Modulation of λ plasmid and phage DNA replication by *Escherichia coli* SeqA protein

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SeqA protein, a main negative regulator of the replication initiation of the *Escherichia coli* chromosome, also has several other functions which are still poorly understood. It was demonstrated previously that in *seqA* mutants the copy number of another replicon, the λ plasmid, is decreased, and that the activity of the λ p_R promoter (whose function is required for stimulation of *ori λ*) is lower than that in the wild-type host. Here, SeqA-mediated regulation of λ phage and plasmid replicons was investigated in more detail. No significant influence of SeqA on *ori λ* -dependent DNA replication *in vitro* was observed, indicating that a direct regulation of λ DNA replication by this protein is unlikely. On the other hand, density-shift experiments, in which the fate of labelled λ DNA was monitored after phage infection of host cells, strongly suggested the early appearance of σ replication intermediates and preferential rolling-circle replication of phage DNA in *seqA* mutants. The directionality of λ plasmid replication in such mutants was, however, only slightly affected. The stability of the heritable λ replication complex was decreased in the *seqA* mutant relative to the wild-type host, but a stable fraction of the λ O protein was easily detectable, indicating that such a heritable complex can function in the mutant. To investigate the influence of *seqA* gene function on heritable complex- and transcription-dependent λ DNA replication, the efficiency of λ plasmid replication in amino acid-starved *relA seqA* mutants was measured. Under these conditions, *seqA* dysfunction resulted in impairment of λ plasmid replication. These results indicate that unlike *oriC*, SeqA modulates λ DNA replication indirectly, most probably by influencing the stability of the λ replication complex and the transcriptional activation of *ori λ* .

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INTRODUCTION

Replication of genetic material is a fundamental process that occurs in all living organisms (Kornberg & Baker, 1992). To ensure survival, this process must be precisely regulated, and this regulation usually occurs at the stage of initiation. In the model prokaryotic organism *Escherichia coli*, initiation of chromosome replication is controlled in the cell cycle (Messer, 2002). The *dnaA* gene product is a replication initiator protein. It binds to the *oriC* region and positively regulates DNA replication initiation. However, in most biological systems, which require precise and versatile regulation to ensure the ability to respond to various growth conditions, there are both positive and negative regulators. The SeqA protein appears to be one of key factors involved in the negative control of *oriC*-initiated replication (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994; Slater *et al.*, 1995; Boye *et al.*, 1996); namely, it is an

inhibitor of the onset of chromosome replication *in vivo* (Slater *et al.*, 1995; Boye *et al.*, 1996).

It has been demonstrated that *in vivo* SeqA limits DnaA activity in replication from *oriC* (von Freiesleben *et al.*, 1994). Nevertheless, it has been proposed that the main role of SeqA is sequestration of newly replicated *origin* sequences, as this protein interacts with hemimethylated GATC motifs, abundant in the *oriC* region, and prevents DnaA binding to this region (Lu *et al.*, 1994; Slater *et al.*, 1995). However, subsequent studies have revealed that SeqA-mediated regulation of replication is significantly more complex. Although SeqA inhibits replication from *oriC* at high concentrations of DnaA *in vitro*, it stimulates this process at low DnaA concentrations (Wold *et al.*, 1998). Other experimental lines have indicated that SeqA inhibits open complex formation at the replication *origin* (Torheim & Skarstad, 1999).

Studies on DNA supercoiling and the localization of SeqA in cells have suggested that this protein may affect

Abbreviation: AGE, agarose gel electrophoresis.

organization of the nucleoid (Hiraga *et al.*, 1998; Weitaio *et al.*, 1999; Skarstad *et al.*, 2001). In *seqA* mutants, negative supercoiling of DNA increases, and purified SeqA protein generates positive DNA supercoils *in vitro* (Klungsoyr & Skarstad, 2004). Therefore, it has been proposed that binding of SeqA changes either the twist or the writhe of the DNA (Klungsoyr & Skarstad, 2004). This ability to affect the topology of DNA suggests that SeqA can take part in the organization of the chromosome *in vivo*. However, it has been proposed that the direct effect of SeqA on replication initiation is rather the result of binding to hemimethylated *oriC* and releasing DnaA molecules from this region (Taghbalout *et al.*, 2000). Interestingly, several lines of evidence lead to the conclusion that for its proper function, SeqA protein aggregation is required (Lee *et al.*, 2001; Fossum *et al.*, 2003; Han *et al.*, 2003, 2004; Odsbu *et al.*, 2005).

As can be concluded from the data mentioned above, the function of SeqA in replication regulation is definitely not simple. The same applies to the biochemical properties of this protein. Even the rules that govern the binding of SeqA to DNA are not completely clear. Using oligonucleotide templates, it has been demonstrated that SeqA binds specifically to hemimethylated GATC sequences, but not to fully methylated and unmethylated GATC motifs (Brendler & Austin, 1999; Kang *et al.*, 1999). However, studies of larger DNA fragments indicate that SeqA may interact specifically with fully methylated *oriC* fragments (Slater *et al.*, 1995; Skarstad *et al.*, 2000). When other (non-*oriC*) DNA fragments, consisting of several hundred base pairs, are studied, specific binding of SeqA to fully methylated GATC sites is also evident (Słomińska *et al.*, 2001, 2003a, b).

Although the structure of a part of the SeqA protein has been resolved (Guarne *et al.*, 2002), and structure–function studies have been performed (Fujikawa *et al.*, 2003, 2004), some unexpected effects of mutations in the *seqA* gene have been reported, which strongly suggest that SeqA is involved in the regulation of processes as various as transcription initiation (Słomińska *et al.*, 2001, 2003a; Lobner-Olesen *et al.*, 2003), protein degradation (Torheim *et al.*, 2000; Słomińska *et al.*, 2003c), and biological functions of cellular membranes (Węgrzyn *et al.*, 1999). Moreover, it has been proposed that the function of this protein in the regulation of DNA replication initiation may be linked to *E. coli* chromosome segregation and cell division (Bach *et al.*, 2003).

Since viruses are intracellular parasites, they use various host proteins for their propagation. Although the use of products of cellular genes by viruses may sometimes appear unusual, studies of such processes have often led to the discovery of important properties of host proteins. This applies also to bacteriophages and their hosts, including one of the most intensively studied models in molecular biology, phage λ . Examples of the processes described above are the use of *E. coli* LamB protein, normally

allowing uptake of maltose and maltodextrins, for phage adsorption on the cell surface (Vinga *et al.*, 2006), employment of heat-shock proteins in λ DNA replication and morphogenesis (Polissi *et al.*, 1995; Taylor & Węgrzyn, 1995), and involvement of the replication initiator protein DnaA in the regulation of transcription from the λ *p_R* promoter (Szalewska-Pałasz *et al.*, 1998; Glinkowska *et al.*, 2003). Therefore, we aimed to investigate the role of SeqA in the control of replication of the bacteriophage λ genome and plasmids derived from this phage. In fact, previous studies have demonstrated that *seqA* gene function is involved in the regulation of bacteriophage λ development, particularly through the facilitation of transcription stimulation from certain promoters, including *p_R*, and that this is crucial for the expression of replication genes and for transcriptional activation of *ori λ* (Słomińska *et al.*, 2001, 2003a; Węgrzyn, 2006). Moreover, λ plasmid copy number is found to be decreased in *seqA* mutants relative to wild-type bacteria (Słomińska *et al.*, 2001), and it has been suggested that SeqA interferes with DnaA-mediated regulation of DNA replication initiation from *ori λ* (Słomińska *et al.*, 2003b; Glinkowska *et al.*, 2001). On the other hand, the distribution of SeqA-binding sequences (GATC) at *oriC* and *ori λ* is substantially different (GATC motifs are abundant at *oriC* but not at *ori λ* ; for reviews, see Messer, 2002; Węgrzyn, 2006). Thus, we assumed that studies of the effects of SeqA on λ DNA replication would provide important information, useful to understand in more detail both the functions of this protein and the regulation of bacteriophage λ development.

METHODS

Bacterial strains, phages, plasmids and culture media used in *in vivo* experiments. Previously described *E. coli* wild-type strain MG1655 (Jensen, 1993) was used. The $\Delta seqA::Tn10$ (called $\Delta seqA$ in this report) and *dnaA46* $\Delta seqA::Tn10$ derivatives of this strain were constructed previously by Węgrzyn *et al.* (1999). The in-frame deletion mutant *seqA* Δ 10 has already been described (Slater *et al.*, 1995). The MG1655-derived $\Delta relA251::kan$ $\Delta seqA::Tn10$ strain was constructed by P1 transduction of the $\Delta seqA::Tn10$ allele from the above-described strain to the $\Delta relA251::kan$ recipient (Xiao *et al.*, 1991).

Bacteriophage λ d857S7 (Goldberg & Howe, 1969) was used. The following plasmids derived from this phage were employed: pKB2 (Kur *et al.*, 1987), pCB104 (Boyd & Sherratt, 1995), pRLM4 (Wold *et al.*, 1982) and pKBlin (Herman-Antosiewicz *et al.*, 1998b). All of them consist of the λ replication region and a different antibiotic-resistance gene.

LB medium (Sambrook *et al.*, 1989) and minimal medium 2 (MM-2) (Węgrzyn & Taylor, 1992) were used in the *in vivo* experiments.

Proteins, antibodies and protein fraction. Bacteriophage λ O and P proteins were prepared from *E. coli* strain MM294 (Tabor & Richardson, 1985) bearing plasmids pGP1-2 and pEW1. The latter plasmid was constructed by replacement of the *EcoRI*–*SstII* fragment from pIK12 (Konieczny & Marszałek, 1995) with an analogous fragment of pKB2 (Kur *et al.*, 1987). Thus, overexpression of the O and P genes was possible in the T7 RNA polymerase/promoter system (Tabor & Richardson, 1985). λ O and P proteins were purified as

described previously (Żylicz *et al.*, 1984). SeqA protein, purified according to a published method (Skarstad *et al.*, 2000), was a kind gift from Dr Kirsten Skarstad, Institute of Cancer Research, Oslo, Norway, as were anti-SeqA antibodies.

Fraction II, an extract containing proteins necessary for DNA replication, was prepared according to an already-published procedure (Fuller *et al.*, 1981). *E. coli* C600 strain (Appleyard, 1954) was grown in LB medium at 37 °C to OD₆₀₀=1.0. The cells were collected by centrifugation, washed in buffer A (25 mM HEPES/KOH, pH 7.6, 5 mM EDTA) and then resuspended thoroughly in the same buffer (2 ml buffer A per 1 ml cell paste). The cell paste was transferred to a polypropylene tube, quickly frozen in liquid nitrogen and stored at -70 °C. The frozen cell suspension was thawed at 0–4 °C, and KCl, lysozyme and DTT were added to final concentrations of 150 mM, 0.5 mg ml⁻¹ and 2 mM, respectively. Following incubation at 0 °C for 30 min, the cells were frozen again in liquid nitrogen, thawed at 0–4 °C and centrifuged in a Beckman 50.2 Ti rotor at 30 000 r.p.m. for 30 min at 4 °C. The supernatant was collected and ammonium sulfate (0.28 g per ml cleared lysate) was slowly added to the lysate with stirring over a 60 min period at 0 °C. The precipitate was collected by centrifugation at 23 000 r.p.m. for 20 min at 4 °C and packed into a dialysis tube. The pellet was resuspended in 100 μ l buffer B (25 mM HEPES/KOH, pH 8.0, 0.1 mM EDTA, 2 mM DTT) and dialyzed against 200 ml buffer B at 0 °C for 30 min. The fraction extract was frozen in aliquots in liquid nitrogen and stored at -70 °C.

In vitro DNA replication. The standard reaction mixture (final volume 25 μ l) consisted of: 40 mM HEPES/KOH, pH 7.6, 11 mM magnesium acetate, 50 μ g BSA ml⁻¹, 40 mM creatine phosphate, 20 μ g creatine kinase ml⁻¹, 2 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dCTP, 0.1 mM dTTP with [³H]dTTP (150 c.p.m. pmol⁻¹), 8.6 μ g PEG 6000 ml⁻¹, 250 ng supercoiled pRLM4 plasmid DNA [purified by ultracentrifugation in a cesium chloride/ethidium bromide gradient, as described elsewhere (Sambrook *et al.*, 1989)], 300 ng λ O protein and 80 ng λ P protein. SeqA protein was either absent or added to the concentrations indicated. The mixtures were assembled in an ice bath. The reactions were started by the addition of 1.5 μ l fraction II and then the samples were incubated at 32 °C for 2 h. In some experiments, 1.5 μ l undiluted anti-SeqA serum was added to fraction II and incubated for 12 min in an ice bath before mixing with other components. Total ³H-labelled nucleotide incorporation was measured by determining the radioactivity (in a scintillation counter) of samples after TCA precipitation and filter binding.

Density-shift experiments. Density-shift experiments were performed according to a previously published method (Węgrzyn *et al.*, 1995b). Briefly, bacteria were grown in a 'light' MM-2 overnight at 37 °C, and after dilution (1:50, v/v) with fresh medium the cultivation was prolonged to OD₅₀₀=0.2. The bacteria were sedimented, washed with TM buffer (10 mM Tris/HCl, pH 7.2, 10 mM MgSO₄) and suspended with 0.1 volume of this buffer. After 60 min incubation at 37 °C, the [³H]thymidine-labelled phage (9 $\times$ 10⁻⁵ c.p.m. p.f.u.⁻¹) was added to m.o.i.=10 and incubation was continued for 15 min. The suspension was sedimented, resuspended in the original volume of prewarmed (to 37 °C) 'heavy' minimal medium (containing [¹⁵N]NH₄Cl and [¹³C]glucose instead of NH₄Cl and glucose, respectively), and further incubation was performed at 37 °C. Samples of the infected culture were withdrawn at the indicated times, and total DNA was isolated and ultracentrifuged in the CsCl density gradient, as described previously (Węgrzyn *et al.*, 1995b). Fractions were collected from the bottom of the tube and the radioactivity of each fraction was measured in a scintillation counter.

2D-agarose gel electrophoresis (AGE). Analysis of λ plasmid replication intermediates by 2D-AGE was performed according to a

method described elsewhere (Viguera *et al.*, 1996), with modifications described subsequently (Śrutkowska *et al.*, 1999). Before electrophoresis, plasmid pCB104 was digested with *Eco*RI, *Eco*RV or *Ssp*I/*Pvu*II.

Electron microscopy. Electron microscopy analysis of replicating plasmid DNA molecules was performed as described previously (Burkardt & Lurz, 1984; Śrutkowska *et al.*, 1998).

Estimation of O protein stability. The stability of the λ O protein in *E. coli* cells was investigated, as described previously (Węgrzyn *et al.*, 1995a), by [³⁵S]methionine labelling of bacteria growing in MM-2 medium for 20 min, followed by chasing with an excess of unlabelled L-methionine (1 mg ml⁻¹), cell lysis, immunoprecipitation with anti- λ O serum, SDS-PAGE, autoradiography and densitometry.

Plasmid DNA replication in amino acid-starved bacteria. Bacteria were grown in MM-2 medium, and isoleucine starvation was induced by addition of L-valine to a final concentration of 1 mg ml⁻¹. Samples of bacterial cultures were withdrawn at indicated times, plasmids were isolated from cells by alkaline lysis, and the amount of plasmid DNA was estimated after AGE and densitometric analysis of plasmid bands on an electrophoregram, as described previously (Herman-Antosiewicz *et al.*, 1998b).

RESULTS

In vitro λ DNA replication in the presence and absence of SeqA

The SeqA protein regulates replication initiation from *ori*C directly, as demonstrated in studies of the *in vitro* replication of *E. coli* minichromosomes (Slater *et al.*, 1995; Wold *et al.*, 1998; Torheim & Skarstad, 1999). Since a decreased copy number of plasmids derived from bacteriophage λ in *seqA* mutants has been reported previously (Słomińska *et al.*, 2001), we asked whether the influence of SeqA on replication of these replicons was direct or indirect. Note that the only specific effect of SeqA reported to date that could influence λ DNA replication was the regulation of *p_R* promoter activity (Słomińska *et al.*, 2001).

In vitro replication experiments were performed using a λ plasmid supercoiled DNA template, purified λ O and P proteins, and a fraction of replication proteins (called fraction II). In such an experimental system, transcription from *p_R* is effective due to the presence of RNA polymerase in fraction II (Wold *et al.*, 1982; for a review, see Taylor & Węgrzyn, 1995). In our experiments we used λ plasmid DNA template isolated from wild-type *E. coli* cells growing exponentially under standard laboratory conditions. DNA isolated from such bacteria should theoretically be a mixture of hemimethylated and fully methylated molecules. However, assuming that replication of a λ plasmid molecule takes less than 1 min, and considering the kinetics of DNA methylation, it appeared that fully methylated plasmid DNA was predominant in the preparations. On the other hand, it has previously been demonstrated that both the *in vivo* and *in vitro* effects of the $\Delta seqA$ mutation and SeqA protein on λ plasmid copy number and *p_R* activity, as well as the binding of SeqA to the λ DNA

replication region, are of equal efficiency and of the same specificity with respect to both hemimethylated and fully methylated DNA (Słomińska *et al.*, 2001, 2003b).

We observed no significant effects of SeqA on *in vitro* replication initiated from *ori* λ . First, because our Western-blotting analysis revealed that fraction II contains some SeqA protein (data not shown), we tested the effects of neutralization of this protein by anti-SeqA antibodies on λ DNA replication. No significant differences were observed in the efficiency of λ DNA synthesis in the presence and absence of anti-SeqA serum (synthesis in the presence of the serum was $86 \pm 6\%$ of that measured in the absence of anti-SeqA antibodies). Second, in contrast to previous *in vivo* studies (Słomińska *et al.*, 2001), the *in vitro* results presented in this report indicated a slight inhibition rather than stimulation of λ DNA replication by SeqA. Similar results were obtained irrespective of whether SeqA was pre-incubated with DNA template or added together with other replication proteins (Fig. 1). These results show that a direct influence of SeqA on λ DNA replication is unlikely.

Bacteriophage λ replication in infected *E. coli* *seqA* mutant

There are two modes of phage DNA replication after infection of the *E. coli* host by λ (Węgrzyn & Węgrzyn, 2002, 2005). Early after infection, phage DNA replicates according to the circle-to-circle (θ) mode, which is switched later on to the rolling-circle (σ) mode. Since only a few of many (50–100 on average) copies of a circular λ genome that appears after several rounds of θ replication switch to σ replication, the chance is small that parental DNA strands of a DNA molecule that was injected from a phage capsid into the host cell enter rolling-circle replication (Węgrzyn & Węgrzyn, 2002, 2005). This has

Fig. 1. *In vitro* replication of λ plasmid (pRLM4) in the presence of various amounts of the SeqA protein. SeqA was added to the reaction mixture simultaneously with other proteins (●) or incubated with DNA template for 15 min prior to addition of fraction II (○). The amount of λ DNA synthesized in the absence of SeqA was taken as 100% and corresponds to 75 pmol.

been employed in density-shift experiments to monitor the modes of λ DNA replication and detect the early appearance of the σ replication intermediates (Węgrzyn *et al.*, 1995; Barańska *et al.*, 2001). If wild-type host cells are infected with λ virions bearing radioactively labelled DNA, and further cultivation is performed in a heavy but non-radioactive medium (a medium containing heavy isotopes of C and N), followed by cell lysis and ultracentrifugation in a CsCl density gradient, the radioactivity can only be observed in the fully light and heavy-light fractions. On the other hand, in mutants causing an earlier switch from θ to σ replication and/or predominant rolling-circle replication, parental λ DNA strands are likely to be a part of σ intermediates and the radioactivity moves toward the fully heavy position (Fig. 2a).

In this type of experiment, 60 min after phage infection of the wild-type cells, we could observe parental λ DNA-specific radioactivity only at positions fully light (DNA molecules that did not enter replication) and heavy-light (parental DNA molecules after one or more rounds of θ replication). However, in the λ -infected *seqA* mutant, a part of the radioactivity derived from parental λ DNA moved toward the fully heavy position (Fig. 2b). These results suggested that in the *seqA* mutant the rolling-circle replication starts earlier and is more abundant than in wild-type bacteria.

Directionality of λ DNA replication in the *seqA* mutant

It has been proposed previously that initiation of the σ mode of bacteriophage λ DNA replication is preceded by one round of unidirectional θ replication (in contrast to bidirectional replication, which predominates at early stages of phage development) (Barańska *et al.*, 2001). Therefore, to determine whether the SeqA-mediated delay in the θ to σ switch results from a SeqA-driven stimulation of bidirectional replication, we investigated the directionality of λ plasmid replication in wild-type cells and *seqA* mutants. One should note that λ plasmid copy number is decreased in *seqA* mutants (Słomińska *et al.*, 2001) and that plasmids form multimers. Nevertheless, we assumed that these facts did not influence the interpretation of the results significantly.

Analysis of the results of 2D-AGE experiments indicated that there were few differences in the directionality of λ plasmid replication between wild-type and $\Delta seqA$ strains (Fig. 3a, b). Interestingly, the *seqA* mutation partially suppressed the effects of *dnaA* gene dysfunction on λ DNA replication directionality demonstrated previously (Barańska *et al.*, 2001). In contrast to the *dnaA46* single mutant, in which a high predominance of unidirectional replication has been reported (Barańska *et al.*, 2001), in the double mutant *dnaA46* $\Delta seqA$, both unidirectional and bidirectional replication intermediates could be clearly visualized (data not shown). The suppression was however partial, as in contrast to the wild-type host, in which

Fig. 2. Fate of DNA of infecting λ phage in *E. coli* *seqA*⁺ and Δ *seqA* hosts. (a) Diagram of experiments and possible results. Radioactively labelled parental phage DNA (dashed-line circles) starts its replication in bacteria growing in the heavy, non-radioactive medium. After the first round of replication, two molecules of λ DNA appear, each consisting of one radioactive light strand (dashed circles) and one non-radioactive heavy strand (solid circles). Next, several rounds of θ replication result in the formation of many (50–100 on average) λ DNA molecules, but only two of them can contain a radioactive strand, and thus radioactivity can be detected only at the heavy-light position after DNA isolation and ultracentrifugation on a CsCl density gradient. Since only a few of these 50–100 λ DNA molecules enter σ replication at late stages of phage development, there is a very low probability that any of these rolling-circle molecules would contain a radioactive strand, and as such they are not detected in these experiments [(a), upper panel]. The lower panel of (a) shows a situation in which σ replication starts early after infection or rolling-circle molecules are highly predominant in the population of λ DNA molecules. Such replication intermediates are likely to contain a light, radioactive parental DNA strand and a newly synthesized, heavy, non-radioactive tail, which leads to the transfer of the radioactivity toward the fully heavy position during density-gradient ultracentrifugation. (b) Experimental results. *E. coli* *seqA*⁺ and Δ *seqA* strains growing in the minimal light medium (at 30 °C) were infected with phage λ c/857S7 (m.o.i.=10) labelled with [³H]thymidine (9×10^{-5} c.p.m. p.f.u.⁻¹), and further incubation was performed in heavy medium (containing [¹³C]glucose and [¹⁵N]NH₄Cl instead of glucose and NH₄Cl, respectively). Samples were withdrawn at the indicated times, and total DNA was isolated and centrifuged in a CsCl density gradient. Five-drop fractions were collected from the bottom of the tube, and the radioactivity in each fraction was estimated in a scintillation counter. Arrows indicate the positions of fully heavy, heavy-light and fully light DNA (from left to right in each sub-panel). The results of the experiment with the *seqA* mutant at time 0 were analogous to those obtained for the wild-type host (i.e. only a single peak at the fully light position was detected; data not shown). The experiments were repeated three times with roughly the same distribution of the peaks.

Fig. 3. 2D-AGE of the λ plasmid (pCB104). The results obtained after isolation of plasmid DNA from cultures of *E. coli* *seqA*⁺ (a) and Δ *seqA* (b) hosts bearing pCB104 and growing at 30 °C are presented in the left-hand panels. Plasmid DNA was digested with *EcoRI* before electrophoresis. Schemes of the electrophoretic profiles are shown in the corresponding right-hand panels. Theoretical schemes based on the electrophoretic profiles of replication according to the unidirectional leftward θ mode (Uni-L), the unidirectional rightward θ mode (Uni-R), the bidirectional θ mode (Bi) and a combination of molecules replicating according to all these modes (All) are shown below. The schemes were obtained using a computer model for the analysis of DNA replication intermediates by 2D-AGE (Viguera *et al.*, 1998). Consistent data were obtained after digestion of pCB104 with *EcoRV* or *SspI* and *PvuI* (data not shown). (c) Schemes of pCB104 digested with particular enzymes, with *oriλ* marked as a bubble. The theoretical patterns of electrophoretic profiles in the case of bidirectional and unidirectional replication of pCB104 were predicted based on the assumption that replication forks initiate synchronously at *oriλ* and travel at the same rate in each direction.

and Δ *seqA* hosts), and a partial suppression of the effect of *dnaA46* mutation by the Δ *seqA* allele (roughly 10 % of bidirectional and 90 % of unidirectional replication in the *dnaA46* mutant, and 30 % of bidirectional and 70 % of unidirectional replication in the *dnaA46* Δ *seqA* double mutant; data not shown).

Effect of *seqA* mutation on stability of the λ heritable replication complex

It has been demonstrated previously that after initiation of a new round of λ DNA replication, the once-formed replication complex is not disassembled but rather inherited by one of two daughter DNA copies (Węgrzyn *et al.*, 1992, 1996a; Węgrzyn & Taylor, 1992). Interestingly, subsequently, a similar phenomenon has been proposed to occur in eukaryotic cells also (Duncker *et al.*, 2002; Li & DePamphilis, 2002). In λ , the heritable replication complex consists of λ O and P proteins and at least two host-encoded proteins, DnaB and DnaK (Potrykus *et al.*, 2002), and occurs in both bacteriophage λ -infected cells and those bearing λ plasmids (Węgrzyn *et al.*, 1995a, 1996a). The existence of the heritable λ replication complex can be monitored by measurement of the stability of the λ O protein, which is rapidly degraded in a free form in *E. coli* cells but is protected from proteolysis by other components of the complex (Węgrzyn *et al.*, 1992, 1995a, 1996b, 1998).

To monitor the stability of the λ O protein in λ plasmid-bearing wild-type and *seqA* strains, the cells were pulse-labelled with radioactive methionine, and following chasing with an excess of unlabelled methionine, samples of bacterial culture were withdrawn and analysed. After cell lysis, immunoprecipitation with anti-O serum, and SDS-PAGE, the radioactivity of protein bands was estimated. We found that a stable fraction of the λ O protein,

roughly equal frequencies of unidirectional and bidirectional replication could be deduced (Fig. 3a), in the *dnaA46* Δ *seqA* cells the accumulation of simple-Y and late double-Y structures, as well as short bubble arcs, suggested a somewhat more frequent unidirectional (preferentially leftward) replication than a bidirectional one (data not shown).

The results obtained in 2D-AGE experiments were generally confirmed in electron microscopic studies. λ plasmids were isolated from bacterial cells and linearized with restriction enzymes, and molecules with replication intermediates were analysed under the electron microscope. We found little influence of the *seqA* mutation on the directionality of λ plasmid replication (roughly 50 % of bidirectional and unidirectional replication in both *seqA*⁺

corresponding to the heritable replication complex, existed in both wild-type and *seqA* mutant hosts. However, this fraction was less abundant in the mutant (Fig. 4), suggesting that SeqA is required for fully pronounced stabilization of the λ replication complex.

It has been demonstrated that the $\Delta seqA$ ($\Delta seqA::Tn10$) mutation also results in impairment of expression of the cistronic gene *pgm* (Lu *et al.*, 1994). Recently, Hardy & Cozzarelli (2005) have found that deletion of *pgm* affects chromosome topology, without affecting plasmid supercoiling. Nevertheless, since changes in DNA topology can affect the stability of the *ori λ* -bound replication complex (Węgrzyn *et al.*, 1998), we repeated the experiments described above using the *seqA Δ 10* mutant, in which the in-frame deletion does not influence expression of *pgm*. The results of experiments with this mutant were analogous to those obtained using $\Delta seqA::Tn10$ (data not shown), indicating that dysfunction of *pgm* has no significant influence on the investigated processes.

Replication of λ plasmids in the *seqA* mutant during the relaxed response

During amino acid starvation, when new replication complexes cannot be formed due to inhibition of protein synthesis, replication of λ plasmids may occur solely due to the activity of the heritable replication complex (Węgrzyn & Taylor, 1992). However, transcriptional activation of *ori λ* (transcription initiated at the *p_R* promoter and passing through the *origin* region) is necessary for initiation of λ plasmid replication *in vivo*. Thus, synthesis of λ DNA is inhibited in amino acid-starved wild-type cells, due to guanosine tetraphosphate (ppGpp)-mediated impairment of

p_R activity, but proceeds in *relA* (relaxed) mutants, unable to produce ppGpp in starved cells (Szalewska-Pałasz *et al.*, 1994). Because of this, λ plasmid replication in the amino acid-starved *relA* host reflects the efficiency of functioning of the heritable replication complex and the effectiveness of *p_R*-initiated transcriptional activation of *ori λ* .

We found that in *relA* $\Delta seqA$ double-mutant cells growing exponentially in a minimal medium, the relative kinetics of replication of λ plasmid DNA was at a level similar to that observed in the *relA seqA⁺* host [one should note a lower plasmid copy number in $\Delta seqA$ cells, as demonstrated previously (Słomińska *et al.*, 2001) and confirmed in this report]. However, amino acid starvation caused an impairment in the plasmid DNA synthesis in the double (*relA* $\Delta seqA$) mutant relative to the *relA seqA⁺* bacteria (Fig. 5). In contrast to the almost complete inhibition of λ plasmid replication in the wild-type host (caused by accumulation of ppGpp and inhibition of transcription from *p_R*), some increase in the amount of λ DNA occurred in the *relA* $\Delta seqA$ host during the relaxed response, but the efficiency of this process was significantly lower than that in the *relA seqA⁺* bacteria (Fig. 5).

DISCUSSION

Although the SeqA protein is one of major negative regulators of *E. coli* chromosome replication (see Introduction), little is known about the putative involvement of this

Fig. 4. Relative O protein levels in *E. coli seqA⁺* (○) and $\Delta seqA$ (●) hosts bearing λ plasmid (pKBlin). Bacterial cultures were pulse-labelled with [³⁵S]methionine and chased with an excess of non-radioactive methionine at time 0. The y axis shows the O protein level as a percentage of that of the corresponding *E. coli* strain at time 0.

Fig. 5. Replication of λ plasmid (pCB104) in amino acid-starved *E. coli* wild-type (*relA⁺ seqA⁺*) host (○), and $\Delta relA seqA⁺$ (■) and *relA⁺ ΔseqA* (●) mutants. The results of experiments with the *relA⁺ ΔseqA* strain were analogous to those obtained for *relA⁺ seqA⁺* (data not shown). Isoleucine starvation was induced by the addition of L-valine up to 1 mg ml⁻¹ at the time indicated by the arrow. The y axis shows the amount of plasmid DNA relative to that of the corresponding *E. coli* strain at time 0.

factor in the control of the replication of other replicons. Examples of studies of the possible role of SeqA in the regulation of replication initiated from *origins* different from *oriC* include relatively preliminary observations only, namely the demonstration of the binding of this protein to bacteriophage P1 *ori* (Brendler *et al.*, 1995), and the findings that the copy number of plasmids derived from bacteriophage λ is decreased in a *seqA* mutant host (Słomińska *et al.*, 2001) and that the $\Delta seqA$ mutation can suppress incompatibility between λ plasmids and certain *dnaA*(ts) mutants (Słomińska *et al.*, 2003b; Glinkowska *et al.*, 2003). Here, we investigated the role of SeqA in the regulation of λ DNA replication in more detail.

Since SeqA regulates replication from *oriC* directly (by binding specifically to the *origin* region, and influencing initiator protein function and changes in nucleoprotein structures in this region), which can be demonstrated *in vitro* (Slater *et al.*, 1995; Wold *et al.*, 1998; Torheim & Skarstad, 1999), we asked whether the effect of this protein on λ DNA replication is also direct. However, in contrast to a stimulation of λ plasmid replication *in vivo*, deduced from the negative effects of the $\Delta seqA$ mutation on plasmid copy number, a small negative effect of SeqA was observed in *in vitro* experiments (Fig. 1). Thus, in the absence of stimulation of λ DNA replication by SeqA *in vitro*, we conclude that this protein controls the λ replicon indirectly. One might argue that since SeqA interacts with cell membranes, which are missing in the *in vitro* replication assay, such an experimental system could be incomplete. Although we cannot exclude such a possibility, the fact that SeqA can clearly influence *oriC*-initiated replication *in vitro* (Wold *et al.*, 1998; Torheim & Skarstad, 1999), together with our results (this report), makes the putative hypothesis of a direct involvement of this protein in λ DNA replication unlikely.

Despite the apparent lack of direct effects, there appears to be a significant role for SeqA in the regulation of λ DNA replication. This can be concluded from the results of our density-shift experiments, which indicated considerable differences in the distribution of replication intermediates between wild-type and *seqA* hosts infected by bacteriophage λ (Fig. 2). The movement of the parental radioactively labelled phage DNA toward the fully heavy position suggests that σ replication intermediates appear earlier and are more abundant in the mutant, implying a role for SeqA in the switch from circle-to-circle to rolling-circle replication during bacteriophage development. The lack of the signal at the fully light position in the experiment with the *seqA* mutant (60 min after infection), in comparison with the wild-type host, is also intriguing. In theory, this could arise from the stimulation of either θ or σ replication. However, since *seqA* inactivation has a negative effect on the replication of λ plasmids (Słomińska *et al.*, 2001; see also Fig. 5 in this report), which replicate exclusively according to the θ mode, these results may support the hypothesis that SeqA delays the rolling-circle replication of phage λ DNA.

Because it has been proposed previously that σ replication of λ DNA is preceded by one round of unidirectional θ replication (Barańska *et al.*, 2001), the lack of a marked effect of the *seqA* mutation on the directionality of λ plasmid replication (Fig. 3) might appear surprising. Nevertheless, this suggests that other processes and/or factors can also influence the mechanisms of the switch from an early to a late mode of DNA replication during bacteriophage λ lytic development. One might be the stabilization of the λ replication complex, which has in fact been postulated previously (Żylicz *et al.*, 1998). The nucleoprotein complex formed at *ori λ* contains the O protein, which is protected from proteolysis by other components of this complex, and inherited by one of the two daughter copies of λ DNA after each round of θ replication (Węgrzyn *et al.*, 1992, 1996a; Węgrzyn & Taylor, 1992; Potrykus *et al.*, 2002). Since a free (unbound) form of the O gene product is rapidly degraded in *E. coli* cells ($t_{1/2}$ ~1–2 min) (Węgrzyn *et al.*, 1992), the efficiency of formation and persistence of the heritable replication complex may be deduced from the stability of the O protein in *in vivo* pulse-chase experiments. Interestingly, the stable fraction of the O protein was significantly less abundant in the *seqA* mutant relative to wild-type bacteria (Fig. 4), indicating an involvement of SeqA in either the formation of the stable λ replication complex or its survival in cells. The mechanism of this phenomenon remains to be elucidated; nevertheless, it is worth noting that SeqA binds to a region of the λ *p_R* promoter and regulates its activity (Słomińska *et al.*, 2001), and that a decrease in the abundance of the stable O protein fraction is also observed in cells bearing a derivative of λ plasmid (pTC λ 2) in which *p_R* is replaced by another promoter (*p_{tet}*) (Herman-Antosiewicz *et al.*, 1998a) [the activity of the *p_{tet}* promoter has been demonstrated to be SeqA independent (Słomińska *et al.*, 2001)]. Moreover, another regulator of *p_R* activity, the DnaA protein (Szalewska-Pałasz *et al.*, 1998; Glinkowska *et al.*, 2003), has also been suggested to be a factor that influences the stability of the λ replication complex (Herman-Antosiewicz *et al.*, 1998a), and an interplay between DnaA and SeqA at the *p_R* promoter region has been clearly demonstrated (Słomińska *et al.*, 2003b). In this light, *seqA* dysfunction-mediated suppression of the effects of certain *dnaA* alleles on the transformation of *E. coli* cells with λ plasmids, demonstrated previously (Glinkowska *et al.*, 2001), and on the directionality of λ plasmid replication, suggested in this report, may be important to understand the regulation of λ DNA replication initiation. It is tempting to speculate that a nucleoprotein structure much larger than that suggested previously is formed at the λ DNA region encompassing *p_R* and *ori λ* . SeqA and DnaA proteins might be involved in the formation of such a structure, which would control replication initiation from *ori λ* by both facilitating stabilization of the heritable replication complex and regulating *p_R*-dependent transcriptional activation of the *origin*. A precedent for the formation of this kind of 'super-complex' has already been described; namely, the ParB

protein of phage P1 is capable of forming large complexes, due to spreading along DNA, which affect promoters located downstream (Rodionov & Yarmolinsky, 2004). In this light, it is worth mentioning that the filamentous form of SeqA is able to induce negative DNA supercoiling (Odsbu *et al.*, 2005). This might potentially affect both p_R activity and λ O protein stability.

The lack of drastic effects of SeqA on *in vitro* λ DNA replication (Fig. 1), together with the moderate effect of *seqA* dysfunction on λ plasmid and phage replication *in vivo*, suggests that this protein functions in the modulation of this process rather than being essential. However, such modulation can play an important regulatory role under different environmental and physiological conditions. Interestingly, bacteriophage λ DNA replication based on an unstable, rather than stable, replication complex has been reported in UV-irradiated bacteria (Węgrzyn & Węgrzyn, 2000), strengthening the hypothesis presented above.

If SeqA indeed modulates the efficiency of λ DNA replication by regulating the stability of the replication complex and the transcriptional activation of *ori λ* , one should observe the most significant effects of *seqA* mutations on replication, which is totally dependent on these processes. Such replication occurs as the sole mode of λ plasmid DNA synthesis during the relaxed response of bacterial cells to amino acid starvation (Taylor & Węgrzyn, 1995; Szalewska-Pałasz *et al.*, 1994). Under these conditions, new λ replication complexes cannot be formed due to the lack of amino acids, and the previously formed, heritable replication complex requires p_R -initiated transcription to initiate DNA synthesis. In accordance with the above hypothesis, we observed a significant impairment of λ plasmid replication in the amino acid-starved *relA seqA* double mutant relative to the *relA seqA*⁺ host (Fig. 5).

In conclusion, unlike the *E. coli* chromosome, the replication of which is negatively regulated by the sequestering of *oriC* due to binding of the SeqA protein to hemimethylated GATC motifs (Taghbalout *et al.*, 2000; Guarne *et al.*, 2005), replication of λ DNA is moderately stimulated by SeqA rather than inhibited. This stimulation is indirect, brought about by stabilization of the heritable replication complex and stimulation of the transcriptional activation of *ori λ* , but may nevertheless play an important regulatory role, especially under various stress conditions. This also indicates that SeqA may differentially control the replication of various replicons, employing a large spectrum of molecular mechanisms. Such a function supports the proposal that this protein is a global regulator of processes occurring in bacterial cells. In fact, types of processes that have been demonstrated to be SeqA regulated in studies of bacteriophage λ , such as transcription regulation and the stabilization of protein complexes, may represent more general phenomena occurring in the host (*E. coli*) cells rather than being restricted solely to its parasite (phage λ).

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