-scale inversions have been previously reported in *E. coli* (Hill and Harnish 1981; Louarn *et al.* 1985; Xia and Enomoto 1986) and other bacteria (Liu and Sanderson 1995; Gibbs and Meyer 1996; Itaya 1997; Römling *et al.* 1997; Daveran-Mingot *et al.* 1998). In all cases, inversions occurred between inverse-ordered repeated sequences, such as IS elements and *rrn* operons. Some studies deliberately placed known sequences in inverse order at various locations to generate inversions and observe their effects (Segall *et al.* 1988; Miesel *et al.* 1994). In most cases, growth properties of the inversion strains were indistinguishable from their parent, but some inversions were detrimental (Hill and Gray 1988; Rebollo *et al.* 1988; Segall *et al.* 1988). Roth

et al. (1996) hypothesized that selection maintains symmetry between the replication origin and the termination sequences located roughly opposite on the chromosome. The observation that only inversions maintaining this symmetry are found in natural populations is consistent with this view, as is the conservation of gene order between *E. coli* and Salmonella. In *E. coli* K-12, the replication origin, *oriC*, is located at 84.5 min, while *terC* at 34.6 min is the first termination site to impede clockwise replication and *terA* at 28.9 min is the first to do so for the counterclockwise fork (Hill 1996), after 50.1 and 55.6 min, respectively. The inversion endpoints in the evolved population Ara − 1 are at 14.1 and 46.9 min, and those in Ara + 1 are located at 62.2 and 99.7 min. In Ara + 1, *oriC* is moved counterclockwise by 7.1 min, such that clockwise and counterclockwise replication should be impeded after 57.2 and 48.5 min, respectively. In Ara − 1, the termination loci have been moved and flipped in their orientation, such that *terA* should impede clockwise replication after 47.6 min, while *terC* should impede the counterclockwise fork after 58.1 min. (These distances are based on the map of K-12, and they may be somewhat longer or shorter depending on any insertions or deletions in B *vs.* K-12.) Thus, in both cases, asymmetry seems to have increased slightly relative to the ancestral state, which suggests that the inversions may be deleterious. If so, they were presumably fixed by hitchhiking with a beneficial mutation that occurred in the same background. However, owing to the problem of constructing otherwise isogenic strains with and without large inversions, it will be difficult to test their effects.

In conclusion, the work reported here has identified a number of IS-mediated mutations that became fixed during long-term evolution experiments with populations of *E. coli*. Some of the affected genes can now be directly manipulated to determine whether or not they were targets of natural selection in the experimental environment.

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