Silencing of the gene coding for the ε subunit of DNA polymerase III slows down the growth rate of *Escherichia coli* populations

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Abstract Chromosome replication in *Escherichia coli* is accomplished by the multimeric enzyme DNA polymerase III; the relevance, in vivo, of the ε subunit (encoded by *dnaQ*) for processivity and fidelity of DNA polymerase III has been evaluated. To this aim, *dnaQ* has been conditionally silenced by means of in vivo expression of different antisense RNAs. Unexpectedly, the presence of the Shine–Dalgarno sequence is essential for the effectiveness of antisense constructs. Silencing of *dnaQ* induces a severe decrease in growth rate not paralleled by high mutation frequencies, suggesting that the ε subunit primarily affects the processivity of DNA polymerase III.

Key words: Mutator strains; Replication; Antisense RNA; DNA polymerase; *Escherichia coli*

1. Introduction

Chromosome replication in *Escherichia coli* is accomplished by DNA polymerase III, a multimeric enzyme composed of 10 different subunits [1]; biochemical studies have revealed that the $5' \Rightarrow 3'$ polymerase and the $3' \Rightarrow 5'$ exonuclease activities reside in the α and ε subunits of the holoenzyme, respectively [2,3]. *E. coli* mutator strains feature defective replicative and/or post-replicative mismatch repair and, accordingly, their genome is exposed to high mutational loads [4,5]. In particular, *dnaQ* (*mutD*) strains are the strongest known mutators [6]: their phenotype is due to mutations mapping in *dnaQ* (coding for the ε subunit of DNA polymerase III), resulting in defective $3' \Rightarrow 5'$ exonuclease activity of the holoenzyme [3,7–9]. Deletion of DnaQ activity has been shown to hamper the isolation of viable *E. coli* strains, indicating a link between ε exonuclease activity, mutation frequency, and cell viability [10]. Moreover, the isolation of viable strains lacking DnaQ activity has been shown to depend on the concomitant presence of a suppressor mutation in *dnaE*, coding for the α subunit of DNA polymerase III [2]. Nevertheless, the ε subunit is also considered to increase the processivity of DNA polymerase III holoenzyme [11]; in addition, the interaction between α and ε subunits is paralleled by a mutual stimulation of their activities [12].

The construction of vectors useful for the transcription, in vivo, of antisense RNAs has been recognized long ago as an efficient tool to control the expression of prokaryotic genes [13]. In particular, it has been shown that antisense RNAs can be successfully used in *E. coli* to repress both host genes (e.g. by means of a transcript complementary to the 5′ region of the mRNA of the *lac* operon) and genes of infecting phages [13,14]. The antisense strategy can also be used to identify essential bacterial genes. Upon insertion in reverse orientation of chromosome fragments into appropriate expression vectors, the growth of the corresponding transformants can be studied under conditions inducing or not the transcription of the different antisense constructs. By this means, the genome of *Staphylococcus aureus* has recently been inspected [15]. Approximately 150 different *S. aureus* transformants fully viable in the absence and unviable in the presence of antisense transcription have been detected, and the corresponding targeted essential genes have been identified [15].

According to these observations, the study of *dnaQ* functions by means of an antisense strategy appears particularly appealing, especially when considering the essential role of this protein in the cell cycle of *E. coli*. A study of fidelity and processivity functions featured by ε subunit in vivo is presented here. In particular, the conditional expression of antisense RNAs directed against *dnaQ* has been attempted. The effect of *dnaQ* silencing upon mutation frequency and growth rate of *E. coli* populations is reported.

2. Materials and methods

2.1. Strains, plasmids and growth media

E. coli TOP10 (F⁻, *mcrA*, Δ(*mrr-hsdMRS-mcrBC*), Δ*lacX74*, ϕ80*lacZ*Δ*M15*, *deoR*, *endA1*, *recA1*, *araD139*, Δ(*araA-leu*)*7697*, *galU*, *galK*, *rpsL*, *nupG*, also known as DH10B [16]), was grown in LB broth. *E. coli* CSH109 (*ara*, Δ(*gpt-lac*), *rpsL*) [17] and CSH126 (*ara*, Δ(*gpt-lac*), Δ(*recA-srl*), *rpsL*) [17] were grown in minimal (M9) medium containing peptone (10 g/l). Growth media were supplemented, when appropriate, with ampicillin and/or streptomycin at 50 and 30 μg/ml, respectively. Plasmid pBAD/HisB (Invitrogen) was used to clone different regions of *dnaQ* in reverse orientation, yielding pBAD-AR1, pBAD-AC1, pBAD-AC2, and pBAD-ARC1 (Fig. 1A).

2.2. Growth rates and mutation frequency assays

Single colonies were grown in LB broth or M9/peptone medium at 37°C under continuous shaking (180 rpm) for 8 h. The corresponding cultures were then diluted (1:1000) in fresh medium and grown overnight. Finally, each bacterial population was subcultured (1:1000) and propagated for ca. 10 generations in the absence (repressing conditions) or in the presence (inducing conditions) of arabinose. The growth of bacterial cultures was spectrophotometrically evaluated (600 nm). Initial and final population densities were estimated by

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colony counting, and the actual number of generations that had occurred in the cultures was accordingly calculated. To determine mutation frequencies per generation, aliquots of the different cultures were plated on Petri dishes containing LB supplemented with rifampicin or nalidixic acid (100 and 30 μg/ml, respectively).

2.3. Cloning

Fragments of the regulative and/or of the coding region of *dnaQ* (accession number X0407 [9]) were amplified by means of polymerase chain reaction (PCR) using *E. coli* TOP10 genomic DNA as template and the following primers sets: 5′-GATAGCGTAGAATTCCGCCG-3′ (for, AR1 and ARC1) and 5′-CCAATCTCCATGGTCTTGTGG-3′ (rev, AR1); 5′-ATGAACCGAATTCGTGCGCACT-3′ (for, AC2), and 5′-CTGACGTCCATGGCGCTGAA-3′ (rev, AC2 and ARC1). Fragment AC1 was amplified from *dnaQ* previously cloned into the expression vector pTrc-His with the following primers: 5′-GAGATCTGGAATTCCACGCCAG-3′ (for) and 5′-ACTCGTAGTCCATGGAGCCGA-3′ (rev). The fragments and pBAD/HisB were digested with *Eco*RI and *Nco*I restriction enzymes, and each corresponding construct obtained by ligation was then used to transform *E. coli* TOP10. Correct cloning was verified by sequencing. To alter the Shine–Dalgarno sequence of pBAD, the MORPH site-specific plasmid DNA mutagenesis kit (Eppendorf) was used with the mutagenic oligonucleotide 5′-CCCGTTTTTTTGGGCTAACTCTAGAAATTAACCATGGTCTTGTGGC-3′. All DNA manipulations were performed according to standard procedures [18].

2.4. Northern blotting

A DNA fragment spanning bases 1–759 of *dnaQ* mRNA was amplified by means of PCR using *E. coli* TOP10 genomic DNA as template and the following primers: 5′-CCATCAACTGCAGACGGTTG-3′ (for), 5′-CCTGACGTAGAATTCGCTGA-3′ (rev). The fragment and pUC19 were digested with *Eco*RI and *Pst*I restriction enzymes, and the corresponding construct obtained by ligation (pUC19-MQ759) was used to transform *E. coli* TOP10. Biotinylation of the *dnaQ* probe was obtained by PCR amplification of pUC19-MQ759 using universal primers in the presence of 0.15 mM biotin-N_4-dCTP, 0.05 mM dCTP, and 0.2 mM dATP, dGTP, and dTTP. Total RNA preparation, electrophoresis, and Northern blotting were performed according to standard procedures [18]. The presence of transcripts complementary to the biotinylated probe was revealed by incubating the membranes with alkaline phosphatase conjugated to streptavidin, and using CDP-Star as substrate (KPL, Gaithersburg, MD, USA).

2.5. RNA structure determination

The structure of antisense RNAs was determined using the STAR genetic algorithm [19]. The best structures predicted by this algorithm for nascent antisense RNAs are reported by means of the software RNAViz [20].

3. Results and discussion

Four different fragments of the regulative and/or coding regions of the *dnaQ* gene were amplified by PCR and cloned in reverse orientation into the expression vector pBAD, containing the regulatory elements of the arabinose operon [21,22]. Upon transformation of appropriate *E. coli* strains, the conditional expression of the corresponding antisense RNAs can be regulated by the addition of arabinose to the culture medium (Fig. 1A). Accordingly, *E. coli* TOP10 (Δ*araA-leu*) was transformed with each of the four different plasmids, and the mutation frequencies (as determined by the appearance of rifampicin-resistant clones) of induced and of

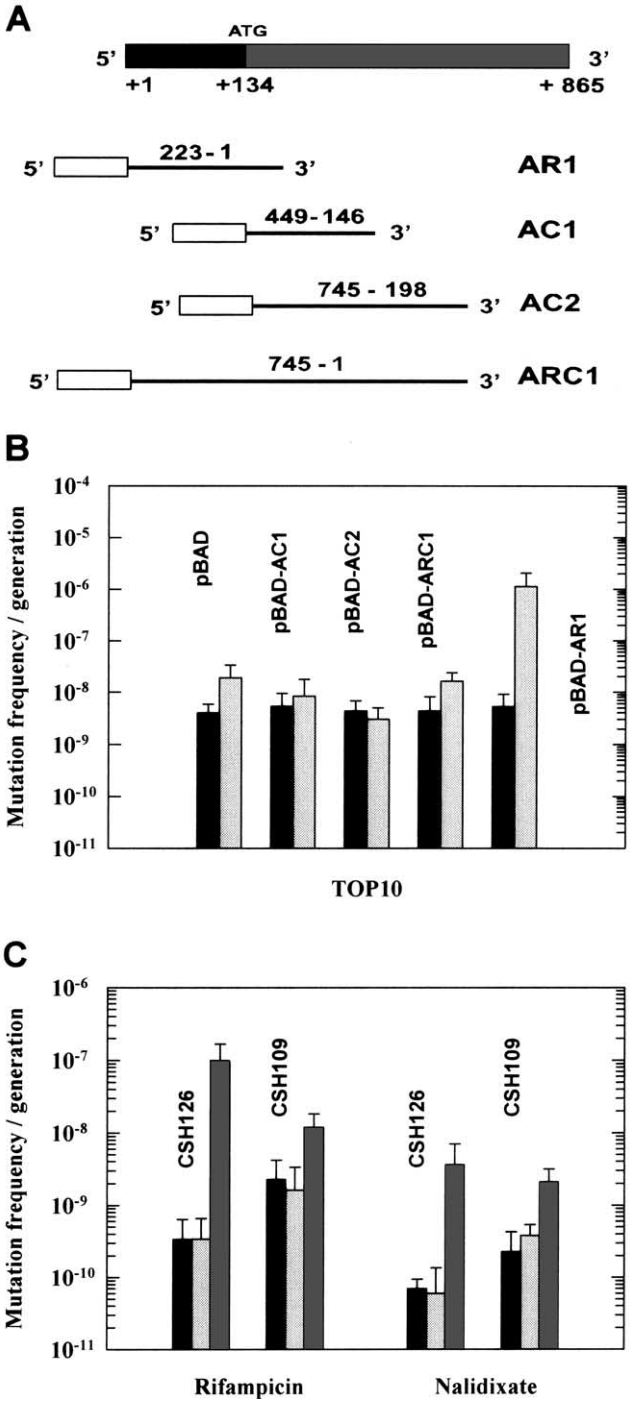

Fig. 1. Messenger RNA of *dnaQ*: regulative and coding regions are indicated with black and gray bars, respectively (A). Amplification of *dnaQ* with the primers indicated (see Section 2.3) leads to the AR1, AC1, AC2, and ARC1 fragments containing *Nco*I and *Eco*RI sites, useful for their insertion in reverse orientation into the expression vector pBAD. Numbers indicate the 5′ and the 3′ base of each construct excluding the restriction sites. Upon induction of transcription, the antisense RNAs contain a portion (empty bars) of the regulative region of the *araBAD* operon and the Shine–Dalgarno sequence AGGAGG (A). Mutation frequencies of *E. coli* TOP10 populations containing the indicated plasmids and grown under non-inducing (black bars, no arabinose) or inducing (gray bars, 13.3 mM arabinose) conditions (B). Mutation frequencies as determined by the appearance of rifampicin-resistant clones. Error bars represent standard deviation ($n = 3$). Mutation frequencies of *E. coli* CSH109 and of CSH126 populations (black bars) grown in the absence of arabinose and of the corresponding populations containing pBAD-AR1 and grown under non-inducing (light gray bars, no arabinose) or inducing (dark gray bars, 13.3 mM arabinose) conditions (C). Mutation frequencies as determined by the appearance of rifampicin- or nalidixate-resistant clones. Error bars represent standard deviation ($n = 3$).

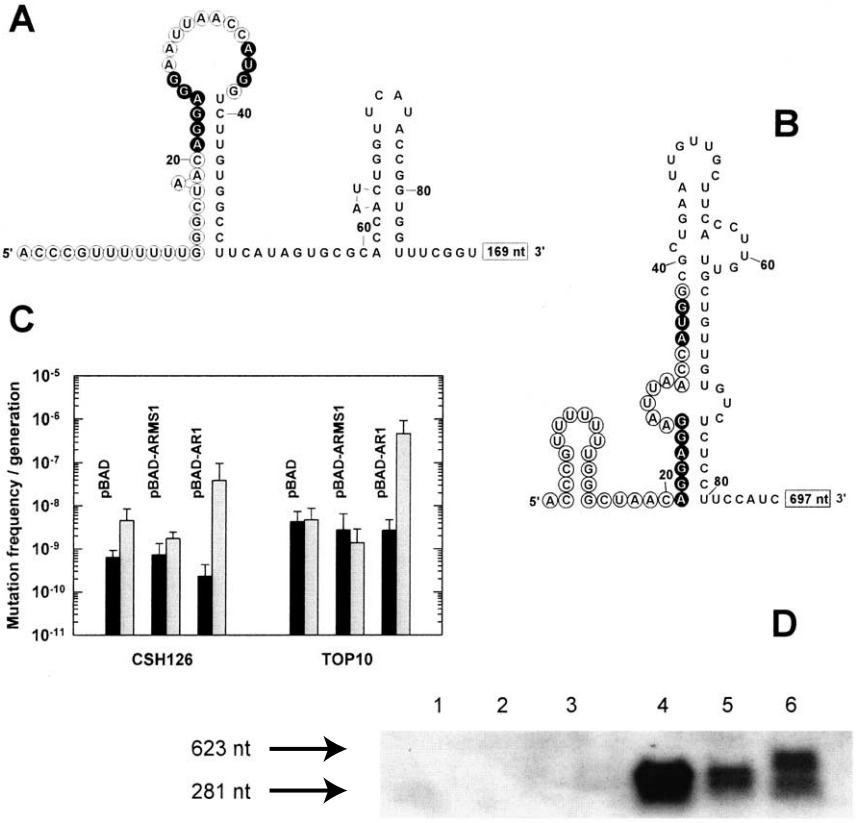

Fig. 2. Structure of the nascent antisense RNAs encoded by pBAD-AR1 (A) and by pBAD-ARC1 (B). Circled bases represent the fragment of the transcript conferred by the vector. The Shine–Dalgarno sequence and the AUG start codon are enclosed in black circles. The structures of the nascent antisense RNAs encoded by pBAD-AC1 and by pBAD-AC2 feature complete pairing of the Shine–Dalgarno sequence and of the AUG start codon (not shown). Mutation frequencies of *E. coli* CSH126 and TOP10 populations containing the indicated plasmids (C). Conditions and symbols as in Fig. 1B. Northern blotting analysis of total RNA isolated from uninduced and induced *E. coli* TOP10 populations containing pBAD-AR1, pBAD-ARMSD1, and pBAD-ARC1 (D, lanes 1 and 4, 2 and 5, 3 and 6, respectively). Induction with 13.3 mM arabinose for 3 h. Arrows indicate the corresponding position of molecular mass markers.

uninduced populations were evaluated. Uninduced cultures feature mutation frequencies of ca. 5×10^{-9}/generation, and significant differences are not observed within this group (Fig. 1B). The induced population bearing pBAD-AR1 mutates at a frequency, equal to 10^{-6}/generation, significantly higher than the other induced and uninduced populations (Fig. 1B). Interestingly, the longest transcript, expressed by pBAD-ARC1 and containing both the pBAD-AR1 and the pBAD-AC2 fragment, does not induce mutagenesis (Fig. 1B). This observation is presumably due to structural and/or kinetic reasons (see below).

Upon the induction of the SOS response (triggered by the protein RecA, see [23]), *E. coli* can express DNA polymerases IV and V [24,25]. To assess whether or not these mutagenic polymerases affect the mutation frequency observed upon silencing of *dnaQ* by pBAD-AR1, the mutation frequency in the presence of this vector was determined in two genetic backgrounds, i.e. *E. coli* CSH109 (*ara*), and CSH126 (*ara*, Δ*recA-srl*). Interestingly, the frequency of the appearance of rifampicin-resistant clones is significantly higher in induced populations of CSH126 than in the corresponding populations of CSH109 (Fig. 1C). When the mutation frequency is determined as the appearance of clones resistant to nalidixic acid, a significant difference is not observed between the two strains, while irrespective of the antibiotic and of the genetic background the induced populations feature significantly higher mutations frequencies when compared with the uninduced populations (Fig. 1C). These observations suggest that silencing of *dnaQ* is mutagenic irrespective of the SOS response.

A structural analysis (according to [19]) of the different antisense RNAs reveals that only pBAD-AR1 leads to the expression of a nascent transcript featuring a partially unpaired Shine–Dalgarno sequence and an unpaired AUG start codon (Fig. 2A,B). It is also important to note that the silencing of *dnaQ* triggered by the expression of AR1 antisense is unlikely due to the concomitant expression of a potentially toxic polypeptide: the translation initiation site is indeed followed by a stop signal after six codons (Fig. 2A). In contrast, peptides of higher molecular mass are potentially expressed upon induction of the non-mutagenic constructs (stop signals are located at the 40th, 40th, and 19th codons of ARC1, AC2, and AC1, respectively).

The structural and functional features of anti-*dnaQ* transcripts suggest that the presence of the ribosome binding sequence is responsible for the competence in silencing of the AR1 antisense. To test this hypothesis, the Shine–Dalgarno sequence of pBAD-AR1 (AGGAGG) was mutated into the corresponding hexamer TCTAGA, yielding pBAD-ARMSD1. The silencing effectiveness of this construct was evaluated in two genetic backgrounds, namely *E. coli* TOP10 (Δ*araA-leu*) and CSH126 (*ara*). Comparison with the mutation frequency

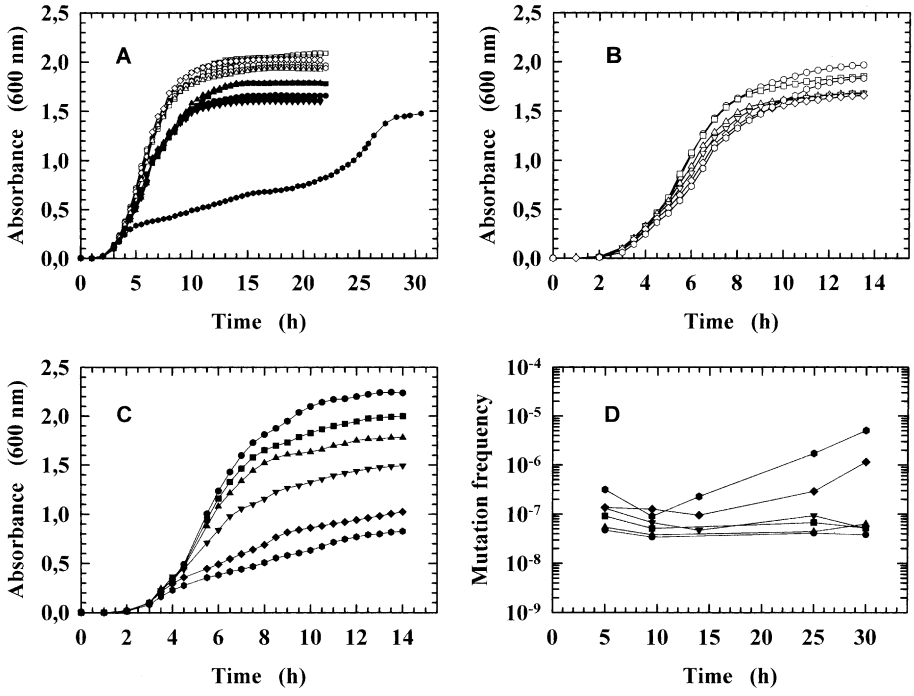

Fig. 3. Kinetics of growth at 37°C (A) of *E. coli* TOP10/pBAD (circles), TOP10/pBAD-AR1 (hexagons), TOP10/pBAD-AC1 (triangles down), TOP10/pBAD-AC2 (diamonds), TOP10/pBAD-ARC1 (triangles up), and TOP10/pBAD-ARMSD1 (squares), in LB medium in the absence (empty symbols) or in the presence of 13.3 mM arabinose (filled symbols). Kinetics of growth at 37°C of *E. coli* TOP10/pBAD (B) and of TOP10/pBAD-AR1 (C) in LB medium in the absence (circles) or in the presence of 0.013 (squares), 0.13 (triangles up), 0.26 (triangles down), 1.3 (diamonds), and 13.3 (hexagons) mM arabinose. Mutation frequencies (D) at 37°C (as determined by the appearance of rifampicin-resistant clones) of populations of *E. coli* TOP10/pBAD-AR1 grown in LB medium in the absence or in the presence of 0.013, 0.13, 0.26, 1.3, and 13.3 mM arabinose (symbols as in C).

observed in the presence of the parent plasmid (Fig. 2C) suggests that the interaction with ribosomes is a prerequisite for the effectiveness of *dnaQ* silencing by means of the antisense strategy presented here. In particular, this interaction could stabilize the AR1 antisense and/or affect the transcription of this construct. To assess the interplay between the structure and the cellular concentration of different anti-*dnaQ* transcripts, total RNA was isolated from *E. coli* TOP10 populations containing pBAD-AR1, pBAD-ARMSD1, or pBAD-ARC1. Northern blotting analysis (performed comparing equal amounts of total RNA) reveals that the level of AR1 transcript is dramatically higher than that of ARMSD1 and of ARC1 (Fig. 2D). Moreover, the expression of pBAD-ARC1 yields fragments featuring molecular masses lower than expected (cf. Fig. 2B,D). These observations are in agreement with previous findings reporting the instability of mRNAs whose binding to ribosomes is hindered [26].

Both *E. coli* CSH109 and CSH126 bear an unknown mutation mapping in the arabinose operon *araBAD* [17,27]. *E. coli* TOP10, bearing the deletion Δ(*araA-leu*) and therefore incapable of arabinose metabolism, was then used to study the effect of *dnaQ* silencing on growth rate and mutation frequency. Expression of the mutagenic antisense RNA encoded by pBAD-AR1 is paralleled by a very slow growth rate (at 37°C) of its host (Fig. 3A). Moreover, the growth rate of *E. coli* TOP10 containing any of the plasmids coding for non-mutagenic antisense RNAs is not significantly affected by the expression of these constructs (Fig. 3A). These observations could suggest that, upon *dnaQ* silencing, a high mutational load hampers the growth of the bacterial population [10,28,29]. To study in detail the interplay between mutation frequency and growth rate, the appearance of rifampicin-resistant clones was determined in *E. coli* TOP10 populations at different phases of growth at the expense of LB supplemented with variable amounts of arabinose. The addition of arabinose to the culture medium does not drastically affect either the growth rate (Fig. 3A,B) or the mutation frequency (Fig. 1B) of populations containing pBAD, pBAD-AC1, pBAD-AC2 or pBAD-ARC1. In contrast, arabinose strongly decreases the growth rate of populations bearing pBAD-AR1 (Fig. 3C). In particular, the generation time increases from

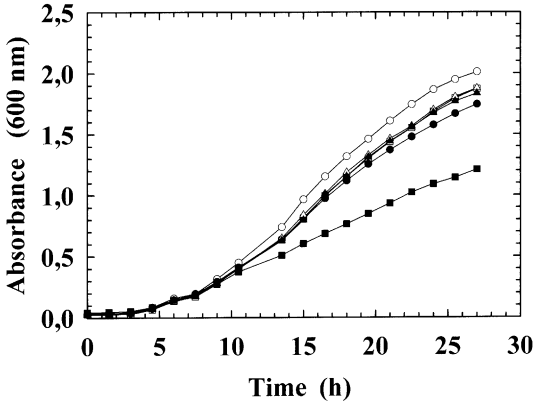

Fig. 4. Kinetics of growth at 20°C of *E. coli* TOP10/pBAD (circles), TOP10/pBAD-AR1 (squares), and TOP10/pBAD-ARC1 (triangles) in LB medium in the absence (empty symbols) or in the presence of 13.3 mM arabinose (filled symbols).

40 min under non-inducing conditions to 73, 181, and 260 min in the presence of 0.26, 1.33 and 13.3 mM arabinose, respectively. Concomitantly, mutation frequency is positively correlated with the arabinose concentration in the growth medium, and, surprisingly, high mutational loads are only observed at 25–30 h of culture (Fig. 3D). Altogether, these observations suggest that the ε subunit primarily controls the processivity of DNA polymerase III, independently of its proofreading function.

The growth rate of *E. coli* populations is known to depend on the number and on the processivity of replication forks simultaneously engaged in chromosome replication [30]. To analyze the effect of *dnaQ* silencing upon cultures featuring long generation times, the growth rate at 20°C of different *E. coli* TOP10 populations was evaluated. Under these conditions, the growth rate of *E. coli* TOP10 is significantly affected by the expression of the AR1 antisense transcript. In particular, the generation time increases from 157 min under non-inducing conditions to 323 min when arabinose is present in the culture medium (Fig. 4). This observation is not paralleled by any significant increase of the mutation frequency upon induction of AR1 expression (at 20°C, data not shown), suggesting a complete apparent uncoupling between processivity and fidelity functions of the ε subunit in vivo. Moreover, the generation time of uninduced *E. coli* TOP10/pBAD-AR1 populations decreases ca. four-fold upon increasing the temperature from 20 to 37°C, and a slight (20%) decrease is featured by the corresponding induced populations. This indicates that the silencing of *dnaQ* tightly controls the generation time of *E. coli* populations.

According to the results of the present work, the ε subunit primarily affects the processivity of DNA polymerase III. At 37 and at 20°C slower growth rates of *E. coli* populations are indeed observed upon silencing of *dnaQ*, and the corresponding slow and error-prone replication of chromosomes is not paralleled by a significant increase in mutation frequency. This situation is most likely due to the recruitment of post-replicative mismatch repair systems [23], whose effectiveness presumably increases upon the increase in generation time. Only at 37°C and upon holding *dnaQ* silencing for 25–30 h does mutation frequency increase, suggesting an intervening saturation of the post-replicative mismatch repair system. Finally, the present work demonstrates that silencing of *dnaQ* can be used to construct fully conditional mutator strains: attempts to evolve bacterial populations by this means are currently in progress.

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