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MiniReview

Generation of genetic diversity by DNA rearrangements in resting bacteria

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Abstract: Transpositional DNA rearrangements importantly contribute to the genomic plasticity of bacteria, their viruses and plasmids. Interestingly, enzymatically mediated transposition is not limited to propagating bacteria, but it also occurs in prolonged periods of rest. As revealed with RFLP analysis, genetic polymorphism increases steadily upon storage of bacteria in the resting phase. These results are discussed here in the wider context of accumulating knowledge on molecular mechanisms contributing to overall spontaneous mutagenesis, which is the result of a multitude of specific, often enzyme-mediated processes of variation generation.

Key words: Non-propagating bacteria; Transposition; Genetic variation; Spontaneous mutagenesis; Restriction fragment length polymorphism; Microbial evolution

Introduction

In the past decades, knowledge on life functions of bacteria has accumulated rapidly, at least for selected laboratory strains such as *Escherichia coli* K12. Most of the successful genetic and physiological experiments are routinely carried out under well controlled laboratory conditions which often include exponential growth in well defined media. Among biological functions thus studied are recombination processes. While bacteria do not necessarily require genetic recombination for their reproduction, recombination is generally

recognized to serve both for repair of DNA damage and for the procreation of genetic variation as a prerequisite of biological evolution. The haploid nature of bacteria and of their viruses highly facilitates studies on genetic variation, since new mutations become phenotypically manifested rapidly. In addition, bacteria present the advantage of forming large populations in a short time which represents ideal conditions for population genetic studies.

In this review we discuss the application of molecular genetic methodology to bacteria kept under different life conditions. We refer mainly to investigations carried out in our laboratory both with resting bacteria and with bacterial populations submitted to periodic changes of their life conditions. Emphasis is thereby given to the

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identification of genetic rearrangements which may occur either in the chromosome or in plasmids. The latter open the possibility to also study lethal mutations and to identify molecular mechanisms involved in their production. On the basis of evidence gained in such studies, knowledge on the overall relevance of enzyme-mediated DNA rearrangements for genetic variation and therefore for biological evolution can be refined.

The life of microorganisms is not limited to exponential growth

Bacteria reproduce exponentially by cell division as long as unlimited sources of nutrition and energy are available. In the presence of a good growth medium, *E. coli* bacteria routinely grow with generation times of 30 min. This leads every 5 h to an increase in the cell number by a factor of 1000. If an exponentially growing population of bacteria would be maintained in an unlimited volume of growth medium, a total of 10^{30} individual cells could accumulate from an inoculum with a single cell within only two days. 10^{30} roughly corresponds to the total number of living cells on the planet earth. This number is based on the known average cell volume and on an estimation of the total volume of all organisms living on and near the surface of the earth. To do so, we assume that all living cells, if densely packed, would form a layer of 20 cm thick over the entire planet. (However, if this layer was 2 m thick, the number of cells would be 10^{31} , which does not make a principal difference.)

In view of these considerations it is obvious that only a minor part of the life of bacteria can be devoted to exponential growth. More common are alternating phases of growth and rest, whereby the periods of rest must represent the more important part in terms of time. Still relatively little is known on physiological functions expressed and maintained in the resting phase and on how a reduced physiological activity can manage to keep the bacteria alive during periods of starvation [1–3].

Possibly an extreme condition of starvation is found in stab cultures. Stabs offer a quite conve-

nient way of long-term storage of bacterial cultures. A typical stab culture is made as follows. A complete, but not too rich, growth medium in soft agar is introduced into a small vial of a few ml volume. The medium is inoculated with a loopful of a suspension of bacteria to be preserved by stabbing the platinum loop all the way down to the bottom of the solid medium. Upon overnight incubation at 37°C, the inoculum gives rise to a turbid trace through the solid medium. At this time the tube is closed either by a paraffinated cork or by an airtight screw cap. Upon storage at room temperature, many bacterial strains are maintained alive during several decades. Under these conditions there is obviously no possibility for extensive growth of the bacteria. At most they can undergo very residual growth and this might occur at the expense of dying cells. As will be shown below, genetic variation increases in stab cultures as a function of time of storage. This variation is largely attributable to the transposition of mobile genetic elements residing in the bacterial genome. Transposition is an enzyme-mediated process [4]. Genetic variation observed in resting bacteria thus testifies on specific physiological activities under very nutrient-limiting living conditions.

Transposition of mobile genetic elements is a major source of lethal mutagenesis both in resting bacteria and in bacteria undergoing alternating phases of growth and rest

The study of lethality of mutations has suffered for a long time from being limited to statistical investigations. However, microbial genetics does offer a means to specifically study the cause of the appearance of individual lethal mutations. The experimental strategy is based on the detection of loss of function exerted by accessory elements which are inessential for the life of the host cell. An interesting application of this strategy is the monitoring of the loss of the ability of lysogenic bacteria to produce plaque forming bacteriophage upon induction of the vegetative cycle of phage reproduction. This strategy has been successfully applied to P1 lysogens of *E. coli* K12

[5]. The P1 prophage in P1 lysogens is carried as a plasmid. Only a few phage genes are needed for maintenance of the plasmid in lysogenic bacteria. In contrast, about 60 of the genes carried on the P1 genome are essential for vegetative reproduction of the phage [6]. If any of these essential genes is hit by a lethal mutation, the prophage can normally still be maintained and propagated in its plasmid form. However, upon induction of phage reproduction, no viable phage particles can be produced by such mutant prophages. Production of active phage particles by individual subclones of P1 lysogens can easily be monitored by classical replica plating techniques with the use of appropriate indicator bacteria. This method was applied to subclones of P1 lysogens isolated from stab cultures and it was shown that some subclones were unable to produce active phage particles upon induction although the P1 plasmid was still present. Often these lethal effects could be attributed to deletion formation mediated by a residential *IS1* element on the P1 prophage. Almost as frequently, an IS element had transposed from the host chromosome onto the P1 prophage and thereby inactivated one or several genes essential for phage reproduction [5].

In further studies, a P1 derivative devoid of a residential *IS1* element was used and the search for lethal mutants was extended to bacterial cultures kept under alternating cycles of growth and rest [7]. Under these conditions, the influence of transposition on the genetic variation could not only be confirmed but also quantitated. As for stab cultures, bacterial populations submitted to alternating cycles of growth and rest accumulated an increasing number of lethal mutations. As an example, a lysogen was kept under alternating growth and rest by a 20-fold dilution every few days. After a total of about 100 generations, 0.3% of individual colonies had lost their ability to produce plaque forming phage. In all cases, the prophage was still present and, in 95% of all mutants analysed, the prophage had suffered the insertion of an IS element which had been transposed from the host chromosome into the prophage. Hence, transposition of mobile genetic elements is the major cause of lethal mutagenesis in bacteria both upon prolonged rest and upon

rest periodically interrupted by phase of growth. The latter condition may best mimic living conditions encountered by bacteria in their natural environment.

The physical mapping of the insertion sites of IS elements in the genome of lethal P1 mutants resulted in interesting insights into the criteria for target selection by IS elements. Indeed, the overall distribution of identified insertion sites on the 90 kb long P1 genome is far from being random [8]. Rather there are some hot regions for IS insertion, and in addition not all IS elements behave in the same way. The IS element encountered most often in these studies was *IS2*. This mobile genetic element showed a pronounced regional target specificity. Indeed, a particular region of the P1 genome was about 20-fold more often used for *IS2* insertion than would correspond to the average. Other regions were only infrequently hit. However, in the preferred region of insertion, individual insertion sites of *IS2* were almost randomly distributed, and different insertion targets showed no sequence homology among each other [9,10]

In contrast to *IS2* and most other mobile genetic elements such as *IS1*, *IS3*, *IS5* and $\gamma\delta$ which were encountered in these studies, *IS30* showed a very pronounced target specificity. This element was identified three times to have caused phage lethality. In these three independent strains the *IS30* element resided exactly in the same location on the P1 genome between the same two base pairs [11]. In two cases, the element was carried in one direction and in the third case in inverted orientation. It should be mentioned, however, that under experimental conditions allowing selection of *IS30* insertion, other sites for *IS30* insertion in the P1 genome were also identified [11]. Hence, *IS30* insertion does not only occur at unique specific DNA sequences.

Structure and functions of *IS30*

In many respects, *IS30* resembles other IS elements. Its length is 1221 bp, the ends of which form inverted repeats of 26 bp length with three mismatches [12]. A long open reading frame pre-

ceded by an *E. coli* transcription promoter covers most of the *IS30* sequences and encodes the transposase [13]. Expression of the transposase gene appears to be controlled by several strategies. These include premature transcription termination internal to the transposase gene [14] and inhibition of expression by an antisense RNA which is produced from the complementary DNA strand and located roughly in the middle of the transposase gene (A. Arini, M. Keller and W. Arber, to be published).

Interestingly, the gene product of the large open reading frame of *IS30* is bifunctional. It not only mediates *IS30* transposition, it also catalyses site-specific recombination. Targets for such recombination are the ends of the *IS30* element. This was seen in investigations with an artificially made *IS30*-mediated transposon which consisted of a chloramphenicol resistance determinant carried between two *IS30* elements in the same orientation [15]. The observed instability of this transposon was explained by the fact that, once in a while, a site-specific deletion formation between the two internal ends of the flanking *IS30* elements removed the centrally located selectable gene. The resulting product was called *IS30* dimer or $(IS30)_2$. This is a tandem form of *IS30* with a

spacer between the two *IS30* elements of usually two base pairs which were left over upon the deletion formation. Interestingly, the $(IS30)_2$ structure was highly active in transposition. Indeed, with frequencies in the order of 10^{-1} per generation, again *IS30*-mediated site-specific deletion formation yielded a unique copy of *IS30* at the former site of residence of $(IS30)_2$. Alternatively, and with frequencies of about 10^{-3} per generation, the intermediate $(IS30)_2$ form underwent inverse transposition in which the two base pair spacer segment was lost and the target underwent a two base pair duplication which is characteristic for *IS30* transposition. If such transposition occurred intramolecularly, the result was sometimes deletion formation and sometimes DNA inversion. Intermolecular inverse transposition of $(IS30)_2$ to a target site carried on another DNA molecule mimicked cointegration, but the spacer base pairs were lost and target duplication had occurred.

Figure 1 summarizes the main pathways followed by *IS30* in its *IS30*-mediated DNA rearrangement processes. The Fig. also includes a proposed hypothesis that a single *IS30* element might become duplicated in a rare event either upon DNA replication or by dimerization of the

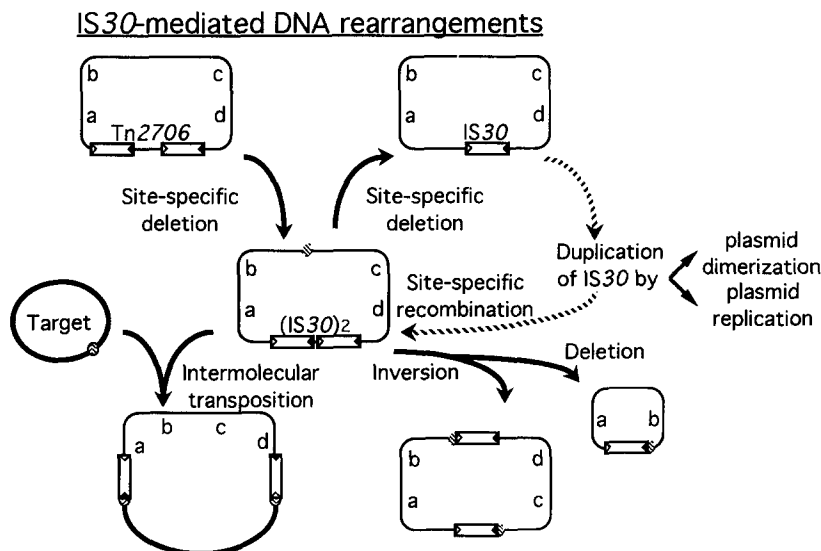


Fig. 1. *IS30*-mediated DNA rearrangements. The drawing schematically shows *IS30*-mediated pathways of DNA rearrangements and to the right the postulated (dashed arrows) possibilities for the generation of $(IS30)_2$ dimers from a single *IS30* element. This model refers to experimental evidence obtained with multicopy plasmids after [15].

carrier DNA molecules. The resulting structure would resemble the artificially made *IS30* transposon shown on the left of the Fig. and this could eventually give rise to the highly transposable $(IS30)_2$.

Once $(IS30)_2$ is formed, it has a half-life which is somewhat longer than the generation time of the carrier DNA. As explained in Fig. 1, different pathways can lead to the loss of the $(IS30)_2$ structure. While site-specific deletion formation reproducibly results mostly in identical stable structures, the variability in the inverse transposition process is obvious because of the flexibility in the selection of the target for insertion. For these reasons, $(IS30)_2$ once formed can give rise in the progeny of the host cell to a burst of DNA rearrangements with multiple possibilities for differently rearranged DNA sequences.

Restriction fragment length polymorphism (RFLP) can serve as an indicator for increasing genetic variation of resting populations of bacteria

Structural plasticity of the bacterial chromosome can be revealed by an RFLP analysis. The underlying strategy is to analyse individual subclones from a bacterial population originally derived from a single cell. Cultures grown from individual subclones serve for the extraction of DNA which is then cleaved by a restriction enzyme, such as *EcoRV*. The DNA fragments are separated by electrophoresis and then submitted to Southern hybridization. Various chromosomal DNA sequences of an approximate length of 1 kb can serve as hybridization probes. In the studies to be discussed here, hybridization probes were either prepared from internal sequences of IS elements or from unique chromosomal genes.

When this method was applied to single colonies obtained from a freshly cloned bacterial culture, practically no polymorphism could be detected. In contrast, old bacterial cultures showed a pronounced polymorphism. This was very clearly seen with old stab cultures, particularly those which were stored for 10–30 years at room temperature. We refer here to a study



Fig. 2. Pedigree of 118 subclones isolated from a 30 years old stab culture as deduced from the RFLP patterns of their isolated DNA probed with 8 different IS sequences after [16]. 55 subclones displayed each a unique RFLP pattern. For the rest, the number of subclones showing the same RFLP pattern is indicated.

carried out with subclones isolated from the *E. coli* K12 standard strain W3110 after its storage for 30 years in a stab collection [16]. In this bacterial population the degree of genetic polymorphism was such that it was difficult to identify the postulated parental form of the chromosome (for illustration, see Fig. 2). As a matter of fact, the studied subclones had undergone an average of 12 ± 2 structural alterations. A very high polymorphism was revealed upon use of IS5 sequences as a hybridization probe. Most other residential IS elements also contributed to genetic polymorphism, while little or no polymor-

phism was seen with unique chromosomal gene sequences as hybridization probes. Interestingly, IS30 remained quite stable in part of the subclones, while about one third of the analyzed subclones had undergone a burst of IS30 transposition as testified by the gain of several new DNA fragments able to hybridize with IS30 sequences. A number of independent arguments indicated that the RFLP observed upon use of IS sequences as hybridization probes was a direct result of transpositional DNA rearrangement activities rather than of other sources of mutagenesis. Therefore, transpositional DNA rearrangements must be an important source of genetic plasticity in *E. coli* bacteria. This is in line with data obtained for spontaneous mutations affecting single genes [17,18].

In the experimental approach described here, one looked at viable cells rather than at lethal effects. Therefore these experiments yielded results complementary to those of experiments carried out with the P1 prophage in which lethal mutations had been screened for, as explained above. One can conclude from these two approaches that IS transposition plays a role in the production both of lethal mutants and of non-lethal spontaneous sequence alterations which may or may not affect the physiology of the concerned bacteria. When subclones with specific RFLP patterns were characterized for their physiological properties, some of the subclones did not show any detectable alterations of their traits. Others had lost certain abilities and/or showed negative selection coefficients in competition experiments with a standard subclone of the same original bacterial strain. In several instances, positive selection coefficients were found and in these cases a specific RFLP pattern was usually present in several different subclones studied. This indicates that these specific mutants had undergone residual growth in the stab which might have been correlated with an acquired better fitness under the encountered living conditions. In contrast, most of the other studied subclones showed unique RFLP patterns.

An interesting result from the comparison of genetic plasticity of bacterial strains kept for various times in a stab collection indicates that RFLP

increases linearly with time of storage of a culture. This suggests that resting bacteria are steadily subjected to enzyme-mediated DNA rearrangements during their stay in the resting phase. This conclusion has obviously an interesting ecological and evolutionary implication, i.e. spontaneous mutagenesis is not limited to growing bacteria, but also occurs in resting microorganisms. Similar conclusions were reached a long time ago by completely different experimental approaches not allowing to identify the molecular mechanism of mutagenesis [19].

Genetic variation results from the action of a multitude of different molecular mechanisms including DNA rearrangements and gene acquisition

Transposition is only one of the different sources of genetic variation. A more complete picture of spontaneous genetic alteration is drawn in Fig. 3. The specific molecular mechanisms causing spontaneous mutagenesis are grouped into four categories [20,21]. Reproductive infidelity is a major source of nucleotide substitution and of other local variation of DNA sequences. The second category englobes both external and internal environmental mutagens. Depending on their specificity of action, their effects can be

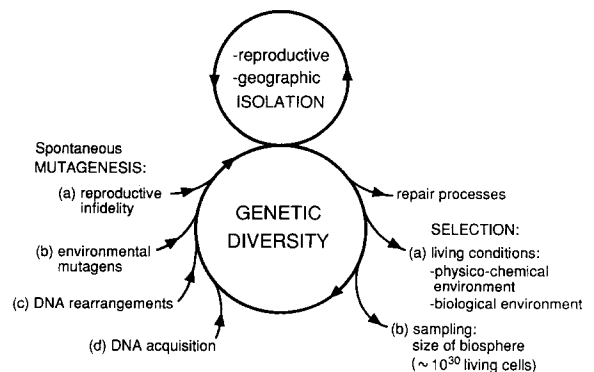


Fig. 3. Schematic representation of overall genetic diversity resulting from a steady production of new DNA sequences by various molecular mechanisms on the one hand and from the elimination of newly formed sequences by repair processes and most importantly by natural selection on the other hand.

local or involve extended sequences. A third category is labelled DNA rearrangements. Besides the already discussed transpositional DNA rearrangements, homologous, site-specific and 'illegitimate' recombinations contribute to this source of genetic variation. Interestingly, IS elements present in more than one copy in DNA molecules passively offer means to increase genetic diversity by presenting their sequences inserted at different locations as extended homologies, at which general recombination can act. This can result in sequence duplications. Site-specific recombination systems contribute to the formation of unique genetic variants by the occasional use of secondary crossover sites [22,23]. This process can be an important evolutionary source of gene fusions and operon fusions. The fourth category of sources of spontaneous mutagenesis is DNA acquisition, i.e. the horizontal or lateral transfer of DNA sequences from one strain of bacteria to another. The well studied transfer processes include transformation, conjugation and phage-mediated transduction. DNA acquisition is probably one of the most efficient ways available to microorganisms to extend their genetic capacities. This is convincingly documented by the efficient and rapid spread of drug resistance traits to enterobacteria following the onset of the worldwide use of antibiotics in human and veterinary medicine, which represented a drastic change in selective conditions.

Figure 3 also symbolically illustrates the respective roles of selection and of repair processes which both reduce the genetic diversity generated by the spontaneous alterations of DNA sequences. Finally, both reproductive and geographic isolations limit, to some extent, gene flow and this can have its influence on the overall genetic diversity both in negative as well as in positive ways.

Is biological evolution the result of accumulated errors or of specific biological functions promoting genetic variation?

Although the answer to this question remains to some degree a matter of attitude of the ob-

server of the evolutionary process, it becomes more and more clear from molecular genetic studies that several of the major contributions to genetic variation are brought about by enzyme-mediated molecular processes. If enzymes are involved, one should accept that these enzymes are also the result of a long evolutionary process in which steady selection had been exerted on the functions of gene products. For genes whose products are mainly involved in the evolutionary process, selection must be exerted at the level of populations rather than at the level of individuals. For instance, the widespread presence of transposable genetic elements and site-specific recombination systems in living organisms may have been useful in the past evolutionary development of these organisms. This would argue against the elimination of the genetic information for such activities from evolving populations. Rather, a steady presence of a certain number of different generators of sequence varieties might provide means for adaptation and successful evolution. Gene products involved would be expected to act as variation generators, irreducibly as far as the specific DNA sequences resulting from the process are concerned. This is exactly what transposition as well as other DNA rearrangement systems do, at least in parts of their activities. Since the natural formation of mutants is not directed towards a particular goal of evolution, variation generators act to some degree randomly and the products of genetic variation are most of the time not providing increased fitness. Rather, individual cells having suffered a genetic alteration may often show decreased fitness or are lethally affected. Therefore biological functions which meet evolutionary needs and become only relevant at the population level might appear undesirable if one considers individual lives separately, e.g. that of a bacterial cell from one generation to the next. For these reasons, one could classify biological functions into two large categories: those which meet the needs of individual lives and those which meet the evolutionary needs. In the first category we classify all housekeeping genes and other functions needed for the accomplishment of life processes between one generation and the next.

In contrast, the second category of biological functions is not required for individual lives, but they accomplish long-term duties for maintenance and evolutionary progress of populations. Of course, some gene products can serve both purposes.

Conclusions

Methods of molecular genetics and population genetic analysis are appropriate means to refine our knowledge on the evolutionary process of microorganisms. Many of these methods can be applied either to pure cultures kept under defined life conditions, to cultures submitted to a sequence of different life conditions as well as to mixed competing populations under various selective forces. While one cannot observe mutagenesis processes exerted with quite low probability in individual cells, it is possible to conclude on specific variation effects by a molecular analysis of the progeny of cells having suffered a genetic alteration. The application of this strategy of analysis can give insights into biological functions acting as variation generators. It is likely that individual variation generator systems act with specific efficiency only at some specific life conditions. It has now become clear that some of these systems are steadily active in the resting phase of microorganisms. Results obtained with the approach described are expected to contribute to the comprehension of genetic adaptation and evolution in natural environments in which many different life conditions alternate steadily.

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