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Reduced LPS phosphorylation in *Escherichia coli* lowers the elevated ori/ter ratio in *seqA* mutants

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Summary

The *seqA* defect in *E. coli* increases the ori/ter ratio and causes chromosomal fragmentation, making *seqA* mutants dependent on recombinational repair (the *seqA recA* co-lethality). To understand the nature of this chromosomal fragmentation, we characterized Δ*seqA* mutants and isolated suppressors of the Δ*seqA recA* lethality. We demonstrate that our Δ*seqA* alleles have normal function of the downstream *pgm* gene and normal ratios of the major phospholipids in the membranes, but increased surface lipopolysaccharide (LPS) phosphorylation. The predominant class of Δ*seqA recA* suppressors disrupts the *rfaQGP* genes, reducing phosphorylation of the inner core region of LPS. The *rfaQGP* suppressors also reduce the elevated ori/ter ratio of the Δ*seqA* mutants, but, unexpectedly, the suppressed mutants still exhibit the high levels of chromosomal fragmentation and SOS induction, characteristic of the Δ*seqA* mutants. We also found that co-lethality of *rfaP* with defects in the production of acidic phospholipids is suppressed by alternative initiation of chromosomal replication, suggesting that LPS phosphorylation stimulates replication initiation. The *rfaQGP* suppression of the *seqA recA* lethality provides genetic support for the surprising physical evidence that the *oriC* DNA forms complexes with the outer membrane.

Keywords

seqA; *recA*; *rfaP*; ori/ter ratio; LPS; chromosomal fragmentation

Introduction

There are two classes of events leading to chromosomal fragmentation: 1) DNA damage; 2) malfunctioning of the replisomes (Kuzminov, 1995a; b). A third class of chromosome-fragmenting events was recently proposed to be overinitiation of the chromosomal DNA replication (Bidnenko *et al.*, 2002; Grigorian *et al.*, 2003; Simmons *et al.*, 2004; Nordman *et al.*, 2007). Fragmented chromosomes are incompatible with life and need to be reassembled by recombinational repair, catalyzed in bacteria by the RecBC, RecA and RuvABC enzymes (Kuzminov, 1999). Therefore, mutants with increased chromosomal fragmentation, due either to the increased DNA damage (Bradshaw and Kuzminov, 2003; Kouzminova and Kuzminov, 2004) or to the various replisome defects (Seigneur *et al.*, 1998; Flores *et al.*, 2001; Grompone *et al.*, 2002), are all dependent on RecA for viability or, to say it the other way around, they are co-lethal with the *recA* defect. One such recently-isolated RecA-dependent mutant inactivates the *seqA* gene for the negative regulator of initiation of the chromosomal replication, raising the suspicion that the chromosomal fragmentation in this case is due to replication overinitiation (Kouzminova *et al.*, 2004).

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Initiation in bacteria is accomplished by binding the DnaA initiator protein to the replication origin, *oriC* (Fig. 1). Since both the origin DNA and DnaA are constantly present in the cell, unscheduled initiation must be actively prevented. Two separate regulatory cycles in the initiation of DNA replication in *E. coli* are known. In the DnaA cycle, unscheduled initiation is prevented by a transient inactivation of DnaA after initiation via Hda+DnaN-stimulated hydrolysis of the associated ATP (Fig. 1 left). DnaA is then "rejuvenated" by exchanging its ADP for ATP in the presence of acidic phospholipids of the cell envelope. Finally, ATP-bound DnaA, with the help of DiaA (Ishida *et al.*, 2004;Keyamura *et al.*, 2007), binds to a fully methylated *oriC* DNA in preparation for origin firing (Fig. 1, center). The operation of the DnaA cycle is grossly affected by titration of the free DnaA excess to the multiple chromosomal DnaA-binding sites (Christensen *et al.*, 1999;Hansen *et al.*, 2007), among which *datA* locus alone binds ~60% of the protein (Kitagawa *et al.*, 1996;Kitagawa *et al.*, 1998). In addition, DnaA binding in the promoter of its own gene autoregulates the overall amount of the protein in the cell (Atlung *et al.*, 1985;Braun *et al.*, 1985).

In parallel to the DnaA cycle, there is the *oriC* cycle, in which unscheduled initiation is prevented by sequestering the replication origin by SeqA (reviewed by (Waldminghaus and Skarstad, 2009)), a 21 kDa dimeric protein that forms spiral filaments in vitro (Guarné *et al.*, 2005) and a few foci in dividing cells in vivo (Hiraga, 2000) (Fig. 1 right). SeqA binds properly-spaced pairs of hemi-methylated GATC sites in DNA and, therefore, is proposed to transiently bind and organize any newly-replicated DNA (Brendler *et al.*, 2000), as GATC sites throughout the chromosome remain hemimethylated for about two minutes after replication fork passage (Campbell and Kleckner, 1990). The *oriC* DNA contains several properly-spaced pairs of GATC sites (Kaguni, 2006) and binds SeqA strongly (Kang *et al.*, 1999; Skarstad *et al.*, 2000). Although SeqA itself does not have membrane-association domains, the SeqA-bound replication origin is found associated with the membrane fraction until the *oriC* DNA becomes completely methylated (Slater *et al.*, 1995; d'Alençon *et al.*, 1999), which, in contrast to the rest of the chromosome, takes up to 13 minutes after the replication start (Campbell and Kleckner, 1990). Thus, hemimethylated *oriC* remains inaccessible to DnaA, preventing unscheduled initiation (Fig. 1 right). Similar to the DnaA cycle, operation of the *oriC* cycle could be affected at the cellular level due to titration of SeqA by its multiple transient chromosomal contacts, although this aspect has never been experimentally addressed.

The *seqA* mutant cells have increased number of origins (von Freiesleben *et al.*, 1994; Boye *et al.*, 1996; Riber *et al.*, 2006) and therefore are presumed to overinitiate chromosomal replication (reviewed in (Waldminghaus and Skarstad, 2009)). One benefit of finding a co-lethal combination, like *seqA recA*, is that it offers a strong selection for suppressors — mutants that return viability to co-lethal combinations. These suppressors frequently reveal specific damaging steps that poison the original co-lethal combinations. The *seqA recA* co-lethality proved to be readily suppressed, and we expected these suppressors to inactivate various positive factors in the initiation of chromosomal replication. Assuming that chromosomal fragmentation is the lethal event in the *seqA* mutants, we further expected that the depression of replication initiation by suppressors of the *seqA recA* co-lethality would also translate into reduced chromosomal fragmentation. We did isolate the expected suppressors in the potential initiation factors that reduced both the initiation and the chromosomal fragmentation (ER and AK, unpublished). However, the majority of suppressors of the *seqA recA* co-lethality were of a different kind, indicating that the lethality can in principle be relieved without decreasing chromosomal fragmentation.

Results

Characterization of the Δ*seqA* allele

The *seqA* gene in the *E. coli* chromosome is upstream of *pgm* in a two-gene operon (Lu and Kleckner, 1994) (Fig. 2A). The Pgm protein is the phosphoglucomutase that catalyzes the reversible transformation of glucose-1-phosphate (an intermediate in maltose, galactose and glycogen metabolism and a precursor to polysaccharide wall biosynthesis) into glucose-6-phosphate (the starting point of glycolysis and gluconeogenesis) (Joshi and Handler, 1964; Adhya and Schwartz, 1971; Lu and Kleckner, 1994). To avoid the two imperfections of the previously-constructed Δ*seqA*::*tet* allele of Lu and Kleckner, — an incomplete removal of the *seqA* ORF and the significant polar effect on *pgm* (Lu *et al.*, 1994; Lu and Kleckner, 1994), — we precisely deleted the *seqA* ORF by replacing it with a kanamycin-resistance cassette (the Δ*seqA20*::*kan* allele) and later removed most of the insert (the Δ*seqA21* allele) (Fig. 2A). Since the *pgm* mutants are sensitive to 1% SDS (Lu and Kleckner, 1994), we tested for any polar effects on *pgm* in our two Δ*seqA* alleles by plating *E. coli* on media supplemented with this detergent. We found that, while both the original Δ*pgm*::*tet* and Δ*seqA*::*tet* mutants of Lu and Kleckner cannot grow on 1.5% SDS, both our Δ*seqA* mutants can, although they form much smaller colonies than wild type cells (Fig. 2B and not shown), suggesting a change in the outer membrane.

We also tested for functional phosphoglucomutase activity by iodine staining. When a *pgm* mutant is grown on minimal media with galactose as the sole carbon source, maltodextrin accumulates as an amylose intermediate and is secreted by the cell. As a result, colonies of *pgm* mutants form a dark blue border when overlaid with a solution containing 0.1% iodine and 1% potassium iodide, whereas colonies of *pgm*+ cells do not (Adhya and Schwartz, 1971). Our Δ*seqA20*::kan and Δ*seqA21* mutant colonies did not form a border, confirming their *pgm*+ status, whereas both the Δ*seqA*::*tet* and the Δ*pgm*::*tet* mutants did (Fig. 2C). To avoid working with a double *seqA pgm* mutant, Lu and Kleckner complemented their experimental strain with the pgm^+ gene on a plasmid (Lu *et al.*, 1994). Indeed, when we tested the Δ*seqA*::*tet* pML14 combination with iodine staining, the blue border no longer appeared (Fig. 2C).

Inactivation of *rfaQ*, *rfaG*, and *rfaP* suppresses the *seqA recA* lethality

To understand the nature of chromosomal fragmentation in the Δ*seqA* mutants, we used insertional mutagenesis to isolate suppressors of the *seqA recA* synthetic lethality. The Δ*seqA* Δ*recA* double mutant was barely viable at 42°C (and completely dead at lower temperatures) (Fig. 3A), which disqualified the double deletion mutant for suppression analysis due to the anticipated high background of spontaneous suppressors. Since we (and others before (Lu *et al.*, 1994)) noticed that the Δ*seqA* single defect was more severe at lower temperatures, we combined the Δ*seqA* allele with *recA629*(Cs), a cold sensitive allele of *recA*, which is deficient at 28°C, but only moderately defective at 42°C (Knight *et al.*, 1984). As expected, the double Δ*seqA21 recA629*(Cs) mutant grew well at 42°C, but failed to grow at 28−30°C (Fig. 3B and 4A), although a M9-based medium or anaerobic conditions relieved the lethality slightly (Fig. 3B).

After insertional mutagenesis, we selected for colonies that were able to grow at 30°C and, using the kanamycin-resistance determinant of the insert, verified suppressors after P1 transduction into the original Δ*seqA recA*(Cs) double mutant. Strong suppressors were able to form visible colonies after one day incubation, while weaker ones took two days. Out of multiple suppressors isolated (E.R. and A.K., unpublished), approximately three quarters took two days to grow and were highly mucoid. Sequencing identified 18 of these weak suppressors as insertions in *rfaQ*, *rfaG*, and *rfaP* (also known as *waaQ*, *waaG*, and *waaP*

(Heinrichs *et al.*, 1998)), the first three genes of the *rfaQGPSBIJYZK* operon (Fig. 4B), responsible for lipopolysaccharide (LPS) biosynthesis (Schnaitman and Klena, 1993). Another weak (mildly mucoid) suppressor was a single hit to *gmhB*, involved in the synthesis of an LPS precursor. LPS forms the outer leaflet of the outer membrane in Gram-negative bacteria, its core covering the surface of the cell as scales of protective armor (Fig. 4B inset) (Vaara, 1992; Nikaido, 1996). All three *rfa* genes function in the biosynthesis of the LPS core (see below). While all three are weak suppressors, the *rfaG* and *rfaP* mutant colonies appear larger at 30°C due to the production of the slime capsule of colanic acid (Fig. 4A). Secretion of this polysaccharide is a response to the cell envelope stress and is characteristic of the *rfaGP* mutants at lower temperatures (Parker *et al.*, 1992). To avoid problems due to this colanic acid capsule production, we handled our *rfa* mutants at 42°C.

The *rfa* inactivation is not in response to Pgm overproduction

Isolation of significant numbers of the *rfa* suppressors again raised a possibility of the *pgm* defect, — this time of *pgm* overexpression, — in our Δ*seqA* mutants. Indeed, too much of phosphoglucomutase is known to shift the balance of glucose metabolism in *E. coli* from glycolysis towards the production of UDP-glucose (Mao *et al.*, 2006) which, in some bacteria (but not in others) translates into higher yield of polysaccharides (discussed in (Boels *et al.*, 2003)). This may create cell envelope stress, which could be, somehow, alleviated by the *rfa* defect. To test for the possibility that the *rfa* suppressors are isolated in response to a change in *pgm* gene expression in our Δ*seqA* mutants, we selected suppressors of the triple *recA629*(Cs) Δ*seqA* Δ*pgm* mutant, expecting no *rfa* hits if this explanation were true. However, the triple mutant was suppressed by the same *rfa* inserts at about the same frequency as the double Δ*seqA recA* mutant (not shown), indicating that the *rfa* suppressors are not in response to changes in expression of the phosphoglucomutase gene.

Gene specificity of the *rfa* suppression

Since insertions in *rfaQ* and *rfaG* would interfere with the *rfaP* expression, it was possible that the *rfaP* defect was the only real suppressor of the Δ*seqA recA* lethality. Reducing this possibility was the fact that most of our insertions in *rfaQ* and *rfaG* have their kanamycin-resistance gene co-oriented with the *rfa* operon (Fig. 4B), so no major disruption of the *rfaP* expression was expected at least in those cases. To clarify which of the three genes were contributing to the suppression, we constructed a precise deletion of all three genes and then verified that the triple Δ*seqA recA629*(Cs) Δ*rfaQGP* mutant is still suppressed and can grow at 30°C (Fig. 4C). We then complemented the triple mutant with different combinations of the *rfaQ*+, *rfaG*+ and *rfaP*+ genes on a low-copy number plasmid (Fig. 4C). As expected, all three genes together abolished the suppression; moreover, the clones with the *rfaQ* or *rfaG* genes deleted, either singly or together, still mostly abolished the suppression (Fig. 4C). In contrast, inactivation of the single *rfaP*+ gene, or the *rfaG*+ and *rfaP*+ genes together restored the suppression (Fig. 4C), pointing to the *rfaP* status as the significant variable. Although we tentatively concluded that inactivation of *rfaP* alone is sufficient to suppress the Δ*seqA recA* lethality, we proceeded with further characterization of this suppression with all three mutations, to be on the safe side.

The *rfaP* and *rfaG* mutations compensate for the increased LPS phosphorylation in the *seqA* mutants

The *E. coli* LPS has three main components: the membrane anchor lipid A, the core region, and the O-antigen (Fig. 5A). Laboratory *E. coli* strains, such as K-12 and its derivatives that we work with, lack their O-antigen and are said to have a “rough” phenotype (non-smooth surface of colonies). If a strain also has a modified core region of its LPS, it is considered a “deep rough” mutant, in which destabilization of the outer membrane results in sensitivity to detergents and hydrophobic antibiotics (Schnaitman and Klena, 1993). RfaQ adds the branch

heptose III residue on heptose II of the core (Yethon *et al.*, 1998), RfaG builds the first glucosyl group on heptose II (Parker *et al.*, 1992), while RfaP phosphorylates heptose I (Parker *et al.*, 1992;Yethon *et al.*, 1998) (Fig. 5A). It should be noted that the *rfaP* defect prevents phosphorylations of heptose II (by RfaY), as well as addition of the heptose III branch to heptose II (Yethon *et al.*, 1998), while the *rfaG* defect decreases phosphorylation of the core LPS in an unknown way (Yethon *et al.*, 2000). Therefore, all three *rfa* mutants that we have isolated as suppressors of the *seqA recA* lethality are "deep rough" mutants, although the *rfaP* mutant strains have the strongest defects, while the *rfaQ* mutant strains have the mildest defects of the three.

To verify that our *rfaG* and *rfaP* suppressors indeed decrease the LPS core phosphorylation, we grew Δ*seqA21 recA629*(Cs) and its *rfaQ, rfaG,* and *rfaP* derivatives in the MOPS (reduced phosphate) minimal medium and labeled the cells with ^{32}P-orthophosphoric acid for five minutes. Inorganic phosphorus is expected to incorporate primarily into RNA, DNA, LPS and phospholipids, but can also be found in polyphosphates (Brown and Kornberg, 2004). We employed an isolation / separation protocol that removed phospholipids and also hydrolyzed RNA, leaving three species: DNA, LPS without ester-attached fatty acid tails and polyphosphates, which can be separated from each other (Luciana Amado and A.K., unpublished). To this end, the material produced by the "total DNA extraction protocol" was run in an alkaline agarose gel. Under these conditions, RNA is completely hydrolyzed by the alkaline buffer, chromosomal DNA forms a band right below the wells, LPS forms a faster migrating oval below the DNA band, while polyphosphates form an even faster-migrating smear (Fig. 5B).

We found that, compared to the wild type cells, *rfaP* single mutants do have lower phosphate content in their LPS (or a lower LPS/DNA ratio, which is less likely, but we did not distinguish between the two). Unexpectedly, we also found that *seqA* single mutants and the *seqA recA* double mutants have higher LPS-phosphate content (or a higher LPS/DNA ratio) (Fig. 5B), while the *rfaQGP* suppressors lower this content/ratio to either wild type levels (*rfaQ*) or to the single *rfaP* mutant levels (*rfaG* and *rfaP*) (Fig. 5B and C). Thus, one of the proximal changes in the *seqA* mutants that leads to their synthetic lethality with *recA* could be this increased LPS phosphorylation, compensated by the *rfaQGP* defect.

The decrease in LPS phosphorylation in the *rfa* mutants should lead to the weakening of the outer armor of the cell (in which individual scales are linked together via the Mg^{2+}-phosphate interactions), making mutants sensitive to anionic detergents (Vaara, 1992; Nikaido, 1996). In fact, the *rfaG* and *rfaP* mutants could not grow on LB, supplemented with 1% SDS, whereas the *rfaQ* mutants could grow, but slower than the *seqA* mutants, and only above 30°C (Fig. 5D and data not shown). Conversely, if the increased LPS phosphorylation is the real cause of the *seqA recA* inviability, shielding the extra negative LPS charge with magnesium should alleviate the lethality, — and it indeed did so completely (Fig. 5E). Magnesium supplementation also eliminated mucoidy of the *rfa* mutants (Fig. 5E), suggesting that it is the reduced LPS phosphorylation and the resulting weakening of magnesium cross-linking in these mutants that causes cell envelope stress.

No change in phospholipids in the Δ *seqA* mutants

Since the *rfa* mutations, by reducing phosphorylation, decrease the negative charge of LPS, the recent demonstration that the *seqA* defect changes the phospholipid composition of the *E. coli* membranes (Daghfous *et al.*, 2006), was also of interest. The three major phospholipids in *E. coli* are the zwitterionic (neutral) phosphatidylethanolamine (PE) and the two acidic species, phosphatidylglycerol (PG) and cardiolipin (CL) (Fig. 6A) (Cronan and Vagelos, 1972). The *seqA* defect was reported to dramatically decrease the fraction of PE, while increasing the fraction of both PG and CL (Daghfous *et al.*, 2006), in effect,

increasing the acidity of the phospholipid membranes. As mentioned in the introduction (Fig. 1), in vitro, acidic phosholipids PG and especially CL stimulate the ADP—>ATP exchange in the DnaA replication initiation protein, associated with the *oriC* DNA, thus recharging it for the new initiation round (Sekimizu and Kornberg, 1988; Crooke *et al.*, 1992). The defect in production of PG in the *E. coli pgsA* mutant apparently inhibits replication initiation, because this lethal phenotype was once reported to be suppressed by inactivation of the *rnhA* gene (Xia and Dowhan, 1995). The *rnhA* defect also suppresses the replication initiation problems of the *dnaA* and Δ*oriC* mutants by permitting alternative replication initiations all around the chromosome (Kogoma and von Meyenburg, 1983). Therefore, if confirmed, the increased acidity of phospholipids in the *seqA* mutants would have increased the initiation potential, supporting an additional mechanism for replication overinitiation in the *seqA* mutants unrelated to the lack of the origin sequestration (Fig. 1, compare the DnaA cycle versus the *oriC* cycle). This possible additional DnaA activation in *seqA* mutants would explain our isolation of the *rfa* suppressors of the *seqA recA* lethality as a compensation of this change in the overall membrane charge, especially so that the DnaA activation by acidic lipids is not specific for a particular lipid chemistry or even lipid structure (Castuma *et al.*, 1993; Garner and Crooke, 1996). Accordingly, the *rfa* suppressors, by decreasing the charge of the outer membrane, could have reduced the overall membrane charge, shifting the ADP/ATP in the DnaA population towards the inactive DnaA species and, thus, reducing unscheduled initiations.

We analyzed the phospholipid composition in wild type and Δ*seqA* mutants of *E. coli* after labeling phospholipids with ^{32}P and separating them in one-dimensional TLC (Fig. 6B) (Cronan, 1968). A typical phospholipid composition in wild type cells is 80−85% PE, the remaining 15−20% being distributed between PG and CL (Cronan and Vagelos, 1972). We confirmed this composition for wild type cells (Fig. 6C). However, we did not detect any significant differences between two different wild type strains and their Δ*seqA* derivatives in phospholipid composition, although we did detect a significant increase in the acidic species as the cells moved from growth phase into the stationary phase (Fig. 6C). We conclude that the *rfa* suppressors are not compensating for any changes in the phospholipid composition.

Ori/ter ratio, SOS induction and chromosomal fragmentation in *seqA rfa* mutants

At the chromosomal level, the major difference between the *seqA* mutants versus wild type cells is the greatly increased ori/ter ratio in the former (which is >6 at 30°C, compared to <2 in the *seqA*+ parent) (Fig. 7A). Thus, the expected general consequence of suppression of the *seqA recA* lethality is reduction of the high ori/ter ratio of the Δ*seqA* mutants (Fig. 7A). The ori/ter ratio analysis of the *seqA rfa* double mutants indeed showed a significant reduction in the ratio (to 3−4) for all three *rfa* suppressors, although not to the wild type levels (Fig. 7A, center). A trivial reason for the decreased ori/ter ratio could be a slower growth of the *rfaQGP* mutants: as already mentioned, growing the Δ*seqA recA629* double mutant on a minimal medium or in anaerobic conditions did relieve the lethality slightly (Fig. 3B). However, simply increasing the doubling time of the *seqA* mutants to match the doubling time of *seqA rfa* double mutants by supplementing the growth medium with sub-static concentrations of tetracycline (0.05−0.1 μg/ml) did not reduce the ori/ter ratio of the former (not shown). Thus, the *rfa* suppressors are likely to reduce the ori/ter ratio in the chromosomal DNA of *seqA* mutants directly.

The Δ*seqA* mutants have increased chromosomal fragmentation, detectable in the *recBC*-deficient background, which is presumed to be the ultimate reason for the *seqA recA* co-lethality (Kouzminova *et al.*, 2004). Thus, we expected that the viability of the *seqA recA rfa* triple mutants would reflect decreased chromosomal fragmentation in the double *seqA rfa* mutants. To quantify chromosomal fragmentation, we introduced the *rfa* suppressors into the *seqA recBC*(Ts) double and *recBC*(Ts) single mutants. Surprisingly, we found no significant

alleviation of the high chromosomal fragmentation levels in the Δ*seqA21 recBC*(Ts) mutant by the *rfaQ*, *rfaG*, or *rfaP* suppressors (Fig. 7B, center). At the same time, the *rfa recBC*(Ts) double mutants (*seqA*+) showed the *recBC*(Ts) levels of chromosomal fragmentation (Fig. 7B, right). We conclude that 1) lowering of the ori/ter ratio does not necessarily translate into reduced chromosomal fragmentation; 2) the *rfaQGP* defect allows the *seqA recA* mutants to tolerate the levels of chromosomal fragmentation which are apparently lethal for the RfaQGP+ strains.

The increased chromosomal fragmentation in the *seqA* mutants leads to a significant SOS induction (a transcriptional response of bacterial cells to chromosomal damage) (Kouzminova *et al.*, 2004). Consistent with the high chromosomal fragmentation in the *seqA rfa* mutants, SOS induction in the double *seqA rfa* mutants was still high and was even higher than in the single Δ*seqA* mutant (Fig. 7C, center), suggesting even more DNA damage. One possibility was that the *rfa* suppressors, while decreasing the SOS induction due to the *seqA* defect, caused some SOS response by themselves, which is not a reaction to double-strand breaks (absent in single mutants (Fig. 7B)). This indeed was found to be the case for the *rfaG* and *rfaP* single mutants, but the magnitude of induction was not enough to account for the values in the *seqA rfa* double mutants (Fig. 7C, right). We conclude that the *seqA* and *rfa* defects have an additive effect on SOS induction, suggesting that the *rfa* suppressors do not decrease SOS in the *seqA* mutants, but, instead, contribute their own induction.

Genetic analysis of the *rfaP* suppression of the *seqA recA* lethality

The increased number of the replication origins in the Δ*seqA* mutants is interpreted to reflect replication overinitiation (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994), the reason for this overinitiation being the increased accessibility of *oriC* to binding by the DnaA initiator protein (Fig. 8A, the Δ*seqA* shunt). If overinitiation is indeed the problem of *seqA* mutants, the current understanding (Fig. 1) predicts two possible types of suppression mechanisms: one acting via the *oriC* loop (Fig. 8A, right) (for example, by providing alternative means of sequestering the origin), the other acting via the DnaA loop (Fig. 8A, left) (for example, via decreasing the ADP-DnaA —> ATP-DnaA exchange). We sought to distinguish between the two ideas genetically, by combining the *rfaQGP* mutants with defects in the replication initiation.

First, employing a mutant DnaA, called DnaA(Cs) (or DnaA(cos)), that does not need the membranes to exchange ADP for ATP (Fig. 8A, the DnaA(Cs) shunt) because of the lower affinity to either nucleotide (Katayama, 1994), we tested the idea that the *rfa* suppression works through the *oriC* loop. As a result of its defect, DnaA(Cs) protein binds *oriC* more aggressively and is thought to overinitiate chromosomal replication in otherwise wild type strains to such an extent that the cells are inhibited (since the *dnaA*(Cos) defect was isolated as a suppressor of a dnaA(Ts) defect, *dnaA*(Cs) cells are inhibited only at lower temperatures) (Kellenberger-Gujer *et al.*, 1978;Simmons *et al.*, 2004). The *dnaA*(Cs) mutant inhibition at low temperatures is suppressed by a defect in the positive initiation factor, the DNA adenine methylase Dam (Katayama *et al.*, 1997;Nordman *et al.*, 2007). We have confirmed this observation (Fig. 8B) and also showed that the same is true for the defect in DiaA (Ishida *et al.*, 2004) (Fig. 8B), the only other positive factor to the right of the DnaA(Cs) shunt (Fig. 8A). On the other hand, we found that inactivation of Hda has no effect on the DnaA(Cs)-overproducing strain (Fig. 8B), perhaps because the Hda-promoted ATP hydrolysis cannot inhibit the DnaA(Cs) protein, which exchanges nucleotide cofactors freely. Somewhat unexpectedly, inactivation of the other negative replication initiation regulator, SeqA (Fig. 8A), has no effect on the DnaA(Cs)-overproducing strain either (Fig. 8B). Even more surprisingly, we found that the *rfaP* mutant somewhat exaggerated the inhibition of the DnaA(Cs)-producing strain, instead of alleviating it, as *dam* and *diaA*

defects did (Fig. 8B). This result suggests the *rfa* suppression works outside of the *oriC*-cycle, — one possibility was that it works in the DnaA-cycle, by negatively affecting a step to the left of the DnaA(Cs) shortcut (Fig. 8A).

To test the idea that the *rfaQGP* suppression works through the DnaA loop, for example by affecting DnaA interaction with membranes, we tested if the *rfaQGP* defect would exacerbate the temperature-sensitivity of the *dnaA*(Ts) mutants. However, we found no significant interaction between the *rfa* mutants and four different *dnaA*(Ts) strains (*dnaA5, 46, 177* and *508*, Fig. S1A and data not shown), — perhaps because the mutant DnaA proteins were not affected in the nucleotide exchange. Therefore, we introduced the *rfaP* defect into the *dnaA* T174P and *dnaA* A345S mutants that spontaneously hydrolyze ATP and thus underinitiate (Gon *et al.*, 2006); likewise, no interaction between these *dnaA* defects and the *rfaP* defect was apparent, with the exception of the peculiar suppression of mucoidy at low temperature (Fig. S1B). We interpret the results of this epistatic analysis to mean that 1) LPS phosphorylation acts outside of the two known cycles of regulation of replication initiation (Fig. 1 and 8A); 2) there is no direct interaction between DnaA and LPS of the type proposed for acidic phospholipids.

To test whether LPS phosphorylation affects replication initiation at all, we introduced the *rfaP* mutant into the double *pgsA lpxB* (formerly *pgsB*) mutant that synthesizes reduced amounts of phosphatidylglycerol and cardiolipin at low temperatures and stops their synthesis altogether at 42°C (Nishijima and Raetz, 1979; Nishijima *et al.*, 1981). Since, as mentioned above, these two acidic phospholipids promote the nucleotide exchange in DnaA in vitro (Sekimizu and Kornberg, 1988; Crooke *et al.*, 1992), the inability of *pgsA lpxB* mutant to form colonies at 42°C may be in part due to a defect in replication initiation, especially since a similar inviability of a different *pgsA* null mutation was reported to be suppressed by providing alternative means for initiation of chromosomal replication through the *rnhA* defect (Xia and Dowhan, 1995) (although we were unable to confirm this observation (Fig. S2 and S3)). We found that we can build the triple mutant *pgsA lpxB rfaP* at 28°C, but the mutant was severely inhibited at 34°C (mostly due to the *pgsA lpxB* defect) and essentially non-viable at 23°C (due to the *rfaP* defect, which is worse at lower temperatures (Parker *et al.*, 1992)) (Fig. 8C). We confirmed that the triple mutant's growth problems were due to the replication initiation defect, by inserting an IPTG-induced plasmid replication origin near *oriC*, — the strains with the plasmid origin grew much better both in the presence and in the absence of IPTG (Fig. 8D, rows 3 and 6). On the basis of our results we propose that *rfaQGP* inactivation suppresses the *seqA recA* co-lethality by lowering the ori/ter ratio to manageable levels via decreasing replication initiation, working outside of the two known regulation cycles and without direct interactions with DnaA itself.

Discussion

To get insights into the mechanisms of chromosomal fragmentation in Δ*seqA* mutants we designed a genetic system for isolation of suppressors of the *seqA recA* lethality. We found that *seqA* mutants have increased LPS phosphorylation, whereas a major class of Δ*seqA recA* suppressors, *rfaQGP*, has reduced LPS phosphorylation, suggesting an initial cause of both lethality and its suppression, but not a mechanism. As expected, the *rfaQGP* suppressors did reduce the elevated ori/ter ratio of the Δ*seqA* mutants, but, unexpectedly, the suppressed strains still exhibited the high levels of chromosomal fragmentation, characteristic of the non-suppressed *seqA* mutants. We also found that the *rfaP* defect is co-lethal with the defect in the production of acidic phospholipids, and the co-lethality is suppressed by alternative initiation of chromosomal replication, suggesting a role of LPS phosphorylation in replication initiation. We failed to find genetic evidence for direct interactions between LPS and the DnaA initiator protein, though.

The finding that the *rfaQGP* suppressors improve growth of the *seqA* mutants despite the same level of chromosomal fragmentation is surprising. In other words, chromosomal fragmentation does not seem to be as lethal as the increased ori/ter ratio. Other suppressors of the *seqA recA* lethality restore viability because they concurrently reduce both the ori/ter ratio *and* chromosomal fragmentation (Fig. 7BC, the *ybfE* control; E.R. and A.K., unpublished). A face-value explanation of the unexpectedly high levels of chromosomal fragmentation in the *rfa*-suppressed *seqA* mutants is that the increase in ori/ter ratio and the chromosomal fragmentation are not always causally-related, as is currently assumed, but can affect cell viability independently of each other. We are not aware of any additional evidence to corroborate this point, however. Another explanation is that formation of subchromosomal fragments in the *seqA* mutants is not lethal per se, and an unknown signaling pathway is required to stop the cell growth by the accumulating subchromosomal fragments, while the *rfaQGP* defects interfere with this signaling independently of lowering the ori/ter ratio. For example, the cell cycle in a lower eukaryote, the budding yeast, can be permanently halted by one double-strand DNA break in a non-essential DNA molecule, while the *rad9* defect in the DNA damage-signaling pathway alleviates this lethal block (Bennett *et al.*, 1993). If the RfaQGP proteins are indeed involved in signaling between DNA damage and the bacterial cell cycle, this will be the first example of such function. Yet another way to explain the high chromosomal fragmentation in the *rfaQGP*-suppressed *seqA* mutants is to propose two different mechanisms of this fragmentation, with one mechanism producing the bulk of fragmentation, which is not lethal, while the other mechanism being responsible for a small fraction of lethal fragmentation events. According to this explanation, it is the second (lethal) mechanism that the inactivation of *rfaQGP* blocks.

Another interesting aspect of the *rfa* suppression of the replication overinitiation defect is that LPS forms the outer leaflet of the outer membrane, whereas DNA metabolism is clearly cytoplasmic. Remarkably, both SeqA and origin DNA have been isolated from *outer* membrane extracts (Ogden *et al.*, 1988) (Chakraborti *et al.*, 1992) (Slater *et al.*, 1995) (d'Alençon *et al.*, 1999), although in some cases, the origin is associated with a particular intermediate (neither inner nor outer membrane) fraction (Chakraborti *et al.*, 1992). Since it is known that: 1) the DnaA protein is activated for initiation by acidic phospholipids (Sekimizu and Kornberg, 1988; Crooke *et al.*, 1992; Xia and Dowhan, 1995), but this activation is not dependent on a particular lipid chemistry (Castuma *et al.*, 1993; Garner and Crooke, 1996); 2) as just mentioned, the origin DNA is found in complex with the outer membrane (LPS), rather than with the inner one (phospholipids); 3) the *rfaQGP* mutants reduce phosphorylation of LPS, — we imagined that LPS could interact with DnaA protein directly, while its reduced phosphorylation (= acidity) would translate into a reduced activation of DnaA, explaining the mechanism of the *rfaQGP* suppression of the overinitiation in *seqA* mutants. Since we found 1) no evidence for direct interaction between DnaA and LPS; 2) increased ori/ter ratio in mutants with increased LPS phosphorylation (*seqA*) 3) decreased ori/ter ratio in mutants with decreased LPS phosphorylation (*rfaQGP*), LPS phosphorylation could stimulate replication initiation outside of the two known regulation cycles (Fig. 1 and 8A) via yet-to-be identified factors.

From the perspective of the cell envelope structure, our *rfa* suppressors offer a genetic confirmation for the previous reports of the direct contacts between DNA and the outer membrane. For such patently cytoplasmic entities as SeqA and the replication origin DNA to have contacts with the component of the outer surface of the cell, there must be places in the cell, where the outer membrane comes in contact with the cytoplasm. Such places are most likely junctions connecting the outer and the inner membranes. Two types of such junctions are generally acknowledged, although their existence is still debated (Nikaido, 1996; Oliver, 1996). The first type of junctions are periseptal annuli; these are circumferential zones of adhesion of the two membranes positioned either at the poles or in

the middle of the cell (Macalister *et al.*, 1983; Cook *et al.*, 1986). The second type is represented by much smaller and dispersed junctions, numbered 200−400 per cell, that have been seen only in growing cells and are known as “Bayer patches” (Bayer, 1968). They are proposed to be sites of fusion of the two membranes, where LPS, capsules, and proteins of the cell wall are synthesized (Bayer, 1979) (Fig. 9). Additionally, chromosomal DNA has been photographed interacting with these junctions (Bayer, 1979). It is interesting to note that the *rfaQGP* suppression of the *seqA recA* lethality provides genetic evidence to support the idea that parts of the outer membrane, most likely continuous with the inner membrane, are in direct contact with the cytoplasm in general and with the chromosomal DNA in particular (Fig. 9).

Experimental Procedures

Growth conditions

Cells were grown in LB (10g Tryptone, 5g Yeast Extract, 5g NaCl, 250 μl 4M NaOH per 1 liter) or on LB agar (LB supplemented with 15 g of agar per 1 liter). M9 minimal plates (Miller, 1972) contained 1× M9 salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and were supplemented per 1 liter with 10 mg thiamine (B1), 15 g agar and 2 g galactose or glucose. MOPS minimal phosphate medium was as described (Neidhardt *et al.*, 1974). Ampicillin (100 μg/ml), kanamycin (50 μg/ml), spectinomycin (100 μg/ml) and chloramphenicol (10 μg/ml) were added when the strains carried the corresponding antibiotic-resistance.

Bacterial strains and plasmid

E. coli strains used in these experiments were all K-12, and most of them were derivatives of AB1157 (Table 1). L-216 has an IPTG-dependent ColE1-derived replication origin, inserted in the chromosome at position 4,054 Mbp (Elena Kouzminova, personal communication; (Kouzminova and Kuzminov, 2008)). Precise deletion-replacement alleles of selected genes were created and confirmed by PCR by the method of Datsenko and Wanner (Datsenko and Wanner, 2000). Alleles were moved between strains by P1 transduction (Miller, 1972). The *rec* mutants were confirmed by their characteristic UV-sensitivities. The *dnaA* T174P and A345S alleles were confirmed by sequencing. The plasmids are described in Table 2.

Spot test for synthetic lethality

Growth at the “non-permissive” temperature was first assayed by diluting an overnight culture 100-fold, growing it to 5×10^8 cells/ml, diluting 0.2 μl in 5 ml of 1% NaCl, and spotting by 10 μl. Since preliminary results indicated that the viability of saturated cultures was similar to that of rapidly growing cultures, in subsequent assays we spotted 10 μl of a 10^{-6} dilution of a saturated culture. Plates were incubated at either 42°C (permissive temperature for the *recA629*(Cs) allele) or 30°C (the non-permissive temperature). Colonies were given approximately 24 hours to grow at 42°C and 48 hours at 30°C.

Iodine Staining

Mutants in MG1655 background were grown on M9 galactose minimal medium for two days (wild type) or for four days (*pgm* mutant) until colonies were approximately 1 mm in diameter. The plates were overlaid with 3 ml top M9 iodine agar containing 1× M9 salts, 7.5 g agar, 10 g potassium iodide, and 1 g iodine per 1 liter. Within seconds, *pgm* mutants formed a dark blue border, which then disappeared within 20 minutes (Adhya and Schwartz, 1971).

Insertional mutagenesis

pRL27 is a plasmid containing a hyperactive Tn5 transposase gene under the *tetA* promoter and a separate insertional cassette consisting of kanamycin resistance and the *pir*-dependent origin of replication *oriR6K*, bracketed by Tn5 inverted repeats (Larsen *et al.*, 2002). Electroporation of this plasmid in a *pir*- background causes the mini-transposon to insert randomly into the chromosome. The Δ*seqA21 recA629* cells were electroporated with 10 ng of pRL27 and outgrown for 1 hour and 20 minutes at 42°C before being plated on LB kan (10 μg/ml) at 30°C. Colonies were streaked after two days of growth onto LB supplemented with 50 μg/ml kanamycin next to the original Δ*seqA21 recA629* double mutant and the single *recA629* mutant (with a *kan*-insert at an irrelevant gene to provide kanamycin-resistance) as a positive control. If the strain was able to form colonies, a P1 lysate was made at 42°C and used to transduce the parental Δ*seqA21 recA629* double mutant, also at 42°C. 10 μl of a 10^{-6} dilution of the transductants was used to test the ability to grow at the non-permissive temperature of 30°C. The confirmed suppressors linked to the kanamycin-resistance insertion were identified as before (Kouzminova *et al.*, 2004; Ting *et al.*, 2008), after digesting chromosomal DNA with MluI (which has no sites in the insertion cassette), circularizing the digested fragments by ligation and transforming into DH5α pir^{+}. The plasmid with interrupted gene was selected for by plating with kanamycin, and the gene was identified using primers (Bradshaw and Kuzminov, 2003) facing outwards from the insertion element's ends.

Pulsed-field gel electrophoresis

Overnight LB cultures were diluted to OD_{600}=0.02 in LB and grown in the presence of 2.5 − 10 μCi of ^{32}P orthophosphoric acid for one hour at 22° and three hours at 37°. All cultures were then brought to OD_{600}=0.35. Cells from 0.5−1 ml aliquots were spun down, washed in 1 ml of TE and resuspended in 60 μl of TE. To this cell suspension, 2.5 μl of proteinase K (5 mg/ml) and 65 μl of 1.2% agarose in Lysis buffer (see below) was added, and mixed by pipetting. 110 μl of the mixture was then pipetted into the plug mold and allowed to solidify. The plugs were incubated overnight at 60°C in Lysis buffer (1% sarcosine, 50 mM Tris-HCl, and 25 mM EDTA). Samples were loaded into a 1.0% agarose gel in 0.5× TBE buffer and run at 6.5 V/Cm with a pulse time of 90 seconds for 7 hours, 105 seconds for 8 hours, and 125 seconds for 8 hours in Gene Navigator (Pharmacia). The gel was vacuum dried onto a piece of chromatography paper (Fisher) for two hours at 80°C and then exposed to a PhosphorImager screen until signals from the wells reached between 300,000 and 900,000 counts. If unlabeled, the gel was stained for 30 minutes in 0.5 μg/ml ethidium bromide and de-stained for 30 minutes in deionized water before pictures were taken.

SOS induction

To determine the level of SOS induction in the cell, the Δ*seqA20*::kan mutation was P1 transduced into AK43 (Kouzminova *et al.*, 2004), a strain containing a *mud*-derived construct with the *lacZ* gene fused under the *sfiA* promoter (Ossanna and Mount, 1989). To generate Δ*seqA21* P*sfiA*—>*lacZ*, we P1 transduced the Mud into Δ*seqA21*. Other mutants were later P1 transduced into this *seqA* P*sfiA*—>*lacZ* reporter background. When the cells are under SOS-induced stress, either by the mutation or external DNA damage, the promoter is expressed, and the level of β-galactosidase can be quantitatively measured by the modified protocol of Miller (Miller, 1972), using 200 μl of culture (Kouzminova *et al.*, 2004). As a positive control, wild type cells containing the P*sfiA*—>*lacZ* fusion were treated with 100 ng/ml Mitomycin C, a cross-linking agent. At these Mitomycin C concentrations, cells continue slower growth.

Dot-hybridization to determine ori/ter ratio

Total DNA was extracted from saturated and exponentially growing (OD_{600}=0.6) cultures by phenol/chloroform method (Kouzminova and Kuzminov, 2006). 2 μg of this DNA was denatured in 400 μl of 0.1M NaOH for 15 minutes at 37°C and spotted in duplicate on a positively charged Nylon membrane (Amersham) using a vacuum manifold. After cross-linking the DNA to the membrane with UV, the membrane was divided in two with one half hybridizing to an origin-proximal probe and the other half to a terminus-proximal probe (Kouzminova and Kuzminov, 2006).

Phospholipid extraction

Total phospholipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959). ^{32}P labeled cells were pelleted, resuspended in the remaining liquid, and mixed with 4 ml methanol and 2 ml chloroform. After an hour tumble, 2 ml chloroform and 2 ml deionized water were added, and the suspension was mixed and centrifuged to separate the phases. The aqueous phase was removed, and 4 ml 2M KCl was used for another extraction, followed by yet another extraction with 3 ml of water. The remaining 3 ml of organic phase was dried under a stream of nitrogen and dissolved in 100 μl 2:1 chloroform:methanol (or 1 ml was dried and dissolved in 33 μl of the chloroform/methanol mixture). Running lanes were generated by scraping on a Silica-G TLC plate (Analtech), which was then baked at 100°C for 30−60 minutes, and 8−12 μl of sample was loaded per lane. The samples were run in a buffer containing 97.5 ml chloroform, 32.5 ml methanol and12 ml glacial acetic acid until the front reached the top of the plate. The TLC plate was exposed to a PhosphorImager screen until signals from the phosphatidylglycerol spots exceeded 500,000 counts.

Detection of LPS phosphorylation

Bacterial cultures were grown in MOPS-minimal phosphate-limiting medium to OD of 0.35−0.4 and pulse labeled with ~10 μCi ^{32}P orthophosphate for 5 minutes before centrifugation, and then processed according to the “total DNA isolation protocol” (Kouzminova and Kuzminov, 2006). Cells were resuspended in 50 μl of 30% sucrose in TE buffer and lysed for 5 minutes at 70°C after addition of 350 μl of 2% SDS in TE and careful mixing. The lysate was extracted with 400 μl of phenol, followed by 400 μl of phenol:chloroform (1:1), and 400 μl of chlorofom. The aqueous phase was ethanol precipitated twice and dissolved in 40 μl of TE buffer. 20 μl of this sample was combined with 20 μl of 2X alkaline agarose loading dye, and 20 μl was separated on a 1.1% alkaline agarose gel (Maniatis *et al.*, 1982) for 620 minutes at 20V (1.4 V/cm).

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References

Adhya S, Schwartz M. Phosphoglucomutase mutants of *Escherichia coli* K-12. J. Bacteriol. 1971; 108:621–626. [PubMed: 4942754]

Atlung T, Clausen ES, Hansen FG. Autoregulation of the *dnaA* gene of *Escherichia coli* K12. Mol. Gen. Genet. 1985; 200:442–450. [PubMed: 2995766]

Atlung T, Løbner-Olesen A, Hansen FG. Overproduction of DnaA protein stimulates initiation of chromosome and minichromosome replication in *Escherichia coli*. Mol. Gen. Genet. 1987; 206:51–59. [PubMed: 3033441]

Bach T, Krekling MA, Skarstad K. Excess SeqA prolongs sequestration of *oriC* and delays nucleoid segregation and cell division. EMBO J. 2003; 22:315–323. [PubMed: 12514137]

Bach T, Skarstad K. Re-replication from non-sequesterable origins generates three-nucleoid cells which divide asymmetrically. Mol. Microbiol. 2004; 51:1589–1600. [PubMed: 15009887]

Bachmann, BJ. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12.. In: Neidhardt, FC., editor. Escherichia coli and Salmonella typhimurium. Cellular and Molecular Biology. American Society for Microbiology; Washington, D.C.: 1987. p. 1190-1219.

Bayer ME. Areas of adhesion between wall and membrane of *Escherichia coli*. J. Gen. Microbiol. 1968; 53:395–404. [PubMed: 4181162]

Bayer, ME. The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection.. In: Inouye, M., editor. Bacterial Outer Membranes. John Wiley & Sons; New York: 1979. p. 167-202.

Bennett CB, Lewis AL, Baldwin KK, Resnick MA. Lethality induced by a single site-specific double-strand break in a dispensable yeast plasmid. Proc. Natl. Acad. Sci. USA. 1993; 90:5613–5617. [PubMed: 8516308]

Bidnenko V, Ehrlich SD, Michel B. Replication fork collapse at replication terminator sequences. EMBO J. 2002; 21:3898–3907. [PubMed: 12110601]

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y. The complete genome sequence of *Escherichia coli* K-12. Science. 1997; 277:1453–1462. [PubMed: 9278503]

Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Ca, J. Biochem. Physiol. 1959; 37:911–917.

Boels IC, Kleerebezem M, W.M. d.V. Engineering of carbon distribution between glycolysis and sugar nucleotide biosynthesis in *Lactococcus lactis*. Appl. Environ. Microbiol. 2003; 69:1129–1135. [PubMed: 12571039]

Boye E, Stokke T, Kleckner N, Skarstad K. Coordinating DNA replication initiation with cell growth: differential roles for DnaA and SeqA proteins. Proc. Natl. Acad. Sci. USA. 1996; 93:12206–12211. [PubMed: 8901558]

Bradshaw JS, Kuzminov A. RdgB acts to avoid chromosome fragmentation in *Escherichia coli*. Mol. Microbiol. 2003; 48:1711–1725. [PubMed: 12791149]

Braun RE, O'Day K, Wright A. Autoregulation of the DNA replication gene *dnaA* in *E. coli* K-12. Cell. 1985; 40:159–169. [PubMed: 2981626]

Brendler T, Abeles A, Austin S. A protein that binds to the P1 origin core and the *oriC* 13mer region in a methylation-specific fashion is the product of the host *seqA* gene. EMBO J. 1995; 14:4083–4089. [PubMed: 7664748]

Brendler T, Sawitzke J, Sergueev K, Austin S. A case for sliding SeqA tracts at anchored replication forks during *Escherichia coli* chromosome replication and segregation. EMBO J. 2000; 19:6249–6258. [PubMed: 11080170]

Brown MR, Kornberg A. Inorganic polyphosphate in the origin and survival of species. Proc. Natl. Acad. Sci. USA. 2004; 101:16085–16087. [PubMed: 15520374]

Camara JE, Breier AM, Brendler T, Austin S, Cozzarelli NR, Crooke E. Hda inactivation of DnaA is the predominant mechanism preventing hyperinitiation of *Escherichia coli* DNA replication. EMBO Reports. 2005; 6:736–741. [PubMed: 16041320]

Campbell JL, Kleckner N. *E. coli oriC* and the *dnaA* gene promoter are sequestered from dam methyltransferase following the passage of the chromosomal replication fork. Cell. 1990; 62:967–979. [PubMed: 1697508]

Castuma CE, Crooke E, Kornberg A. Fluid membranes with acidic domains activate DnaA, the initiator protein of replication in *Escherichia coli*. J. Biol. Chem. 1993; 268:24665–24668. [PubMed: 8227025]

Chakraborti A, Gunji S, Shakibai N, Cubeddu J, Rothfield L. Characterization of the *Escherichia coli* membrane domain responsible for binding oriC DNA. J. Bacteriol. 1992; 174:7202–7206. [PubMed: 1429444]

Christensen BB, Atlung T, Hansen FG. DnaA boxes are important elements in setting the initiation mass of *Escherichia coli*. J. Bacteriol. 1999; 181:2683–2688. [PubMed: 10217754]

Cook WR, MacAlister TJ, Rothfield LI. Compartmentalization of the periplasmic space at division sites in gram-negative bacteria. J. Bacteriol. 1986; 168:1430–1438. [PubMed: 3536880]

Cronan JE. Phospholipid alterations during growth of *Escherichia coli*. J. Bacteriol. 1968; 95:2054–2061. [PubMed: 4876126]

Cronan JE, Vagelos PR. Metabolism and function of the membrane phospholipids of *Escherichia coli*. Biochim. Biophys. Acta. 1972; 265:25–60. [PubMed: 4552305]

Crooke E, Castuma CE, Kornberg A. The chromosome origin of *Escherichia coli* stabilizes DnaA protein during rejuvenation by phospholipids. J. Biol. Chem. 1992; 267:16779–16782. [PubMed: 1512219]

Czonka LN, Clark AJ. Deletions generated by the transposon Tn*10* in the *srl-recA* region of the *Escherichia coli* K-12 chromosome. Genetics. 1979; 93:321–343. [PubMed: 395024]

d'Alençon E, Taghbalout A, Kern R, Kohiyama M. Replication cycle dependent association of SeqA to the outer membrane fraction of *E. coli*. Biochimie. 1999; 81:841–846. [PubMed: 10572297]

Daghfous D, Chatti A, Marzouk B, Landoulsi A. Phospholipid changes in *seqA* and *dam* mutants of *Escherichia coli*. C. R. Biol. 2006; 329:271–276. [PubMed: 16644499]

Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA. 2000; 97:6640–6645. [PubMed: 10829079]

Flores M-J, Bierne H, Ehrlich SD, Michel B. Impairment of lagging strand synthesis triggers the formation of a RuvABC substrate at replication forks. EMBO J. 2001; 20:619–629. [PubMed: 11157768]

Garner J, Crooke E. Membrane regulation of the chromosomal replication activity of *E. coli* DnaA requires a discrete site on the protein. EMBO J. 1996; 15:3477–3485. [PubMed: 8670850]

Gon S, Camara JE, Klungsøyr HK, Crooke E, Skarstad K, Beckwith J. A novel regulatory mechanism couples deoxyribonucleotide synthesis and DNA replication in *Escherichia coli*. EMBO J. 2006; 25:1137–1147. [PubMed: 16482221]

Grigorian AV, Lustig RB, Guzman EC, Mahaffy JM, Zyskind JW. *Escherichia coli* cells with increased levels of DnaA and deficient in recombinational repair have decreased viability. J. Bacteriol. 2003; 185:630–644. [PubMed: 12511510]

Grompone G, Seigneur M, Ehrlich SD, Michel B. Replication fork reversal in DNA polymerase III mutants of *Escherichia coli*: a role for the beta clamp. Mol. Microbiol. 2002; 44:1331–1339. [PubMed: 12028381]

Guarné A, Brendler T, Zhao Q, Ghirlando R, Austin S, Yang W. Crystal structure of a SeqA-N filament: implications for DNA replication and chromosome organization. EMBO J. 2005; 24:1502–1511. [PubMed: 15933720]

Hansen FG, Christensen BB, Atlung T. Sequence characteristics required for cooperative binding and efficient in vivo titration of the replication initiator protein DnaA in *E. coli*. J. Mol. Biol. 2007; 367:942–952. [PubMed: 17316685]

Heinrichs DE, Yethon JA, Whitfield C. Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. Mol. Microbiol. 1998; 30:221–232. [PubMed: 9791168]

Hiraga S. Dynamic localization of bacterial and plasmid chromosomes. Annu. Rev. Genet. 2000; 34:21–59. [PubMed: 11092821]

Ishida T, Akimitsu N, Kashioka T, Hatano M, Kubota T, Ogata Y, Sekimizu K, Katayama T. DiaA, a novel DnaA-binding protein, ensures the timely initiation of *Escherichia coli* chromosome replication. J. Biol. Chem. 2004; 279:45546–45555. [PubMed: 15326179]

Joshi JG, Handler P. Phosphoglucomutase. I. Purification and properties of phosphoglucomutase from *Escherichia coli*. J. Biol. Chem. 1964; 239:2741–2751. [PubMed: 14216423]

Kaguni JM. DnaA: controlling the initiation of bacterial DNA replication and mor. Annu. Rev. Microbiol. 2006; 60:351–375. [PubMed: 16753031]

Kang S, Lee H, Han JS, Hwang DS. Interaction of SeqA and Dam methylase on the hemimethylated origin of *Escherichia coli* chromosomal DNA replication. J. Biol. Chem. 1999; 274:11463–11468. [PubMed: 10206949]

Katayama T. The mutant DnaAcos protein which overinitiates replication of the *Escherichia coli* chromosome is inert to negative regulation for initiation. J. Biol. Chem. 1994; 269:22075–22079. [PubMed: 8071330]

Katayama T, Akimitsu N, Mizushima T, Miki T, Sekimizu K. Overinitiation of chromosome replication in the *Escherichia coli dnaA*cos mutant depends on activation of *oriC* function by the *dam* gene product. Mol. Microbiol. 1997; 25:661–670. [PubMed: 9379896]

Katayama T, Crooke E, Sekimizu K. Characterization of *Escherichia coli* DnaAcos protein in replication systems reconstituted with highly purified proteins. Mol. Microbiol. 1995; 18:813–820. [PubMed: 8825085]

Katayama T, Kornberg A. Hyperactive initiation of chromosomal replication in vivo and in vitro by a mutant initiator protein, DnaAcos, of *Escherichia coli*. J. Biol. Chem. 1994; 269:12698–12703. [PubMed: 8175680]

Katayama T, Kubota T, Kurokawa K, Crooke E, Sekimizu K. The initiator function of DnaA protein is negatively regulated by the sliding clamp of the *E. coli* chromosomal replicase. Cell. 1998; 94:61–71. [PubMed: 9674428]

Kato J, Katayama T. Hda, a novel DnaA-related protein, regulates the replication cycle in *Escherichia coli*. EMBO J. 2001; 20:4253–4262. [PubMed: 11483528]

Kellenberger-Gujer G, Podhajska AJ, Caro L. A cold sensitive *dnaA* mutant of *E. coli* which overinitiates chromosome replication at low temperature. Mol. Gen. Genet. 1978; 162:9–16. [PubMed: 353526]

Keyamura K, Fujikawa N, Ishida T, Ozaki S, Su'etsugu M, Fujimitsu K, Kagawa W, Yokoyama S, Kurumizaka H, Katayama T. The interaction of DiaA and DnaA regulates the replication cycle in *E. coli* by directly promoting ATP DnaA-specific initiation complexes. Genes Dev. 2007; 21:2083–2099. [PubMed: 17699754]

Kitagawa R, Mitsuki H, Okazaki T, Ogawa T. A novel DnaA protein-binding site at 94.7 min on the *Escherichia coli* chromosome. Mol. Microbiol. 1996; 19:1137–1147. [PubMed: 8830270]

Kitagawa R, Ozaki T, Moriya S, Ogawa T. Negative control of replication initiation by a novel chromosomal locus exhibiting exceptional affinity for *Escherichia coli* DnaA protein. Genes Dev. 1998; 12:3032–3043. [PubMed: 9765205]

Knight KL, Aoki KH, Ujita EL, McEntee K. Identification of the amino acid substitutions in two mutant forms of the RecA protein from *Escherichia coli*: RecA441 and RecA629. J. Biol. Chem. 1984; 259:11279–11283. [PubMed: 6088537]

Kogoma T, von Meyenburg K. The origin of replication, *oriC*, and the *dnaA* protein are dispensable in stable DNA replication (*sdrA*) mutants of *Escherichia coli* K-12. EMBO J. 1983; 2:463–468. [PubMed: 11894964]

Kouzminova EA, Kuzminov A. Chromosomal fragmentation in dUTPase-deficient mutants of *Escherichia coli* and its recombinational repair. Mol. Microbiol. 2004; 51:1279–1295. [PubMed: 14982624]

Kouzminova EA, Kuzminov A. Fragmentation of replicating chromosomes triggered by uracil in DNA. J. Mol. Biol. 2006; 355:20–33. [PubMed: 16297932]

Kouzminova EA, Kuzminov A. Patterns of chromosomal fragmentation due to uracil-DNA incorporation reveal a novel mechanism of replication-dependent double-strand breaks. Mol. Microbiol. 2008; 68:202–215. [PubMed: 18312272]

Kouzminova EA, Rotman E, Macomber L, Zhang J, Kuzminov A. RecA-dependent mutants in *E. coli* reveal strategies to avoid replication fork failure. Proc. Natl. Acad. Sci. USA. 2004; 101:16262–16267. [PubMed: 15531636]

Kurokawa K, Nishida S, Emoto A, Sekimizu K, Katayama T. Replication cycle-coordinated change of the adenine nucleotide-bound forms of DnaA protein in *Escherichia coli*. EMBO J. 1999; 18:6642–6652. [PubMed: 10581238]

Kushner SR. In vivo studies of temperature-sensitive *recB* and *recC* mutants. J. Bacteriol. 1974; 120:1213–1218. [PubMed: 4612007]

Kuzminov A. Collapse and repair of replication forks in *Escherichia coli*. Mol. Microbiol. 1995a; 16:373–384. [PubMed: 7565099]

Kuzminov A. Instability of inhibited replication forks in *E. coli*. BioEssays. 1995b; 17:733–741. [PubMed: 7661854]

Kuzminov A. Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage λ. Microbiol. Mol. Biol. Rev. 1999; 63:751–813. [PubMed: 10585965]

Kuzminov A, Stahl FW. Stability of linear DNA in *recA* mutant *Escherichia coli* cells reflects ongoing chromosomal DNA degradation. J. Bacteriol. 1997; 179:880–888. [PubMed: 9006046]

Larsen RA, Wilson MM, Guss AM, Metcalf WW. Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. Arch. Microbiol. 2002; 178:193–201. [PubMed: 12189420]

Løbner-Olesen A, Marinus MG, Hansen FG. Role of SeqA and Dam in *Escherichia coli* gene expression: a global/microarray analysis. Proc. Natl. Acad. Sci. USA. 2003; 100:4672–4677. [PubMed: 12682301]

Løbner-Olesen A, Skarstad K, Hansen FG, von Meyenburg K, Boye E. The DnaA protein determines the initiation mass of *Escherichia coli* K-12. Cell. 1989; 57:881–889. [PubMed: 2541928]

Lu M, Campbell JL, Boye E, Kleckner N. SeqA: a negative modulator of replication initiation. Cell. 1994; 77:413–426. [PubMed: 8011018]

Lu M, Kleckner N. Molecular cloning and characterization of the pgm gene encoding phosphoglucomutase of *Escherichia coli*. J. Bacteriol. 1994; 176:5847–5851. [PubMed: 8083177]

Macalister TJ, Macdonald B, Rothfield LI. The periseptal annulus: An organelle associated with cell division in Gram-negative bacteria. Proc. Natl. Acad. Sci. USA. 1983; 80:1372–1376. [PubMed: 16593288]

Maniatis, T.; Fritsch, EF.; Sambrook, J. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory; Cold Spring Harbor: 1982.

Mao Z, Shin HD, Chen RR. Engineering the E. coli UDP-glucose synthesis pathway for oligosaccharide synthesis. Biotechnol. Prog. 2006; 22:369–374. [PubMed: 16599548]

Miller, JH. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 1972.

Neidhardt FC, Bloch PL, Smith DF. Culture medium for enterobacteria. J. Bacteriol. 1974; 119:736–747. [PubMed: 4604283]

Nikaido, H. Outer Membrane.. In: Neidhardt, FC., editor. Escherichia coli and Salmonella. Cellular and Molecular Biology. ASM Press; Washington, D.C.: 1996. p. 29-47.

Nishijima M, Bulawa CE, Raetz CR. Two interacting mutations causing temperature-sensitive phosphatidylglycerol synthesis in *Escherichia coli* membranes. J. Bacteriol. 1981; 145:113–121. [PubMed: 7007311]

Nishijima M, Raetz CR. Membrane lipid biogenesis in *Escherichia coli*: identification of genetic loci for phosphatidylglycerophosphate synthetase and construction of mutants lacking phosphatidylglycerol. J. Biol. Chem. 1979; 254:7837–7844. [PubMed: 381294]

Nordman J, Skovgaard O, Wright A. A novel class of mutations that affect DNA replication in *E. coli*. Mol. Microbiol. 2007; 64:125–138. [PubMed: 17376077]

Ogden GB, Pratt MJ, Schaechter M. The replicative origin of the *E. coli* chromosome binds to cell membranes only when hemimethylated. Cell. 1988; 54:127–135. [PubMed: 2838178]

Oliver, DB. Periplasm.. In: Neidhardt, FC., editor. Escherichia coli and Salmonella. Cellular and Molecular Biology. ASM Press; Washington, D.C.: 1996. p. 88-103.

Ossanna N, Mount DW. Mutations in *uvrD* induce the SOS response in *Escherichia coli*. J. Bacteriol. 1989; 171:303–307. [PubMed: 2536658]

Parker B, Marinus MG. A simple and rapid method to obtain substitution mutations in *Escherichia coli*: isolation of a *dam* deletion/insertion mutation. Gene. 1988; 73:531–535. [PubMed: 2854098]

Parker CT, Kloser AW, Schnaitman CA, Stein MA, Gottesman S, Gibson BW. Role of the *rfaG* and *rfaP* genes in determining the lipopolysaccharide core structure and cell surface properties of *Escherichia coli* K-12. J. Bacteriol. 1992; 174:2525–2538. [PubMed: 1348243]

Raetz, CRH. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles.. In: Neidhardt, FC., editor. Escherichia coli and Salmonella. Cellular and Molecular Biology. ASM Press; Washington, D.C.: 1996. p. 1035-1063.

Riber L, Olsson JA, Jensen RB, Skovgaard O, Dasgupta S, Marinus MG, Løbner-Olesen A. Hda-mediated inactivation of the DnaA protein and *dnaA* gene autoregulation act in concert to ensure homeostatic maintenance of the *Escherichia coli* chromosome. Genes Dev. 2006; 20:2121–2134. [PubMed: 16882985]

Rotman E, Kuzminov A. The *mutT* defect does not elevate chromosomal fragmentation in *Escherichia coli* because of the surprisingly low levels of MutM/MutY-recognized DNA modifications. J. Bacteriol. 2007; 189:6976–6988. [PubMed: 17616589]

Russell DW, Zinder ND. Hemimethylation prevents DNA replication in *E. coli*. Cell. 1987; 50:1071–1079. [PubMed: 3304662]

Schnaitman CA, Klena JD. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. 1993; 57:655–682. [PubMed: 7504166]

Seigneur M, Bidnenko V, Ehrlich SD, Michel B. RuvAB acts at arrested replication forks. Cell. 1998; 95:419–430. [PubMed: 9814711]

Sekimizu K, Kornberg A. Cardiolipin activation of DnaA protein, the initiation protein of replication in *Escherichia coli*. J. Biol. Chem. 1988; 263:7131–7135. [PubMed: 2835364]

Simmons LA, Breier AM, Cozzarelli NR, Kaguni JM. Hyperinitiation of DNA replication in *Escherichia coli* leads to replication fork collapse and inviability. Mol. Microbiol. 2004; 51:349–358. [PubMed: 14756777]

Simmons LA, Kaguni JM. The DnaAcos allele of *Escherichia coli*: hyperactive initiation is caused by substitution of A184V and Y271H, resulting in defective ATP binding and aberrant DNA replication control. Mol. Microbiol. 2003; 47:755–765. [PubMed: 12535074]

Skarstad K, Løbner-Olesen A, Atlung T, von Meyenburg K, Boye E. Initiation of DNA replication in *Escherichia coli* after overproduction of the DnaA protein. Mol. Gen. Genet. 1989; 218:50–56. [PubMed: 2550764]

Skarstad K, Lueder G, Lurz R, Speck C, Messer W. The *Escherichia coli* SeqA protein binds specifically and co-operatively to two sites in hemimethylated and fully methylated *oriC*. Mol. Microbiol. 2000; 36:1319–1326. [PubMed: 10931282]

Slater S, Wold S, Lu M, Boye E, Skarstad K, Kleckner N. *E. coli* SeqA protein binds *oriC* in two different methyl-modulated reactions appropriate to its roles in DNA replication initiation and origin sequestration. Cell. 1995; 82:927–936. [PubMed: 7553853]

Su'etsugu M, Shimuta TR, Ishida T, Kawakami H, Katayama T. Protein associations in DnaA-ATP hydrolysis mediated by the Hda-replicase clamp complex. J. Biol. Chem. 2005; 280:6528–6536. [PubMed: 15611053]

Su'etsugu M, Takata M, Kubota T, Matsuda Y, Katayama T. Molecular mechanism of DNA replication-coupled inactivation of the initiator protein in *Escherichia coli*: interaction of DnaA with the sliding clamp-loaded DNA and the sliding clamp-Hda complex. Genes Cells. 2004; 9:509–522. [PubMed: 15189445]

Ting H, Kouzminova EA, Kuzminov A. Synthetic lethality with the *dut* defect in *Escherichia coli* reveals layers of DNA damage of increasing complexity due to uracil incorporation. J. Bacteriol. 2008; 190:5841–5854. [PubMed: 18586941]

Vaara M. Agents that increase the permeability of the outer membrane. Microbiol. Rev. 1992; 56:395–411. [PubMed: 1406489]

von Freiesleben U, Rasmussen KV, Schaechter M. SeqA limits DnaA activity in replication from *oriC* in *Escherichia coli*. Mol. Microbiol. 1994; 14:763–772. [PubMed: 7891562]

Waldminghaus T, Skarstad K. The *Escherichia coli* SeqA protein. Plasmid. 2009; 61:141–150. [PubMed: 19254745]

Xia W, Dowhan W. In vivo evidence for the involvement of anionic phospholipids in initiation of DNA replication in *Escherichia coli*. Proc. Natl. Acad. Sci. USA. 1995; 92:783–787. [PubMed: 7846051]

Yethon JA, Heinrichs DE, Monteiro MA, Perry MB, Whitfield C. Involvement of *waaY*, *waaQ*, and *waaP* in the modification of *Escherichia coli* lipopolysaccharide and their role in the formation of a stable outer membrane. J. Biol. Chem. 1998; 273:26310–26316. [PubMed: 9756860]

Yethon JA, Vinogradov E, Perry MB, Whitfield C. Mutation of the lipopolysaccharide core glycosyltransferase encoded by *waaG* destabilizes the outer membrane of *Escherichia coli* by interfering with core phosphorylation. J. Bacteriol. 2000; 182:5620–5623. [PubMed: 10986272]

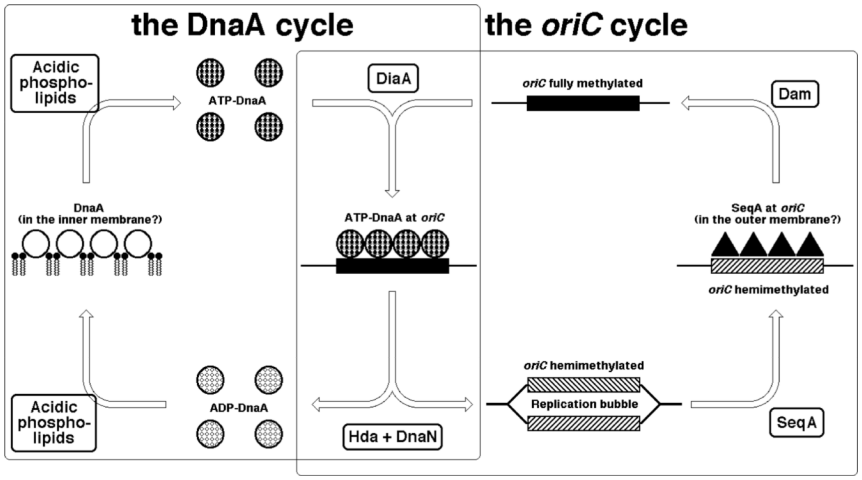

Fig. 1. Initiation of DNA replication in *E. coli* and its control via two separate inactivation-reactivation cycles, the "DnaA cycle" and the "*oriC* cycle"

Explanations are in the figure body: framed text identifies activities helping with the corresponding transitions, whereas unframed text explains specific stages. References: Hda (Kato and Katayama, 2001; Su'etsugu *et al.*, 2005), Δ*hda* (Camara *et al.*, 2005; Riber *et al.*, 2006), DnaN (Katayama *et al.*, 1998; Su'etsugu *et al.*, 2004), DnaA(Cs) (Kellenberger-Gujer *et al.*, 1978; Katayama and Kornberg, 1994; Katayama *et al.*, 1995), DnaA overproduction (Atlung *et al.*, 1987; Løbner-Olesen *et al.*, 1989; Skarstad *et al.*, 1989; Kurokawa *et al.*, 1999), DnaA and acidic phospholipids (Sekimizu and Kornberg, 1988; Crooke *et al.*, 1992; Xia and Dowhan, 1995), DiaA (Ishida *et al.*, 2004; Keyamura *et al.*, 2007), SeqA (Brendler *et al.*, 1995; Slater *et al.*, 1995; Kang *et al.*, 1999; Bach *et al.*, 2003; Bach and Skarstad, 2004), *oriC*::SeqA in the outer membrane (Ogden *et al.*, 1988; Chakraborti *et al.*, 1992; Slater *et al.*, 1995; d'Alençon *et al.*, 1999), Δ*seqA* (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994; Løbner-Olesen *et al.*, 2003), Dam (Russell and Zinder, 1987; Katayama *et al.*, 1997; Kang *et al.*, 1999; Løbner-Olesen *et al.*, 2003).

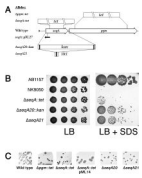

Fig. 2. Characterization of the Δ*seqA* alleles for possible *pgm* defect

A. The scheme of the *seqA-pgm* operon and the mutant alleles used in this study. The two genes, as well the *kan* and the FRT inserts are shown to scale; the *tet* and the pRL27 inserts are not to scale. The arrow of the pRL27 insert indicates the direction of the kanamycin-resistance gene. **B.** The SDS-test to reveal the *pgm* defect. Cultures grown in LB were serially diluted and spotted by 10 μl on an LB plate and by 5 μl on an "LB supplemented with 1.5% SDS" plate (the detergent causes the spot spreading). AB1157 is the wild type progenitor for all our experimental strains. NK9050 is the original Δ*seqA*::*tet* mutant from Kleckner's lab and the only non-AB1157 strain in this set. The strains are: Δ*seqA*::*tet*, ER17; Δ*seqA20*::*kan*, ER15; Δ*seqA21*, ER16. **C.** The iodine staining test for the *pgm* defect. Wild type, MG1655; Δ*pgm*::*tet*, ER29; Δ*seqA*::*tet*, ER22; Δ*seqA20*, ER23; Δ*seqA21*, ER24.

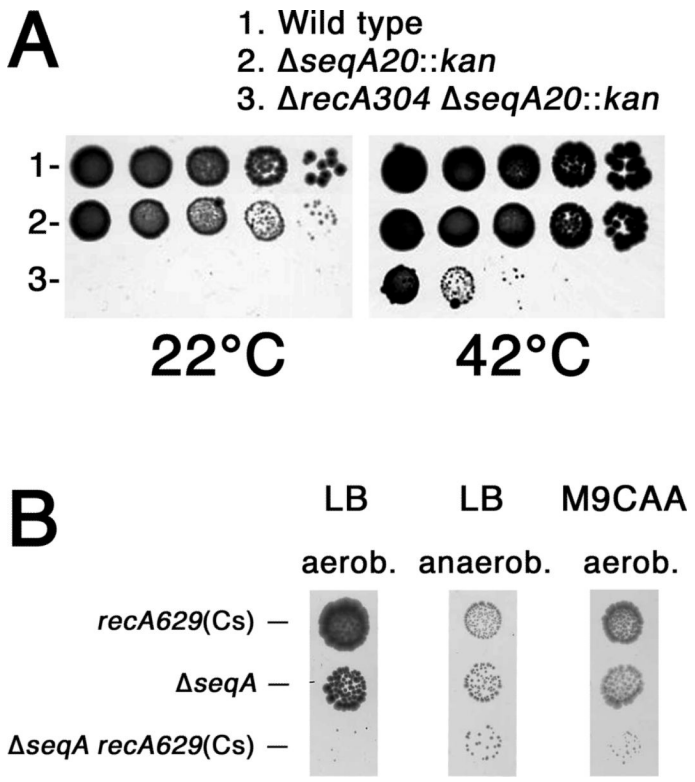

Fig. 3. The co-lethality of Δ*seqA recA* double mutants

A. The Δ*seqA* mutants grow slower at low temperatures. Cultures grown at 42°C were serially diluted and spotted by 10 μl on LB plates. One was incubated at 22°C for two days, the other at 42°C for one day. Wild type, AB1157; Δ*seqA20*::*kan*, ER15; Δ*recA304* Δ*seqA20*::*kan*, ER18. **B.** The double Δ*seqA recA629*(Cs) mutant cannot grow at 28°C on LB aerobically, but can grow either on LB anaerobically, or (very slowly) on a M9-based medium aerobically. The strains are: *recA629*(Cs), AK20; Δ*seqA*, ER16; Δ*seqA recA629*(Cs), ER36.

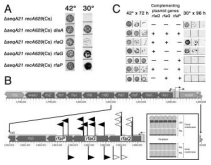

Fig. 4. The *rfa* suppressors of the Δ*seqA recA* lethality

A. The three types of colony morphology of the suppressors of Δ*seqA recA* synthetic lethality. All the strains grow similarly at 42°C, and the differences are only expressed at 30°C. The non-suppressed double mutant does not grow; a non-*rfa* suppressor (*diaA* is shown as an example — E.R. and A.K., unpublished) shows normal colony size and morphology; the *rfaQ* suppressor forms small, non-mucoid colonies; the *rfaG* and *rfaP* suppressors form apparently bigger, mucoid colonies. The strains are: from top to bottom: ER94, ER96−99. **B.** The position and orientation of the suppressing inserts. The inserts are shown as flags pointing in the direction of their kanamycin genes. Black flags, mucoid suppressors; white flags, non-mucoid suppressors (see "A"). Multiple inserts at some positions, suggesting hot-spots for pRL27 insertion, are all independent and include those in the opposite orientation. Small arrows on stems upstream of genes indicate promoters. The gene diagrams were generated with the help of the EcoCyc site (http://ecocyc.org/). **Inset:** The scheme of the double membrane of *E. coli*. LPS, lipopolysaccharide (shown without the O-antigen); PhL, phospholipids. The inner membrane comprises two phospholipid layers; the outer membrane has a phospholipid layer towards the periplasm and the LPS layer ("armor") facing the outside. **C.** Inactivation of which of the three *rfa* genes causes the suppression? The suppressed strain Δ*seqA recA* Δ*rfaQGP* (ER75) was transformed either with an empty vector (pK80) or with its various derivatives, carrying the indicated combinations of the *rfaQ*+, *rfaG*+ and *rfaP*+ genes ("−" means the gene is absent, "+" means the wild type gene is present). Growing cultures were serially diluted and spotted on plates, that were incubated at the indicated temperatures for the indicated times. Plasmids from top to bottom: pK80 (vector), pPB2, pPB3, pPB4, pPB5, pPB6 and pER20.

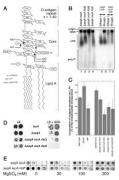

Fig. 5. Changes in the LPS core phosphorylation in the *rfaQ, rfaG* and *rfaP* mutants

A. The proposed overall structure of the *E. coli* lipopolysaccharide (after (Raetz, 1996)). The structure is one of several alternatives due to heterogeneity in the composition of the core domain (Raetz, 1996). Asterisks indicate alternative groups. For the explanation of the abbreviated names of the sugars, see Fig. 3 of (Raetz, 1996). P, phosphate; Etn, ethanolamine. The positions where the products of the *rfaG, rfaP, rfaQ* and *rfaY* genes add side chains are indicated by the arrows. **B.** A representative image of an alkaline agarose gel electrophoresis to separate phosphate-incorporating cellular species. The mobility of the three major species, DNA, LPS and poly-phosphate (poly-P), is indicated on the left. In lanes 5 and 7, the same material as used in lanes 1 and 4, was treated with DNase I, to reveal DNA. In lanes 6 and 8, the same material was treated with alkaline phosphatase (phosph.) to remove polyphosphates. Polyphosphates are also sensitive to incubation in the DNase I buffer. The strains are: Δ*seqA recA*, ER94; Δ*seqA recA rfaQ*, ER97; Δ*seqA recA rfaG*, ER98; Δ*seqA recA rfaP*, ER99. **C.** Quantification of the LPS phosphorylation. The data were derived from gels like in "B" by measuring the percent of the total radioactivity in the lane localized to the LPS part of the lane. The data are averages of 4−6 measurements, done on different days, ± SE. A single exception is the triple Δ*seqA recA* Δ*rfaQGP* mutant, for which only two measurements were done (thus, the significant SE). The strains are like in "B", with the addition of: Wild type, AB1157; Δ*seqA*, ER16; *rfaP*, ER77; Δ*seqA recA* Δ*rfaQGP*, ER95. **D.** The *rfa* mutants are more sensitive to SDS than their Δ*seqA* parent. Growing cultures were serially diluted; 10^{-5} and 10^{-6} dilutions were spotted on LB plates with (5 μl) or without (10 μl) 1% SDS and incubated at 37°C for two days. The strains are: *recA*, AK20; Δ*seqA*, ER16; Δ*seqA recA rfaQ*, ER37; Δ*seqA recA rfaG*, ER42. **E.** Magnesium supplementation eliminates both the *seqA recA* lethality and the *rfa* mucoidy. Serial dilutions of 42°C-growing cultures were spotted and incubated at 30°C for 48 hours on LB agar, supplemented with the indicated concentration of $MgSO_4$. The strains are: *seqA recA*, ER36; *seqA recA rfaP*, ER45.

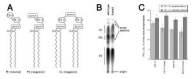

Fig. 6. Composition of phospholipids in the Δ*seqA* mutants

A. A schematic diagram of the three major phospholipids of the *E. coli* membranes. The chemical structure of glycerol, phosphate (P(−)) and ethanolamine (E(+)) are not shown for clarity. PE, phosphatidylethanolamine (no overall charge); PG, phosphatidylglycerol (overall negative charge); CL, cardiolipin (overall negative charge). **B.** A representative TLC separation of phospholipids from wild type (AB1157) and Δ*seqA* (ER16) mutant cells. In our quantification, we separately measured PE versus "everything above PE". Then we calculated the percentage of "everything above PE" (= PG + CL) of the total signal in the lane. **C.** The fraction of PG + CL in the total phospholipids. The measurements were done in two different wild type strains and corresponding Δ*seqA* mutants, separately for growing versus stationary cultures. The values are averages of 4−7 independent measurements, done on different days, ± SE. The strains are: AB1157 Δ*seqA*, ER16; MG1655 Δ*seqA*, ER24.

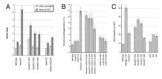

Fig. 7. Ori/ter ratio, SOS induction and chromosomal fragmentation in the *seqA rfa* double mutants

All the values are averages of three to eight independent determinations, done on different days, ± SE. **A.** The ori/ter ratios in the Δ*seqA* mutants. Total DNA from correspondent strains, either from overnight (saturated) or growing cultures, was deposited on hybridization membrane and hybridized to either origin-specific or terminus-specific probes. The resulting signals were then normalized to the signal from the wild type overnight culture (taken for "1"). The values for the Δ*rfaQGP* strain are from a single determination (thus, no error bars). The strains are: wild type, AB1157; Δ*seqA21*, ER16; Δ*seqA21 rfaQ*, ER79; Δ*seqA21 rfaG*, ER80; Δ*seqA21 rfaP*, ER81; Δ*rfaQGP*, ER78; Δ*seqA* Δ*rfaQGP*, ER82. **B.** The chromosomal fragmentation was quantified as before, after labeling of the chromosomal DNA with ^{32}P-orthophosphate ((Rotman and Kuzminov, 2007) and see "Methods"). The *ybfE* suppressor is one of the non-*rfa* suppressors (E.R. and A.K., unpublished) and is shown as a positive control for the expected reduction in fragmentation. The strains are: wild type, AB1157; Δ*seqA21*, ER16; *recBC*(Ts), SK129; Δ*seqA21 recBC*(Ts), ER89; Δ*seqA21 recBC*(Ts) *rfaQ*, ER90; Δ*seqA21 recBC*(Ts) *rfaG*, ER91; Δ*seqA21 recBC*(Ts) *rfaP*, ER92; Δ*seqA21 recBC*(Ts) *ybfE*, ER93; *recBC*(Ts) *rfaQ*, ER103; *recBC*(Ts) *rfaG*, ER104; *recBC*(Ts) *rfaP*, ER105. **C.** The level of SOS induction in Δ*seqA* and *rfa* strains. The corresponding mutants were transduced with a *sfiA*::*lacZ* fusion construct, and β-galactosidase activity at 30°C was taken as a measure of the SOS induction, as before (Kouzminova *et al.*, 2004). As a positive control for SOS induction, wild type cells were treated with sub-inhibitory doses of mitomycin C. The *ybfE* suppressor is again shown as a positive control for the expected reduction of the SOS response. The strains are: Wild type, AK43; Wild type + MC, AK43, grown in the presence of 100 ng/ml of mitomycin C as a control for SOS induction; Δ*seqA21*, ER83; Δ*seqA21 rfaQ*, ER84; Δ*seqA21 rfaG*, ER86; Δ*seqA21 rfaP*, ER87; Δ*seqA21 jbfE*, ER88; *rfaQ*, ER100; *rfaG*, ER101; *rfaP*, ER102.

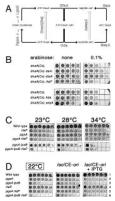

Fig. 8. Genetic analysis of the *rfaP* suppression of the *seqA recA* lethality

A. A simplified version of the DnaA and *oriC* cycles (Fig. 1). The DnaA(Cs) and Δ*seqA* shunts are shown by open arrows. On the very left, instead of acidic phospholipids, the two gene products involved in production of phosphatidylglycerol (PgsA and LpxB) are shown. **B.** Epistatic analysis with the *dnaA*(Cs) defect. The plasmid expressing DnaA(Cs) under the arabinose promoter was introduced into the wild type cells or the corresponding single mutants. Serial dilutions of exponentially growing cultures were spotted on LB without any sugar or on LB with 0.1% arabinose, to induce *dnaA*(Cs) expression. The strains are: *dnaA*(Cs), MC1061 pLS120; *dnaA*(Cs) *dam*, ER106 pLS120; *dnaA*(Cs) *diaA*, ER107 pLS120; *dnaA*(Cs) *rfaP*, ER108 pLS120; *dnaA*(Cs) *hda*, ER152 pLS120; *dnaA*(Cs) *seqA*, ER154 pLS120. **C.** Lethality of the *pgsA lpxB rfaP* triple mutant (*lpxB* is formerly known as *pgsB*). Serial dilutions of exponentially growing cultures were spotted on LB and incubated at the indicated temperatures. The strains are: wild type, R477; *rfaP*, ER109; *pgsA*, MN1; *pgsA rfaP*, ER110; *pgsA lpxB*, MN7; *pgsA lpxB rfaP*, ER111. **D.** The effects of the IPTG-dependent replication origin (*lac*/CE-*ori*) on the viability of *pgsA* and *pgsA rfaP* combinations. The left panel: the six strains from “C” (the order of spotting is different). The central panel: the derivatives of the “six strains” with the IPTG-inducible ColE1 replication origin in the chromosome, plated without IPTG. The right panel: same as the central panel, but with IPTG (the plasmid replication origin is induced). The strains in the central and the right panels are ER139−143 and ER150.

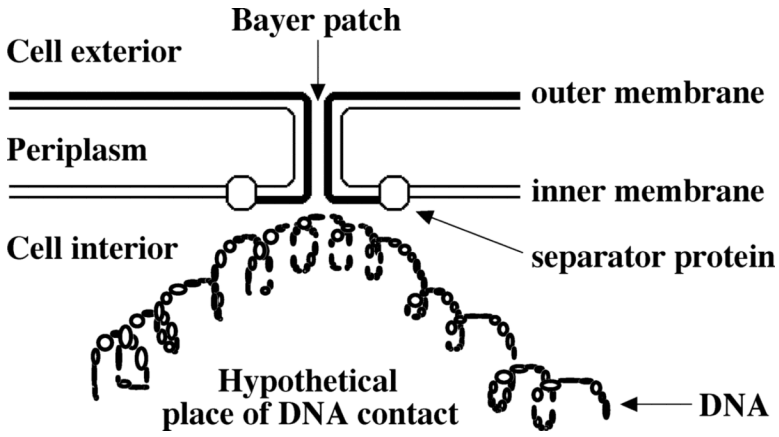

Fig. 9.

A hypothetical structure of Bayer patch (Bayer, 1979), where DNA can come in contact with the outer membrane. Explanations in the text.

Table 1

Bacterial strains.

Strain #	Relevant Genotype[a]	Reference / Derivation
	Previous studies	
AB1157[b]	wild type	(Bachmann, 1987)
AK20	(*recA629*Cs Φ80)	(Kuzminov and Stahl, 1997)
AK43	P*sfiA*—>*lacZ* (MuΔX *cat*)	(Kouzminova *et al.*, 2004)
CAG12119	*malE*::Tn*10-kan*	Carol Gross
ER15	Δ*seqA20*::*kan*	(Rotman and Kuzminov, 2007)
ER16	Δ *seqA21*	(Rotman and Kuzminov, 2007)
ER26	P*sfiA*—>*lacZ* Δ*seqA20*::*kan*	(Rotman and Kuzminov, 2007)
ER46	Δ*seqA20*::*kan recBC*(Ts)	(Rotman and Kuzminov, 2007)
GM3819	Δ*dam16*::*kan*	(Parker and Marinus, 1988)
JC10287	Δ(*srlR-recA*)*304*	(Czonka and Clark, 1979)
JW5397−1	Δ*hda-744*::*kan*	*E. coli* Genetic Stock Center #11363
L-216	*dnaA46*(Ts) *lac*/CE–>ori::*bla*	Elena Kouzminova
MC1061[c]	wild type	(Simmons *et al.*, 2004)
MG1655[d]	wild type	(Blattner *et al.*, 1997)
MN1	R477 *pgsA444*	(Nishijima *et al.*, 1981)
MN7	R477 *pgsA444 pgsB1*	(Nishijima *et al.*, 1981)
NK9050	Δ*seqA*::*tet*	(Lu *et al.*, 1994)
R477	wild type	(Nishijima *et al.*, 1981)
SK129	*recB270*(Ts) *recC271*(Ts)	(Kushner, 1974)
W1485	Δ*pgm*::*tet*	(Lu and Kleckner, 1994)
	This study	
ER17	AB1157 Δ*seqA*::tet	AB1157 × P1 NK9050
ER18	Δ*seqA20*::kan Δ*recA304*	JC10287 pEAK2[e] × P1 ER15
ER22	MG1655 Δ*seqA*::tet	MG1655 × P1 NK9050
ER23	MG1655 Δ*seqA20*::kan	MG1655 × P1 ER15
ER24	MG1655 Δ*seqA21*	ER23, kan removed by
pCP20		
ER29	MG1655 Δ*pgm*::tet	MG1655 × P1 W1485
ER36	Δ*seqA21 recA629*(Cs) Φ80	ER16 × P1 AK20
ER37	Δ*seqA21 recA629*(Cs) *rfaQ3* Φ80	pRL27 mutagenesis of ER36
ER40	Δ*seqA21 recA629*(Cs) *ybfE11* Φ80	pRL27 mutagenesis of ER36
ER42	Δ*seqA21 recA629*(Cs) *rfaG21* Φ80	pRL27 mutagenesis of ER36
ER43	Δ*seqA21 recA629*(Cs) *rfaP22* Φ80	pRL27 mutagenesis of ER36
ER44	Δ*seqA21 recA629*(Cs) *rfaG23* Φ80	pRL27 mutagenesis of ER36
ER45	Δ*seqA21 recA629*(Cs) *rfaP31* Φ80	pRL27 mutagenesis of ER36
ER72	Δ*seqA21 recA629*(Cs) malE::Tn10 Φ80	ER36 × P1 CAG12119
ER73	Δ*seqA21 recA629*(Cs) *rfaG35* Φ80	pRL27 mutagenesis of ER36

Strain #	Relevant Genotype[a]	Reference / Derivation
ER75	Δ*seqA21 recA629*(Cs) Δ*rfaQGP*::cat Φ80	ER36 × P1 PB-QGP
ER76	*recA629*(Cs)	AB1157 × P1 AK20, 42°C
ER77	AB1157 *rfaP31*	AB1157 × P1 ER45
ER78	AB1157 Δ*rfaQGP*::cat	AB1157 × P1 PB-QGP
ER79	Δ *seqA21 rfaQ72*	ER16 × P1 PB-BT
ER80	Δ *seqA21 rfaG35*	ER16 × P1 ER73
ER81	Δ *seqA21 rfaP31*	ER16 × P1 ER45
ER82	Δ*seqA21* Δ*rfaQGP*::*cat*	ER16 × P1 PB-QGP
ER83	P*sfiA*—>*lacZ* Δ*seqA21*	ER16 × P1 AK43
ER84	P*sfiA*—>*lacZ* Δ*seqA21 rfaQ3*	AK43 × P1 ER37
ER85	P*sfiA*—>*lacZ* Δ*seqA21 rfaG21*	AK43 × P1 ER42
ER86	P*sfiA*—>*lacZ* Δ*seqA21 rfaG23*	AK43 × P1 ER44
ER87	P*sfiA*—>*lacZ* Δ*seqA21 rfaP22*	AK43 × P1 ER43
ER88	P*sfiA*—>*lacZ* Δ*seqA21 ybfE11*	AK43 × P1 ER40
ER89	Δ*seqA21 recBC*(Ts)	ER46, kan removed by
pCP20		
ER90	Δ*seqA21 recBC*(Ts) *rfaQ72*	ER89 × P1 PB-BT
ER91	Δ*seqA21 recBC*(Ts) *rfaG62*	ER89 × P1 PB-D
ER92	Δ*seqA21 recBC*(Ts) *rfaP31*	ER89 × P1 ER45
ER93	Δ*seqA21 recBC*(Ts) *ybfE11*	ER89 × P1 ER40
ER94	Δ*seqA21 recA629*(Cs)	ER16 × P1 ER76
ER95	Δ*seqA21 recA629*(Cs) Δ*rfaQGP*::*cat*	ER94 × P1 PB-QGP
ER96	Δ*seqA21 recA629*(Cs) *diaA96*	ER94 × P1 BP-CP
ER97	Δ*seqA21 recA629*(Cs) *rfaQ72*	ER94 × P1 PB-BT
ER98	Δ*seqA21 recA629*(Cs) *rfaG35*	ER94 × P1 ER73
ER99	Δ*seqA21 recA629*(Cs) *rfaP31*	ER94 × P1 ER45
ER100	P*sfiA*—>*lacZ rfaQ3*	AK43 × P1 ER37
ER101	P*sfiA*—>*lacZ rfaG23*	AK43 × P1 ER44
ER102	P*sfiA*—>*lacZ rfaP31*	AK43 × P1 ER45
ER103	*recBC*(Ts) *rfaQ98* Φ80	SK129 × P1 PB-CU
ER104	*recBC*(Ts) *rfaG21* Φ80	SK129 × P1 ER42
ER105	*recBC*(Ts) *rfaP31* Φ80	SK129 × P1 ER45
ER106	Δ*dam16*::kan pLS120	MC1061 pLS120 × P1 GM3819
ER107	*diaA69* pLS120 Φ80	MC1061 pLS120 × P1 PB-AL
ER108	*rfaP31* pLS120	MC1061 pLS120 × P1 ER45
ER109	*rfaP31*	R744 × P1 ER45
ER110	*pgsA444 rfaP31*	MN1 × P1 ER45
ER111	*pgsA444 pgsB1 rfaP31*	MN7 × P1 ER45
ER139	*lac*/CE–>ori::*bla*	R477 × P1 L-216
ER140	*pgsA444 lac*/CE–>ori::*bla*	MN1 × P1 L-216
ER141	*pgsA444 pgsB1 lac*/CE–>ori::*bla*	MN7 × P1 L-216
ER142	*rfaP31 lac*/CE–>ori::*bla*	ER109 × P1 L-216

Strain #	Relevant Genotype[a]	Reference / Derivation
ER143	*pgsA444 rfaP31 lac*/CE–>ori::*bla*	ER110 × P1 L-216
ER150	*pgsA444 pgsB1 rfaP31 lac*/CE–>ori::*bla*	ER111 × P1 L-216
ER152	Δ*hda-744*::*kan* pLS120	MC1061 pLS120 × P1 JW5397−1
ER154	Δ*seqA20*::*kan* pLS120	MC1061 pLS120 × P1 ER15
PB-D	Δ*seqA21 recBC*(Ts) *rfaG62* Φ80	pRL27mutagenesis of ER36
PB-AL	Δ*seqA21 recA629*(Cs) *diaA69* Φ80	pRL27mutagenesis of ER36
PB-BT	Δ*seqA21 recA629*(Cs) *rfaQ72* Φ80	pRL27mutagenesis of ER36
PB-CP	Δ*seqA21 recA629*(Cs) *diaA96* Φ80	pRL27mutagenesis of ER36
PB-CU	Δ *seqA21 recA629*(Cs) *rfaQ98* Φ80	pRL27mutagenesis of ER36
PB-QGP	AB1157 Δ*rfaQGP*::*cat*	Deletion-replacement

[a] All strains have the AB1157 background (footnote “a”) unless mentioned otherwise.

[b] a complete genotype of AB1157: F– λ– rac- *thi-1 hisG4 Δ(gpt-proA)62 argE3 thr-1 leuB6 kdgK51 rfbD1 araC14 lacY1 galK2 xylA5 mtl-1 tsx-33 glnV44 rpsL31*

[c] a complete genotype of MC1061: *araD139* Δ(*ara-leu*)*7697* Δ(*lac*)*X74 galU galK hsdR2*(r_K^-, m_K^+) *strA mcrA mcrB1*

[d] a complete genotype of MG1655: F- lambda- *rph-1 ilvG rfb-50*

[e] The *recA* defect was complemented with the pEAK2 plasmid for the purpose of P1 transduction. The plasmid was cured at 37°C once the construct was confirmed.

Table 2

Plasmids.

Plasmid	ori / drug resistance	other genes	Reference / Derivation
Previous studies			
pEAK2	pSC101(Ts) / *bla*	*recA*	(Kouzminova and Kuzminov, 2004)
pK80	pSC101 / *aad*	–	(Kuzminov and Stahl, 1997)
pLS120	pBR322 / *bla*	p_{araBAD}—> *dnaA*(Cs)	(Simmons and Kaguni, 2003)
pML14	pACYC177 / *cat*	*pgm*	(Lu *et al.*, 1994)
pRL27	R6K / *kan*	P*tetA*→*tnp*(Tn*5*)	(Larsen *et al.*, 2002)
This study			
pPB2	pSC101 / *aad*	*rfaQGP*	pK80::*rfaQGP* (PCR BamHI/EcoRI)
pPB3	pSC101 / *aad*	*rfaQG*	pPB2 BamHI/BglII
pPB4	pSC101 / *aad*	*rfaQ*	pPB2 BamHI/NcoI
pPB5	pSC101 / *aad*	*rfaP*	pPB2 SstII/NspV
pPB6	pSC101 / *aad*	*rfaQ*, *rfaP*	pPB2 SstII/FspI
pER20	pSC101 / *aad*	*rfaGP*	pPB2 PflFI/PvuII