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Review

Stress responses and replication of plasmids in bacterial cells

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Abstract

Plasmids, DNA (or rarely RNA) molecules which replicate in cells autonomously (independently of chromosomes) as non-essential genetic elements, play important roles for microbes grown under specific environmental conditions as well as in scientific laboratories and in biotechnology. For example, bacterial plasmids are excellent models in studies on regulation of DNA replication, and their derivatives are the most commonly used vectors in genetic engineering. Detailed mechanisms of replication initiation, which is the crucial process for efficient maintenance of plasmids in cells, have been elucidated for several plasmids. However, to understand plasmid biology, it is necessary to understand regulation of plasmid DNA replication in response to different environmental conditions in which host cells exist. Knowledge of such regulatory processes is also very important for those who use plasmids as expression vectors to produce large amounts of recombinant proteins. Variable conditions in large-scale fermentations must influence replication of plasmid DNA in cells, thus affecting the efficiency of recombinant gene expression significantly. Contrary to extensively investigated biochemistry of plasmid replication, molecular mechanisms of regulation of plasmid DNA replication in response to various environmental stress conditions are relatively poorly understood. There are, however, recently published studies that add significant data to our knowledge on relations between cellular stress responses and control of plasmid DNA replication. In this review we focus on plasmids derived from bacteriophage λ that are among the best investigated replicons. Nevertheless, recent results of studies on other plasmids are also discussed shortly.

Review

Introduction

Stable maintenance in the host cells is the most important process for any bacterial plasmid. There are different mechanisms used by various plasmids to achieve this. First, plasmids must replicate often enough to produce their copies in amounts allowing their distribution to both daughter cells after division of a mother cell. On the other hand, to prevent energetic exhaustion of the host leading to cell death, the frequency of plasmid replication should not be too high. Second, after a replication round, daughter plasmid molecules should be partitioned efficiently. Third, emergence of plasmid-less cells in a population of bacteria that originally carried plasmids is a serious disadvantage from the point of view of these extra-chromosomal genetic elements, as cells devoid of plasmids usually grow faster than those bearing such 'additional' molecules. Thus, plasmid-less cells would win

the competition with plasmid-harboring bacteria in the absence of an environmental pressure favoring cells that bear plasmid DNA. Therefore, some plasmids developed mechanisms of killing bacteria that have lost them.

Genetic structures of plasmid replicons, biochemistry of replication initiation of different plasmids, mechanisms of partitioning of plasmid molecules, and processes of post-segregation regulation of plasmid stability in bacterial cell lines were reviewed recently in several excellent articles [1–7]. Therefore, these problems will not be discussed in detail in this review. Here, we focus on regulation of plasmid replication in response to different cellular stresses. As a model, we have chosen plasmids derived from bacteriophage λ, called λ plasmids. Recently published results provided new data allowing us to understand better the replication of these plasmids in cells growing under various environmental conditions. Moreover, some other recent studies on this type of regulation in other plasmid replicons will be discussed.

λ plasmids

Bacteriophage λ is a temperate virus that infects *Escherichia coli* cells. This phage played a crucial role in the development of molecular biology and still is an extremely useful model in studies on molecular mechanisms of regulation of basic cellular functions, serving as a paradigm for many general biological processes [8–11]. Moreover, genetically modified λ phages and fragments of their genomes are widely used tools in genetic engineering and biotechnology [12–17].

Typical λ plasmid consists of a fragment of bacteriophage λ genome which contains all genes and regulatory sequences necessary for initiation of its replication in *E. coli*. The first plasmids of this type were isolated as derivatives of bacteriophage λ genome, produced by *in vivo* recombination events [18]. Currently, such plasmids are constructed using genetic engineering methods, and often contain, apart from the λ replication region, a genetic marker, e.g. an antibiotic-resistance gene. Since the structure of λ plasmids, basic functions of λ replication genes and mechanism of replication initiation from *ori*λ were reviewed recently [19–22], in this article we provide only basic information about initiation of λ DNA replication, which is necessary to understand regulatory mechanisms operating under various stress conditions.

Replication of λ plasmid DNA is initiated at the *ori*λ region, located in the middle of the *O* gene (Fig. 1). This gene codes for the replication initiator protein, which binds to the replication origin, forming the nucleoprotein structure called 'O-some'. The host-encoded DnaB helicase is delivered to the O-some by another λ replication protein, the *P* gene product. The *ori*λ-O-P-DnaB structure, called 'pre-primosome' is stable but inactive in promoting DNA replication, as strong interactions between P and DnaB proteins prevent helicase activity of the latter component. Action of the heat shock proteins: DnaK, DnaJ and GrpE, is necessary to liberate DnaB from P-mediated inhibition, though the P protein seems to be still present in the complex. Recent studies (see next chapter) suggest that DnaK also remains bound to the *ori*λ-O-P-DnaB complex. The process of chaperone-dependent pre-primosome remodeling is coupled with transcriptional activation of *ori*λ, a transcription proceeding in the replication *origin* region. Transcriptional activation of the *origin* is necessary for efficient initiation of λ DNA replication *in vivo* even if all the replication proteins are provided. It seems that changes in DNA topology caused by movement of RNA polymerase during transcription play a crucial role in stimulation of the replication initiation. λ p_R promoter is a natural start site of transcription that produces mRNA for synthesis of λ replication proteins (O and P) and acts to activate *ori*λ. The final step in the initiation of λ DNA replication is binding of DNA polymerase III holoenzyme and accessory replication proteins (DNA gyrase, SSB and other) encoded by the host to the *ori*λ region.

Replication of λ plasmids in amino acid-starved cells

Amino acid starvation induces specific response of bacterial cells called the stringent response [23]. Under these conditions, product of the *relA* gene is activated to produce specific signal nucleotides, guanosine 5'-triphosphate-3'diphosphate (pppGpp) and guanosine 5'-diphosphate-3'-diphosphate (ppGpp). These nucleotides interact with RNA polymerase causing dramatic changes in the efficiency of transcription from various promoters. Promoters for synthesis of stable RNAs are the most sensitive promoters to (p)ppGpp. Some promoters are not affected by this nucletide, whereas other promoters can be either inhibited or stimulated in amino acid-starved wild-type bacteria [23].

λp_R promoter was found to be negatively regulated by ppGpp [24–26]. Decrease in the activity of p_R results in impaired transcriptional activation of *ori*λ and inhibition of λ plasmid DNA replication. Such a regulation is logical, as one can imagine that inhibition of processes that require extensive energy consumption (like DNA replication) should be an advantage for the host cell endangered by starvation.

Interestingly, *E. coli* mutants in the *relA* gene, which are defective in production of (p)ppGpp in amino acid-starved cells (such a response of bacteria to amino acid starvation is called the relaxed response), can still support replication of λ plasmids [27,28]. This discovery was unexpected, as previous studies [29,30] strongly suggested

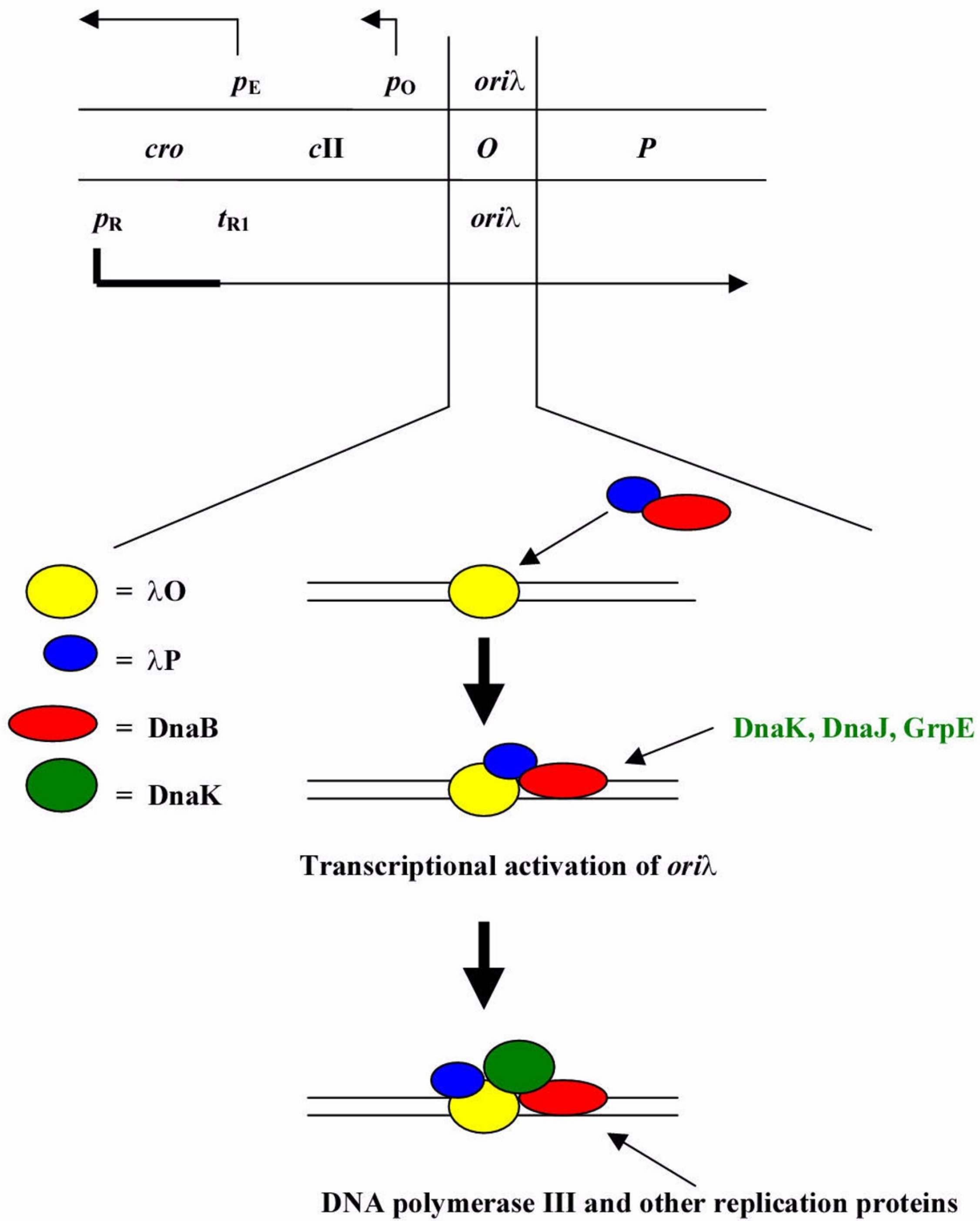

Figure 1
Replication region of bacteriophage λ genome and assembly of the replication complex. Genes, promoters and terminators present in the replication region are indicated. Transcripts are represented by arrows. The scheme is not drawn to scale. See text for details.

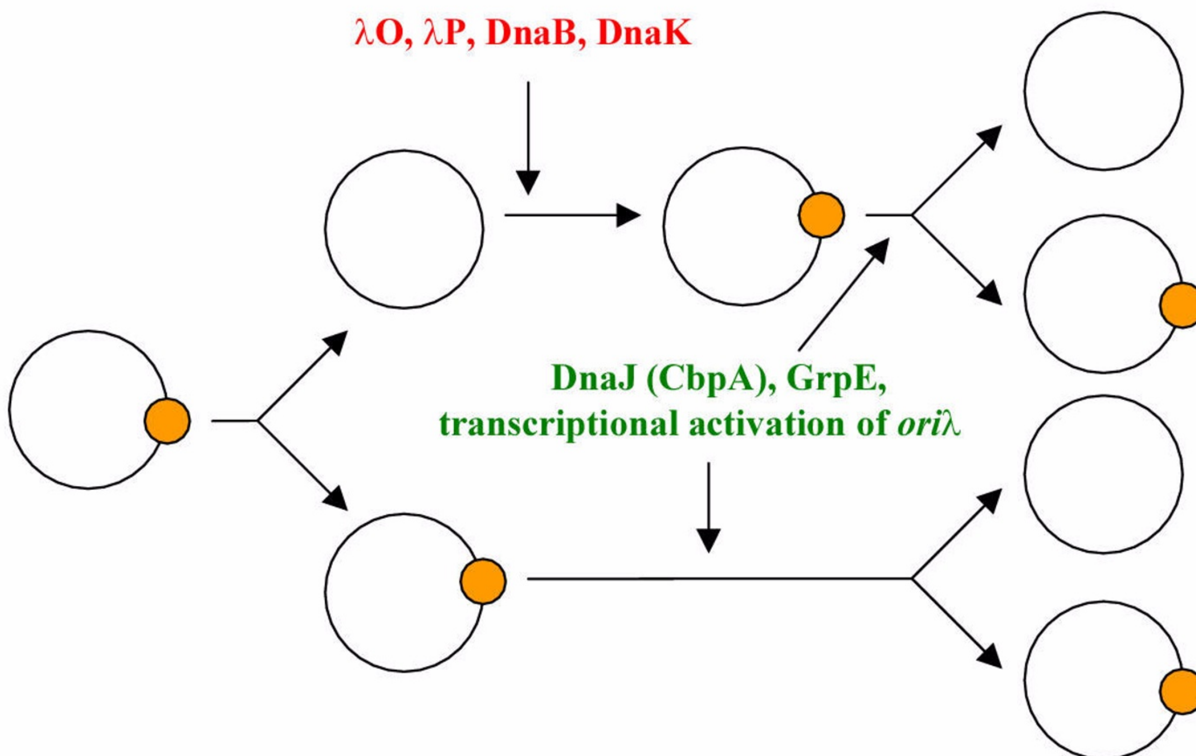

Figure 2
Two pathways of λ plasmid replication. Large circles represent plasmid DNA molecules. Small filled (orange) circles indicate heritable λ replication complexes. See text for details.

that the initiator of DNA replication, λ-encoded O protein, is extremely unstable in *E. coli* cells. It was proposed that after each replication round this protein is degraded and synthesis of new O protein molecules is necessary for initiation of subsequent plasmid replication [31]. Therefore, one could predict that inhibition of O protein synthesis in amino acid-starved cells, which was demonstrated experimentally [32], should result in prevention of replication initiation from *ori*λ. Such an inhibition occurred in amino acid-starved wild-type cells, but not in *relA* mutants. λ plasmid replication during the relaxed response is strongly dependent on the activity of the O protein [28,33], so this replication initiator must still function while its synthesis is blocked. More detailed studies revealed that O protein is protected from proteolysis (performed by ClpP/ClpX protease [34,35]) by other components of the λ replication complex, formed at *ori*λ [32,36,37]. Such a complex is a stable structure, inherited by one of two daughter plasmid DNA copies after each replication round, and may function in subsequent replication events [32,38].

Studies on replication of λ plasmids in amino acid-starved cells, summarized above, led to a new model for regulation of replication of these replicons (Fig. 2). According to this model, there are two pathways of λ plasmid replication. The first pathway is based on the activity of the heritable replication complex present only on one daughter copy after a replication round. On the plasmid copy devoid of the heritable complex, a new replication complex must be assembled. Both heritable and newly assembled complexes require activities of heat shock proteins (DnaK, DnaJ or its homologue CbpA [39], and GrpE) and transcriptional activation of the origin to initiate a new replication round (see ref [22] for detailed review). Heritable replication complexes exist in both wild-type cells and *relA* mutants. However, in amino acid-starved bacteria able to produce (p)ppGpp, activity of the p_R promoter is significantly decreased, which results in inefficient transcriptional activation of *ori*λ and inhibition of the replication initiation. On the other hand, during the relaxed response, in the absence of high (p)ppGpp amounts the p_R promoter is fully active, and replication carried out by the heritable replication complex can proceed.

Results of previous *in vivo*[36] and *in vitro*[40] experiments suggested that the heritable λ replication complex contains O, P and DnaB proteins. Recent studies revealed that this complex consists of these proteins and in addition of the DnaK protein [41]. Replication carried out by the heritable complex may proceed either bidirectionally or unidirectionally (leftward or rightward), similarly to the replication driven by a newly assembled complex [42]. The replication complex is inherited randomly after a replication round in an equal extent by a copy containing parental DNA *l* strand or parental *r* strand [43].

Studies on regulation of λ plasmid replication in amino acid-starved cells had also biotechnological implications. Lack of amino acids causes inhibition of cell growth. Therefore, under these conditions, efficient replication of λ plasmid molecules results in amplification of plasmid DNA in cells. In fact, efficient amplification of λ plasmids was observed in *relA* mutants, both during amino acid starvation and limitation [44,45]. Since derivatives of λ plasmids are used as cloning vectors [15,16], their amplification may be useful in biotechnological laboratories.

Growth rate-dependent regulation of λ plasmid copy number

Bacteria can be cultivated in various media supporting different growth rates. Plasmid copy number in bacterial cells depends mainly on the frequency of plasmid replication initiation, which may vary considerably depending on cellular growth rate. It was found that λ plasmid copy number is higher in cells growing faster [46]. It seems that in slowly growing cells, O protein level is the limiting factor for replication initiation at *ori*λ. This hypothesis is based on experiments which indicated that dysfunction of *clpP*, *clpX* or both of these genes (coding for components of the O-degrading ClpP/ClpX protease) completely abolishes differences in λ plasmid copy number observed in wild-type host growing in various media, supporting different growth rates [46]. Artificially increased levels of the O protein also resulted in more efficient replication initiated at *ori*λ, but only in bacteria growing slowly in poor media [47]. Clearly, the pathway of λ plasmid replication based on newly assembled replication complexes (compare Fig. 2) must be affected under these conditions.

The mechanism of this regulation is not clear. Activity of the p_R promoter, from which the *O* gene is transcribed, was found to be dependent on cellular growth rate, though differences in the transcription efficiency between various growth rates were less pronounced than those in λ plasmid copy number [46]. One might speculate that DnaA and SeqA proteins, which are regulators of bacterial chromosome replication initiation and also stimulators of the p_R promoter [48–52], play a role in response of this promoter activity to different growth rates. Perhaps less efficient translation in slowly growing bacteria might also account for lower O protein levels under these conditions.

Heat shock and λ plasmid replication

Heat shock proteins DnaK, DnaJ (which may be replaced by its homologue CbpA) and GrpE are absolutely necessary for initiation of DNA replication from *ori*λ (for reviews see refs. [19–22]). These proteins remodel the preprimosome to liberate DnaB helicase from the P-mediated inhibition, and perhaps are also involved in proper installment of the helicase in the replication forks. Recent results indicate that one of these proteins, DnaK, is a component of the λ heritable replication complex [41].

On the basis of the facts presented above, one could predict that heat shock might stimulate replication of λ plasmids. However, this is not the case as it was demonstrated that λ plasmid copy number is decreased at 42°C relative to lower temperatures (e.g. 30 or 37°C) [44]. More detailed studies revealed that the replication pathway dependent on the function of the heritable replication complex is impaired by heat shock. Namely, the heritable replication complex, which under standard laboratory conditions is a stable structure able to function for many cell generations, is disassembled relatively shortly after transfer of bacteria from 30 to 43°C [53]. This disassembly was found to be dependent on GroEL and GroES heat shock proteins [53]. In fact, this was the first demonstration, supported by subsequent studies [54], that the GroEL/GroES molecular chaperone system is engaged in an *in vivo* disassembly of a highly organized protein structure.

The mechanism of the GroEL/GroES-dependent, heat shock-induced disassembly of the heritable λ replication complex has been elucidated. Heat shock causes partial and transient relaxation of supercoiled plasmid DNA. Subsequent DNA gyrase-dependent re-supercoiling provokes dissociation of the replication complex, which is then disassembled by the GroEL/GroES chaperone system [55]. Under specific conditions (e.g. absence of the λ Cro repressor, which is coded by a gene present on most λ plasmids), the heritable replication complex may survive heat shock due to formation of a more stable structure as a result of interaction with other proteins [55]. One of them is the host-encoded DnaA protein [56].

In summary, heat shock proteins have both positive and negative functions in the regulation of λ plasmid replication. Interestingly, the re-supercoiling of plasmid DNA after its partial relaxation caused by heat shock, which is required for the heritable replication complex to dissociate from DNA, is dependent on DnaK function [57]. This makes the influence of heat shock proteins on the regulation of λ plasmid replication even more complicated

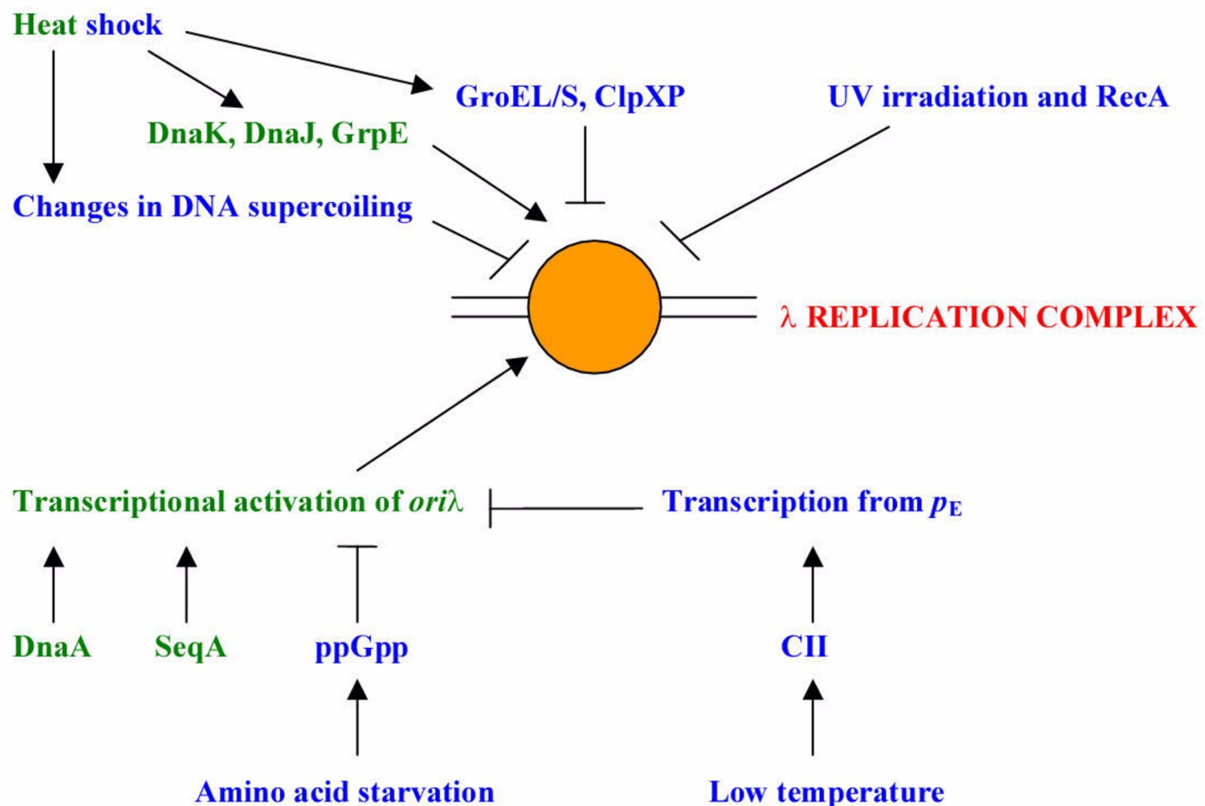

Figure 3
Effects of various factors on formation and stability of the λ heritable replication complex. The complex is symbolized by large orange circle. Positive regulators are marked in green, and negative regulators are marked in blue. Stimulation processes are shown as arrows and inhibitory actions are shown by blunt-ended lines. See text for details.

(compare Fig. 3). Furthermore, ClpP and ClpX proteins, which form a protease that degrades the O replication initiator protein, belong also to heat shock protein family.

Combination of prolonged (several hours) cultivation of bacteria at increased temperature (42–43°C) and amino acid starvation has deleterious effects on plasmids. Namely, plasmid DNA degradation was observed under these conditions [58]. This degradation was discovered in the course of studies on λ plasmid replication, but was then demonstrated to be independent of the plasmid replicon [58]. Mechanism of this phenomenon remains unknown, though one might speculate that some bacterial addiction modules that are activated upon amino acid starvation, like *mazEF*[59], could be involved.

Replication of λ plasmids at low temperatures

Lytic development of bacteriophage λ is blocked at a temperature as low as 20–25°C [60,61]. In fact, the burst size of this phage in *E. coli* cells increases gradually over the temperature range from 20 to 37°C [62]. At low temperatures, phage DNA replication was found to be inhibited [62,63]. Unexpectedly, it was demonstrated that plasmids derived from bacteriophage λ replicate normally at 25°C [62].

What is the mechanism of different λ phage and λ plasmid DNA replication regulation at low temperatures? It was demonstrated that λ phage-encoded CII protein activates the p_E promoter (see Fig. 1) more efficiently at lower than at higher temperatures [62,63]. Enhanced transcription from p_E may interfere with initiation of transcription from p_R, which is oriented in opposite direction relative to p_E

(Fig. 1). Low activity of p_R results in inefficient production of λ replication proteins. Therefore, formation of new replication complexes is impaired. Note that in the case of phage infection, naked viral DNA is injected into a host cell, and all replication complexes must be assembled *de novo*. When λ plasmids were investigated, plasmid-bearing cells were transferred from standard temperature (usually 37°C) to low temperatures. Therefore, previously assembled heritable λ replication complexes existed at the time of the temperature shift, and could function at low temperatures irrespectively of the new replication complex formation inhibition [62].

Effect of the SOS response on formation and stability of λ replication complexes

DNA damage induces specific bacterial stress response, called the SOS response [64]. UV irradiation is one of the factors effectively inducing the SOS response. It was demonstrated that UV irradiation of the host cells prevents formation of the stable λ replication complex, while it does not inhibit λ DNA replication [65]. Stable replication complexes could be formed in UV-irradiated *recA* mutants [65] suggesting that the SOS response, rather than UV irradiation *per se*, is responsible for changes in the process of the λ replication complex assembly. Interestingly, exposure of bacteria to UV light did not affect stability of the replication complex assembled prior to the irradiation [65]. These results indicate that the stable (heritable) λ replication complex, although sensitive to heat shock, is resistant to some other environmental stresses, like cold shock (see the preceding paragraph) or factors causing DNA damage.

Efficient replication of λ DNA under normal growth conditions and in UV-irradiated cells suggests that formation of at least two types of λ replication complexes is possible. The stable (heritable) complex can be assembled under normal growth conditions and an unstable (though still functional for single replication initiation) complex may be assembled in UV-irradiated cells. Interestingly, considerable differences in initiation of DNA replication from *ori*λ between cells cultivated under normal growth conditions and after UV irradiation were reported previously [66]. Normally, the *ori*λ region has to be under superhelical tension to be a suitable substrate for replication initiation machinery [19,20]. Therefore, once a round of replication is in progress, the daughter *ori*λ sequences would not be under such a tension, and thus they would be inactive. However, in UV-irradiated bacteria the requirement for supercoiled *origin* region to initiate replication appears to be alleviated, as re-initiation of replication from *ori*λ at relaxed, still replicating, daugther molecules was observed [66]. These results may support the idea presented above that at least two different kinds of replication complexes can effectively operate at *ori*λ.

Some recent studies on replication of other plasmids under various stresses

Plasmids derived from bacteriophage λ were described in this article as models in studies on effects of stress conditions on the regulation of replication of extrachromosomal genetic elements. However, it is obvious that cellular stress responses significantly affect replication of most, if not all, kinds of plasmids. For example, similarly to λ replicons, many plasmids require functions of the heat shock proteins [1]. Biochemical aspects of requirements of various stress proteins for replication of different plasmids have recently been reviewed [1–6], and will not be discussed in detail here. Nevertheless, we are still far from complete understanding of regulatory processes that control replication of different plasmids under stress conditions. Below we discuss briefly some recent studies that addressed this problem and added significant information to our still incomplete knowledge about plasmid physiology.

Replication of several plasmids (derivatives of ColE1-like replicons, pSC101, R1, RK2, R6K, F, and phage P1-derived plasmids) was investigated in amino acid-starved bacterial cells. These studies were reviewed recently in detail [67] and here they will be only summarized briefly. In most cases, inhibition of plasmid DNA replication was observed in amino acid-starved wild-type cells. However, different phenomena were observed during the relaxed response, i.e. replication of some replicons was inhibited in amino acid-starved *relA* mutants, whereas replication of other replicons proceeded efficiently under these conditions. Such a replication led to amplification of plasmids in bacteria. Interestingly, in many cases efficiency of plasmid DNA replication during the relaxed response depended on the nature of lacking amino acid (for details see ref. [67] and references therein). Suggestions about possible regulatory mechanisms of replication control of some plasmids in amino acid-starved *relA* mutants were presented [67].

It has been demonstrated that plasmid pSY10, which is present in marine cyanobacteria belonging to the genus *Synechococcus*, is maintained at a high copy number when host cells grow in seawater and at low copy number in cells cultured in freshwater [68]. Therefore, regulation of replication of this plasmid must depend on salinity of environment. More detailed experiments revealed that transcription of the *repA* gene, coding for the plasmid replication initiator protein, is depressed in cells growing in freshwater relative to cyanobacteria cultured at higher salinity [68]. Moreover, a putative repressor of the *rep* gene promoter was discovered, and this protein was found to be synthesized only at low salinity [68]. This protein, which is encoded by the host chromosome, is an excellent

candidate for the main regulator of pSY10 replication in response to changes in salinity of environment.

Some plasmids encode their own stress proteins. For example, genes of small heat shock proteins are expressed from plasmids pER16, pER35 and pER36, naturally occurring in *Streptococcus thermophilus* strains [69]. These proteins enhance the viability of bacteria in extreme environments and it was suggested that the presence of plasmid-encoded heat shock proteins may enhance survival of cells under specific conditions.

Interesting combined effects of temperature upshift and high-level production of recombinant proteins on replication of ColE1-like plasmids were observed [70]. Namely, under conditions normally causing amplification of plasmid DNA at increased temperatures, simultaneous overexpression of recombinant proteins that form inclusion bodies attenuated plasmid DNA amplification [70]. In fact, effects of enhanced production of recombinant proteins on stability of ColE1-like plasmids in *E. coli* strains were reported previously [71], and more recent studies revealed that features of recombinant proteins produced in bacteria affect efficiency of plasmid maintenance [72]. Molecular mechanisms of these regulations remain to be elucidated.

As discussed above, contrary to plasmids derived from bacetriophage λ (see the preceding paragraphs), temperature upshift causes amplification of ColE1-like plasmids [70]. Increased rate of initiation of bacterial chromosme replication under such conditions was also observed previously [73]. More recent studies demonstrated that this heat-induced chromosome replication is initiated at *oriC* (normal site for initiation of nucleoid replication) and requires RNaseH1 and RecA proteins but not RNA polymerase activity and *de novo* protein synthesis [74]. It was suggested that this phenomenon might be explained by a heat-inducible thermodynamic alteration of the *oriC* structure and/or of membrane fluidity [74].

As mentioned in preceding paragraphs, overexpression of recombinant genes may inhibit plasmid DNA replication [70]. Such an inhibition may be the triggering signal for the cellular SOS response [75]. In fact, the SOS response may be induced by a single plasmid-encoded protein, for example the replication initiator protein of plasmid pSC101, RepA [76]. It was proposed that such an induction of the SOS response may be helpful in detecting an imbalance between the cellular level of the RepA protein and plasmid-borne RepA-binding sites [76]. This mechanism should lead to inhibition of the host chromosome replication and should delay host cell division when RepA is in relative excess. This may help to limit variation in plasmid copy number and to prevent formation of plasmid-less cells.

Conclusions

Bacterial stress responses influence replication of various plasmids in host cells. Although biochemical roles of some specific stress proteins in replication of certain plasmids were elucidated, mechanisms of responses of plasmid regulatory systems to many environmental stresses remain largely unknown. However, elucidation of these mechanisms is necessary to understand physiology of plasmids and to use these extrachromosomal genetic elements more effectively in genetic engineering and biotechnology. Plasmids derived from bacteriophage λ provide examples of replicons whose mechanisms controlling frequency of their replication initiation under various stress conditions have been partially explained. We hope that further studies will allow us to understand these mechanisms even in more detail and that they will provide new and important information about physiology of replication of other plasmids.

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