An analysis of restriction fragment length polymorphism (RFLP) using eight residential insertion sequence (IS) elements as hybridization probes reveals that the genome of resting bacteria is more dynamic than it was long believed. *Escherichia coli* strains stored in agar stab for up to 30 yr accumulate a genetic variation which is correlated to time of storage. This spontaneous mutagenesis is often IS-specific, with particularly high activity for IS5, and thus suggests that transpositional DNA rearrangements are a major cause for the observed genetic polymorphism. The RFLP patterns indicate a burst of IS30 transposition to occur occasionally. Mutation rate is estimated by two different methods to roughly $10^{-5}$ IS-related DNA rearrangements per bacterial chromosome per hour of storage for the eight IS elements studied. A pedigree derived from the RFLP data reveals that populations had evolved independently in each stab and showed no signs of convergence. Relics of an assumed ancestral population were still present in the stab cultures, but the elder stabs provided mostly mutants. These results indicate that cells placed under nutritional deprivation might have a highly plastic genome and suggest that such plasticity might play an adaptive role.

**Introduction**

Original experiments by Luria and Delbrück (1943) and later by Lederberg and Lederberg (1952) revealed that mutants arise spontaneously in microbial populations before any selection for the mutated trait is applied. Since then, the concept of spontaneous mutation as a random modification of the DNA sequence has evolved (see, e.g., Drake 1991 for a review). The prevailing view is that spontaneous mutations arise primarily as errors during replication of the genetic material (see, e.g., Brock and Madigan 1991, p. 7). However, it was shown long ago that mutations may occur in bacteria during the stationary phase, that is, in the absence of extensive replication (Ryan 1955, 1959). More recently, a number of investigations have suggested that starvation of bacteria might alter their mutation rate (Bucala et al. 1984; Cairns et al. 1991; Boe 1990; Foster and Cairns 1992; Hall 1992), although this notion is still very controversial (Lenski and Mittler 1993; MacPhee 1993).

Many molecular mechanisms generating genetic variation are mediated by specific enzymes and thus have the possibility to be regulated (Arber 1991, 1993 for discussion). Systematic studies of the mutational spectrum within genes of *Escherichia coli* reveal a major contribution of mobile genetic elements to spontaneous mutagenesis. For example, transposition of insertional sequence (IS) elements was shown to produce ca. 60% of the mutations in gene *supF*, while deletions account for ca. 15% (Rodriguez et al. 1992). In another study insertions of the IS7 element represent 12% of *lacI* mutations (Schaaper et al. 1986). At the genome level, about 95% of spontaneously arising lethal mutants in the prophage P1 are due to transposition (Arber et al. 1980). Transposition of several IS elements may not require the replication of the chromosome (Galas and Chandlee 1989 for transposition mechanisms), and it can therefore also occur in nondividing bacteria. It has been proposed on several occasions that transposition of mobile genetic elements might depend on environmental factors and bacterial physiology (Shapiro and Higgins 1989; Mittler and Lenski 1990; Turner et al. 1990).

If any relation exists between nutritional deprivation and genome instability, such dependence should be easier to determine in extreme situations (e.g., long time storage of bacteria in stab cultures). Indeed, a recent study to determine the amount of transposition at the level of the entire bacterial chromosome revealed an impressively high level of spontaneous IS-related DNA rearrangements to have occurred during 30 yr of starvation (Naas et al. 1994). Since the history of the inoc...
ulation of this stab culture was not documented and because the stab had been renewed twice, the identification of the time when the mutations arose remained an important question. Several subclones were observed up to 12 times in a sample of 118 colonies, and they displayed “evolved patterns” compared to a putative ancestor. Alternative explanations were discussed to explain similarities among subclones: genetic convergence (i.e., the multiple occurrence of the same mutational event) or cryptic growth of some of the arising mutants. The latter explanation was supported by a slightly higher growth rate in broth for these mutants, although it could not be excluded that similar patterns might also arise because of hot spots for IS insertions.

Both questions are addressed in this paper. First, the kinetics of IS-related DNA rearrangements is studied by comparing the diversity of mutants revealed by restriction fragment length polymorphism (RFLP) in stabs of the same *E. coli* K-12 strain (W3110) stored for various lengths of time. Second, a pedigree was made with all subclones analyzed pooled in a unique matrix to determine the trends of genetic differentiation and whether identical mutants did arise in different stabs.

The results show an accumulation of mutants to correlate with the time of storage in stabs, suggesting that IS-related rearrangements may accumulate in the genome of a resting bacterium. No signs of convergent evolution are seen in independent bacterial populations. The pedigree gives a direct view of bacterial evolution in a so-called “strain” during 30 yr of storage. The number of mutants which arose under conditions of nutritional deprivation is discussed as well as the role of mobile elements as mutators.

### Material and Methods

**Bacterial Strains, Storage, and Sampling**

Stabs of the *Escherichia coli* K-12 strain W3110 used in this study are listed in table 1. All cultures used for inoculation of agar stabs of our collection originated from a stock of the California Institute of Technology which was used in experiments with phage λ (Arber 1958). The oldest stab of strain W3110 which provided viable colonies is marked to be 30 yr old, but it has been renewed twice, in 1965 and 1972 (Naas et al. 1994 for complete description of origin and storage). The 9–13-yr-old stabs were inoculated with cultures used in microbial genetics courses. These tryptone broth cultures had been inoculated with either samples from a stab culture or a single colony from such samples (no records are available on the precise manipulations for each specific inoculum). At rare occasions, some of these stabs were opened to collect material and then tightly closed again. No records exist when this was done, nor do we know how much this manipulation might have affected the bacterial physiology in the stab.

In 1990 several new stabs were prepared with a culture grown from a single colony obtained from our standard laboratory strain of W3110. One of these stabs was analyzed after a few days (age 0 yr), another one after 1 yr (age +1 yr). Stab K-215 has an independent origin. It was prepared in about 1968 by Norton Zinder at Rockefeller University, New York, and was given to us in 1990.

In view of the analysis, the stabs were sampled by vigorously suspending a loopful of agar in 5 ml of LB (Luria broth) and plating immediately 0.2 ml or dilutions on LA (Luria broth agar) plates. Depending on the stab culture, 34–118 individual colonies were collected on plates after overnight incubation at 37°C.

**RFLP Analysis**

Methods used in this study have been described elsewhere (Naas et al. 1994). In brief, bacteria from single colonies were grown in LB; chromosomal DNA was extracted from each culture and digested with *EcoRV*. The resulting DNA fragments were separated by electrophoresis on agarose gel and blotted onto a nylon membrane. The blot was hybridized successively with eight 32P-labeled probes derived from internal fragments of IS elements (IS1, IS2, IS3, IS4, IS5, IS30, IS150, and IS186) and autoradiographed. The electrophoretic mobility of each hybridization fragment was carefully measured and compared to size markers (1-kb Ladder<sup>®</sup>, BRL).

### Table 1

<table>
<thead>
<tr>
<th>Age</th>
<th>Inoculation</th>
<th>Renewed</th>
<th>Size</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>−30</td>
<td>1960</td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>118</td>
<td>W. Arber stab 3164</td>
</tr>
<tr>
<td>−22</td>
<td>1968&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>72</td>
<td>N. Zinder stab K215</td>
</tr>
<tr>
<td>−15</td>
<td>1975</td>
<td>0</td>
<td>60</td>
<td>W. Arber stab 2087c</td>
</tr>
<tr>
<td>−13</td>
<td>1977</td>
<td>0</td>
<td>34</td>
<td>W. Arber stab 3597</td>
</tr>
<tr>
<td>12</td>
<td>1978</td>
<td>0</td>
<td>60</td>
<td>W. Arber stab 3736</td>
</tr>
<tr>
<td>−11</td>
<td>1979</td>
<td>0</td>
<td>72</td>
<td>W. Arber stab 3758</td>
</tr>
<tr>
<td>−9</td>
<td>1981</td>
<td>0</td>
<td>60</td>
<td>W. Arber stab 3773</td>
</tr>
<tr>
<td>0</td>
<td>1990</td>
<td>0</td>
<td>60</td>
<td>This study</td>
</tr>
<tr>
<td>+1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1990</td>
<td>0</td>
<td>60</td>
<td>This study</td>
</tr>
</tbody>
</table>

**NOTE.**—The age, date of inoculation, and the number of studied subclones (size) are given for each stab studied in the Arber collection as well as for one stab in the Zinder collection. Genetic differentiation among subclones recovered from these stabs was studied by RFLP, using IS elements as probes for hybridization.

<sup>a</sup> See Naas et al. (1994), agar plug directly transferred to a fresh stab (1965 and 1975).

<sup>b</sup> Date not precisely known ± 2 yr.

<sup>c</sup> Analyzed in 1991.
The hybridization data of 596 individual colonies (subclones) from 9 different stabs (see table 1) were pooled. They included the results of the analysis of 118 subclones from a 30-yr-old stock already described by Naas et al. (1994), and they revealed a total of 245 different hybridizing fragments. A matrix (fragments \times subclones) was established by scoring a subclone 1 if the hybridization fragment was present and 0 if absent. This large matrix (245 \times 596) is not represented here, but it can be provided on request to the authors. A reduced version of this matrix (536 subclones, excluding stab K215 from the Zinder collection) was used to reconstruct a pedigree with the program ANCESTOR as described previously (Naas et al. 1994), except that the operator (W. Fitch) was not told from which stab the subclones originated.

The matrix was also used to calculate the variance per fragment among individuals. This variance was summed for all fragments and used as an index of genetic variability per stab. This variance has the advantage of being independent of the frequency at which a fragment is observed and of the sample size. However, because most observed variability is due to IS-related DNA rearrangements (Naas et al. 1994), it may overestimate the variation in the case of conservative transposition which is counted as two steps (loss of a fragment and gain of another). Moreover, no data are available on covariance in the RFLP, because of, for instance, the co-insertion of two IS elements in the same restriction fragment. This might be a problem when calculating the variance per population. We have assumed that these biases were equal in the different samples; therefore, the variance was used only to calculate a regression against time storage, but no significance was attributed to its intrinsic value.

The pedigree describes a number of mutation steps (1→0 and 0→1) necessary to explain the relationship among subclones. Genetic variability may also be evaluated after dividing the sum of these steps by the number of subclones analyzed in a given stab. A linear regression of this value for each stab against time of storage was also calculated. It is not a direct measurement and it might be affected by the basic assumptions used in parsimony methods for deriving phylogenetic trees. It provided, however, a regression against time similar to that given by the variance, and its value gives an estimate of the number of mutational steps per genome per time unit.

**Results**

A total of 596 subclones of strain W3110 were isolated from 9 stab cultures (table 1), and the RFLP of their chromosomal DNAs was analyzed. In the subcultures from the Arber collection, the copy number of a given IS element was from 1 copy (IS150) up to 19 copies (IS30) as determined by direct counting on autoradiographs. In the subclones from the stab of the Zinder collection, a maximum of 22 copies of IS30 was observed. For all IS elements together, 245 different locations (fragment of a given size revealed by a given probe) were distinguished (table 2). The strong \( \mu \) number variation of some IS elements among subclones...
of the same stab population (table 2) indicated that transposition events must have made major contributions to chromosomal polymorphism as was already outlined (Naas et al. 1994). From the simple observation of Southern blots, it was clear that the degree of polymorphism increased with the time of storage of the stab cultures. This was more systematically studied after coding of the results.

Accumulation of Genetic Variability with Time of Storage

Variance was computed for each stab as a measure of genetic variability. The linear regression of variance per stab against time of storage indicated a constant accumulation of the number of mutants with the age of stabs (fig. 1). This included samples of our collection as well as an independent stab from a U.S. laboratory which also fit well the regression. One stab made in 1977 looked as an exception to good fit of the regression and showed a much lower variance than that expected for its age. More precisely, the number of different patterns observed in this stab culture (11 of 34 subclones studied) was in the same range as in other stab cultures, but these patterns were not very different from each other, and one pattern was observed 22 times. The short distances among the subclones of this stab in the pedigree and the abundance of the putative ancestral type (see below) indicated that genetic variability did not accumulate to the same degree as in other stabs. No obvious reasons for this could be discovered. Sixty other subclones of this stab were analyzed with the IS5 probe only (data not shown) which also revealed a low genetic variability. Removing this stab from the regression did not affect the slope (0.24) and resulted in a correlation coefficient of 0.98 (based on eight counts) instead of 0.83 in its presence, indicating for the majority of stabs an approximately linear increase of genetic variability with time of storage.

The eight IS elements contributed differently to genetic variability in the population of a stab (table 2). When the increase of variance with time of storage was studied with each IS element separately, approximate linearity was also observed with IS2, IS3, and IS5, but not with IS30. The amount of polymorphism revealed with IS1, IS4, IS150, and IS186 was not sufficient to test significantly the linearity of their individual contribution to genetic variability. For IS30, three stabs (12, 22, and 30 yr old) displayed a burst of new patterns which contributed importantly to genetic variation in these stabs. On the other hand, the other stabs revealed only a few or no mutants with IS30. Therefore the increase in genetic variation as revealed with IS30 was not linear with time of storage (fig. 1).

Nature of Genetic Variations

The strongest contribution to genetic variability came from IS5, followed by IS30 and, to a lesser extent, by IS2, IS3, and IS150. The three other elements (IS4, IS186, and IS1) contributed only to a few changes among the 596 subclones studied (table 2). These considerably different contributions of specific IS elements may relate both to the intrinsic transposition efficiencies and target specificities of the different IS elements and to the particular physiological conditions in the stab kept at room temperature. They also indicate that the observed RFLP does not depend in most cases on mutations in restriction sites which would affect all IS elements unspecifically. The important genetic variability which was revealed
with a probe of IS5 confirms previous observations of a high mutagenesis of this element in stabs (Green et al. 1984; Naas et al. 1994). Only in the stab analyzed after a few days of storage was there no mutant for this element in a sample of 60 subclones. The patterns observed with IS30 were of two kinds and influenced largely the genetic structure of the population in a stab: there were “regular” patterns identical to those described for the strain W3110 (three copies of IS30 plus a deleted element giving a weaker hybridization fragment; Umeda and Ohtsubo 1990) and new patterns revealing several additional IS30 copies at different locations. One of the hybridization bands of the new patterns appeared with double intensity. Such an increase in the copy number of IS30 indicates that these mutations were caused by transposition. The subclones of the stab culture from the Zinder collection revealed generally a higher number of copies for IS30. No precise information is available on the origin of this stab culture, in particular on the IS30 copy number when it was inoculated, but again this element contributed to a large amount of genetic differentiation.

**Distribution of Mutants among Stabs**

All subclones scored in the different stabs from the Arber collection were mixed in a large matrix which was used to reconstruct a tree without taking into account the origin of the subclone (i.e., the stab from which it was taken). This was done by a “blind” labeling of the subclones. Because the stab from Rockefeller University had a different origin and displayed specific patterns for IS30, it was not used to reconstruct the tree in which it would have been an outgroup. After the pedigrees were drawn, all subclones which belong to a given stab culture were given the same color (fig. 2). It is not common to show networks instead of trees, but these data pushed us to do so (but see Templeton et al. 1992). They arise simply by connecting every pair of taxa with only one
Mutations Accumulate in Resting Bacteria

It is common knowledge that some bacteria recovered from stab cultures could show altered phenotypes acquired during storage. Our previous data (Naas et al. 1994) showed that a large number of IS-related rearrangements occurred in a 30-yr-old stab, leading to a highly polymorphic population of cells. The present data show that bacteria kept in stab cultures have a dynamic genome: that is, mutations continuously arise during storage. The stabs of different ages revealed similar mutation rates indicating that their value does not depend on the length of the storage (or starvation) but that only
the number of mutations depends on this length. The analysis of mutation rates with fine tools (e.g., the fluctuation test: Luria and Delbrück 1943) allows one to screen billions of individuals in growing cultures to obtain a few mutants. With a much smaller sample size, our method reveals that populations from old stabs contained mostly mutants, many of these mutants showing more than one mutation.

The change in the genetic structure of the population can be further analyzed with the pedigree reconstructed from the RFLP patterns. The first mutations which occur in a homogeneous clone should arise, if random, in different individuals. This was observed in the pedigree with the 1-yr-old stab. When the time increases, the chance to get more than one mutation per individual also increases for bacteria which are almost nonreplicating. This was also observed in old stabs which showed some lineages of successive mutants with many intermediates missing. For instance, a maximum of 22 steps from the putative ancestor is observed in the oldest stab (Naas et al. 1994). However, the presence of some intermediates indicates that at least some residual growth occurred in the stabs. This also suggests that besides the genome, the population in the stab is also dynamic, meaning that during storage some mutants could increase in number compared to the rest of the population. This hypothesis is supported by previous data on the selective coefficients of the subclones isolated in the 30-yr-old stab which revealed four better fit mutants (Naas et al. 1994). The case would be similar to periodic selection which occurs in bacteria growing in a chemostat (Novick and Szilard 1950) or in bacteria entering the stationary phase (Zambrano et al. 1993). A consequence would be that bacteria kept under stab conditions for long periods have in fact a residual cryptic growth at the expense of dead bacteria or unused nutrients. The fact that some "evolved" patterns (i.e., those which accumulated mutations and are thus displayed at the bottom of the pedigree) are represented by 6-12 subclones (see Naas et al. 1994 for details on the pedigree of the 30-yr-old stab) could also be explained by the same natural selection. For instance, these patterns with multiple subclones isolated from the 30-yr-old stab were shown to grow faster in a broth similar to stab medium (Naas et al. 1994), suggesting that they were more able to benefit from the resources in the stab. However, we cannot estimate the extent of cryptic growth and cell turnover in stabs, although we believe it to be very low.

More Mutations in Stabs or Less Selection?

The mutation rate in our study (0.08 mutational steps per subclone analyzed per year) was similar to previous data with all IS elements in a 30-yr-old stab (0.13; Naas et al. 1994) and with IS3 only (0.08; Green et al. 1984). This could be translated into mutation rates ranging from 2 to 9 \times 10^{-6} IS-related DNA rearrangements per cell per hour in our different stabs. This is slightly higher than the overall mutation rate calculated for a single gene in growing bacteria (0.7-1 \times 10^{-6} mutation per cell per generation, after Rodriguez et al. 1992). Obviously, conditions of exponential growth and of storage in stabs are not really comparable, nor are data for mutations into one gene comparable to DNA rearrangements in the complete genome. Nevertheless, assuming that growing bacteria have nearly two generations per hour (ca. 10^7 genes) and that 60% of the mutations are transposition of IS elements (Rodriguez et al. 1992), a first approximation would suggest more mutations per time unit occur on the genome in a growing culture than in stabs. According to another line of thought, the longer the nutritional deprivation, the greater the stress, which might increase the number of new mutants. If so, the increase in the number of mutants would not be linear but, for example, exponential. This was not supported by our observations when the mutation rate seemed constant independently of the length of storage. The data on IS30 are maybe an exception because only old stabs displayed mutants, whereas transpositions of this element are difficult to observe in growing cultures (M. Keller, A. Arini, and W. Arber, unpublished manuscript). A similar case was reported to us with IS200 in Salmonella typhimurium for which transposition is observed only after 2 yr in stabs and never in growing cultures (J. Casadesus, personal communication). Another factor differently affecting mutations which arise during the exponential
phase of growth and those originating during storage in stabs is the intensity of natural selection. Because of the reduced level of propagation, and in view of the fact that only a small proportion of the genome is expressed (Siegele and Kolter 1992), a number of deleterious mutations may remain viable in stabs but would be progressively eliminated in a growing culture. This view is supported by the data on growth rates of the subclones isolated in the 30-yr-old stab (Naas et al. 1994). Furthermore, preliminary results with populations consisting of a mixture of mutants from these stabs show that most of them disappear "naturally" within 1 d from a growing culture (J. Meier, T. Naas, and M. Blot, unpublished manuscript).

Mutations arising during the stationary phase have became an important issue in the last few years, since it was proposed that bacteria might modify their mutational process when starved (Foster 1993 for a review). Our results do not refute this possibility but also suggest a need to review critically the role of selective processes on the maintenance of mutants versus wild type (Lenski and Mittler 1993 for discussion) and therefore on an apparent increase in the occurrence of mutants during starvation. Our previous results indicated that a large proportion of the IS-related DNA rearrangements occurring in the genome affect fitness (mostly negatively, but at rare occasions positively), but only a minority give rise to a visible phenotype (Naas et al. 1994). Therefore, when a mutation assay is based on phenotypes with comparisons between the growth and the stationary phases, it is not only the mutation rate induced by selection (i.e., starvation) which is measured but also the natural selection imposed on the entire genome of the mutants, independently of the observed trait. The present results indicate that the genome of resting bacteria is more dynamic than it was long believed and suggest that the control of mutation rates depending on the environment might be an adaptation providing their genomic plasticity to these bacteria.

Which Is Strain W3110?

In this study, IS-related DNA rearrangements were screened for, and numerous patterns were observed as a consequence of mutations. Some of these mutants had lost some of the W3110 strain phenotypes such as prototrophy, but a majority of the subclones were indistinguishable by their phenotypes (Naas et al. 1994). Until now, only phenotypes but not chromosomal polymorphism had been taken into account for defining bacterial strains. A total of 260 different patterns were observed of the 596 subclones analyzed. Which of them is strain W3110? The question is important because this strain is used for the *Escherichia coli* sequencing project as well as for the genetic map, and divergence might have accumulated among the different stocks of this strain. For instance, the strain W3110 from the Zinder collection displayed RFLP very different from the stabs of the Arber collection, particularly with IS30 and IS5. The extent of genetic differentiation among stocks of strain W3110 could thus be measured with the RFLP method, as it was done in the ECOR collection of natural isolates (Sawyer et al. 1987) to determine whether a standard strain needs to be defined.

On the other hand, the putative ancestor present 30 yr ago and the routine strain used now in our laboratory differ only by a new IS30 copy and a band shift with IS5. One reason for this similarity might be that old stab cultures were never taken to restart a culture in our laboratory but only young stabs, or better, bacteria frozen in glycerol. A complementary explanation might be that among the large variety of subclones arising by mutation, few are well adapted to laboratory conditions of growth in rich media, while most others disappear in liquid cultures. This assumption is supported by competitions among mutants recovered from an old stab. When mixed together, most disappear rapidly from LB cultures while one subclone takes over the population (J. Meier, T. Naas, and M. Blot, unpublished manuscript). A comparison of strain W3110 among laboratories would be helpful to determine whether a stabilizing selection maintains the stock homogeneous. The authors would thus appreciate receiving such strains (please no old stabs).

Outlooks

Bacterial populations that are not in a growth phase have long been neglected in physiology and genetic studies even though starvation is probably the most frequent state in nature (Postgate 1989; Kolter et al. 1993). Bacteria such as *Escherichia coli* have not evolved special programs such as sporulation to resist starvation but have means to change their physiology during stressful periods of nutritional deprivation (Kaprelyants et al. 1993 for discussion). These changes include drastic modifications in the expression of many genes under the control of a sigma factor (Siegele and Kolter 1992), some of these genes being involved in the stability of the genome (Almiron et al. 1992). We have documented a large amount of genetic variation arising under conditions of starvation. These mutations, described as IS-related rearrangements, were often transposition of IS elements which depend on the expression of particular enzymes encoded on the elements themselves (Naas et al. 1994). The consequences of transposition can be neutral, deleterious, or, on rare occasions, beneficial to the host (Blot 1994 for discussion), and preliminary results suggested that a few mutants recovered from the stabs display enhanced fitness while many others have suffered deletere.
arious mutations (Naas et al. 1994). Having the possibility to enhance the mutation rate during a starvation period is potentially a good strategy because one of the new genetic rearrangements might be beneficial under these environmental conditions (Fitch 1982 for discussion). Using transposable elements for such a strategy guarantees producing a plastic population of rearranged genomes because reversions by chance must occur later in some of the bacteria which would survive. Our data support this speculative scenario and may provide understanding of how transposable elements may earn their keep in microbial populations.

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