

Role of plasmid multimers in mutation to tetracycline resistance

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Summary

As an additional system for analysing mutations that appear to be specifically induced or directed, we have used a plasmid that contains the *mnt* repressor gene inserted as an operon fusion with the *tet* gene of the plasmid pBR322. Thus, the *mnt* gene product acts as a negative transcriptional regulator of *tet* gene expression. Mutations inactivating the Mnt repressor are recessive while those destroying operator recognition (O^c) are dominant in conferring tetracycline resistance on the host. When resistance mutations were isolated on plates with high levels of tetracycline they were preferentially *mnt*⁻ and the plasmids were monomers. Pre-exposure to low concentrations increased the frequency of resistant mutants by 100- to 1000-fold, and the mutations were now mostly O^c , located on one unit of a plasmid multimer. Recessive repressor mutations on one unit would not have been selected. We suggest that the high frequency of mutation in tandem multimeric plasmids may be caused by the formation of single-stranded and hence highly mutable regions by homologous pairing out of register. The role of tetracycline in promoting mutations is discussed.

Introduction

Several cases of adaptive mutation apparently directed by selective agents in the culture medium have been reported. The first observation was from an *Escherichia coli* strain in which the *araC* regulatory gene was placed upstream of the *lacZ* structural gene but separated from it by a defective phage Mu (Shapiro, 1984). When arabinose was present as an inducer, the excision of Mu

resulted in a mutant cell able to grow on lactose; these mutants have been termed Lac(Ara)⁺, and the parental strain as Lac(Ara)⁻ (Cairns *et al.*, 1988). The Lac(Ara)⁺ mutation did not occur when cells were grown under non-selective conditions (the frequency was lower than 10⁻¹¹) but when plated on medium containing arabinose and lactose, Lac(Ara)⁺ colonies appeared after several days of incubation (Shapiro, 1984; Cairns *et al.*, 1988).

Recent work has shown that these apparent directed mutations are, in some cases (Mittler and Lenski, 1990) but not in others (Cairns, 1990), the result of an increase in the frequency of phage Mu excision owing to starvation stress in general, rather than a result of a specific induction of Lac(Ara)⁺ variants. But even with this example, there remain other examples of adaptive mutation that do appear to be directed by selection pressure; for example, the reversion of a *lac*_{am} mutation in certain strains of *E. coli* (Cairns *et al.*, 1988) the precise excision of Tn3 from the *ilv* operon (Boe, 1990); the activation of the cryptic *bgl* operon (Hall, 1988); and the reversion of point mutations in the *trp* operon (Hall, 1990).

Mutations to antibiotic resistance are generally isolated as colonies that appear after the parent strain has been plated in the presence of the antibiotic. Since most of these mutations require a certain time to be phenotypically expressed, only cells that were already resistant before the plating, *i.e.*, pre-adaptive mutants, can ordinarily give rise to colonies. However, if the parent strain is exposed to low concentrations of the antibiotic before being plated on the inhibitory concentration, directed mutational events might be detected.

We have investigated the formation of tetracycline resistant mutants in a strain of *E. coli* (JM103; Yanisch-Perron *et al.*, 1985) harbouring a plasmid, pPY97, that carries the *mnt* (maintenance of lysogeny of phage P22) repressor gene in a fusion that places the *tet* (tetracycline-resistance) gene under the control of the *mnt* gene product (Rewinski and Marinus, 1987). Mutations that inactivate the *mnt* repressor, or O^c mutations in the operator that destroy Mnt recognition, both allow transcription, rendering the host tetracycline-resistant; the former are recessive and the latter dominant. This system is easy to analyse and is sensitive to many different kinds of spontaneous mutations (Rewinski and Marinus, 1987). Surprisingly, we found that the pre-adaptive mutations occurred in the *mnt* gene on monomeric plasmids, while

the post-adaptive mutations were almost exclusively isolated in the operator region on multimeric plasmids. The importance of the multimeric plasmids suggests a novel mechanism for mutations.

Results

Isolation of tetracycline-resistant mutants

When tested in liquid medium, low concentrations of tetracycline (0.5–1 mg l⁻¹) resulted in a 10 to 50% reduction in growth rate of the JM103 strain harbouring pPY97. When challenged with high concentration (8 mg l⁻¹) growth was immediately arrested. The colony-forming units on plates containing the low concentration of tetracycline were reduced by a factor of 10 to 100 compared with medium without tetracycline when the plates were examined after overnight incubation. Prolonged incubation increased the number of colony forming units. On plates containing high concentration of tetracycline only mutants resistant to tetracycline were able to form colonies overnight.

The mutations obtained on plasmid pPY97 that render the host strain (JM103) tetracycline resistant were isolated from independent cultures as follows: pre-adaptive mutants were isolated by plating cells on Luria–Bertani (LB) medium not containing tetracycline, incubated overnight, and replica plated to a medium containing a high concentration of tetracycline (8 mg l⁻¹). About 10⁸ parental cells gave rise, on the average, to 10 mutant colonies. There was a positive correlation between the number of cells plated and the number of mutants isolated, but this was not linear. This indicates that the number of mutants obtained is limited, not only by the number of cells plated, but also by the nutritional capacity of the plate.

Presumptive post-adaptive mutations were isolated by plating serial dilutions on LB medium containing 0.5 to 1 mg tetracycline l⁻¹, incubating overnight, and replica plating on medium containing 8 mg l⁻¹ of tetracycline. About 10⁶ parental cells gave rise, on average, to about 100 mutant clones. There appeared to be a linear correlation between the number of parental cells plated on the low concentration of tetracycline and the number of mutants obtained.

A number of independent experiments showed that the cells pre-exposed to low concentrations gave rise to 100- to 1000-fold more colonies resistant to high concentrations of tetracycline when normalized to the number of parental cells being plated (the actual mutation rates cannot be calculated from these experiments).

When plasmids were extracted from several of the post-adaptive mutants and reintroduced into the JM103 strain the resulting transformants could be established directly on 8 mg tetracycline l⁻¹. This rules out the pos-

sibility that the observed post-adaptive mutants cannot grow when suddenly challenged with a high concentration of tetracycline but can become physiologically adapted to tetracycline when pre-exposed to low concentrations.

Characterization of pre-adaptive and post-adaptive mutants

Fifty-five pre-adaptive and post-adaptive mutants from independent cultures were analysed in detail. Plasmids were extracted and transformed into an indicator strain (GM4331) that revealed whether the mutation mapped in the operator or in the *mnt* repressor gene. In addition, new plasmid lysates were made from this host and analysed on agarose gels to detect large insertions or deletions, and to determine the degree of plasmid multimerization. Plasmid multimerization state was determined from the mobility on agarose gels containing 0.4 mg ethidium bromide l⁻¹ (Summers and Sherratt 1984).

Of the 55 pre-adaptive mutants, 52 were *mnt*⁻ and three were *O*^c (inferred from the fact that they were not *mnt*⁻). Most were due to insertions (probably an IS element) in the *mnt* gene. All mutant plasmids were present predominantly as monomers, as would be expected because, the *mnt*⁻ mutations are recessive to *mnt*⁺. Consequently an *mnt*⁻ dimer requires a double mutation to express tetracycline resistance.

In contrast, 51 out of 55 of the post-adaptive mutants had mutations in the operator and, surprisingly, in 49 of 51 *O*^c mutants the plasmids were present as dimers, trimers, or tetramers. When these multimers were resolved to monomers by cutting at a unique restriction site (*Eco*RI) and religation, they all segregated into parental type (sensitive to tetracycline) and mutant (*mnt*⁺, *O*^c) plasmids. The dimers segregated into mutant and wild type in the ratio of 1:1, trimers in the ratio of 1:2, and tetramers in the ratio of 1:3. Evidently, each multimer had mutated in only one of the plasmid copies. These mutants could be recovered because *O*^c is dominant.

Sequencing of six post-adaptive *O*^c mutants showed that three were small deletions (2, 4, and 11 base-pairs) starting in the putative loop of the operator, two were single base changes, and one was a double base change in the vicinity of the putative loop of the operator. This suggests that most of the mutations occur in one step, though the double point mutation could signify that some of the apparently directed mutations are the result of two-step mutations as has been discussed by Lenski *et al.* (1989).

Mutation rate and plasmid multimerization state

The data above suggest that the frequency of mutations is coupled to the multimeric state of the plasmid DNA. Monomeric plasmids have a low mutation rate and the

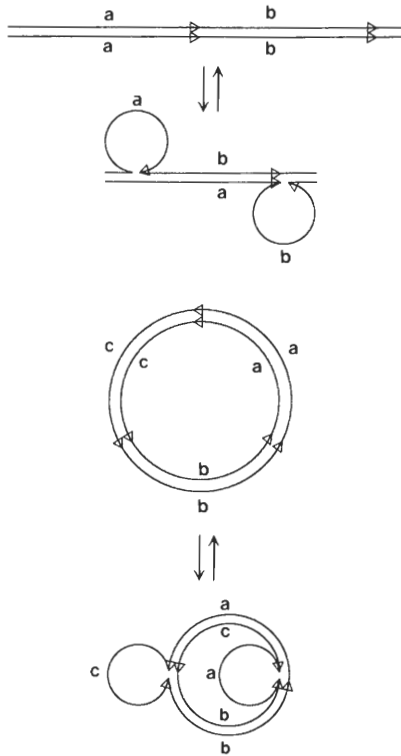


Fig. 1. A model to explain the high mutability of DNA in direct repeats or plasmid multimers. If a strand in one of the repeats can base pair with the complementary strand of one of the other repeats, one copy of the repeated sequence is rendered single-stranded. Since single-stranded DNA appears to be more prone to mutations, this could stimulate the formation of heteroduplex DNA, harbouring a mutation in one strand only. The plasmid multimer is presented as a trimer, but could, in principle, also be a dimer, tetramer, or a higher order of multimer. The repeats have been designated a, b, and c for clarity.

mutations are located in the *mnt* gene. Multimeric plasmids have a high mutation rate in the operator region.

A possible explanation for the high rate of O^c mutations on multimeric plasmids is that DNA present as direct repeats is more mutable than non-repeated DNA. To test this hypothesis directly, monomers, dimers and trimers of pPY97 were isolated from an agarose gel and transformed into a *recF* strain in which plasmid recombination is reduced (Fishel *et al.*, 1981). The mutation rate in cultures carrying each plasmid type was determined by fluctuation tests. The location of the mutations (*mnt* or O^c) was tested by transforming the indicator strain, GM4331, with 20 independently isolated mutant plasmids of each plasmid type. In addition, the plasmid multimerization state was determined by agarose gel electrophoresis of the mutant plasmids. The mutation rates are shown in Table 1. As seen, the rate of O^c mutations increased by about a factor of 10 when the plasmid multimerization state increased from monomer to dimer, or from dimer to trimer.

Discussion

The data presented in this paper show that the rate of O^c mutations depends on the plasmid multimerization state. As shown in Table 1, the rate of O^c mutation increased by about a factor of 10 when the plasmid state increased from monomer to dimer or from dimer to trimer. This increase cannot be due to variation in the number of operator sites, since the origin-counting hypothesis for regulation of plasmid copy-number (*cf.* Summers and Sherratt, 1984) predicts that the total number does not increase with plasmid multimerization. For example, a dimer is regulated to have half the copy number of a monomer, resulting in the same gene dose per cell. Inferred from the yield of plasmid DNA in lysates we found this to be the case for pPY97 (data not shown).

Most of the pre-adaptive mutants isolated in JM103 were *mnt*⁻ mutations on monomeric plasmids. This was unexpected because Rewinski and Marinus (1987) found that only four out of 518 tetracycline-resistant mutants were *mnt*⁻ using AB1157. The plasmid pPY97 is apparently more multimerized in AB1157 than in JM103. Thereby the isolation of the dominant O^c mutants is favoured in AB1157. The reason for the low frequency of plasmid recombination in JM103 is not known, but it may be due to the *sbcB15* allele in this strain.

Since DNA *in vivo* is supercoiled it is possible that direct repeats are in a single-stranded conformation part of the time as indicated in Fig. 1. It would also be expected that the higher order plasmid multimers would spend a longer time in a partly single-stranded form. Since single-stranded DNA is much more susceptible to mutation (*cf.* Davis (1989)), this could explain why the rate of O^c mutations increases with the plasmid multimerization state. Recessive *mnt*⁻ mutations were presumably also present in such multimers, perhaps in great excess over O^c (as in monomers), but they would not have been recovered.

Several models have been proposed to explain directed mutations (Cairns *et al.*, 1988, Stahl, 1988, Davis, 1989, Hall, 1990). Our suggestion for the mutability

Table 1. Mutation rates of O^c mutations in a *recF* strain harbouring pPY97 as a monomer, dimer, or trimer.

Plasmid state	Mutation rate
Monomer ^a	6×10^{-9}
Dimer	4×10^{-8}
Trimer	6×10^{-7}

a. Corrected for *mnt*⁻ mutations and dimerization: 20 independent mutants of each plasmid type were analysed. Only the plasmid monomers gave rise to *mnt*⁻ mutations (three out of 20 were *mnt*⁻). Even though the strain was *recF*, four out of 20 of the mutations from the strain initially harbouring monomers were isolated as dimers.

of tandem-duplicated DNA is related to the model proposed by Davis (1989) that since transcription requires strand-separation of the DNA template, thereby rendering parts of it single-stranded, transcription induced by substrates can create specific regions of increased mutability.

Plasmid multimers may represent a model system for DNA present in direct repeats. If direct repeats of DNA are more prone to mutation, the evolutionary importance is evident, since duplications not only provide supplementary copies that can evolve, but may also provide a mechanism, as long as they remain direct repeats, for accelerating that evolution. This finding may also have relevance to carcinogenesis. Oncogenes, for examples, are often found to be amplified in advanced tumors. Since mutational events in proto-oncogenes converts them to oncogenes, one could speculate that DNA amplification of proto-oncogenes is followed by a high mutability, thereby increasing the risk of developing a tumour (for a recent review on gene amplification, see Stark *et al.* 1989).

The role of tetracycline in the isolation of the mutations described in this paper is uncertain. Although the mutations were isolated as a consequence of the selective pressure, *i.e.* depending on the concentration of tetracycline used for their isolation, we would emphasize that there is no proof that they were the result of a directed or induced mutational event. At least four alternative explanations can be offered: (i) low concentrations of tetracycline would enrich for pre-existing, dominant O^c mutants, and not for recessive *mnt⁻* mutants, (ii) low concentrations of tetracycline might act as a mutagen, (iii) it could increase the plasmid copy number, thereby increasing the chance for a mutational event to occur, or (iv) promote the formation of single-stranded loops of DNA in direct repeats. At present, the available experimental data do not allow us to distinguish among these possibilities.

Experimental procedures

Bacterial strains and plasmid

Spontaneous mutations to tetracycline resistance were isolated on plasmid pPY97. It is a derivative of pBR322 that has the *mnt* repressor gene and its operator binding site of phage P22 inserted to produce an operon fusion with the *tet* gene. In this construct, the *tet* gene is transcribed from a promoter (p_{ant}) that is negatively regulated by the *mnt* gene product (Rewinski and Marinus, 1987). The *mnt* gene product acts as a dominant, *trans*-acting, negative transcriptional regulator of *tet*-gene expression by binding to an operator located at the startpoint of p_{ant} transcription (for more details, see Rewinski and Marinus, 1987).

The mutants were obtained in the *E. coli* strain JM103 (Δ (*lac-pro*), *thi*, *rpsL*, *supE*, *endA*, *sbcB15*, *hsdR4/F'traD36*, *proAB⁺*, *lacI^qZ* Δ -M15) (Yanisch-Perron *et al.*, 1985) and in an

AB1157-derived *recF* strain JC9239 (*recF143*, *thr-1*, *ara-14*, *leuB6*, Δ (*gpt-proA*)62, *lacY1*, *tsx-33*, *supE44*, *galK2*, *hisG4*, *rpsL31*, *kdgK51*, *xyl-5*, *mtl-1*, *argE3*, *thi-1*) (Horii and Clark, 1973).

mnt⁻ mutations were identified in strain GM4331 (also derived from AB1157), a lysogen containing a lambda phage with a p_{ant} -*lacZYA* transcriptional fusion (M.G. Marinus, unpublished data). If this strain is transformed with an *mnt⁺* plasmid the colonies on McConkey lactose plates are white; conversely, if the plasmid is *mnt⁻* the colonies are red.

Media and growth conditions

AB medium (Clark and Maaløe, 1967) was used as minimal medium and LB (Bertani, 1951) as complex medium. Proline, arginine, histidine, threonine, and leucine were added when required (50 mg l⁻¹). Ampicillin were added to select for the plasmid (50 mg l⁻¹), and tetracycline as indicated in the text. All experiments were performed at 37°C.

Standard genetic techniques

Plasmid lysates, agarose gel electrophoresis, transformations, and DNA sequencing were carried out according to standard laboratory protocols (Maniatis *et al.* 1982, Sanger *et al.*, 1977).

Fluctuation tests

The fluctuation tests were performed according to Luria and Delbrück (1943): cultures were grown overnight in minimal medium with varying concentrations of glucose as the limiting factor. An overnight culture was diluted in minimal medium and each of 48 wells in a micro-titre plate received 0.1 ml containing 100 to 1000 cells. After incubation overnight 0.1 ml LB medium containing 16 mg tetracycline l⁻¹ was added to each well, and the plates were again incubated overnight to enrich for mutants. Finally, part of the cultures were spotted on solid LB medium containing 8 mg tetracycline l⁻¹ using a device with 48 pins. Cultures harbouring mutants were identified by growth. The culture size at the time of tetracycline addition (usually between 10⁶ and 10⁷ cells) was adjusted by the initial glucose concentration so that between 10 and 30 cultures harboured no mutants.

The mutation rates were calculated from (i) the proportion of cultures that grew and, therefore, must have contained pre-adaptive mutants that could grow in the presence of 8 mg tetracycline l⁻¹, and (ii) the population size at the time of addition of tetracycline. A recent method that also makes use of the frequencies of mutants in the mutant-harboring cultures (Stewart *et al.* 1990) was not used as periodic selection has been observed in AB1157 derivatives (L. Boe, unpublished data) and this may strongly affect the frequency of mutants (*cf.* Atwood *et al.* 1951).

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