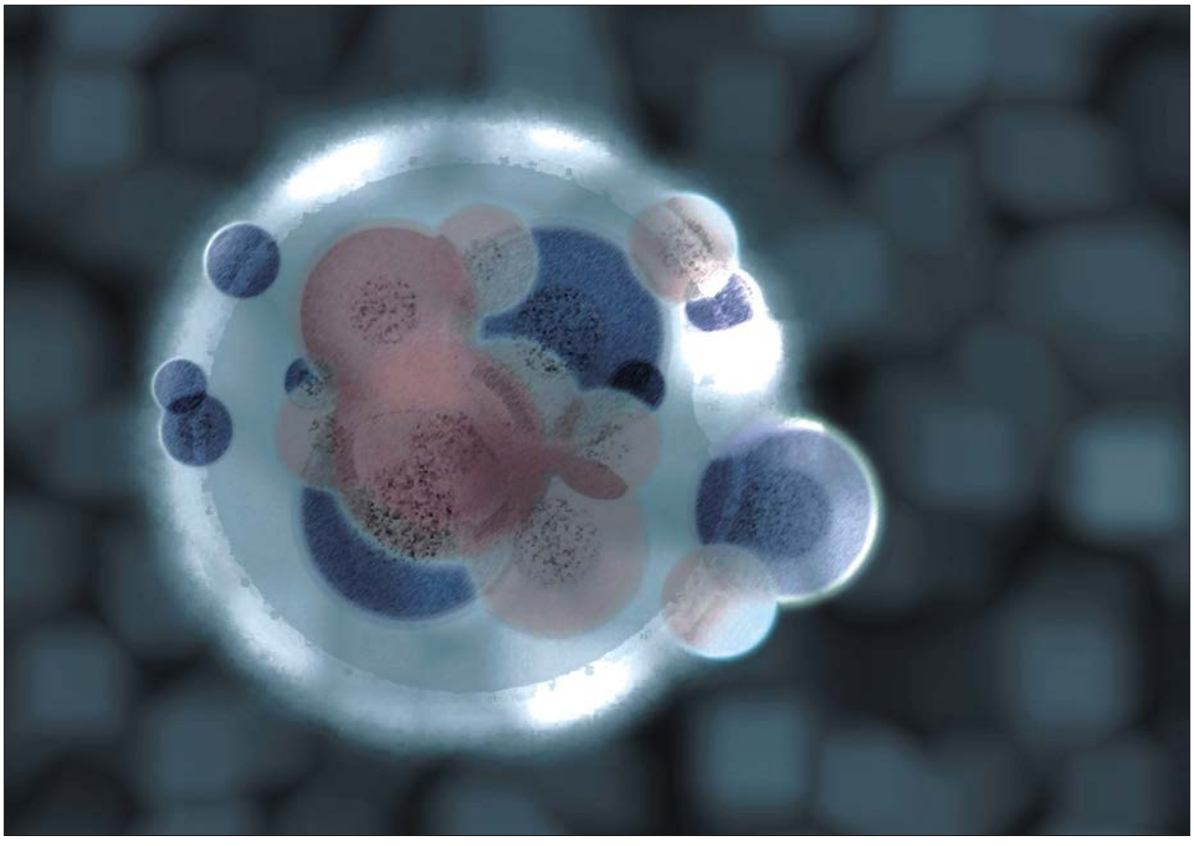

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 5 (2): 222-229, 2005
ISSN 1727-3048

Experimental and Theoretical Considerations of P1-plasmid Replication and Segregation During the *E. coli* Cell Cycle

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Abstract: Two contrasting experimental descriptions of P1-plasmid replication during the cell cycle of *Escherichia coli* have been described. One set of results led to the proposal that replication of P1-plasmid occurs at a specific time during the cell cycle over a wide range of growth rates and follows rules similar to that governing bacterial chromosome replication. Experiments supporting this proposal utilized membrane-elution experiments, radioactive double-labeling of DNA and scintillation counting of purified plasmids. An alternative experimental description of P1-plasmid replication during the cell cycle, also based on membrane-elution methodology but measuring radioactivity incorporated into plasmid DNA by autoradiography and scanning of films, proposed that P1-plasmid replicates at all stages of the cell cycle in rapidly growing cells, but with a slight periodicity or increase in P1 replication probability within the cell cycle of slower growing cells. These discordant experimental results are analyzed. It is concluded that the direct double-label counting approach is to be preferred, as the results are consistent with a large number of experiments, are supported by theoretical considerations and yield a unified view of plasmid replication over a wide range of growth rates. Theoretical ramifications of each view of P1-plasmid replication-cycle-dependent and cycle-independent are compared. An analysis of P1-plasmid segregation is also presented.

Key words: *E. coli,* plasmid replication, membrane-elution method, double-label, P1

INTRODUCTION

Experiments using membrane-elution to age-select cells, incorporation of radiolabeled nucleosides into plasmid DNA and direct counting of radioactivity in plasmid DNA as a measure of the rate of plasmid replication during the cell cycle led to a unitary rule governing P1-plasmid replication. Replication occurred at a particular time during the cell cycle when a particular cell size was reached[1]. This conclusion was based on numerous experiments, carried out over a large range of growth rates, showing that the time of P1-plasmid replication followed rules similar to those governing chromosome replication.

An alternative view of P1-plasmid replication during the bacterial cell cycle has been proposed in which the P1-plasmid can replicate at all stages of the cell cycle (i.e., replication is cycle-independent) at fast growth rates (25 min interdivision time) but that P1-plasmid displays a slight periodicity or increase in replication probability during the cell cycle of slower growing cells (40 and 60 min interdivision times). At more rapid growth rates no periodicity was observed[2].

Here we will examine the experimental bases for these contrasting views as well as the theoretical implications of these contrasting views in order to ascertain which experimental results are to be accepted as a description of plasmid replication during the cell cycle.

There are seven points that require discussion:

1. Comparison of the different experimental approaches to measure radioactivity in isolated plasmid DNA
2. Applicability of temperature arrest and release as a measure of plasmid replication during the cell cycle
3. Problems in using mini-plasmids as surrogates for large, natural plasmids in replication studies
4. Comparison of the number of experiments upon which the different replication hypotheses of P1-plasmid replication are based
5. Theoretical difficulties with a non-specific or random timing of low-copy (e.g., P1) plasmid replication during the bacterial cell cycle

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6. Unification of the pattern of replication over a range of growth rates to produce a consistent description of P1-plasmid replication that accounts for a wide array of data
7. Discussion of addiction/suicide fail-safe mechanisms as they relate to the problem of precise regulation of replication and segregation of the P1-plasmid

Comparison of autoradiography and scintillation counting: The major difference in assay methods between the two studies is the way radioactivity in the isolated plasmid was assayed. In both studies, exponentially growing cells were pulse-labeled with tritiated thymidine, the cells were placed on a membrane, the membrane was inverted and the bound cells were allowed to grow and divide on the membrane. Newborn cells were released at division. Newborn cells come off in reverse age order, reflecting the age of the cells at the time of labeling and subsequent binding to the membrane. The first newborn cells eluted from the membrane come from the division of the oldest cells at the time of labeling and binding to the membrane. With time, newborn cells are subsequently released from cells that were labeled at younger and younger cell-cycle ages. The eluted cells are collected, the plasmids are isolated and the plasmid radioactivity is determined.

If a plasmid replicated at a particular cell-cycle age, for example in mid-cycle at age 0.5 (newborn cells are age 0.0, dividing cells are age 1.0), then one would expect, at the time of pulse-labeling, that labeled plasmid would only be found in cells of approximately age 0.5. All cells on the membrane would have plasmid DNA, but only cells that replicated the plasmid in the presence of the radioactive thymidine would have labeled plasmid. For the example of mid-cycle plasmid replication, cells eluted for less than one-half of a generation would not contain any radioactive plasmid. Then cells that were age 0.5 at the time of pulse-labeling would divide and yield newborn cells containing radioactive plasmid. With further elution, newborn cells from cells younger than age 0.5 at the time of labeling/binding would not contain any radioactive plasmid. A peak of radioactivity would be seen approximately one-half of a generation after the start of elution, indicating that plasmid replication occurred at a specific cell-cycle age, in this case at approximately age 0.5.

This is not a synchrony experiment because the eluted newborn cells are not allowed to grow. Also, perturbations of the growing cells are not relevant to the experiment as thymidine labeling occurred during unperturbed, exponential growth.

If, in contrast to the example above, a plasmid replicated throughout the cell cycle, then newborn cells eluted from the membrane at all times during a cell-cycle of growth on the membrane would contain labeled plasmid, because there would be some cells of every cell-cycle age that would have replicated a plasmid during the labeling period. In this case, there would be no age-specific variation that is, no peak in radioactivity in labeled plasmid in the eluted cells.

As membrane-elution methodology was used in both experiments, the different results are presumably related to subsequent aspects of the experiment, such as the method of plasmid radioactivity assay. Bogan *et al.*[2] comment that the differences from the earlier studies[3] were ascribed to the use of autoradiographic procedures for assaying plasmid replication and the inclusion of both positive and negative controls for replication periodicity in all experiments[4]. Bogan *et al.*[2] suggested that their method, autoradiography coupled with a positive and negative plasmid control in each experiment, is the correct method for measuring plasmid replication during the cell cycle.

We agree with the proposal that the differences between the proposal of cycle-independent and cycle-dependent replication may be due to the use of autoradiography by Bogan *et al.*[2] but in contrast to the acceptance of the superiority of the autoradiographic method, we propose that the direct counting method used by Keasling *et al.*[1] is actually the superior method.

The autoradiographic approach used by Bogan *et al.*[2] involves isolation of the plasmid DNA from eluted cells, separation of the plasmid on an agarose gel, drying the gel and exposing the gel to an X-ray film. After a suitable period of exposure, the film is developed and the degree of film darkening due to radioactive decay of the incorporated tritiated thymidine is taken as a measure of plasmid radioactivity. The densities of the bands were determined by scanning the autoradiograms with a flat-bed scanner and the use of Adobe Photoshop and NIH Image 1.55 for quantitation[2].

Keasling *et al.*[1] also extracted plasmid DNA from eluted cells and separated plasmid DNA using agarose gel electrophoresis. But rather than using autoradiography to determine radioactivity, the plasmid bands were cut out and the radioactivity in the bands were determined by direct scintillation counting. To improve the quantitation of the assay, a counter-label was added to each sample (e.g., if the experimental cells were labeled with tritiated thymidine, then a constant amount of [^{14}C] thymidine labeled cells were added to each sample). The plasmids were thus isolated from a double-labeled sample. Plasmid labeling was therefore quantified by using the ratio of radioactivities in the plasmid band as an accurate measure of tritium label. This simple method eliminated problems such as losses during plasmid isolation or incomplete cutting out of the plasmid band from the agarose gel.

More important, the direct counting method is a linear method of radioactivity determination. The scintillation counter used was linear over many logs of activity and as the radioactivity actually counted was not near the top of the scale, the assay was clearly in the linear range.

In contrast, it is not clear that the autoradiography approach is linear. In order to demonstrate linearity (i.e., demonstrating that the measured darkening of the film was directly proportional to the amount of radioactivity present and the proportionality is independent of the absolute degree of film darkening) it would be important to have test samples of known radioactivity on the gel covering the range of radioactivity in the samples. It should be shown that the darkening of the film with the known samples was linear over the range of assay. Although the description of the assay method by Bogan *et al.*[2] mentioned that the dried gels were exposed for different time periods to get clear radioactive images at similar densities, there is no pictorial evidence to support this approach. In fact, in Fig. 1 of Bogan *et al.*[2] the minichromosome (the standard for cell-cycle-specific replication) is present at extremely low levels with only the peak samples evincing any strong signal, while the P1-plasmid (pZC176) was significantly darker over the entire range of the experiment. It would have been important to present a picture showing that at equivalent autoradiographic densities the pZC176 plasmid (the P1-miniplasmid) gave a less peaked, or unpeaked, pattern compared to the minichromosome.

We have looked at the autoradiographic approach in our laboratory and in our experience the quantitation of the radioactive bands is not as accurate as direct scintillation counting.

Temperature arrest as a measure of cycle-replication pattern: Support for the claim that the P1-plasmid does not replicate in a cell-cycle-specific manner is the use of a temperature shift to release a DNAC mutant from temperature arrest. Comparison of the P1-plasmid replication to minichromosome replication and to replication of a generally accepted randomly replicating plasmid, pBR322, was taken to indicate that the P1-plasmid did not follow the minichromosome pattern[2]. We deem that this assay has no relevance to the normal pattern of replication, as there are many explanations as to why the pattern of P1 replication did not mimic the minichromosome. Furthermore, if P1-plasmid and the chromosome initiate replication at different times during the cell cycle, then one may not expect the minichromosome and the P1-plasmid to have the same pattern of replication upon release from arrest.

But even within this experimental result one can see alternative explanations. The minichromosome, upon temperature release, gave a large peak of replication, going up to a high value of radioactivity and then a low value. The pBR322 did not give a sudden peak but replication was delayed for up to 20 min. What we find most striking is the fact that the P1-plasmid followed the same initial kinetics as the minichromosome (and thus different from the pBR322), but it failed to cease replication, as did the minichromosome[2]. We do not know why replication did not peak and cease. But we suggest that one could take the similarity of the initial kinetics of replication of the P1-plasmid and the minichromosome to indicate similarity in replication control. There may be something about the physiology of the P1-plasmid in the mutant cells when the temperature is lowered that did not allow P1-plasmid to cease replication. The temperature shift results are ambiguous. It is possible, without too much contortion, to have the plasmid follow the minichromosome pattern rather than the pBR322 pattern.

Problems in using mini-plasmids as surrogates for large, natural plasmids in replication studies: Another important difference between the study by Bogan *et al.*[2] and that of Keasling *et al.*[1] was the plasmid choice. Keasling *et al.*[1] used the native, full-length P1 prophage. This plasmid has been studied in detail and its replication and partition functions are well known. Bogan *et al.*[2] used pZC176, a mini-P1-plasmid that has not been described in the literature but that presumably contains all of the P1 replication and partition elements. The description of this mini-P1-plasmid states only that the plasmid contains the *parABS* genes, which are known to be involved in P1 partition, not replication. Inclusion of all elements involved in native P1 replication, segregation and maintenance is essential if a comparison is to be made between the native P1 prophage and a mini-P1-plasmid. In the absence of one or more of these elements, the replication pattern could be changed significantly. Because the mini-P1-plasmid (pZC176) construction was not described and has not been published in the literature, it is difficult to know if this mini-P1-plasmid replicates in the same manner as the native P1 prophage.

Furthermore, it is not known if the antibiotic resistance genes included on the mini-P1-plasmid (Amp^r and Sm^r/Spc^r) were isolated from the P1 origin of replication using transcription terminators to prevent transcriptional read-through into the plasmid origins. Any transcription read-through into the origin of replication could initiate plasmid randomly. Finally, although the copy number of pZC176 was reported to be 1.2 copies/chromosome equivalent (again unpublished data), the reader is given no other details about the nature

of replication. Does the plasmid replicate bi-directionally? Without these details, it is really impossible to compare results using the mini-P1-plasmid with the full P1 prophage.

Because two different plasmids were used in these two conflicting studies we suggest that the experimental differences may be due solely to the different plasmids. Thus, the experimental data in both cases may be accurate but the different conclusions may be due to the different plasmids.

Comparison of number of measurements and range of growth rates: A major difference between the experiments of Bogan *et al.*[2] and Keasling *et al.*[1] is the number of experiments performed. Keasling *et al.*[1] present the results from ten independent experiments over a range of growth rates from a 40 min interdivision time to an interdivision time greater than 300 min, while only three growth rates (25, 40 and 60 min interdivision times) are presented by Bogan *et al.*[2]. In all of the experiments of Keasling *et al.*[1] a peaked pattern of P1-plasmid replication was observed. While one might be able to imagine an ad hoc explanation of how patterns of replication are affected by growth rate, we feel that explanations set up solely in response to a particular situation or problem, without considering wider issues is not the correct approach to understanding plasmid replication. First, there is no precedent for this situation, as chromosomal replication as well as the low-copy F-plasmid replication appear to follow rules that are consistent over a wide range of growth rates.

Overall theoretical analysis of replication pattern: More important are the theoretical implications of the results of Bogan *et al.*[2]. Who propose that P1 replication is not coupled to cell mass as is the minichromosome but instead replicates throughout the cell cycle without a higher probability when a given mass per plasmid is reached. If this analysis correct, they go on to propose that it is likely that cell mass is not the critical parameter but perhaps the cellular concentration of RepA protein is the critical component for initiation of replication.

It is important to consider the implications of cell-cycle-independent replication of P1-plasmid. Given that the average copy number of P1 is known to be approximately one per chromosome equivalent, P1 very likely doubles in copy number during the division cycle, from one plasmid per cell to two plasmids per cell. For a cycle-independent pattern, with a constant replication probability during the cell cycle, one would expect a large fraction of plasmid-free cells. This is because in the population one can envision that during each fraction of the cell cycle a constant fraction of the cells replicate their plasmid once. As the end of the cell cycle approaches, those cells that have not replicated a plasmid even once would suddenly be required to replicate the plasmid in a deterministic manner. Thus, a cycle-independent and a cycle-dependent approach to plasmid replication would be needed to ensure that every plasmid replicates prior to cell division. If the two types of replication patterns did not co-exist, there would be a number of cells that would divide prior to plasmid replication leading to the appearance of plasmid-free cells. (Below we will deal with various fail-safe mechanisms that have been put forward to explain the absence of plasmid-free cells without invoking rigorous replication control or segregation control).

More troubling is the mixing of a cell-cycle-specific pattern at some growth rates and a random pattern at other growth rates. We suggest that a consistent control mechanism is expected, not one dependent on the growth rate of the cell.

This cell-cycle-independent conclusion should be contrasted to the cycle-specific conclusions of Keasling *et al.*[1] where the data plotted over ten experiments fit a pattern of replication during the cell cycle that indicated that the rules governing plasmid replication were similar to the minichromosome and the chromosome. Thus, when the age at initiation of replication for both the plasmid and the minichromosome are plotted against the inverse of the doubling time (i.e., the growth rate), one gets parallel lines. A replotting of the minichromosome data and the P1 data to determine the cell-cycle time of P1 replication as a fraction of time between rounds of minichromosome replication gave a flat line when plotted as a function of the growth rate[1], indicating that the timing of P1-plasmid replication during the cell cycle was always one-half a generation away from minichromosome replication. This result implies that the rule governing P1-plasmid replication is similar to that of the minichromosome or the chromosome and reflects, in some way, the amount of cell mass (or something that is a constant fraction of cell mass) being the triggering material that initiates plasmid replication.

Contrasting views of plasmid replication: If the results of Bogan *et al.*[2] are accepted, with low growth rate cells having a cell-cycle-specific pattern of low-copy plasmid replication and high cell growth rates having a random pattern, there would presumably have to be a particular growth rate at which a switch in controls occurred. It is hard to imagine how this could occur since there does not appear to be any growth rate at which there is a major change in replication pattern for any other plasmid. Thus,

over a range of growth rates both the minichromosome and the chromosome have the same control mechanisms during the cell cycle.

For this reason we suggest that a unitary explanation that presents the same control system over all growth rates[1] is to be preferred over a pattern of replication that changes with growth rate[2].

A detailed and relatively precise argument favoring a mixed pattern for plasmid replication (i.e., one containing both cycle-dependent and cycle-independent patterns) has been proposed by Bogan *et al.*[2]. Because this viewpoint underlines, accentuates and heightens the differences between the conclusions presented here, we shall describe this mixed proposal in detail and discuss why we do not accept this proposal.

The argument is explicitly based on the difference between 1 and >1 copies per newborn cell, yet still considering these to be cases of low-copy-number plasmids. (We agree with this distinction, that low copy numbers can be as high as 6-8 plasmids per cell, with high copy numbers being significantly greater.) Thus, even among low-copy plasmids, the number of plasmids per cell may range from 1-2 during the division cycle of slow growing cells to 4-8 during the division cycle of more rapidly growing cells.

The argument for a mixed pattern assumes that a plasmid has a sharp regulation of its concentration so that the probability of replication per unit time increases when plasmids are diluted below a threshold concentration. If cells typically have 1 copy at the beginning of the cell cycle, the plasmid concentration will be diluted by cell growth until the concentration is so low that replication occurs. Hence, it is argued, in a synchronized population of cells, this single plasmid will replicate at a cell-cycle-specific time point because cells generally reach approximately the same cell size at a particular time during the cell cycle. However, if plasmids are present at more copies per cell, for example the plasmids go from 3 to 6 copies during the division cycle, the same mechanism would automatically spread out the replications throughout the cell cycle because dilution is continuous. This would result in three peaks or instances of replication. Further, this analysis contends, if replication control does not respond sharply to concentration changes, the timing of replication would not be consistent in all cells and there would be no 'peaks' in replication. This analysis is put forth to explain the existence of cycle-specific replication at slow growth rates (with very low plasmid copy number) and the existence of a cycle-independent pattern of replication at more rapid growth rates (with a slightly elevated plasmid copy number).

The argument for this mixed explanation continues by noting that without any ad hoc assumptions about cell-cycle specific mechanisms and assuming the proposed view of plasmid replication, the replication pattern will automatically depend on copy number especially the difference between 1 and >1 copy per cell and thus depend on growth rate. Because copy numbers in turn depend on growth rate, with 1-2 copies per slowly growing cell but as many as 4-6 per rapidly growing cells, this directly explains the experimental difference. The cell-cycle independent result was obtained in rapidly growing cells where there should be many copies[2], while the cycle-dependent result comes from slowly growing cells where the copy number is much lower. What this proposed alternative explanation does not take into account is that the change in copy number as a function of growth rate is explained by the precise pattern of timing of plasmid replication as growth rate increases over a range of growth rates. Just as increasing the bacterial growth rates leads to a continuous increase in the number of chromosomal origins per cell[5,6], the finding that the peak of plasmid P1 replication occurs at a fixed cell-cycle fraction away from chromosome replication[1] rather than at a fixed fraction of cell-cycle age-indicated that one should have a continuous increase in plasmid content per cell as growth rate increases. Thus, it is not that plasmid number increases per cell because cells grow larger and can accommodate more plasmids, but it is the continuous moving back in time of plasmid replication (denoted by the particular timings of the peak of replication at different growth rates) that leads to an increase in plasmid numbers per cell. The arguments for a mixed pattern would be stronger if, for example, it was observed that plasmids always replicated at a fixed time during the cell cycle (e.g., at mid-cycle) and did not move back with increasing growth rate. Then one could possibly conclude that merely plasmid concentration was the operative control element determining plasmid replication during the cell cycle.

The argument raised against this mixed explanation is thus not based merely on a desire to have a uniform pattern for plasmid replication, but rather is based on the totality of the observed replication patterns, with the peak of plasmid replication mimicking the timing of chromosome replication. The continuity of timing of plasmid replication as growth rates are continuously increased is the fundamental indication that plasmid replication during the division cycle is the same over a wide range of growth rates.

Finally, the mixed pattern of P1-plasmid replication assumes that the plasmid and the basic elements that govern plasmid replication actually change properties at some growth rate. For example, one might propose that

replication is controlled by a protein inhibitor and that the dilution of this inhibitor allows replication. Then, the mixed pattern would postulate that the protein inhibitor behaves one way above a particular growth rate and differently below that growth rate. The argument that the plasmid will have timed replication when at low copy number and stochastic replication at higher copy number that is, assuming that the same mechanism is used to control replication at single copy number as at high copy number, replication will be just as stochastic when there is one plasmid in the cell as when there are multiple copies in the cell. Within the cells of a culture the timing of plasmid replication in cells with very low copy number (i.e., one copy per newborn cell) will be spread out at different times during the cell cycle so no peak of replication will be observed. Stochastic copy number control will lead to replication throughout the cell cycle for one copy or for one thousand copies. Thus, the only way for a plasmid to have a mixed pattern would be to have two completely independent replication systems.

Addiction/suicide mechanisms to deal with plasmid-free cells: It has been argued that it is possible to have poor replication or segregation controls since plasmids have potent mechanisms to prevent the survival of plasmid-free segregants. The existence of such mechanisms makes it theoretically possible to have a random replication mechanism (and even a random segregation mechanism) as plasmid-free cells would be killed.

But this type of fail-safe mechanism is only rarely called into play. If, for example, plasmids segregated randomly and a dividing cell had only two plasmids, one would expect that half of the divisions would produce at least one dead cell. Cultures of plasmid containing cells would be expected to accumulate dead cells and to grow at a slower rate than plasmid-free cultures. This is not observed. This means that these addiction/killing modules[7-10] are only rarely called into use and the main mechanism of stable maintenance of P1-plasmid is the regularity of replication and the regularity of segregation.

Supporting this proposal is a microscopic analysis that clearly indicated that the prophage of bacteriophage P1 in its plasmid form is a low copy number plasmid that is segregated to daughter cells by an active partition system[11]. Time-lapse photomicroscopy of the dynamic aspects of plasmid segregation indicated that there is a non-randomness to both the replication and segregation processes.

DISCUSSION

The analysis presented here deals with a particular experimental problem, the determination of when during the division cycle P1-plasmid is replicated. This is an important issue to resolve as there are two contrasting proposals in the literature and without a resolution of the issue there may be problems in the future in the analysis of plasmid replication controls.

Based on the arguments presented above, a summary of permissible patterns of cell-cycle replication of high and low-copy plasmids is presented in Table 1. For high copy plasmids, two patterns are allowed. The high-copy plasmid pBR322 appears to replicate in a random manner throughout the cell cycle, while the high-copy minichromosome plasmid has been shown to replicate in a decidedly cell-cycle-specific pattern[12]. Although we may not understand the precise mechanism allowing the simultaneous or near-simultaneous replication of minichromosomes at a particular time during the cell cycle, one can readily understand the random pattern of high copy plasmids. For example, a plasmid that, on average, has 50 copies in a newborn cell and 100 copies in a dividing cell may have, within each cell, a sequential, cell cycle-independent replication pattern. Thus, one of the fifty plasmids in the newborn cell may replicate to make 51, then another to make 52 and so on until approximately 100 plasmids are produced by the time the cell divides. A cell that by accident of statistical variation had a relatively low number of replication events perhaps yielding only 70 plasmids in the dividing cell would nevertheless have approximately 35 plasmids in each daughter cell at division. In the succeeding cycle some feedback mechanism (i.e., sensing a low-plasmid population and allowing more rapid replication during the next cell cycle) would then allow a replenishment of these plasmids within the daughter cells. There would be a return to the approximately 100 plasmids at division. Thus, for high copy plasmids, even with a poor replication rate,

Table 1: Permissible and non-permissible patterns of high- and low-copy plasmid replication during the bacterial cell cycle

	Cell cycle specific replication pattern	Cell cycle independent (random) replication pattern
High copy plasmids	Allowed (minichromosome)	Allowed (pBR322)
Low copy plasmids	Allowed (F, P1)	Not allowed

Table 2: Permissible and non-permissible patterns of high- and low-copy plasmid segregation at division

	Random segregation	Non-random segregation
High copy plasmids	Allowed	Allowed (not demonstrated)
Low copy plasmids	Not allowed	Required

segregation of plasmid-free cells would not be expected. The probability of all 70 plasmids in the dividing cell going into only one of the daughters is very low.

High-copy plasmids that replicate at a particular time during the cell cycle (e.g., minichromosomes) presumably do not have plasmid-free daughter cells because there is a precise mechanism of replication that leads to a doubling of plasmids in mid-cycle, thus ensuring that the daughter cells all receive at least one plasmid.

For low-copy plasmids different considerations apply. If a low-copy plasmid replicates under a stringent, cell-cycle-specific control mechanism, such as that for the bacterial chromosome, then it would be expected, as for the chromosome, that at some point during the cell cycle all cells would replicate the plasmid. By following a cell-cycle specific pattern of plasmid replication, a newborn cell with one plasmid would be assured of having at least two plasmids in the dividing, mother cell. By having a precise, cycle-dependent segregation mechanism, one can be assured of having one plasmid available to segregate into each daughter cell.

For low-copy plasmids with a random pattern of replication, problems arise when considering the absence of plasmid-free cells. Let us consider a population of cells with 1 plasmid in each newborn cell and with plasmid replication occurring with equal probability at any time during the cell cycle. Thus, approximately 10% of the cells will replicate the plasmid during the first 10% of the cell cycle, another 10% during the next 10% of the cell cycle and so on. When we come to the end of the cell cycle there is a significant probability that some of the cells will not replicate their plasmids until the cell has divided. To ensure that plasmid-free cells would not arise, the plasmid would need a second (deterministic) control mechanism that would trigger replication for all cells that have not yet replicated their plasmids to ensure that there are two plasmids in the dividing cell. Thus, one would find, in low-copy situations, a peak just at the end of the cell cycle. This is not found, nor is it expected if there is a cycle-independent mechanism of replication.

The fail-safe mechanism for plasmid maintenance that is discussed above appears to be only rarely used, as plasmid replication and segregation in normal, growing cells is quite precise[11]. For this reason study propose that it is not possible for a low-copy plasmid to have a random pattern of replication control (Table 1).

The cell-cycle-specific replication pattern of the low-copy P1-plasmid is also consistent with the cell-cycle-specific pattern of F-plasmid replication. As long ago as 1972 it was shown that F-plasmid replicated in a cell-cycle-specific manner[13]. This pattern of replication has been supported by other experiments[14,15]. This pattern of replication control was then questioned[4], again on the basis of autoradiographic assays of plasmid replication. Subsequent study on F-plasmid replication, using membrane-elution, coupled with direct double-label counting[3] indicated that the F-plasmid replicated in a cell-cycle-specific manner over a wide range of growth rates. Moreover, the replication pattern was consistent with replication of F-plasmid being controlled by rules similar to those regulating chromosome replication. That is, the F-plasmid replicated when the cell size reached a particular value. Even more recently, cycle-specific F-plasmid replication was supported by a reanalysis[16] of data from another laboratory on the segregation of plasmids during bacterial growth[17]. The consistency of the replication of low-copy F-plasmid and low-copy P1-plasmid are mutually supportive of the conclusions presented here.

The same theoretical analysis as applied to plasmid replication (Table 1) can also be applied to plasmid segregation (Table 2). Considering the four possibilities arising from high-copy and low-copy plasmid populations having either random or non-random segregation patterns or mechanisms, it is clear that stable propagation of plasmids cannot occur if there is a random segregation of low-copy plasmids. This leads to the requirement of a deterministic mechanism for low-copy (but not necessarily for high-copy) plasmids at division.

To summarize the analysis presented here, both experimental results and theoretical concepts suggested that low-copy plasmids will have specific cycle-related replication patterns and specific non-random segregation patterns. Only when low-copy plasmids have these non-random patterns of replication and segregation will plasmids be stably propagated in all cells in a culture.

ACKNOWLEDGMENT

This study was supported by Grant MCB-0323346 from the National Science Foundation.

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