Insertion Sequence-Related Genetic Variation in Resting *Escherichia coli* K-12

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ABSTRACT

Bacterial subclones recovered from an old stab culture of Escherichia coli K-12 revealed a high degree of genetic diversity, which occurred in spite of a very reduced rate of propagation during storage. This conclusion is based on a pronounced restriction fragment length polymorphism (RFLP) detected upon hybridization with internal fragments of eight resident insertion sequences (IS). Genetic diversity was dependent on the IS considered and, in many cases, a clear consequence of IS transposition. IS*5* was particularly active in the generation of variation. All subclones in which IS*30* had been active testify to a burst of IS*30* transposition. This was correlated with a loss of prototrophy and a reduced growth on rich media. A pedigree of the entire clone could be drawn from the RFLP patterns of the subclones. Out of 118 subclones analyzed, 68 different patterns were found but the putative ancestral population had disappeared. A few patterns were each represented by several subclones displaying improved fitness. These results offer insights into the role of IS elements in the plasticity of the *E. coli* genome, and they further document that enzyme-mediated DNA rearrangements do occur in resting bacterial cultures.

STAB cultures kept in tightly sealed glass tubes at room temperature offer a simple, convenient method of storage of bacterial strains for very long periods. However, it is well known, to most scientists using stabs, that these do not guarantee genetic stability of the stored bacterial strains. This is somewhat surprising since after the short period of exponential growth following inoculation, one expects no further growth–or at most only a very slow residual growth–to occur during storage. Transposition of residential mobile genetic elements is a most common cause of mutation in bacteria (RODRIGUEZ *et al.* 1992). It was postulated that under the steady conditions in stab cultures, transposition of insertion sequence (IS) elements might contribute significantly to genetic instability. This was based on the observations (a) that IS-mediated transposition does not require chromosomal replication [see GALAS and CHANDLER (1989) for transposition mechanisms]; (b) that lethal mutations accumulated in the P1 prophage in stabs were often related to IS*1* transposition (ARBER *et al.* 1978); (c) that the copy number of IS*5* in *Escherichia coli* increases upon prolonged storage in stabs (GREEN *et al.* 1984); (d) that the transposition frequency of some mobile elements seems to increase under starving conditions (HALL 1988; SHAPIRO and HIGGINS 1989; MITTLER and LENSKI 1990). While much is known on the DNA sequences of IS elements and on their molecular biology, less solid knowledge is available on mechanisms governing their persistence in populations [see AJIOKA and HARTL (1989) for a review] and on their effective evolutionary role [see CAMPBELL (1981) and HARTL *et al.* (1984) for discussion].

Eight different IS elements are identified as resident on the chromosome of *E. coli* K-12: IS*1* (OHTSUBO and OHTSUBO 1978); IS*2* (GHOSAL and SAEDLER 1979); IS*3* (TIMMERMAN and TU 1985); IS*4* (KLAER *et al.* 1981); IS*5* (ENGLER and BREE 1981); IS*30* (DALRYMPLE *et al.* 1984); IS*150* (SCHWARTZ *et al.* 1988), IS*186* (KOTHARY *et al.* 1985; SENGSTAG *et al.* 1986). Several transposable elements, most often transposons, have been also introduced into *E. coli* strains as tools for mutagenesis but were not involved in our study. The total length of all known IS copies in *E. coli* K-12 is about 50 kb (*ca.* 1% of the chromosome), and their distribution on the chromosome is not fully at random, since some regions, *e.g.*, near the replication origin, are free of elements (BIRKENBIHL and VIELMETTER 1989). The copy number of studied IS elements and their distribution on the chromosome vary greatly among natural isolates of *E. coli* (BRAHMA *et al.* 1982; GREEN *et al.* 1984; HARTL and SAWYER 1988a,b; SAWYER and HARTL 1986). Nevertheless their locations could be mapped on the chromosome of the strain W3110 (BIRKENBIHL and VIELMETTER 1989, 1991; UMEDA and OHTSUBO 1989, 1990a,b, 1991).

In this report we compare the restriction fragment length polymorphism (RFLP) of subclones made from individual viable cells recovered from a stab culture of the *E. coli* K-12 strain W3110, which had been stored in our collection for the last 30 years. The hybridization probes were internal sequences of various IS elements resident on its chromosome. The *E. coli* strain W3110 is most likely the genetically best known strain for both its physical (KOHARA *et al.* 1987) and genetic (BACHMANN 1990) maps. We show here that this strain has suffered

intense genetic alterations during prolonged storage. Its residential IS elements, particularly IS*5* and IS*30*, but to a lesser extent also others, have caused an impressive genetic variance as revealed in the restriction patterns. Indeed, all tested subclones which were recovered from the stab differed from the input strain by several mutational steps, most of which must be transpositional and thus enzyme-mediated and this under conditions of seriously reduced metabolic activities. Environmental and evolutionary implications of these findings are discussed.

MATERIALS AND METHODS

Strain, storage and sampling: Strain W3110 is a derivative of *E. coli* K-12 (BACHMANN 1987). In the late 1950s a culture of W3110 came from the California Institute of Technology, Pasadena, to the University of Geneva, where it was used in experiments involving λgal transduction (ARBER 1958). In those days laboratory cultures for routine work were usually kept on slants stored in the refrigerator and renewed every few weeks. In the spring of 1960 a stab was prepared with a culture of this strain. Stabs are small glass vials of about 4-ml volume containing 2 ml of growth medium (nutrient broth, Difco, 8 g/liter; Bacto agar, Difco, 7.6 g/liter). The stab is inoculated by stabbing a loopful of the bacterial culture all the way down to the bottom of the solid medium. It is then covered with a cotton plug and incubated at 37° until bacterial growth is visible. Growth is most intense on top of the trace at the surface of the medium. The tube is then sealed with a paraffined cork. Depending on the quality of the cork, stabs kept at room temperature are protected from drying out, and bacteria keep their viability for many years. Our stabs are stored in a closed cupboard at a temperature of 20–25°.

The stab prepared in 1960 with W3110 was only occasionally opened in order to take a small sample of the culture and resealed immediately. At two occasions, in 1965 and in 1972 this stab culture was renewed as follows. The old stab was opened, an agar plug was taken from the surface area with a platine loop and introduced into a fresh stab. Thus the stab culture which we have now analyzed was from a third generation stab. It was taken from the collection in 1990, *i.e.*, after a total storage under stab conditions for 30 years. For this purpose an agar plug was removed from the stab, resuspended in Luria broth (LB) and aliquots were immediately plated on LB-agar. After overnight incubation, 118 colonies were randomly picked regardless of the colony size and grown overnight in 5 ml LB, from which 0.5 ml was stored in 15% glycerol at −70° for the record and 4.5 ml were analyzed (see *DNA preparations, blotting and hybridization*).

In growth experiments, a culture of W3110 used routinely in our laboratory served as reference strain. To estimate the variation that occurred during growth inside the stab culture before sealing, a single colony of this reference strain was inoculated in a stab culture tube. After 1 week of storage the offsprings were analyzed by the same method as for the elder stab subclones.

Prototrophy and growth of subclones: Prototrophy of the bacteria was tested on solid M9 minimal medium (SAMBROOK *et al.* 1989) containing 1 g/liter glucose. Kinetics of growth in nutrient broth, Difco, 8 g/liter, which corresponds to the medium in stabs, were determined by diluting an overnight culture 1:50, incubating at 37° for 4 h and measuring the optical density (600 nm) of aliquots at intervals. The growth rate of each subclone was compared to that of an arbitrarily chosen reference strain W3110 that is routinely used in our laboratory. Selection coefficients were calculated after a method originally designed for chemostat competitions (DYKHUIZEN and HARTL 1983), while in our experiments the two strains compared were grown in separate cultures.

DNA preparations, blotting and hybridization: Bacteria (4.5 ml) grown as described above were centrifuged and the pellet was resuspended in 0.7 ml TE-glucose (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose, pH 8.0) and 0.1 ml of a mixture containing lysozyme, proteinase K and RNase A to a final concentration of 5, 0.05 and 0.5 μg/ml, respectively. After 30 min of incubation at 37°, 0.1 ml SDS (20%) was added and incubation was pursued for another 30 min. DNA was sheared by passing twice through a syringe (0.8 × 40-mm needle), and extracted once with phenol, twice with phenol-chloroform and twice with chloroform. DNA was precipitated at −70° by adding 2.5 volumes of ethanol (100%) and 1/10 (v:v) of Na-acetate (3 M). After centrifugation, the DNA pellet was resuspended overnight in 0.2 ml TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 7.6). DNA concentration was determined by absorbance at 260 nm.

Genomic DNA (10 μg) was digested with *Eco*RV (GAT'ATC) for 6 hr at 37° and the resulting, about 10^3, different fragments were separated by electrophoresis on a 0.6% agarose gel in TBE buffer. DNA was then transferred on a nylon membrane (Pal, Biodyne A) by vacuum transfer (Pharmacia). The Southern blot hybridizations were performed following standard procedures and visualized on Fuji medical X-ray film. Internal fragments of eight IS elements having no *Eco*RV site were used as probes for successive hybridizations (Figure 1). An internal *Hpa*I-*Bgl*II 1.4-kb fragment of IS*50* and an internal 845-bp *Eco*RI fragment of γδ [which is also known as Tn*1000* (GUYER 1978)] were also used to verify the absence of these two elements from the strain W3110. Furthermore, a 1.1-kb *Bgl*I-*Ssp*I internal sequence of the *lacZ* gene derived from plasmid pNM480 (MINTON 1984) was used as a hybridization probe representing a unique chromosomal gene.

Probes were labeled with [α-^{32}P]dATP (Random Labeling Kit, Bio-Rad), and then purified through gel filtration (support G50, Pharmacia) in a 1-ml spun column. For deprobing, the radioactive membranes were washed three times in boiling distilled water for 15 min before a new hybridization with another probe was performed. All DNA methods were after (SAMBROOK *et al.* 1989).

Coding of the RFPL: Each specific fragment that hybridized to a particular IS probe under stringent conditions was scored as one copy of the IS element and its electrophoretic mobility was measured. For the coding, only the presence or the absence of a given band was considered, and not the relative intensities, even in a few cases where particular bands appeared with double intensities as compared to the rest. All of the 118 subclones were compared and all of the observed electrophoretic mobilities were used to define possible hybridization fragments. In individual patterns the scores were 1 or 0 depending on presence or absence, respectively, of a fragment. This resulted in a matrix (fragment × individuals) which was used for establishing the pedigree of the culture.

Pedigree of the culture: IS elements are relatively dynamic in the genome and therefore may serve as a useful, rapidly evolving genetic marker [see SAWYER and HARTL (1986) for discussion]. Since IS elements are inherited, the subclones that share a common ancestor should also tend to share sites at which IS copies are inserted in the chromosome. This property was used to calculate a most parsimonious tree describing the variation of hybridization patterns after prolonged storage, starting with a unique unknown pattern. The tree analysis was done by the procedure of FITCH (1971) which tries to find that tree that accounts for the descent of the sequences in the fewest number of changes. Gains and losses of fragments were

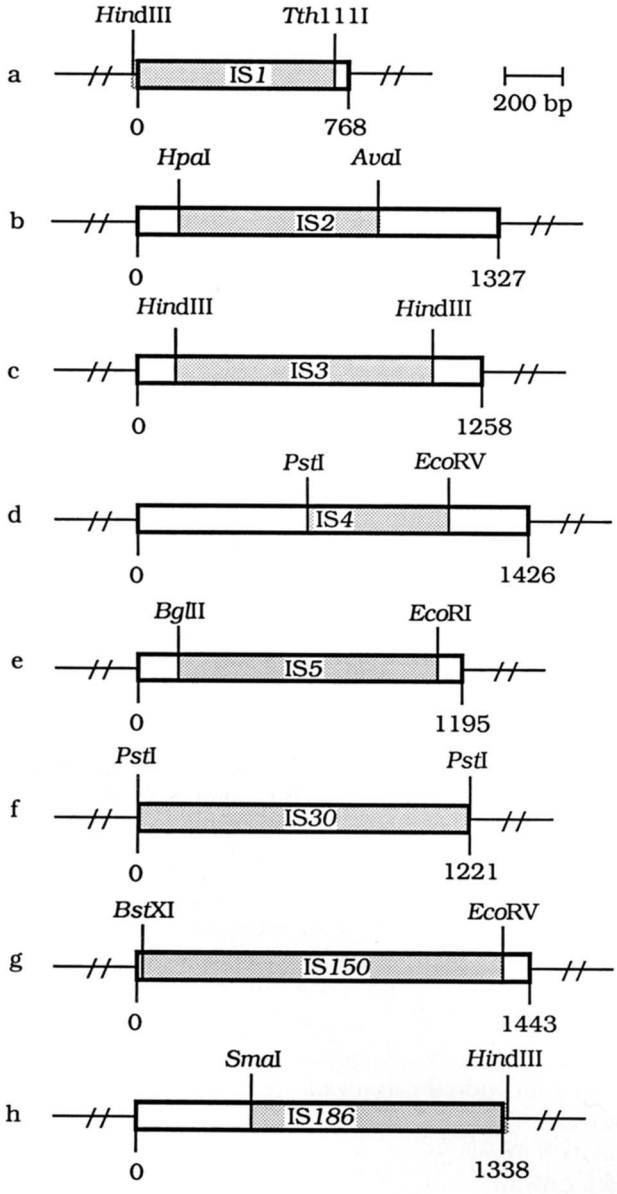

FIGURE 1.—Hybridization probes of IS elements. The restriction fragments of eight IS elements used as probes for hybridization to *E. coli* genomic DNA are shaded. Numbers indicate the nucleotide coordinates in the published sequence of the IS element (see MATERIALS AND METHODS for references). (a) 740-bp *Hin*dIII-*Tth*111I fragment of IS*1* from plasmid pAW929 (unpublished, our collection); (b) 730-bp *Ava*I-*Hpa*I fragment of IS*2* from plasmid pRAB2::IS*2* (RAABE *et al.* 1988); (c) 916-bp *Hin*dIII fragment of IS*3* from plasmid pRAB2::IS*3* (RAABE *et al.* 1988); (d) 525-bp *Pst*I-*Eco*RV fragment of IS*4* from plasmid pFDX934-K61 (unpublished, gift of B. RAK); (e) 940-bp *Bgl*II-*Eco*RI fragment of IS*5* from plasmid pRAB2::IS*5* (RAABE *et al.* 1988); (f) 1200-bp *Pst*I fragment of IS*30* from plasmid pAW304 (STALDER and ARBER 1989); (g) 1141-bp *Bst*XI-*Eco*RV fragment of IS*150* from plasmid pFDX450 (SCHWARTZ *et al.* 1988); (h) 906-bp *Hin*dIII-*Sma*I fragment of IS*186* including 12-bp vector DNA from the plasmid pAW40::IS*186* (SENGSTAG *et al.* 1986).

given equal weight. The program (ANCESTOR) gives strictly bifurcating trees but branches of zero length can be removed to give the polychotomies shown. Thus isolates 1, 13, 14, 25, 35 and 62 (see Figure 3) are all different from the same immediate ancestor (identical to isolate 10) by the change of a single but different site. There are 945 ways that these isolates might be related to each other in a strictly bifurcating tree, all of them equally parsimonious. Such multipliers from other polychotomies yield 1.8×10^{21} trees of the same length (174 changes) out of the 10^{71} possible pedigrees. The ambiguity implied by that large number is already contained within the tree by the unresolved branch order and thus, although there are many equally good trees, they are all subsumed under the tree shown.

RESULTS

Since IS elements are inherited, individual colonies that share a recent common ancestor should also tend to share most of the sites at which IS elements are present in the genome. These shared sites would give rise to restriction fragments of identical size hybridizing with the IS probe on Southern blots. A band shift for a particular individual may result from a point mutation in a restriction site, an insertion, an inversion, a duplication, a transposition or a deletion affecting a fragment. Increase of the copy number of an IS element may reflect a transposition of the IS element or a duplication of a DNA sequence containing the element.

Large RFLP in a stab culture: Of the colonies recovered from a 30 years old stab culture of strain W3110, 118 were analyzed individually. DNA fragments prepared from the chromosome with *Eco*RV were separated by electrophoresis and hybridized to probes from internal sequences of IS elements. Chromosomal DNA showed hybridization to eight probes of resident IS elements, but not to the IS*50* and γδ probes (only 60 individuals were tested to ensure that these two elements were absent from the strain). No polymorphism was detected after hybridization to an internal *lacZ* probe. Most individuals contained fewer than 15 chromosomal copies of a given IS so that direct counting of the number of hybridization bands on gels was reliable. Multiple digestion of the same genomic DNA from some of the studied subclones with other restriction enzymes confirmed that the counting of the IS copy number by this method was reliable (data not shown). In most cases, the intensities of hybridization were homogeneous for a given DNA extract indicating a 1:1 relation between a fragment and an IS copy. Three exceptions were found: (i) with IS*1*, five fragments showed strong signals and two fragments (9.8 and 1.9 kb) weaker signals that likely correspond to the isoforms IS*1*F and IS*1*R, respectively, described for strain W3110 (UMEDA and OHTSUBO 1991); (ii) with IS*30* a weak signal at 1.9 kb is due to a truncated copy of the element (UMEDA and OHTSUBO 1990a); and (iii) with IS*30* some subclones showed a fragment of 5.6-kb length with a double intensity. This fragment was counted as one IS element, although it carries a dimer of IS*30* (see below) which could have arisen by a single DNA rearrangement.

A striking polymorphism was displayed among the subclones, indicating that a high genetic diversity arose

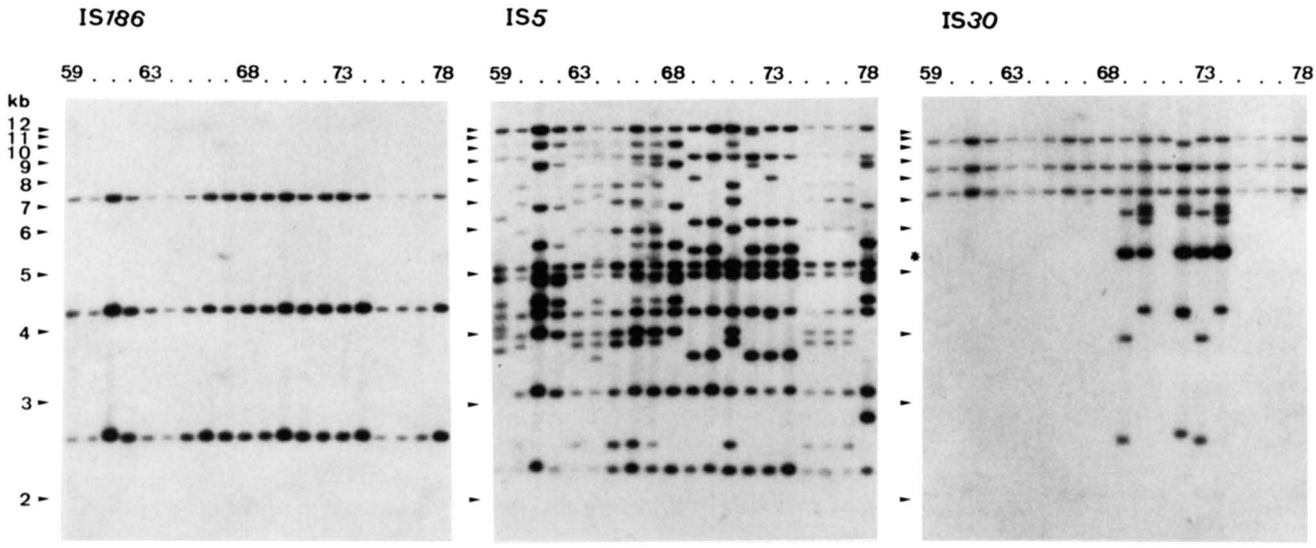

FIGURE 2.—Southern hybridization of *Eco*RV-digested genomic DNA from 20 subclones (representing individuals #59–78) to probes of IS*186*, IS*5* and IS*30*. No polymorphism was observed with IS*186* while the hybridization patterns of electrophoretic bands observed with IS*5* were highly variable. With the IS*30* probe, two types of patterns are noticed: one with four fragments (three strong and one very weak), and one with in addition several new bands including one (marked *) that has a double intensity according to the autoradiography scanning. kb gives the fragment size in kilobase (1-kb ladder, BRL).

in the studied strain. As illustrated in Figure 2, the contributions of the different IS probes to the revealed RFLP were unequal, suggesting an IS-dependent genetic variation. After coding, all data were compiled into a (fragments × individuals) matrix. Because of its size, this matrix is not shown here but it can be provided upon request to the authors.

In a control experiment, 60 individuals from a 1-week-old stab culture of a fresh subclone were also analyzed with the same method. No RFLP was detected, indicating that the preparation of the stab and its short time storage did not induce any significant variance.

Nature of polymorphism: Genetic variation acquired upon prolonged storage was first measured with the copy number per subclone for each IS element (Table 1). For the eight IS probes used, the total copy number varied from 40 to 55 among individual subclones. Considering an average length of an IS to be about 1 kb, this represents a 15-kb difference for the chromosome size within the same clone of bacteria. Although this is not a large proportion (*ca.* 0.3% of the chromosome length), it indicates a fairly good amount of DNA rearrangements. The degree of variation in the number of copies depended on the IS element: while IS*1* was strictly constant in this study, IS*186*, IS*150* and IS*4* showed little variation, but IS*2*, IS*3*, IS*30* and particularly IS*5* displayed a larger degree of variability. The number of patterns per IS reflected a similar variation indicating a large polymorphism within the same culture. Again IS*1*, IS*4*, IS*186* and IS*150* had one major pattern and none or few variants, while the hybridization patterns of IS*2*, IS*3*, IS*5* and IS*30* revealed a higher degree of polymorphism. In that respect, IS*5* displayed a remarkably high polymorphism with 46 different patterns containing from 9 to 19 copies. Such an IS-dependent distribution of the genetic variation indicates that IS-independent mutations, *e.g.*, point mutations in restriction sites and random deletions, are not mainly responsible for the variation observed among individual colonies. Therefore, simple transposition or other IS-mediated DNA rearrangements were most likely the cause of the observed genetic variation.

The frequency of occurrence of each fragment among individual colonies is also a measure of genetic variation (Table 2). For a given IS element some fragments were always present while other fragments were seen only in a fraction of the subclones. Again, the different IS elements did not contribute equally to the differentiation among subclones: IS*1*, IS*4*, IS*150* and IS*186* patterns showed no or little variation while IS*2*, IS*3*, IS*30* and IS*5* patterns were very variable.

The existing maps of IS elements present in *E. coli* K-12 strain W3110 had been constructed from gene libraries obtained from single clones (BIRKENBIHL and VIELMETTER 1989) and offer a reference to analyze the variation among our subclones (Tables 1 and 2). The general patterns observed with the probes of IS*1*, IS*4*, IS*150* and IS*186* are in accordance with these maps, except that one extra copy of IS*1* is resident in our samples. The distribution of the major and stable fragments with the probes of IS*2*, IS*3*, IS*5* and IS*30* are also in accordance with the published data. However, differences exist for the copy numbers and the distribution of fragments for these highly polymorphic markers. For IS*2*, seven fragments were seen in all individuals except one, three fragments in 40–60% of the individuals and

TABLE 1

Distribution of IS elements among 118 individual colonies recovered from an old stab culture of strain W3110

	IS*1*	IS*2*	IS*3*	IS*4*	IS*5*	IS*30*	IS*150*	IS*186*	All 8 IS elements
No. of IS copies per chromosome:[a]									
Standard strain W3110	6	14	6	1	>23	5	1	3	>59
Range in our 118 subclones	7	8–11	4–7	1–2	9–19	4–10	1–2	3	40–55
Subclone #57	7	9	5	1	11	4	1	3	41
Subclone #42	7	8	5	1	12	4	1	3	41
Hybridization patterns:[b]									
Number of patterns	1	8	8	2	46	15	3	2	68[c]
Unique patterns[d]	0	3	3	1	34	7	2	1	51[c]

[a] The range of copy number for each IS element is compared to published data of a standard strain after BIRKENBIHL and VIELMETTER (1989, 1990. Subclones #42 and #57 are the closest to the putative ancestor.

[b] The number of different patterns illustrates the variability related to each IS element.

[c] The total number of patterns differs from the sum of patterns per IS because some individuals showed modifications with more than one IS probe.

[d] Unique patterns are those observed in only one of the 118 subclones.

three other fragments were rare. For IS*3*, four fragments were seen in all individuals except one, three fragments occurred in about half of the individuals and one fragment was rare. For IS*30*, two categories of patterns were observed: individuals which possessed the three major bands of a size similar to that described in the literature (BIRKENBIHL and VIELMETTER 1989) and individuals that carried in addition a diversity of other fragments including a 5.6-kb fragment showing a double hybridization intensity which is likely to contain two copies of the IS*30* element (Fig.2). The occurrence of IS*30* as a dimer has been identified to cause transpositional DNA rearrangements at high rates in *E. coli* (OLASZ *et al.* 1993). The striking increase in the IS*30* copy number in all subclones showing this 5.6-kb fragment is likely to be related to the presence of an IS*30* dimer as a source for a burst of transposition. In accordance with the results of (GREEN *et al.*, 1984), IS*5* showed a high mutator activity in the stab conditions. After 30 years of storage, the transposition frequency of IS*5* can be estimated at 0.013 per copy per year which is in accordance with the value of 0.008 ± 0.002 found previously (GREEN *et al.* 1984).

Pedigree of the old stab culture: Sixty eight different patterns (57%) were found after hybridization of the eight IS probes to the DNA of 118 individuals (Table 1). Fifty one different patterns were represented by one unique individual subclone in the sample surveyed indicating that the polymorphism arose under almost nonreplicating conditions. Based on the parsimony method, a pedigree was designed (Figure 3) which required at least 174 mutational steps to explain the observed distribution of patterns. The average number of changes per observed pattern was 12.1 ± 2.6. The tree was unrooted but contains a major cluster that allows one to assume a location for an ancestor: all individuals shown in the lower part of Figure 3 have had massive modifications of the IS*30* patterns linked to a double copy (see above), while in the upper part individuals displayed unmodified IS*30* patterns. Because nearly half of all the patterns carried the same doublet leading to a burst of IS*30*, it was assumed that the critical event occurred before most events at other IS elements happened. This was used to determine a putative ancestor, located between #57 and the group of #42, 58 and 83 (Figure 3). It is interesting that the assumed genetic structure of the original clone is no more observed at a high density. However this in line with the calculated average number of 12 changes per observed pattern. As an illustration for the variability acquired during storage, individual #72, on the other side of the pedigree, required a minimum of 23 mutational steps from the putative ancestor to be explained.

The high occurrence of unique patterns indicates that many of the observed variations have their origin during the time of storage in the stab culture rather than previously to the inoculation of the stab. Recent experiments with stabs prepared from subclones support this interpretation because the number of mutants accumulates linearily with the time of storage in stabs (T. NAAS, M. BLOT, W. FITCH and W. ARBER, to be published). It is assumed that in stab cultures, a residual growth is possible on the expense of lysed bacteria. Such a cryptic growth should not lead to more than one effective generation for the entire culture and thus should not affect an estimate of the mutation frequency based on the number of changes per pattern per time unit. For 30 years (2.6×10^5 h), this frequency is about 10^{-5} changes per pattern and per hour, and thus corresponds to a high level of mutagenesis. This value does not take into account lethal mutations, due for instance to transposition or deletion in vital genes (REIF and SAEDLER 1975), neither mutations not affecting the distribution of IS elements. Moreover two bottlenecks occurred when the stab was renewed in 1965 and 1972 that may have reduced the genetic diversity within the stab. Therefore, this mutation frequency might be underestimated compared to the actual mutation rate. It also might be that we observed a biased sample of the population, if some mutants had been selected for during storage and led to a larger progeny by differential cryptic growth.

TABLE 2

Size distribution of *Eco*RV fragments containing IS elements

IS*4*		IS*150*		IS*186*		IS*1*		IS*3*		IS*2*		IS*30*		IS*5*[a]	
kb[b]	%[c]	kb	%	kb	%	kb	%	kb	%	kb	%	kb	%	kb	%
7.8	1	**7.0**	100	**7.4**	100	**9.8**	100	9.3	54	**10.0**	100	9.9	98	12.9	1
7.7	100[d]	4.4	1	4.8	99	**8.2**	100	8.4	42	**8.8**	100	9.8	2	**12.1**	100
		1.8	1	4.5	1	**6.5**	100	7.7	41	**7.9**	100	**8.7**	100	10.8	1
				2.6	100	**5.4**	100	**5.2**	100	7.8	39	8.0	2	10.0	51
						4.3	100	4.8	3	**7.6**	100	**7.6**	100	9.1	81
						2.2	100	3.0	99	7.3	40	7.5	1	8.5	58
						1.9	100	**2.8**	100	7.0	64	7.2	13	8.1	2
								1.9	100	**6.5**	100	6.9	42	7.7	28
										6.0	100	6.6	9	7.6	1
										5.3	1	5.6[e]	42	7.4	4
										4.1	99	5.2	25	7.1	22
										3.0	3	4.3	33	6.9	32
										2.9	7	4.0	11	6.6	2
												3.0	1	6.4	42
												2.7	1	6.1	24
												2.5	1	5.9	1
												2.3	14	5.7	34
												2.0	100	5.6	38
														5.5	1
														5.2	100
														5.0	98
														4.8	32
														4.6	2
														4.4	44
														4.1	100
														4.0	2
														3.9	6
														3.8	63
														3.6	23
														3.5	9
														3.4	44
														3.1	94
														2.8	16
														2.5	20
														2.2	100

[a] IS elements are sorted by increasing polymorphism.
[b] Size of *Eco*RV fragments hybrizing with the different IS probes.
[c] Frequency of a given fragment in the population of subclones.
[d] Fragments observed in all subclones (100%) are in boldface.
[e] The 5.6-kb fragment which hybridizes to the IS*30* probe has a double intensity.

Thus these lineages should predominate in the culture and appear as large groups of individuals in one cluster. In fact, most patterns were unique or had one or two siblings, except five subsets of patterns (marked I–V in Figure 3) that contained between 5 and 12 individuals. These groups belonged to different clusters in the pedigree, indicating that they were not relics of the original population but new genetic combinations that appeared in the course of time. Their existence is more likely due to reproduction than to independent identical mutation events. For instance, a mutation that improved fitness might have occurred before group I and II diverged by another neutral IS-related rearrangement.

Physiological studies with genetic variants: On rich broth medium, a great variety in colony sizes at 37° was observed among the individuals. The occurrence of beneficial new genetic combinations during storage was thus addressed by comparing the growth rates, while detrimental mutations were analyzed by testing the different subclones for prototrophy (Figure 3). With 56 subclones (#62–118) the growth rate was determined at 37° in a nutrient broth medium identical to the stab medium except agar. A selection coefficient (s) could be calculated by reference to a culture of W3110 used routinely in the laboratory, and its significance was studied by means of analysis of variance. Nearly half of the tested subclones did not differ significantly from the reference and their selection coefficient was then zero. This was mainly the case for patterns represented by only one subclone. Positive selection coefficients, indicating an advantage, were found for several subclones in the upper cluster and especially for the subsets I, II and IV. Negative selection coefficients were found for about one third of the subclones and only in the lower cluster. These mutants were auxotroph and in most cases showed a long lag phase after dilution as well as a slow growth. With some of these subclones viability could not be maintained for more than two days in liquid cultures.

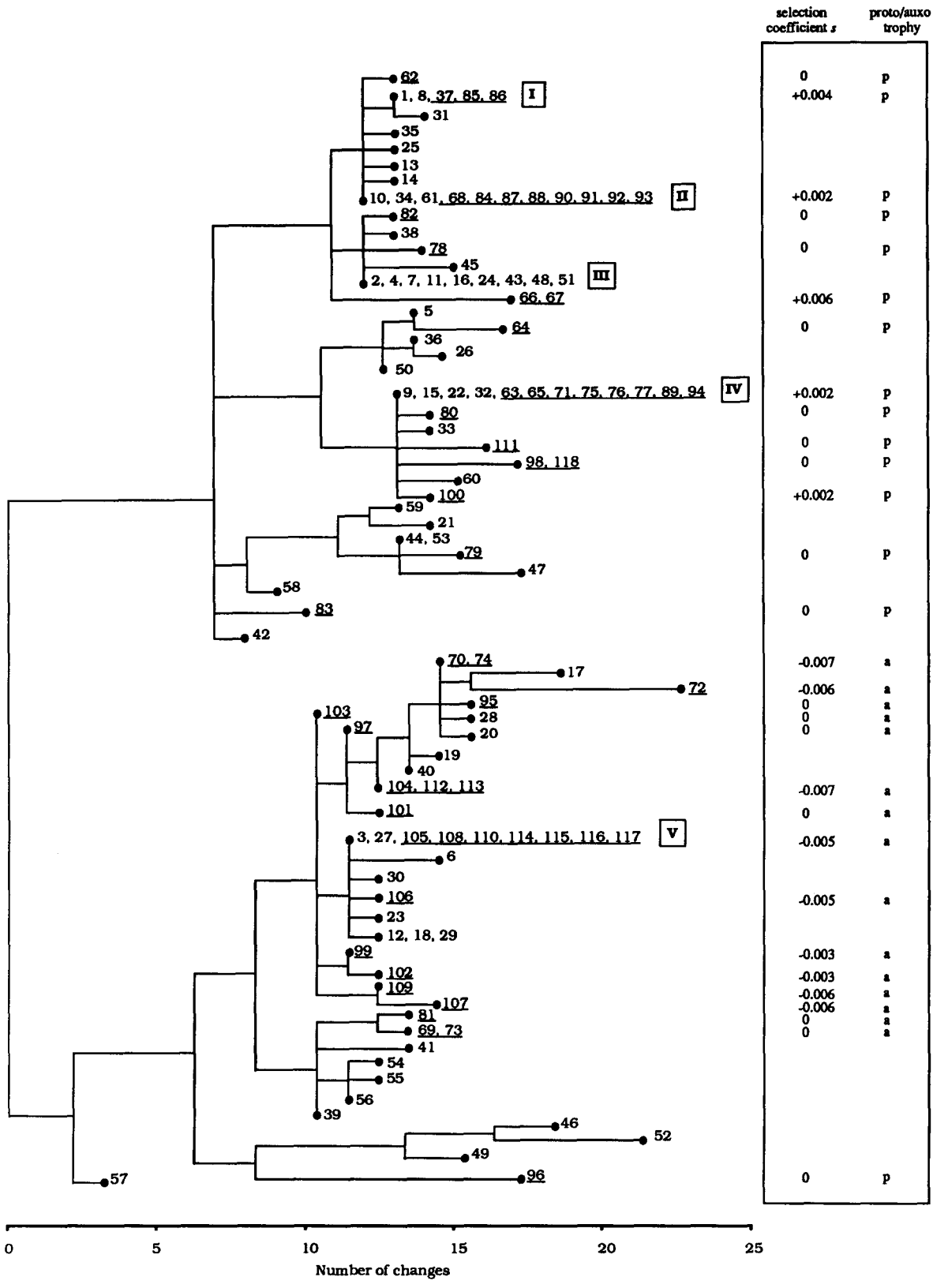

FIGURE 3.—Pedigree of the studied bacterial population after prolonged storage as derived from the observed RFLP. The tree is unrooted but subclones at the left (#57, 42, 58 and 83) are likely to be the closest to the putative ancestor (see text for discussion). Each point represents a defined RFLP pattern after hybridization to IS elements, and individual subclones are indicated by their numbers. Clusters labeled I-V are specific patterns represented by more than five subclones. The horizontal axis represents the minimum number of changes. Subclones tested for their growth rate and prototrophy are underlined and the results are provided on the right: average selective coefficients (s) as a measure of fitness and prototrophy (p) or auxotrophy (a). In a crowded part of the figure, the relevant data for subclones #95, #97 and #103 are given in this order.

Possibly the "burst" of IS*30* transposition shown by these subclones could have caused the growth defects. The fact that the subset V contains nine siblings in the stab culture but shows a lower growth rate at 37° is interesting and indicates that another component of fitness is enhanced for these individuals. We have not yet been able to identify such a component which could perhaps improve viability of resting bacteria.

It is remarkable that all of the subclones in which no IS*30* transposition had occurred (upper cluster of Figure 3) remained prototrophic. In contrast most of the subclones which underwent IS*30* transposition (lower cluster of Figure 3) had become auxotrophic. Subclone #96 is an exception to this rule. Therefore, It is not the burst of IS*30* copies *per se* which caused the loss in growth performance but more likely the loci affected by the DNA rearrangements.

DISCUSSION

Nature and dynamics of DNA rearrangements: The data obtained in the studies reported here confirm, extend and generalize previous observations that IS-mediated transposition contributes importantly to genetic plasticity and that this mutagenic activity is not limited to growing cells but occurs also in resting bacteria. The RFLP method employed is simple, but efficient and reliable. It involves only one restriction enzyme, *Eco*RV, used to fragment chromosomal DNA and about 10 hybridization probes including all known resident IS elements of the *E. coli* K-12 strain W3110. Although the strategy followed cannot reveal all spontaneous mutations occurring to a bacterial population nor the specific mutagenesis mechanism, it is likely to reveal most transpositional DNA rearrangements, any other DNA rearrangements as long as they affect DNA fragments carrying an IS element and the presumably rather rare cases in which a mutation destroys or creates an *Eco*RV cleavage site. A strong variation of the IS copy number points to transposition as a major contribution to the detected structural genome alterations.

The high degree of polymorphism which was revealed upon the analysis of subclones isolated from an old stab culture came as a surprise to us. Such a big variance is not seen with bacterial cultures after prolonged propagation, during which periodic selection is known to reduce genetic diversity in the cell population (NOVICK and SZILARD 1950). It is thus most likely that the variance strongly increased during the 30 years of storage of the stab at room temperature. Unfortunately, the nature of the culture of W3110 which was used in 1960 for the inoculation of the stab remains unknown. Possibly, it had been a routine laboratory culture rather than a fresh subclone. If so, some polymorphism might have already been present at that time. In particular, it remains unknown if the dimeric form of IS*30*, which has been detected in about 40% of the subclones isolated, was already present before the stab was inoculated. But the development of many different unique patterns of restriction fragments must be largely attributed to transposition upon storage of the stab. This hypothesis is supported by recent studies involving stabs which had been inoculated with fresh clones of bacteria. In particular, it is shown that mutants accumulate linearily with time of incubation and the observed patterns are different from a stab to another (T. NAAS, M. BLOT, W. FITCH and W. ARBER, unpublished results).

Our knowledge of bacterial physiology in stab cultures is very poor. There is at most a very slow, residual growth which could occur at the expense of dying cells. This argues for selection conditions differing from those exerted on fast growing bacterial cultures. This situation could explain both the large number of different unique genome structures present and the fact that a few specific structures are represented by several isolates. The latter are likely to have found a fitness advantage in the stab culture and might thus have succeeded to grow to some extent. A majority of such clusters analyzed have indeed shown an increased fitness even in exponential growth.

Among the eight resident IS elements of W3110, IS*5* proved to be the most mobile in the studied stab culture. Most other IS elements also showed transposition activities to a degree depending on the IS considered. To our surprise, IS*1*, although present in seven copies in the chromosome, was absolutely stable. This might be related to the particular physiological condition in the stab kept at room temperature. It is known that at low temperatures IS*1* mainly undergoes deletion formation (REIF and SAEDLER 1975; ARBER *et al.* 1978). Such mutagenesis is likely to be often lethal which could explain that we could not detect any IS*1* activity.

DNA rearrangements affected in multiple ways all viable members of the bacterial population in the stab. In the average, each subclone showed about 12 changes in the RFLP patterns from that of the putative ancestral genome, although individual deviations are considerable from this average. In a crude analysis we could not find any evidence for interdependence of DNA rearrangements involving different IS elements that might correspond to a general response to starvation. Rather, the observed variation should be attributed to a number of independently occurring consecutive events. This conclusion is supported by recent studies with stabs stored for various periods of time (0–20 years) indicating that the number of different IS-related mutants depend on the time of storage. Moreover, the preparation of a stab and its storage for one week does not result in any measurable genetic variance (T. NAAS, M. BLOT, W. FITCH and W. ARBER, unpublished results). However, it is possible that at particular times particular cells might undergo more efficient transposition. Such active phases might depend on a particular physiological state

and it might thus also depend on the environment. Temperature for instance was shown to induce transposition of IS elements in Halobacterium (PFEIFER and BLASEIO 1990). The methods used in this investigation could offer help in the elucidation of these questions. A good example of increased transposition activities is given by IS*30*. It was already known that this element can bring about dimeric forms of IS*30* and that such forms are highly active for transpositional DNA rearrangements (OLASZ *et al.* 1993). The bursts of IS*30* transposition documented in this study show that such transpositional activities can affect the bacterial chromosome indeed.

It may appear needless to state that transposition is enzyme-mediated and that these enzymes must be expressed in stabs to explain their activity. Life in stabs might represent a particular condition of stress. It is thus interesting to note that many IS elements can undergo transposition under these conditions of life. This may be related by their postulated role as evolutionarily relevant variation generators. The documentation of a high degree of variance to be attributed to transposition in stabs and thus to essentially resting cells can represent support for an evolutionary role of mobile genetic elements.

Evolutionary biology of bacteria and IS elements over 30 years: One fundamental question concerning mobile genetic elements addresses their long term maintenance in genomes. Theoretical approaches have opposed a parasitic style of life (DOOLITTLE and SAPIENZA 1980) *vs.* an adaptive role provided to the host (CAMPBELL 1981). Because IS elements only carry genes related to their transposition function, they cannot express beneficial traits that might directly enhance the fitness of their host, as shown for some transposons (BLOT *et al.* 1993). Rather IS elements may play a role in bacterial populations in the long term by creating a diversity of genetic combinations, with occasionally one of them being more fit [see ARBER (1991) for discussion]. From this point of view the pedigree of the stab culture and the fitness measured are very interesting. Less than half of the different mutants seemed neutral at least for their growth rate and they were represented most often by one individual. Nearly another half was disfavored being auxotroph mutants with a lower growth rate, although one of these mutants existed as a group of nine individuals with identical patterns, suggesting that it might have an advantage during storage. Three mutants appeared to have a better growth rate than the other individuals and one pattern showing similar properties has not been tested for growth rate. Thus after 30 years, maybe five new advantageous genetic combinations occurred in the studied sample as a consequence of IS-related rearrangements. If similar kinds of favorable mutations occur at intervals, this might be sufficient to ensure the maintenance of IS elements upon each replacing an ancestor by a better adapted mutant. Evidence for rapid changes in the genetic structure of population during stationary phase (ZAMBRANO *et al.* 1993) illustrates what might have happened at intervals during 30 years of storage.

The observed increase of genetic variation in resting bacteria has its implications for environmental microbiology. Bacteria spend most of their time in a resting state rather than in exponential growth. However, most published data involving quantitative experimentation to explore mutagenesis are based on work done with propagating bacteria. As we suggest here, in part different laws may apply to spontaneous mutagenesis of resting bacteria. The strategies applied in this study may help to understand the role of physiology on the generation of genetic plasticity in bacterial populations. Such investigations are now in process in our laboratory.

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LITERATURE CITED

AJIOKA, J., and HARTL, D., 1989 Population dynamics of transposable elements, pp. 939–958 in *Mobile DNA*, edited by D. BERG and M. M. HOWE. American Society for Microbiology, Washington D.C.

ARBER, W., 1958 Transduction des charactères *gal* par le bactériophage Lambda. Arch. Sci. **11:** 259–338.

ARBER, W., 1991 Elements in microbial evolution. J. Mol. Evol. **33:** 4–12.

ARBER, W., S. IIDA, H. JÜTTE, P. CASPERS, J. MEYER and C. HÄNNI, 1978 Rearrangements of genetic material in *Escherichia coli* as observed on the bacteriophage P1 plasmid. Cold Spring Harbor Symp. Quant. Biol. **43:** 1197–1208.

BACHMANN, B. J., 1987 Derivations and genotypes of some mutant derivatives of *Escherichia coli*-K12, pp. 1190–1219 in *Escherichia coli and Salmonella typhimurium*, edited by F. C. NEIDHARDT. American Society for Microbiology, Washington D.C.

BACHMANN, B. J., 1990 Linkage map of the *Escherichia coli* K-12, Edition 8. Microbiol. Rev. **54:** 130–197.

BIRKENBIHL, R. P., and W. VIELMETTER, 1989 Complete maps of IS*1*, IS*2*, IS*3*, IS*4*, IS*5*, IS*30*, and IS*150* locations in *Escherichia coli* K12. Mol. Gen. Genet. **220:** 147–153.

BIRKENBIHL, R. P., and W. VIELMETTER, 1991 Completion of the IS map in *Escherichia coli*: IS*186* positions on the *Escherichia coli* K12 chromosome. Mol. Gen. Genet. **226:** 318–320.

BLOT, M., J. HEITMAN and W. ARBER, 1993 Tn*5*-mediated bleomycin resistance in *Escherichia coli* requires host genes. Mol. Microbiol. **8:** 1017–1024.

BRAHMA, N., A. SCHUMACHER, J. CULLUM and H. SAEDLER, 1982 Distribution of the *Escherichia coli* K12 insertion sequences IS*1*, IS2 and IS*3* among other bacterial species. J. Gen. Microbiol. **128:** 2229–2234.

CAMPBELL, A., 1981 Some questions about movable elements and their implications. Cold Spring Harbor Symp. Quant. Biol. **45:** 1–9.

DALRYMPLE, B., P. CASPERS and W. ARBER, 1984 Nucleotide sequence of the prokaryotic mobile genetic element IS*30*. EMBO J. **3:** 2145–2149.

DOOLITTLE, W. F., and C. SAPIENZA, 1980 Selfish genes, the phenotype paradigm and genome evolution. Nature **284:** 601–603.

DYKHUIZEN, D. E., and D. L. HARTL, 1983 Selection in chemostats. Microbiol. Rev. **47:** 150–168.

ENGLER, J. A., and M. P. BREE, 1981 The nucleotide sequence and protein-coding capability of the transposable element IS*5*. Gene **14:** 155–163.

FITCH, W. M., 1971 Toward defining the course of evolution: minimum change for specific tree topology. Syst. Zool. **20:** 406–416.

GALAS, D. J., and M. CHANDLER, 1989 Bacterial insertion sequences, pp. 109–162 in *Mobile DNA* edited by D. BERG and M. M. HOWE. American Society for Microbiology, Washington D.C.

GHOSAL, D., and H. SAEDLER, 1979 Nucleotide sequence of the transposable DNA-element IS2. Nucleic Acids Res. **6:** 1111–1122.

GREEN, L., R. MILLER, D. E. DYKHUIZEN and D. L. HARTL, 1984 Distribution of DNA insertion elements IS*5* in natural isolates of *Escherichia coli.* Proc. Natl. Acad. Sci. USA **81:** 4500–4504.

GUYER, M. S., 1978 The gamma-delta sequence of F is an insertion sequence. J. Mol. Biol. **126:** 347–365.

HALL, B. G., 1988 Adaptive evolution that requires multiple spontaneous mutations I. Mutations involving an insertion sequence. Genetics **120:** 887.

HARTL, D., and S. SAWYER, 1988a Multiple correlations among insertion sequences in the genome of natural isolates of *Escherichia coli*, pp. 91–106 in *Transposition*, edited by A. J. KINGSMAN and S. M. KINGSMAN. Cambridge University Press, Cambridge.

HARTL, D. L., and S. A. SAWYER, 1988a Why do unrelated sequences occur together in the genome of *Escherichia coli*? Genetics **118:** 537–541.

HARTL, D., D. E. DYKHUISEN and D. BERG, 1984 Accessory DNAs in the bacterial gene pool: playground for coevolution. Ciba Symp. **102:** 233–245.

KLAER, R., S. KUEHN, E. TILLMANN and P. STARLINGER, 1981 The sequence of IS*4*. Mol. Gen. Genet. **181:** 169–175.

KOHARA, Y., K. AKIYAMA and K. ISONO, 1987 The physical map of the whole *Escherichia coli* chromosome: application of a new strategy for rapid analysis and sorting on a large genomic library. Cell **50:** 495–508.

KOTHARY, R. K., D. JONES and E. P. M. CANDIDO, 1985 IS*186*: an *Escherichia coli* insertion element isolated from a cDNA library. J. Bacteriol. **164:** 957–959.

MINTON, N. P., 1984 Improved plasmid vectors for the isolation of translational *lac* gene fusion. Gene **31:** 269–273.

MITTLER, J. E., and R. E. LENSKI, 1990 Excisions of Mu from *E. coli* MCS2 are not directed mutations. Nature **344:** 173–175.

NOVICK, A., and L. SZILARD, 1950 Experiments with the chemostat on spontaneous mutations of bacteria. Proc. Natl. Acad. Sci. USA **36:** 708–719.

OHTSUBO, H., and E. OHTSUBO, 1978 Nucleotide sequence of an insertion sequence, IS*1*. Proc. Natl. Acad. Sci. USA **75:** 615–619.

OLASZ, F., R. STALDER and W. ARBER, 1993 Formation of the tandem repeat $(IS30)_2$ and its role in IS*30*-mediated transpositional DNA rearrangenments. Mol. Gen. Genet. **239:** 177–187.

PFEIFER, F., and U. BLASEIO, 1990 Transposition burst of the ISHF27 Insertion element family in *Halobacterium halobium*. Nucleic Acids Res. **18:** 6921–6925.

RAABE, T., E. JENNY and J. MEYER, 1988 A selection cartridge for rapid detection and analysis of spontaneous mutations including insertions of transposable elements in Enterobacteriaceae. Mol. Gen. Genet. **215:** 176–180.

REIF, H. J., and H. SAEDLER, 1975 IS*1* is involved in deletion formation in the *gal* region of *Escherichia coli* K12. Mol. Gen. Genet. **137:** 17–28.

RODRIGUEZ, H., E. T. SNOW, U. BHAT and E. L. LOECHLER, 1992 An *Escherichia coli* Plasmid-Based, mutational system in which *supF* mutants are selectable–insertion elements dominate the spontaneous spectra. Mutat. Res. **270:** 219–231.

SAMBROOK, J., T. MANIATIS and E. F. FRITSCH, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

SAWYER, S., and D. L. HARTL, 1986 Distribution of transposable elements in prokaryotes. Theor. Popul. Biol. **30:** 1–16.

SCHWARTZ, E., M. KROEGER and B. RAK, 1988 IS*150*: distribution, nucleotide sequence and phylogenetic relationships of a new *E. coli* insertion element. Nucleic Acids Res. **16:** 6789–6802.

SENGSTAG, C., S. IIDA, R. HIESTAND-NAUER and W. ARBER, 1986 Terminal inverted repeats of prokaryotic transposable element IS*186* which can generate duplications of variable length at an identical target sequence. Gene **49:** 153–156.

SHAPIRO, J. A., and N. P. HIGGINS, 1989 Differential activity of a transposable element in *Escherichia coli* colonies. J. Bacteriol. **171:** 5975–5986.

STALDER, R., and W. ARBER, 1989 Characterization of *in vitro* constructed IS*30*-flanked transposons. Gene **76:** 187–193.

TIMMERMAN, K. P., and C.-P. TU, 1985 Complete sequence of IS*3*. Nucleic Acids Res. **13:** 2127–2139.

UMEDA, M., and E. OHTSUBO, 1989 Mapping of insertion elements IS*1*, IS*2* and IS*3* on the *Escherichia coli* K-12 chromosome. J. Mol. Biol. **208:** 601–614.

UMEDA, M., and E. OHTSUBO, 1990a Mapping of insertion element IS*30* in the *Escherichia coli* K-12 Chromosome. Mol. Gen. Genet. **222:** 317–322.

UMEDA, M., and E. OHTSUBO, 1990b Mapping of insertion element IS*5* in the *Escherichia coli* K-12 chromosome. J. Mol. Biol. **213:** 229–237.

UMEDA, M., and E. OHTSUBO, 1991 Four types of IS*1* with differences in nucleotide sequence reside in the *Escherichia coli* K-12 chromosome. Gene **98:** 1–5.

ZAMBRANO, M. M., D. A. SIEGELE, M. ALMIRON, A. TORMO and R. KOLTER, 1993 Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. Science **259:** 1757–1760.

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