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Directionality of DnaA protein/DNA interaction. Active orientation of the DnaA protein/*dnaA* box complex in transcription termination

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The complex of DnaA protein with its 9 bp consensus binding site, the *dnaA* box 5'-TT(A/T)T(A/C)CA(A/C)A, blocks transcribing RNA polymerase. In a model system, the rate of transcription was monitored distal to the *dnaA* box 5'-TTTTCCACA by the expression of a reporter gene. DnaA-dependent transcription termination occurred irrespective of whether the *dnaA* box region was or was not translated. Only the *dnaA* box orientation 5'-TTTTCCACA on the non-coding strand, but not the reverse orientation, was active in termination. This suggests that DnaA protein contacts only one strand of the DNA duplex. Oligonucleotide-directed mutation of a *dnaA* box present within the *dnaA* coding region resulted in increased expression of *dnaA*. This demonstrates that DnaA protein-directed transcription termination is an element of the autoregulation of the *dnaA* gene.

Key words: autoregulation/*dnaA*/DNA-binding protein/ transcription termination

Introduction

The DnaA protein of *Escherichia coli* is essential for the initiation of replication of the *E.coli* chromosome. Temperature-sensitive *dnaA* mutants are unable to initiate replication of the chromosome (Hirota *et al.*, 1968) and of *oriC* plasmids (von Meyenburg *et al.*, 1979). The DnaA protein binds specifically to a 9 bp consensus sequence, the *dnaA* box, 5'-TT(A/T)T(A/C)CA(A/C)A (Fuller *et al.*, 1984), which is present four times in the 245 bp of the minimal replication origin, *oriC*. Within *oriC*, the DnaA protein functions as a replisome organizer (Sekimizu *et al.*, 1988a; Messer *et al.*, 1988): it recognizes the origin sequence and directs the proteins required for initiation and replication to this site.

DnaA acts also as a repressor of transcription for different genes. One of them is the *dnaA* gene itself. A *dnaA* box is located between the two promoters *dnaA*1p and *dnaA*2p (Hansen *et al.*, 1982b). Both promoters are subject to autoregulation by DnaA protein (Atlung *et al.*, 1985; Braun *et al.*, 1985; Kücherer *et al.*, 1986; Wang and Kaguni, 1987). The *mioC* gene, clockwise adjacent to *oriC*, also contains a *dnaA* box within the promoter region. Binding of DnaA to the box represses the initiation of transcription at the *mioC* promoter (Lother *et al.*, 1985; Rokeach *et al.*, 1986; Stuitje *et al.*, 1986; Løbner-Olesen *et al.*, 1987; Schauzu *et al.*, 1987).

Recently we could demonstrate an additional function for the DnaA protein in the regulation of transcription. The *dnaA* box in the promoter region of *mioC* is located distal to the terminator of the *asnC* gene. The DnaA protein/*dnaA* box complex was found to act as a terminator for *asnC* transcripts. The rate of termination is regulated by the level of DnaA protein in the cell (Gielow *et al.*, 1988; Messer *et al.*, 1988; Schaefer and Messer, 1988). Transcription termination by repressor bound to an operator site distal to the promoter has also been observed for the *lac* repressor (Deuschle *et al.*, 1986).

In this paper we analyse how the rate of transcription termination at the *dnaA* box/DnaA protein complex depends on the orientation of the *dnaA* box and on translation through the region containing the *dnaA* box. By extrapolation from known sequences we estimate that the *E.coli* chromosome contains ~300 *dnaA* boxes. These are located in regulatory regions or within the reading frames of different genes. The *dnaA* gene is one of the genes containing, in addition to the *dnaA* box in the promoter region, a *dnaA* box within the reading frame. This box was mutated without change of the amino acid sequence and the expression of the mutated *dnaA* gene was compared to expression of the wild-type *dnaA* gene.

Results

Plasmid pBXY-gal was used as a model system to determine the effects of the orientation of the *dnaA* box and of translation through the *dnaA* box region on the efficiency of transcription termination by DnaA protein. Plasmid pBXY-gal is derived from pUC19 (Yanisch-Perron *et al.*, 1985) and contains the *lac* promoter, the region coding for the α-complementing *lacZ* peptide including the pUC19 multicloning site, and a *galK* gene, which because of stop codons upstream of the *galK* coding region (McKenney *et al.*, 1981) allows transcription to be monitored independently of the translational status of the *lacZ'* region. A fragment containing the *dnaA* box 5'-TTTTCCACA was inserted into the multicloning site in both orientations. Adjacent restriction sites were manipulated such that either the *dnaA* box was an in-frame insertion allowing translation of *lacZ'* (pBTT, pBRT) or a stop codon was created in front of the *dnaA* box (pBTN,pBRN) (see Materials and methods and Figure 1).

Transcription and translation through the dnaA box at wild-type and at elevated levels of DnaA protein

In the different constructs, translation through the *dnaA* box was measured by the *lacZ* complementing activity, and transcription after the *dnaA* box was quantified using *galK* activity. Both activities were analysed at different intracellular levels of DnaA protein, wild-type level and elevated levels provided by the induction of the *tac* promoter in the co-resident DnaA protein overproducing plasmid pHK27

(Schaefer and Messer, 1988). Both the *lac* promoter on the pB plasmids and the *tac* promoter on pHK27 were induced with 1 mM IPTG starting at $t = 0$.

pBTT: orientation of the dnaA box in the direction of transcription and translated. Upon induction of the *lac* promoter at wild-type levels of DnaA protein, *lacZ* expression increased linearly with time, which is evidence for a translated *dnaA* box. *galK* expression increased with time and reached a plateau level of 132 units 60 min after induction (Figure 2A, pBTT). At elevated DnaA levels due to concomitant induction of *dnaA* expression in plasmid pHK27 and *Plac* in plasmid pBTT, *galK* expression reached a level of 80 units at 60 min (Figure 2B, pBTT), i.e. a 40% reduction compared to *galK* expression at wild-type DnaA levels. The *lacZ* expression reached 19 units at 60 min, i.e. an 85% reduction. The reduction in expression due to an increased intracellular DnaA concentration was thus more pronounced for *lacZ'* than for *galK*.

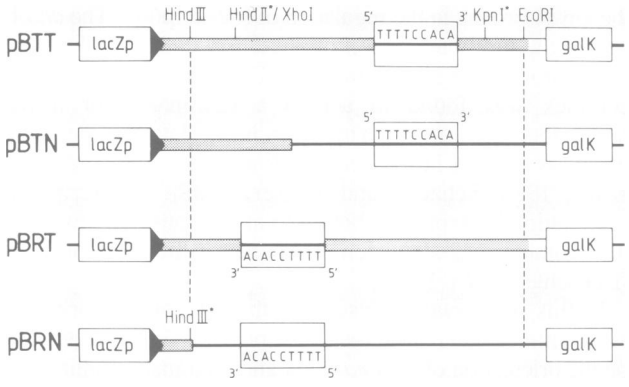

Fig. 1. Schematic representation of the cloning region of pB plasmids. Translated *lacZ'* regions are indicated by stippled bars; stars denote modified restriction sites.

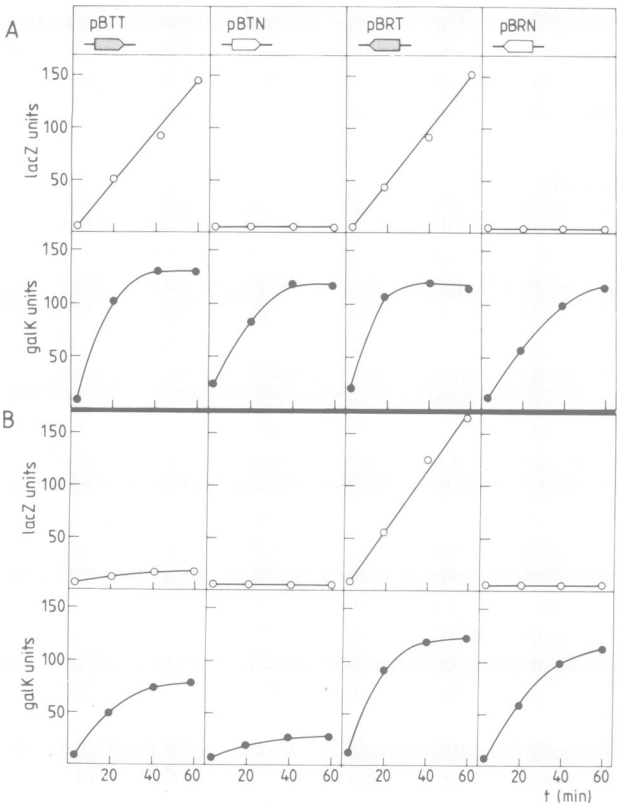

Fig. 2. *lacZ* and *galK* expression at wild-type levels (**A**) and at elevated levels (**B**) of DnaA protein. Symbols at the top indicate orientation and translational status of the *dnaA* box. At $t = 0$ the *lac* promoter on the pB plasmids and the *tac* promoter on pHK27 (B) were induced with 1mM IPTG. *galK* and *lacZ* units were corrected for copy number.

pBTN: orientation of the dnaA box in the direction of transcription and not translated. In pBTN, the *dnaA* box region is not translated due to a stop codon in front of the *dnaA* box, as evidenced by the absence of *lacZ* activity (Figure 2A and B, pBTN). At wild-type DnaA concentration, *galK* expression was indistinguishable from expression in pBTT (Figure 2A). At an elevated DnaA concentration in the cell, pBTN expressed 80% less galactokinase (Figure 2, pBTN).

The results obtained with pBTT and pBTN demonstrate that transcription of *galK* is reduced when DnaA binds to the *dnaA* box irrespective of whether the region containing the *dnaA* box is or is not translated. However, in the absence of translation transcription termination is more efficient.

Reverse orientation of the dnaA box with respect to transcription, pBRT and pBRN. Both plasmids pBRT and pBRN contain the *dnaA* box in the reverse orientation (5'-TGTGGAAAA in transcription direction). pBRT is the translated and pBRN the non-translated version, as verified by the presence or absence of *lacZ* activity (Figure 2, pBRT and pBRN). At wild-type levels of DnaA protein, both plasmids showed a galactokinase expression which was comparable to the expression in pBTT and pBTN (Figure 2A). In striking contrast to those plasmids, overproduction of DnaA protein influenced neither the *galK* nor the *lacZ'* expression on plasmid pBRT and the *galK* expression on pBRN (Figure 2B, pBRT and pBRN). The *dnaA* box (5'-TTTTCCACA) in the non-coding strand (pBTT and pBTN) thus resulted in efficient termination, whereas the reverse orientation (5'-TGTGGAAAA; pBRT and pBRN) was completely inactive.

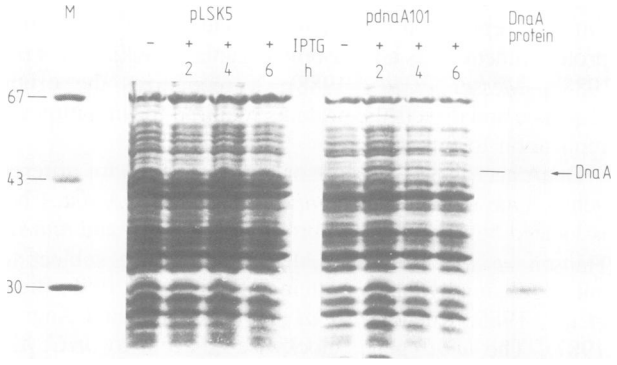

Fig. 3. Induction of DnaA protein in pLSK5 and pdnaA101. SDS–polyacrylamide gel of total cellular proteins stained with Coomassie blue. M: Mol. wt marker (kd); DnaA protein: partially purified DnaA protein preparation.

Effect of transcription termination on dnaA autoregulation

The *dnaA* gene contains, in addition to the *dnaA* box in the promoter region, a *dnaA* box in the terminating orientation within the coding region (positions 1634–1642 in Hansen *et al.*, 1982a). DnaA-mediated transcription termination may, therefore, also be involved in the autoregulation of the *dnaA* gene. DnaA protein levels are elevated ~100 times compared to wild-type in cells carrying a plasmid with *dnaA* under the control of an inducible promoter (Sekimizu *et al.*, 1988b). Despite this it is difficult to demonstrate DnaA overproduction on SDS–polyacrylamide gels of total cellular proteins, possibly due to enhanced termination at this box at elevated concentrations of DnaA protein.

In order to test whether DnaA-protein-mediated transcription termination affects expression of the *dnaA* gene and to improve DnaA overproduction from expression vectors, we replaced the T at the second (invariant) position of the internal *dnaA* box with a C using oligonucleotide-directed mutagenesis (Kunkel, 1985). This transition does not change the amino acid sequence of DnaA protein:

tTTTTCCACAcc → tTCTTCCACAcc
Phe Phe His Thr Phe Phe His Thr

We designate the resulting mutation (T to C at position 1635 in Hansen *et al.*, 1982a) *dnaA*101. Plasmid pLSK5 contains the *dnaA* gene under the control of the *tac* promoter (Schauzu *et al.*, 1987). A fragment containing the *dnaA*101 mutation was used to replace the corresponding wild-type fragment in pLSK5, resulting in plasmid pdnaA101.

For a comparison of the amounts of DnaA protein synthesized upon induction in strains carrying the two DnaA overproducing plasmids pLSK5 and pdnaA101 respectively, cells were induced with IPTG, lysed and total cellular proteins were separated on an SDS–polyacrylamide gel (Figure 3). A strong protein band at the position of DnaA protein, maximally induced within 2 h after IPTG addition, was seen with pdnaA101, whereas little if any induction was visible with pLSK5. A densitometric scanning shows that the DnaA protein band constitutes ~3.5% of total cellular protein with pdnaA101.

These data demonstrate that the expression of the *dnaA* gene is modulated by the binding of DnaA protein to the *dnaA* box within the *dnaA* reading frame. This suggests that also for the chromosomal *dnaA* gene transcription termination at this site is an element of the autoregulation, in addition to DnaA-mediated repression.

Discussion

The DnaA protein/*dnaA* box complex acts as a terminator of transcription and, therefore, constitutes a new element of gene regulation. Transcription termination was observed irrespective of the translational status of the *dnaA* box region, but only in one orientation of the asymmetric *dnaA* box.

Translation of the *dnaA* box region was monitored by *lacZ* complementation. The rate of transcription after the *dnaA* box was measured using *galK* as a reporter gene. The intracellular DnaA concentration was varied from wild-type level to an excess of DnaA protein, provided by induction of a DnaA overproducing plasmid. In the absence of active DnaA protein in a *dnaA*46(Ts) mutant host at 42°C, *lacZ* and *galK* activities were comparable to the wild-type situation (data not shown). This suggests that in wild-type cells very little free DnaA protein was present due to the high copy number of *dnaA* box bearing pB plasmids.

When the *dnaA* box 5′-TTTTCCACA was on the non-coding strand, i.e. oriented in the direction of transcription, transcription termination was increased at elevated DnaA protein levels. The translated *dnaA* box in pBTT resulted in ~40% termination. The untranslated box in pBTN caused ~80% of the transcripts to terminate. Possibly, the primary effect of the interaction of RNA polymerase with the DnaA protein/*dnaA* box complex is pausing of RNA polymerase. In analogy to activation of paused transcription complexes by translation in the *trp* leader region (Landick *et al.*, 1985), translating ribosomes may partially release paused RNA polymerase.

The level of *lacZ* expression was more severely reduced by excess DnaA protein. An increase in DnaA concentration decreased *lacZ* expression to a level close to background in pBTT (Figure 2B), compared to a residual rate of transcription of 60% as measured by *galK* expression. A possible interpretation of this observation is that pausing of RNA polymerase might result in the release of ribosomes from the mRNA and/or ribosomal frameshifting (Weiss *et al.*, 1987), while some transcripts eventually proceed into the *galK* region.

The reverse orientation of the *dnaA* box on the plasmids pBRT and pBRN was completely inactive. *galK* and *lacZ* expression in these plasmids was indistinguishable at the different DnaA concentrations (Figure 2, pBRT and pBRN). This result suggests that the double-strand-specific DnaA protein makes contacts with only one strand of the DNA duplex. If this is the non-coding strand, RNA polymerase passes the DnaA protein/*dnaA* box complex freely. The inherent property of DnaA protein to unwind double-stranded DNA locally (Bramhill and Kornberg, 1988) may help in this process. This interpretation implies that DnaA protein contacts the complement of the conventional representation of the *dnaA* box [i.e. 5′-T(T/G)TG(T/G)A(T/A)AA]. Preferential interaction of DnaA protein with one side of the DNA duplex was also suggested by DNase I footprint analysis of DnaA protein/DNA complexes (Fuller *et al.*, 1984).

In contrast to the *dnaA* box most protein-binding sites for regulatory proteins are symmetric, and the proteins which bind to these sites are oligomers of two or four identical subunits. Contacts of single subunits with one half of a symmetric binding site, however, usually involve both strands of the double helix (Pabo and Sauer, 1984; Anderson *et al.*, 1987). Apparently, DnaA protein represents a different type of regulatory protein.

An alternative explanation for the asymmetry of DnaA-mediated termination would be a release of DnaA protein from the complex due to interaction with RNA polymerase, but only in one orientation of the asymmetric DnaA protein/*dnaA* box complex. Although suppressor mutations suggest a possible interaction between RNA polymerase and DnaA protein (Bagdasarian *et al.*, 1977; Atlung, 1984) we think this interpretation to be less likely.

The orientation of the *dnaA* box is also important for its function in the initiation of DNA synthesis (Seufert *et*

al., 1988). On the other hand, repression occurs in both orientations. Within the *dnaA* promoter region the *dnaA* box is located between promoters *dnaA*p1 and *dnaA*p2 in an orientation which does not permit termination of the p1 transcript. The orientation and position of the box with respect to the two promoters is thus different. Nevertheless, both promoters are subject to repression by DnaA protein (Kücherer *et al.*, 1986).

Mutation of the *dnaA* box within the *dnaA* coding region (*dnaA*101) resulted in increased expression of DnaA protein. Since the mutation is at the wobble position of the Phe codon 250, the resulting protein is wild-type. This mutation thus improves the yield from DnaA-overproducing plasmids, although the mutated *dnaA* box is not completely inactive in DnaA protein binding (unpublished results). More importantly, this result shows that transcription termination within the *dnaA* reading frame is an element of the regulation of *dnaA* gene expression. The *dnaA* gene is thus autoregulated by DnaA protein at two levels: by repression at the promoters *dnaA*1p and *dnaA*2p and by transcription termination.

A cold-sensitive mutation, *dnaA*(Cs), was isolated as an intragenic suppressor of the heat-sensitive mutation *dnaA*46 (Kellenberger-Gujer *et al.*, 1978). The phenotype of this mutant suggested that suppression was due to increased synthesis of a partially active protein. This interpretation was disputed since cloning and sequence analysis of *dnaA*(Cs) revealed three point mutations in the coding region in addition to the *dnaA*46 mutation, but an unchanged promoter region (Braun *et al.*, 1987). One of the *dnaA*(Cs) mutations is a C to T change at position 6 of the internal *dnaA* box. In view of the results reported here, the original interpretation may be correct.

Many genes contain *dnaA* boxes not only in their regulatory regions but also within their coding regions. Apparently, DnaA protein functions as a global regulatory protein linking the expression of many genes to the replication cycle.

An intricate example for interrelated controls is the *E. coli dam* operon. The *dam* gene, coding for Dam methyltransferase, has been shown to be negatively regulated by DnaA protein, due to a *dnaA* box located between *dam* and the upstream gene *damX* (Jonczyk *et al.*, 1989). Since there is no promoter in the vicinity of this *dnaA* box, DnaA-mediated regulation of *dam* must be exerted by the transcription termination process described here. On the other hand, Dam-mediated methylation of the *dnaA* promoter region is required for optimal expression of the *dnaA* gene (Braun and Wright, 1986; Kücherer *et al.*, 1986).

DnaA protein has several important functions in the cell, all of them mediated by the binding to the 9 bp consensus sequence. Within *oriC* it serves as a replisome organizer (Sekimizu *et al.*, 1988a; Messer *et al.*, 1988). It is responsible for the correct timing of the initiation of replication (Løbner-Olesen *et al.*, 1989), and it is a repressor for several genes. The results reported here add another function to this list: transcription termination. In addition, they provide information on the mode of interaction of DnaA protein with the *dnaA* box.

Materials and methods

Bacterial strains and plasmids

Host strain for the plasmids was *E.coli* JM105 [Δ(*lac-pro*), *endA*, *hsd*R4, *rspL*, *sbcB*15, *sup*$^+$, *thi*; F'lac:*lacI*^{Q}, Δ(*lacZ*)M15, *traD*36, *proA*$^+$, *proB*$^+$].

Plasmid pBXY-gal is derived from pUC19 (Yanisch-Perron *et al.*, 1985), replacing the 419 bp *Ssp*I–*Nar*I fragment by a 2004 bp *Ssp*I–*Acc*I fragment of the following configuration: a 1493 bp *Acc*I–*Fok*I fragment from pUTE13 (Schaefer and Messer, 1988) containing the *galK* gene (McKenney *et al.*, 1981) was linked via the filled-in *Fok*I end to the filled-in *Hin*dIII end of a 507 bp *Hin*dIII–*Ssp*I fragment, isolated from pJF118EH (Fuerste *et al.*, 1986), which harbours the *rrnB* terminator (Brosius *et al.*, 1981). The plasmid pBXY-gal with the *lac* promoter, the α-complementing *lacZ'* gene, the polylinker region and the *galK* gene with *rrnB* terminator was used to clone the *dnaA* boxes in both orientations and to create the different mutations in the polylinker region.

For the overproduction of DnaA protein, we used pHK27, a compatible plasmid (pACYC184 replicon) with the *dnaA* gene under the control of the *tac* promoter (Schaefer and Messer, 1988).

Media

The galactokinase (McKenney *et al.*, 1981) and β-lactamase assays (Sargent, 1968) were done with cells grown in M9 minimal medium supplemented with fructose (0.2%), casamino acids (0.5%), thiamine (10 μg/ml) and the necessary antibiotics, ampicillin (50 μg/ml) or chloramphenicol (25 μg/ml) respectively.

In vitro construction of dnaA box galK fusions

We used standard techniques for plasmid preparation and cloning (Maniatis *et al.*, 1982). All enzymes were purchased from Boehringer Mannheim, FRG.

Four plasmids, each with a *dnaA* box, which differ in the orientation of the box and their status of translation, were constructed. The *dnaA* box of three of the plasmids (pBTT, pBTN and pBRT) is contained within a 35 bp *Sau*3A fragment derived from the intergenic region between *mioC* and *asnC* (positions 794–802 in Buhk and Messer, 1983). The fragment was integrated in both orientations into the *Bam*HI site of pBXY-gal. Additional modifications in the polylinker region allowed the adaptions to the reading frames.

The plasmid pBTN harbours the *dnaA* box orientated in the direction of transcription and the box is not translated due to a stop codon in front of the *dnaA* box. Plasmid pBTT is a modified version of pBTN. The *Hin*dIII site was deleted by 'Klenow fill in' reaction and by the insertion of a *Xho*I linker (5'-CCTCGAGG). The protruding ends of the *Kpn*I site were deleted by mung bean nuclease treatment. These modifications result in a translated *dnaA* box in the direction of transcription.

pBRT contains the reverse orientation of the 35 bp *Sau*3A fragment, whereby the *dnaA* box is directed towards the direction of transcription and translated. Plasmid pBRN harbours the *dnaA* box in reverse orientation and the box is not translated. The *Sma*I–*Hin*cII fragment of pBXY-gal was replaced by the oligonucleotide 5'-GGGGCTGTGGAAAACCC. This oligonucleotide contains the same *dnaA* box and the *Sma*I site is regenerated. In addition, the *Hin*dIII site was modified by 'fill in' reaction with Klenow enzyme. All clones were verified by sequencing (Sanger *et al.*, 1977).

Galactokinase, β-lactamase and β-galactosidase assays

The assay procedure for galactokinase (McKenney *et al.*, 1981) and the modifications were as described (Schaefer and Messer, 1988). To determine the relative copy number, the activity of β-lactamase (*bla*) encoded by the plasmids was measured (Sargent, 1968). The values are calculated according to the equation:

$$bla \text{ units} = [\epsilon_{490} \text{ (without extract)} - \epsilon_{490} \text{ (sample)}] \times 3.44^{-1} \times OD_{450}{}^{-1}$$

(ϵ_{490} = extinction at 490 nm, OD_{450} = optical density of the growing culture at 450 nm).

The *galK* units were corrected for copy number according to:

$$galK \text{ units}_{corr} = galK \text{ units} \times bla \text{ units}^{-1}$$

lacZ expression was monitored as described (Miller, 1972). *lacZ* units were corrected for copy number according to:

$$lacZ \text{ units}_{corr} = lacZ \text{ units} \times bla \text{ units}^{-1}$$

In vitro mutagenesis of the dnaA box located within dnaA

The same *dnaA* box as in the plasmids described above is located within the reading frame of the *dnaA* gene in the direction of transcription (positions 1634–1642 in Hansen *et al.*, 1982a). We used plasmid pLSK5 (Schauzu *et al.*, 1987), which contains *lacI*^{Q}, the *tac* promoter fused to *dnaA* and

*rrnB*t1t2, to isolate a fragment containing the *dnaA* box. A 332 bp *Tha*I–*Acc*I fragment from pLSK5, spanning the *dnaA* box and two *Pvu*I sites flanking the box, was trimmed by 'Klenow fill in' reaction and inserted into the *Sma*I site of M13mp8 (Messing, 1983). This phage was used to mutagenize the *dnaA* box by oligonucleotide-directed mutagenesis (Kunkel, 1985). The result, verified by sequence analysis, was an exchange of one base pair within the *dnaA* box, which does not change the amino acid sequence, mutant *dnaA*101. The 225 bp *Pvu*I fragment in pLSK5 containing the wild-type *dnaA* box within the *dnaA* gene was replaced by the corresponding fragment harbouring *dnaA*101. The resulting plasmid is pdnaA101.

dnaA expression

A fresh overnight culture in M9 minimal medium supplemented with glucose (0.2%), casamino acids (0.5%), thiamine (10 μg/ml) and ampicillin (50 μg/ml) of the strain with the respective plasmid was diluted 1/200 in fresh medium. When the culture reached an OD_{590} of 0.4, IPTG (1 mM) was added, and the samples were removed at 2 h intervals. One millilitre was centrifuged and the pellet was resuspended in 30 μl sample buffer. The samples were boiled for 3 min, centrifuged for 10 s and 10 μl of the protein mixture was separated by SDS–PAGE (Laemmli, 1970).

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