DNA Research **14**, 155–168, (2007) doi:10.1093/dnares/dsm017

Distribution of Stable DnaA-Binding Sites on the *Bacillus Subtilis* Genome Detected Using a Modified ChIP-chip Method

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(Received 24 July 2007; revised 10 September 2007; published online 10 October 2007)

Abstract

We developed a modified ChIP-chip method, designated ChAP-chip (Chromatin Affinity Precipitation coupled with tiling chip). The binding sites of *Bacillus subtilis* Spo0J determined using this technique were consistent with previous findings. A DNA replication initiator protein, DnaA, formed stable complexes at eight intergenic regions on the *B. subtilis* genome. Characterization of the binding sequences suggested that two factors—the local density of DnaA boxes and their affinities for DnaA—are critical for stable binding. We further showed that in addition to autoregulation, DnaA directly modulate the expression of *sda* in a positive, and *ywlC* and *yydA* in a negative manner. Examination of possible stable DnaA-binding sequences in other *Bacillus* species suggested that DnaA-dependent regulation of those genes is maintained in most bacteria examined, supporting their biological significance. In addition, a possible stable DnaA-binding site downstream of *gcp* is also suggested to be conserved. Furthermore, potential DnaA-binding sequences specific for each bacterium have been identified, generally in close proximity to *oriC*. These findings suggest that DnaA plays several additional roles, such as control of the level of effective initiator, ATP-DnaA, and/or stabilization of the domain structure of the genome around *oriC* for the proper initiation of chromosome replication.

Key words: ChIP-chip analysis; *Bacillus subtilis*; DnaA protein; DnaA box; high-density tiling chip

1. Introduction

The replication initiation protein of eubacteria, DnaA, binds to an asymmetrical 9 bp consensus sequence (TTATNCACA, DnaA box) that is repeated several times in the replication origin (*oriC*) region, and plays a central role in the formation of the initiation complex for chromosome replication (reviewed by Leonard and Grimwade,[1] Kaguni,[2] Mott and Berger,[3] and Zakrzewska-Czerwinska *et al.*[4]). Additionally, the protein autogenously represses its own expression to maintain the cellular level through binding to DnaA boxes downstream of the *dnaA* promoter in *Escherichia coli*,[5] *Bacillus subtilis*,[6] *Streptomyces lividance*,[7] *Mycobacterium smegmatis*, and *M. bovis*.[8] The timing of chromosome replication initiation is tightly regulated, and involves the cellular DnaA level in *E. coli* (reviewed by Kaguni[2]), *B. subtilis*[6] and *M. smegmatis*.[9] However, the DnaA box sequence is not restricted to the *oriC* and *dnaA* promoter regions. A search for the consensus sequence, TTATNCACA, allowing a one base mismatch along the complete genome sequence of *E. coli* and *B. subtilis*, reveals the existence of 3742 and 4342 sites, respectively.

Indeed, several genes are possibly or conclusively regulated directly by DnaA in *E. coli* (reviewed by Messer and Weigel).[10] DnaA negatively regulates *mioC*, *rpoH*, *uvrB*, and *proS*,[5,11,12] and positively regulates

Edited by Katsumi Isono

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polA expression.[13] Levels of *mioC* and *polA* are controlled in a growth phase-dependent manner, and DnaA possibly contributes to the coupling of expression to growth rate.[13–15] A recent study proposes that repression of *nrdAB* expression by ATP-bound DnaA is associated with coupling of the initiation frequency to elongation rate of chromosome replication,[16] although DnaA has been demonstrated previously to regulate positively *nrdAB* promoter.[17] DnaA might have a dual role in modulating *nrdAB* expression; low levels of DnaA-ATP stimulate the transcription whereas high levels repress it.[18] In addition, a high-affinity DnaA-binding sequence containing five DnaA boxes, *dat* (DnaA Titration), has been identified.[19] Multicopy plasmids harboring the *dat* sequence interfere with the normal replication cycle, although its exact role is unclear.[20] A similar high-affinity DnaA-binding region is involved in regulating the initiation of chromosome replication in *S. coelicolor*.[21] In *B. subtilis*, DnaA functions in repressing the initiation of sporulation in actively replicating cells by controlling the expression of Sda that blocks the signal transduction cascade leading to sporulation.[22,23] The sequence upstream of the *sda* promoter includes five DnaA boxes, and DnaA binding to these is essential for *sda* expression. Transcriptome analysis of replication-inhibited cells and chromatin immunoprecipitation assays reveal that *yllB* (*ftsL*), *dnaB*, *ywlC*, and *yydA* are negatively regulated by DnaA binding to their promoter regions,[23] suggesting that the protein is involved in tolerance against replication stress as a transcriptional regulator. Furthermore, DnaA acts as a transcriptional regulator in *Caulobacter crescentus*.[24,25] In this case, DnaA stimulates the expression of a master regulator of cell cycle progression, *gcrA*, which in turn activates multiple genes, including those involved in chromosome replication and segregation, coordinating the initiation of chromosome replication and cell cycle progression.

Thus, in addition to the initiation of replication and autoregulation of its expression, DnaA appears to participate in the coordination between initiation of chromosome replication and cell cycle progression. However, genome-wide identification of DnaA-binding sites *in vivo* has not yet been attempted. ChIP-chip analysis, a powerful tool for monitoring the distribution of genome-associated proteins *in vivo*, employs chromatin immunoprecipitation in combination with a high-resolution oligonucleotide tiling chip.[26] For instance, we and other groups recently reported the distribution of nucleoid-associated protein, H-NS, on the *E. coli* and *Salmonella* genomes, and found that H-NS preferentially binds to and represses horizontally acquired genes.[27–29] However, ChIP-chip analysis requires a specific antibody that efficiently recognizes the target protein in protein–DNA complexes, which may limit its applicability. Previously, we developed a method to isolate protein complexes with high purity, using a 12 × Histidine-tag under denatured conditions.[30] To improve versatility, this procedure was adapted for the purification of protein–DNA complexes, denoted ChAP-chip (Chromatin Affinity Precipitation coupled with high density tiling chip). The binding sites of Spo0J determined with the modified technique were consistent with previous reports.[31] Clearly, DnaA forms stable complexes at eight intergenic regions on the *B. subtilis* genome, including *oriC*, *oriC*1, and *oriC*2. Characterization of the DnaA-binding sequences revealed that two factors—local density of the DnaA boxes and their affinities to DnaA—are critical for stable binding. Thus, filament formation of multiple ATP-bound DnaA proposed for the bacterial *oriC*–DnaA complex[32] might occur at the stable-binding sites. In combination with transcriptome analysis of cells containing increased or decreased DnaA amounts, our data show that DnaA proteins stably associated with target DNA directly regulate the expression of *sda* in a positive manner, and *ywlC* and *yydA* in a negative manner. Examination of possible stable DnaA-binding sequences in genome sequences of other *Bacillus* species suggested that DnaA-dependent regulation of those genes is maintained in most of bacteria examined, supporting their biological significance. In addition, a possible stable DnaA-binding site downstream of *gcp* is also suggested to be conserved. Furthermore, potential DnaA-binding sequences specific for each bacterium have been identified, generally in close proximity to the *oriC* region. These findings suggest that DnaA plays several additional roles, such as control of the level of effective initiator, ATP-DnaA, and/or stabilization of the domain structure of the genome around *oriC* for the proper initiation of chromosome replication.

2. Materials and methods

2.1. Design of the B. subtilis tiling chip

We retrieved the *B. subtilis* genome sequence and other genetic information from the SubtiList database (http://genolist.pasteur.fr/SubtiList/) for the design of probes, according to the Affymetrix guidelines. Probe sequences were selected for protein coding and intergenic regions using different criteria.[27] Apart from the *rrnA* operon, rRNA genes were excluded from probe design. In addition, *skin* element, two prophage (PNSX and spβ), and seven prophage-like sequences[33] were excluded. The tRNA genes and those encoding proteins with lengths of <100 amino acids were taken as intergenic regions. As a result, 55 430 25-mer sequences on the coding strand were selected for protein coding regions at 25–30 bp intervals, and 72 218 sequences on both strands were selected for intergenic regions at 2–3 bp intervals. In total, 127 648 probe sets (perfect match and mismatch probes) were synthesized on the chip. Chip probe

information is available on our website (http://genome.naist.jp/bacteria/array/dnaA/bsub.html).

2.2. Construction of strains expressing C-terminally histidine-tagged proteins

Primers used for generating plasmids used in this study are presented in Supplementary Table S1.

To construct the pMUTinHis-△spo0J plasmid, a 250 bp fragment encompassing the 3′-region of the *spo0J* gene (except the stop codon) was amplified by PCR from *B. subtilis* 168 genomic DNA using the primer set, spo0J.f–spo0J.r, and cloned between *Eco*RI–*Xho*I sites of pMUTinHis.[30] The resultant plasmid was integrated into the *B. subtilis* chromosome by single crossover to generate *B. subtilis* strains expressing C-terminal histidine-tagged Spo0J (strain SI002).

The *dnaA* gene constitutes an operon with the downstream *dnaN* gene and the intergenic region (*oriC2*) acts as the *B. subtilis oriC*, together with a sequence upstream of *dnaA* (*oriC1*).[34] We generated a strain expressing C-terminal histidine-tagged DnaA without plasmid integration between *dnaA* and *dnaN* (strain SI003), as schematically shown in Fig. 1. A DNA fragment containing the 3′-region of *dnaA*, except the stop codon, was amplified using the primers, 1dnaAHiF and 1dnaALinkR, with a partial 12 × Histidine (His-tag) coding sequence (*dnaA*-side fragment). A fragment containing the His-tag, followed by the stop codon of *dnaA* and its 500 bp downstream sequence (*dnaN*-side fragment) was generated by two-step adaptor PCR, using the primer sets, 2dnaAHisF–2dnaABaR, for initial amplification, and Linker12His–2dnaABaR, for the second PCR. The two fragments were ligated by recombinant PCR using the 1dnaAHiF–2dnaABaR primer set, and cloned within *Hin*dIII and *Bam*HI sites of pMUTinNC.[35] Since the *dnaN* gene essential for cell growth was placed under control of the IPTG-inducible *spac* promoter in the resultant transformants, pMUTinAhisN was used for transformation of *B. subtilis* 168 by single crossover with selection on LB medium containing 0.5 μg/ml erythromycin and 1 mM IPTG (first step in Fig. 1B). To induce a second recombination event for removal of the integrated plasmid, the resultant strain was cultured in competent medium containing 1 mM IPTG. Cells that grew in the absence of IPTG were selected on LB medium without erythromycin and IPTG (second step in Fig. 1B). Cells with insertion of the His-tag sequence were selected from IPTG-independent and erythromycin-sensitive colonies by examining amplification using primers complementary to the His-tag sequence (Linker12His) and a *dnaN* coding sequence (2dnaAbaR), and chromosomal DNA extracted from these colonies. Finally, we confirmed the addition of the 12 × His coding sequence at the 3′ end of *dnaA* and removal of integrated plasmid by sequencing and the resultant strain expressing the His-tagged DnaA was designated SI003. Negligible changes if any in synchrony of initiation of chromosome replication in SI003 cells were confirmed by flow cytometory (Supplementary Fig. S1).

2.3. ChAP-chip analysis

An overnight culture of *B. subtilis* cells expressing histidine-tagged protein in LB liquid medium at 37°C was inoculated into 400 mL LB medium to obtain an initial OD_{600} value of 0.01. During SI002 culture at 37°C, 0.5 μg/mL erythromycin and 1 mM IPTG were added at every step. At OD_{600} of 0.4, the culture was treated with formaldehyde (1% final concentration) for 30 min. Cells were washed with TBS buffer (pH 7.5), and stored at −80°C until use. Next, cells were disrupted by sonication on ice in 3 mL of UT buffer (100 mM HEPES, 50 mM imidazole, 8 M urea, 0.5 M NaCl, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, pH 7.4). After centrifugation at 8000 rpm for 10 min, 200 μL of MagneHis (Promega) was added to the supernatant, followed by overnight incubation at room temperature with gentle shaking. MagneHis was washed five times with UT buffer, and bound proteins eluted with 400 μL of elution buffer (100 mM Tris–HCl, pH 7.5, 0.5 M imidazole, 1% SDS, 10 mM DTT). The eluate was passed through Microcon-100 (Millipore) to remove non-specifically bound uncrossed-linked proteins with a molecular mass lower than 100 kDa. Protein complexes retained on the membrane were washed three times with wash buffer (100 mM Tris–HCl, pH 7.5, 1% SDS, 10 mM DTT), and recovered by the addition of 50 μL buffer. Cross-links were dissociated by heating at 65°C overnight, and DNA purified using Qiaquick (QIAGEN).

Terminal labeling of purified DNA fragments and hybridization to the oligonucleotide chip were performed essentially as described previously.[27] Signal intensities of mismatch probes were subtracted from those of perfect match probes. The signal intensities of DNA in the affinity-purified fraction and those of DNA isolated from the whole cell extract fraction before purification (control DNA) were adjusted to confer a signal average of 500. Signal intensities of DNA in the affinity-purified fraction were subtracted by those of control DNA to obtain protein-binding signals. The distribution of protein-binding signals along the genome coordinate was visualized with the *In Silico* Molecular Cloning program, Array Edition (*In Silico* Biology).

2.4. High-resolution transcriptome analysis

NIS2022 cells harboring the IPTG-inducible *dnaA–dnaN* operon at the *purA* locus[6] were pre-cultured overnight at 30°C on a PAB plate containing 5 μg/ml tetracycline and 10 μg/ml neomycin, with or without 10 μM IPTG. Cells were inoculated into PAB liquid medium with or without 0.1 M IPTG to obtain an initial OD_{600} value of 0.01, followed by culture at 30°C.

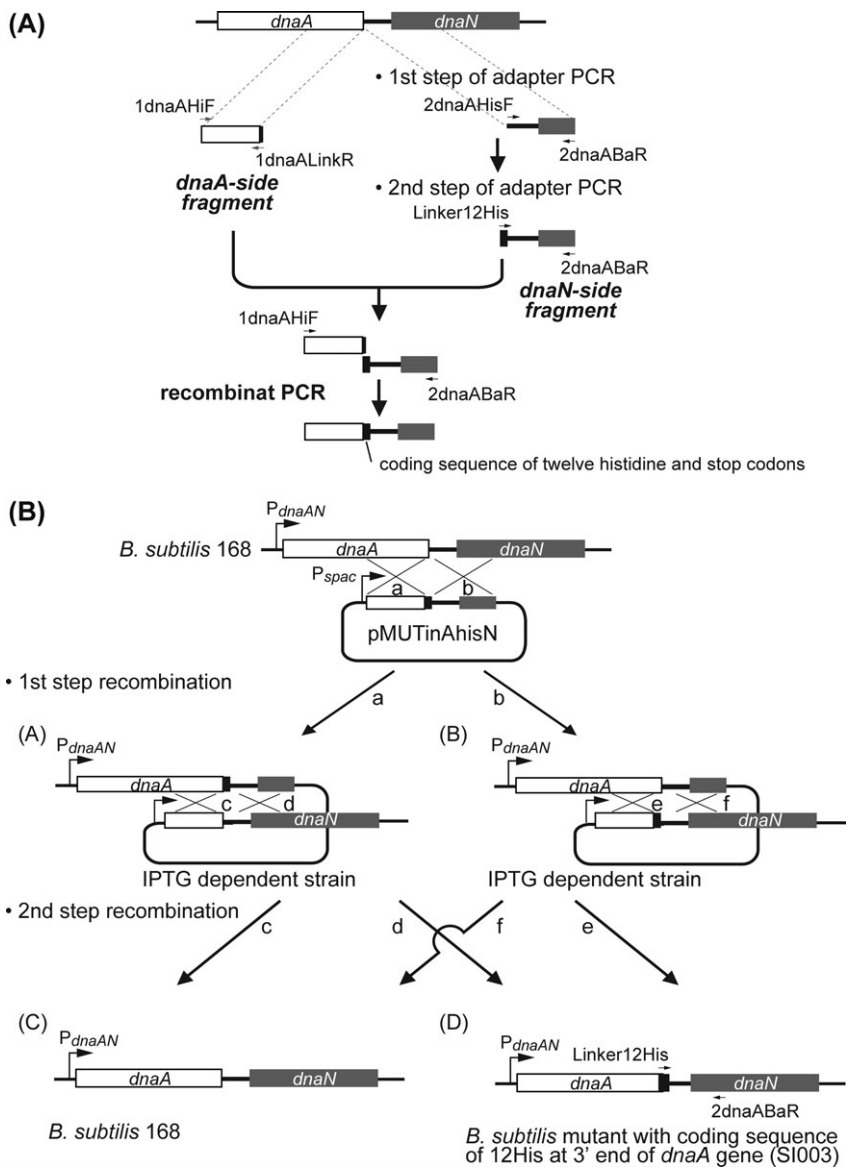

Figure 1. Schematic representation of the generation of strain SI003. (**A**) Adapter PCR and recombinant PCR products used to create the plasmid, pMUTinAhisN. (**B**) Transformation of 168 cells with pMUTinAhisN and removal of the integrated plasmid to generate strain SI003. Integration of the plasmid into the chromosome through recombination at region a or b resulted in cells with structure 1 or 2, respectively. Induction of recombination at region c or d in cells with structure 1 resulted in cells with structure 3 or 4, respectively. On the other hand, recombination at region e or f in cells with structure 2 resulted in cells having structure 4 or 3, respectively. Cells displaying structure 4 were selected for ChAP-chip analysis.

At OD_{600} of 0.4, cells were harvested from 40 mL of culture, and total RNA extracted, as described previously.[36] Expression of DnaA protein in wild-type CRK6000 cells and NIS2022 cells was confirmed by western blotting using a specific antibody, based on an earlier protocol.[6] Synthesis of cDNA, terminal labeling, and hybridization to the oligonucleotide chip were performed using established methods.[27] Amplification of cDNA was avoided to allow the coding strands of each transcript to be distinguished. To compensate for the differences in hybridization efficiency of each 25-mer probe on the chip, we divided the hybridization intensities of cDNA synthesized from total RNA by those of total genome DNA. The distribution of transcriptional signals along the genome coordinate was visualized with the *In Silico* Molecular Cloning program, Array Edition (*In Silico* Biology).

3. Results

3.1. Accurate mapping of known Spo0J-binding sites by ChAP-chip analysis

Previously, we developed a method to purify *in vivo* complexes of cell division proteins using a 12 × histidine-tag under denatured conditions.[30] We anticipated that

the procedure is similarly effective for the purification of *in vivo* DNA–protein complexes. To verify the sensitivity of the tag-based chromatin precipitation protocol, we initially examined the binding sites of Spo0J involved in chromosome partitioning in *B. subtilis*. Spo0J binds at eight specific positions (*parS* sites) on the genome, as observed from the identified binding consensus sequences and chromatin immunoprecipitation analysis.[31] We expressed the Spo0J protein fused to a histidine tag at the C-terminus using SI002 cells. During exponential growth in LB medium under aerobic conditions, Spo0J-12 × His-expressing cells were treated with formaldehyde to cross-link protein and genome DNA, and disrupted by sonication in 8 M urea buffer to generate an average DNA fragment size of ∼500 bp. Protein–DNA complexes were purified with the Ni^{2+}-resin under denatured conditions, and the cross-links removed by heat treatment. DNA fragments co-purified with Spo0J were hybridized with a high-density tiling chip. The hybridization intensities of Spo0J-associated DNA to each oligonucleotide probe on the chip are presented in Fig. 2 (see also detailed map in Supplementary Fig. S2). Our ChAP-chip analysis clearly shows that Spo0J forms a stable complex at eight regions, consistent with previous results.[31] Spo0J-binding signals are more broadly distributed than those of DnaA, as described below (Fig. 1B and Supplementary Fig. S2). This result is consistent with the recent finding that Spo0J is associated with several kilobases of DNA flanking its specific binding sites (*parS*) through a *parS*-dependent nucleation event that promotes lateral spreading along the chromosome.[37] Thus, it appears that our ChAP-chip method is sufficiently sensitive to detect the *in vivo* interaction sites of DNA-binding proteins.

3.2. Identification of high-affinity binding sites of DnaA

To identify the DnaA-binding sites on the *B. subtilis* genome, DnaA was expressed as a fusion protein with a 12 × histidine-tag by the authentic promoter using SI003 cells, and ChAP-chip analysis performed. The hybridization profiles of DnaA-associated DNA clearly disclose eight stable binding sites for DnaA on the *B. subtilis* genome (Fig. 2 and Supplementary Fig. S2). Interestingly, binding sites are mapped only in the intergenic regions (between *rpmH–dnaA*, *dnaA–dnaN*, *yqeG–sda*, *ywlB–ywlC*, *jag–thdF*, *yycS–yydA*, *ywcI–vpr*, and *gcp–ydiF*). Gene organization surrounding the eight binding sites, location of the DnaA boxes, and detailed DnaA-binding signals are depicted in Fig. 3, and the functions of the surrounding genes are summarized in Table 1. The regions upstream and downstream of *dnaA*, *rpmH–dnaA* (*oriC*1), and *dnaA–dnaN* (*oriC*2) comprise *cis*-acting sequences essential for the initiation of chromosome replication,[34] and the upstream sequence contains the *dnaA* promoter that is repressed by the corresponding translated protein.[6] Expression of *sda* is dependent on DnaA binding to the upstream sequence,[22,23] and promoters of *ywlC* and *yydA* were reported to be negatively regulated by DnaA.[23] Multiple DnaA boxes upstream of *thdF* were identified 17 years ago,[38] but with no evidence of actual DnaA binding. Our results additionally suggest a possibility that DnaA regulates the expression of *vpr* and/or *ywcI–secT*. Binding to the *gcp–ydiF* region is uncharacteristic for transcriptional regulation, as it is located at the 3′ end of convergent *gcp* and *ydiF* genes.

DnaA protein binds to single DnaA box *in vitro*.[39,40] However, the DnaA-binding regions identified here contain multiple DnaA boxes. These results suggest that the ChAP-chip analysis can be effectively used to detect stable DnaA–DNA complexes, but not transient or weak binding to dispersed DnaA boxes.

3.3. Effect of DnaA overproduction and depletion on transcription of genes surrounding the stable binding sites

To establish the function of DnaA binding to sequences other than *oriC*, we examined transcriptional profiles in DnaA-overproducing and -depleted cells using the high-density tiling chip employed for ChAP-chip analysis. To this end, we employed NIS2022 cells with an IPTG-inducible *dnaA–dnaN* operon at the *purA* locus, and inactivated the authentic *dnaA* gene by introducing orcher mutations.[6] Expression of the DnaA protein in NIS2022 cells is reduced to One-fifth that in non-induced conditions (0 μM IPTG), and increases fivefold upon induction with 100 μM IPTG, compared with the DnaA concentration in wild-type cells.[6] Notably, under both non-induced and induced conditions, no apparent growth defects were observed, and the overall profiles of gene expression remained unchanged (Supplementary Fig. S3). Transcriptional initiation from the authentic promoter of *dnaA* is clearly repressed upon induction of DnaA expression, and stimulated slightly under non-induced conditions (Fig. 4A). This finding is in agreement with a previous study reporting autoregulation of *dnaA*.[6] A similar change in transcription was observed for the intergenic region between *dnaA* and *dnaN*. The dependence of *sda* expression on DnaA is well characterized.[22,23] We observed *sda* expression and repression in DnaA-induced and non-induced conditions, respectively (Fig. 4B). Interestingly, we identified another transcript covering the *sda* gene, but on the non-coding strand. The newly identified transcript was weakly, but significantly repressed by overexpressed DnaA at the DnaA-binding site. We additionally observed weak, but significant repression of *ywlC*, *yydA*, and *thdF-gidA-gidB-noc* transcription in DnaA-induced cells (Fig. 4C–E), although their de-repression in non-induced cells was not apparent. A sufficient amount of DnaA is present in cells to support growth at a normal rate, even under non-induced

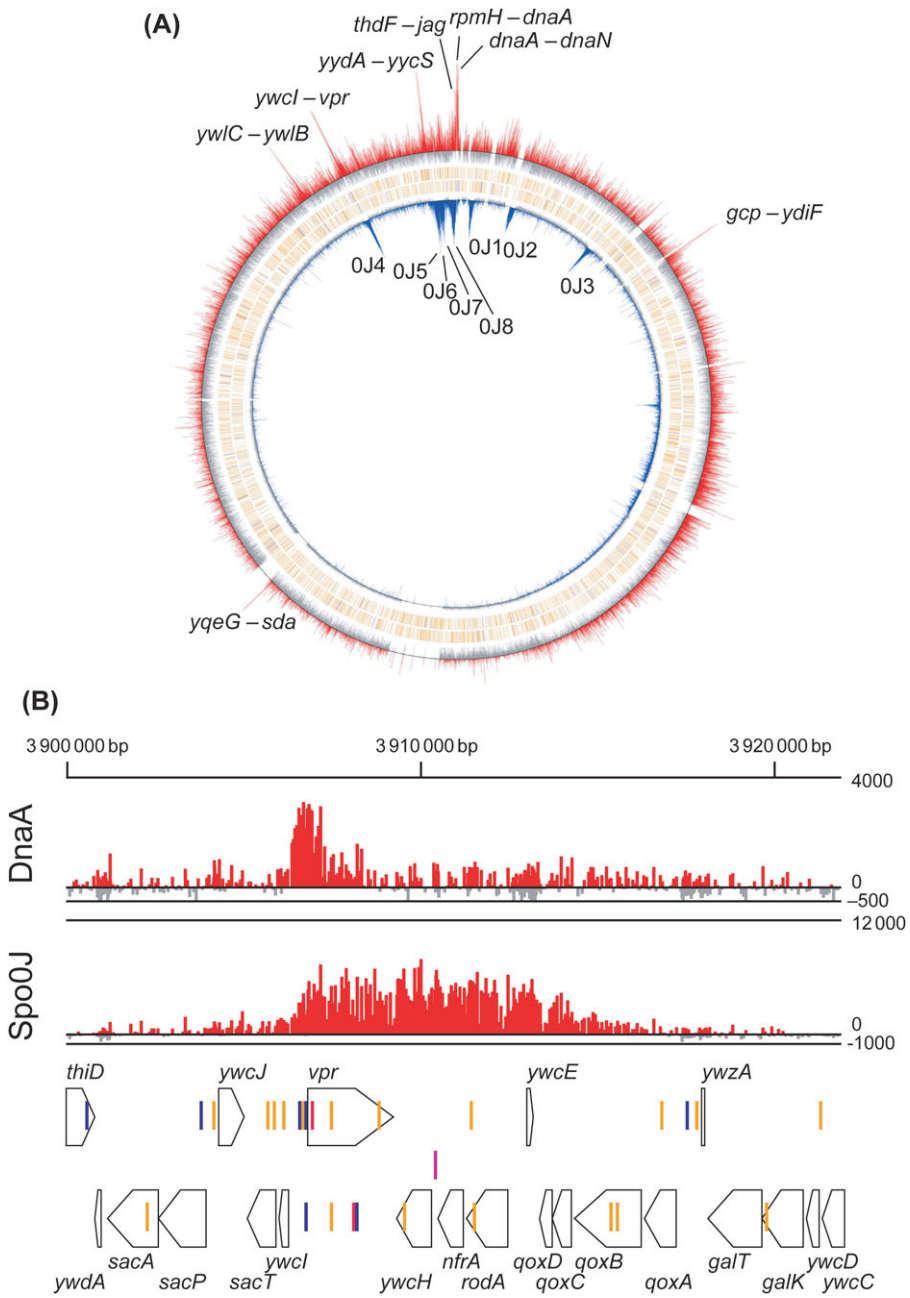

Figure 2. DNA-binding profiles of Spo0J and DnaA proteins on the *B. subtilis* genome revealed using ChAP-chip analysis. (**A**) Spo0J (inner blue bars) and DnaA-binding (outer red bars) signals for each 25-mer probe on the chip were calculated by subtracting signal intensities of control DNA from those of DNA in the affinity-purified fraction, and shown at their corresponding genome coordinates. 0J1–0J8 correspond to the sites previously determined by Lin and Grossman.[31] DnaA boxes are specified as colored vertical lines under DnaA-binding signals: TTATCCACA: red, TTATACACA, TTATAGACA, and TTATATACA: blue, other DnaA boxes having one base mismatch: orange. (**B**) Detailed DnaA and Spo0J binding profiles around the *ywcI–vpr* region, where binding of both proteins was observed, are shown for comparison of binding profiles of two proteins. The arrangement of genes (thick arrows), DnaA-boxes (as in A), and Spo0J binding site (*parS* site, purple vertical line) are shown schematically at the bottom.

conditions, thus those genes may still be repressed by DnaA. The suppression of *ywlC* and *yydA* expression by DnaA is in accordance with reported microarray data.[23] We have demonstrated DnaA-regulated expression of *thdF-gidA-gidB-noc* by northern blot analysis using NIS2022 cells (Ogura *et al.*, unpublished result). These transcriptional alterations were reproducible in two independent experiments (Supplementary Fig. S3). Additionally, the DnaA level affected *rocA*, *rocD*, and *yvdCD* expression positively, and *ykuNOP* and *dhbA* expression negatively (Supplementary Fig. S3 and Supplementary Table S2). The molecular mechanisms and biological significance of these changes are currently unclear. On the other hand, the effects of DnaA on the

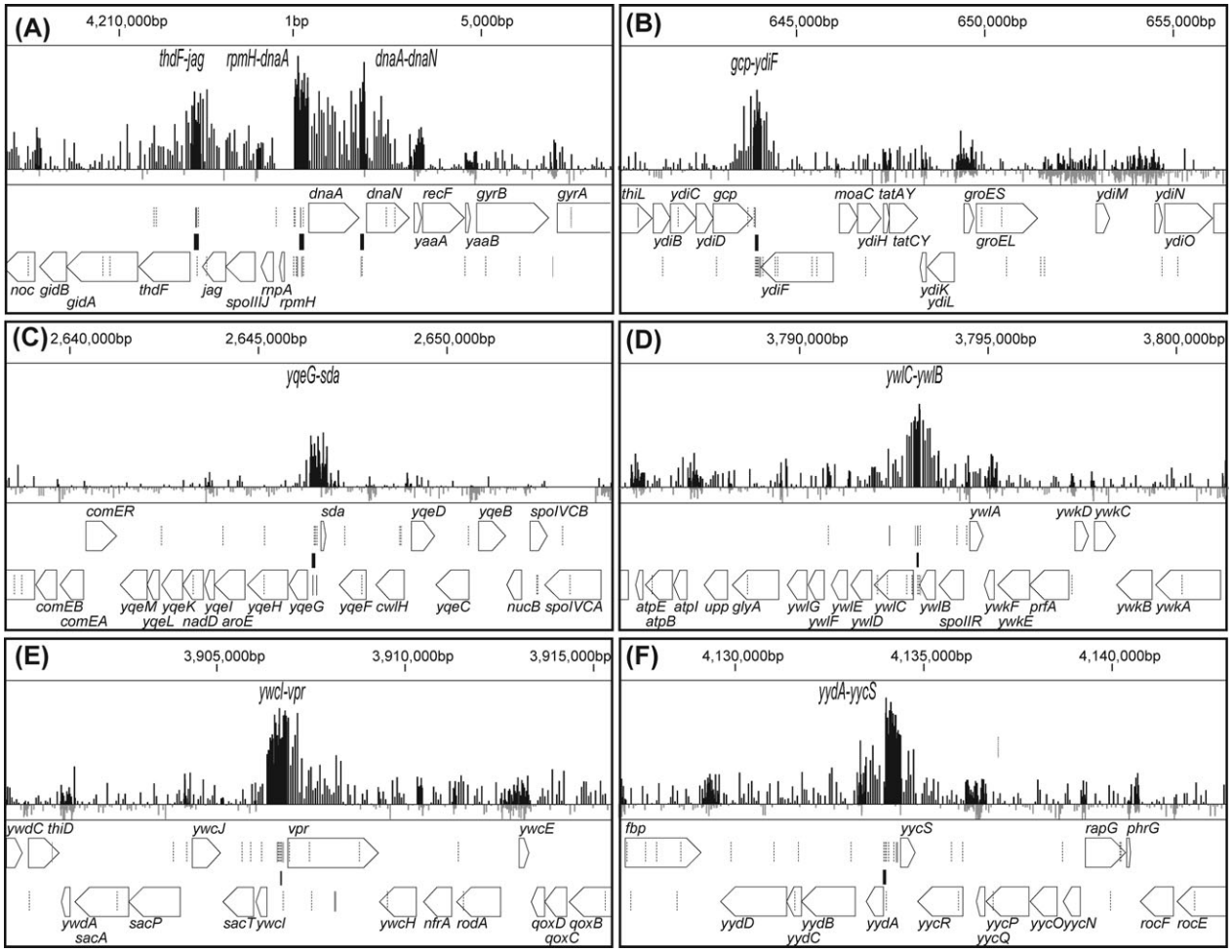

Figure 3. Local DNA-binding profiles of DnaA. Local DNA binding profiles of DnaA are presented, together with genes and DnaA boxes. DnaA boxes are specified as vertical lines under DnaA-binding signals: TTATCCACA: bold lines, TTATACACA, TTATAGACA and TTATATACA: gray lines, other DnaA boxes having one base mismatch: dotted lines. The top, middle, and bottom lines of ChAP-chip signals indicate signal intensities of 4000, 0, and −500, respectively. Closed boxes represent regions having high-affinity successive DnaA box clusters, as described in Table 2.

expression of genes around *ywcI–vpr* and *gcp–ydiF* could not be distinguished due to weak expression.

3.4. Sequence characteristics of stable DnaA-binding sites on the chromosome

Next, we explored the characteristics defining the stable DnaA-binding sites in the *B. subtilis* genome. *Bacillus subtilis* DnaA recognizes DnaA boxes in *E. coli oriC*, and vice versa, *in vitro*. Although the numbers and arrangement of DnaA boxes in *oriC* are different in *B. subtilis* and *E. coli*, the *oriC* regions were incompatible with each other *in vivo*.[39,40] Recently, the crystal structure of the DNA-binding domain of *E. coli* DnaA complexed with a DnaA box (TTATCCACA) was resolved.[41] In the complex, amino acids R399, P423, D433, H434, T435, and H439 form base-specific interactions, and are essential for sequence recognition by DnaA. Interestingly, these residues are conserved in *B. subtilis* DnaA, indicating that DNA-binding specificities are similar for *B. subtilis* and *E. coli* DnaA. Thus, we assume that the consensus sequence of the *B. subtilis* DnaA box is TTATNCACA, similar to the *E. coli* protein, and allow one base mismatch for identifying DnaA boxes. As shown in Fig. 3, stable DnaA-binding sites contain multiple boxes, as observed in *oriC* sequences of eubacteria.[42] Initially, we evaluated the density of DnaA boxes along the genome, using a modified method of0 Mackiewicz *et al.*[42] The density of DnaA box distribution was presented on charts as $b = 1/d$, calculated for each box, where d is the average distance between adjacent DnaA boxes. Mackiewicz and colleagues calculated the b value for three successive DnaA boxes (average of two distances), whereas we additionally calculated for four and five successive boxes, with no clear correlation with the identified binding sites. Recently, the DnaA-binding affinity to DnaA boxes with different fifth nucleotides was evaluated in *E. coli* cells and the affinity was found to decrease in the order: C > A = G > T.[43] Accordingly, we

Table 1. Genes in transcriptional units adjacent to the DnaA-binding sites

Direction[a]	Gene	Essential	Function
	rnpA	yes	Protein subunits of RNase P that cleave tRNA precursors to create the mature tRNA 5′ ends
	rpmH	yes	50S ribosomal protein L34
	dnaA	yes	Chromosomal replication initiator protein
	dnaN	yes	DNA polymerase III beta subunit
	thiL	no	Thiamine-monophosphate kinase
	ydiB	no	The *E. coli* homolog, YjeE, is essential and proposed to be involved in cell wall synthesis
	ydiC	yes	Similar to N-terminal part of Gcp
	ydiD	no	Function-unknown protein having acetyltransferase (GNAT) family domain (Pfam PF00583)
	gcp	yes	Homologous to *S. aureus* Gcp known as essential protein involved in cell wall biosynthesis
	ydiF	no	Similar to *E. coli* Uup, whose mutation induces an increase in precise transposon excision, and translation elongation factor 3 of eukaryote
	yqeM	no	Similar to *E. coli* UbiE and *B. subtilis* MenH
	yqeL	no	Conserved protein with a domain of unknown function, DUF143 (Pfam PF02410)
	yqeK	no	Function-unknown protein with HD domain of metal-dependent phosphatases. The deletion mutant shows an abnormal morphology and biofilm
	nadD	yes	Nicotinate-nucleotide adenylyltransferase
	yqeI	yes	*Escherichia coli* homolog, YhbY, is associated with pre-50S ribosomal subunits.
	aroD	no	Shikimate 5-dehydrogenase involved in shikimate pathway
	yqeH	yes	Essential GTPase required for proper ribosome assembly
	yqeG	no	Function-unknown protein with an acid phosphatase motif B (Pfam PF03767)
	sda	no	Developmental checkpoint protein coupling sporulation and DNA replication
	ywlD	no	Function-unknown protein with an amino acid translocator motif (PF01810)
	ywlC	yes	*Escherichia coli* homolog, YrdC, binds to dsRNA
	ywlB	no	Function-unknown protein with an acetyltransferase domain (Pfam PF00583)
	spoIIR	no	Sporulation protein transcribed by sporulation specific sigma factor, sF
	sacT	no	Transcriptional antiterminator involved in positive regulation of *sacA* and *sacP*
	ywcI	no	Function-unknown and unique protein with one transmembrane domain
	vpr	no	Extracellular serine protease expressed transiently at T_0 in a *phoR*-dependent manner
	yydA	no	Conserved protein with a domain of unknown function (Pfam PF02590)
	yycS	no	Function-unknown and unique protein with one transmembrane domain
	noc	no	Specific effector of nucleoid occlusion, which acts to prevent cell division in the vicinity of the nucleoid
	gidB	no	7-Methylguanosine (m7G) methyltransferase involved in modification of G527 of 16S rRNA
	gidA	no	*Escherichia coli* homolog involved in tRNA modification. The disruptant shows slow growth
	thdF	no	*Escherichia coli* homolog, MnmE, involved in tRNA modification and essential for viability in some genetic backgrounds
	jag	no	Function-unknown protein with RNA and single-stranded nucleic acid binding domains (Pfam PF00013 and PF01424)
	spoIIIJ	no	Homolog of *E. coli* membrane insertase, YidC. Simultaneous inactivation of *spoIIIJ* and its paralogue, *yqjG*, shows lethal phenotype

[a]The location of DnaA-binding site is indicated by circle and direction of the adjacent transcriptional units is indicated by arrow.

assigned the binding affinities as 4, 2, 1.5, 1, and 1 for TTATCCACA, TTATACACA, TTATAGACA, TTATATACA, and other DnaA boxes (having one base mismatch from TTATNCACA), and the binding affinity of DnaA for four successive DnaA boxes was expressed as $A =$ (sum of affinities of four successive DnaA boxes)$/d$. As the result, we found that the A value is well correlated with the DnaA-binding sites; eight binding sites identified here exclusively have A value >0.3 (Fig. 5A). Sequence characteristics of the eight DnaA-binding regions, including number of DnaA boxes and the A value, are summarized in Table 2. Our results suggest that two factors, specifically, closely clustered DnaA boxes of probably four or more, and the affinity of DnaA boxes for the protein, are essential for stable DnaA-binding in *B. subtilis* cells.

3.5. Distribution of probable DnaA-binding sites in other Bacillus species

We estimated the stable DnaA-binding sites in the genome sequences of other *Bacillus* species, using the defined A value. Interestingly, regions with high A values locate in a similar arrangement on the genome in all the bacteria examined (Fig. 5B–I). However, close examination of possible DnaA-binding regions in various *Bacillus* species revealed their conserved and diverse characteristics (Table 3). As expected, multiple DnaA-binding sequences upstream and downstream of *dnaA* (*oriC*) are essentially conserved in all bacteria. Notably, in *B. licheniformis*, the *dnaA*–*dnaN* region contains only three DnaA boxes, and its A value is below the threshold. DnaA-binding sequences upstream of *sda*, *ywlC*, and *yydA* are also present in most bacteria,

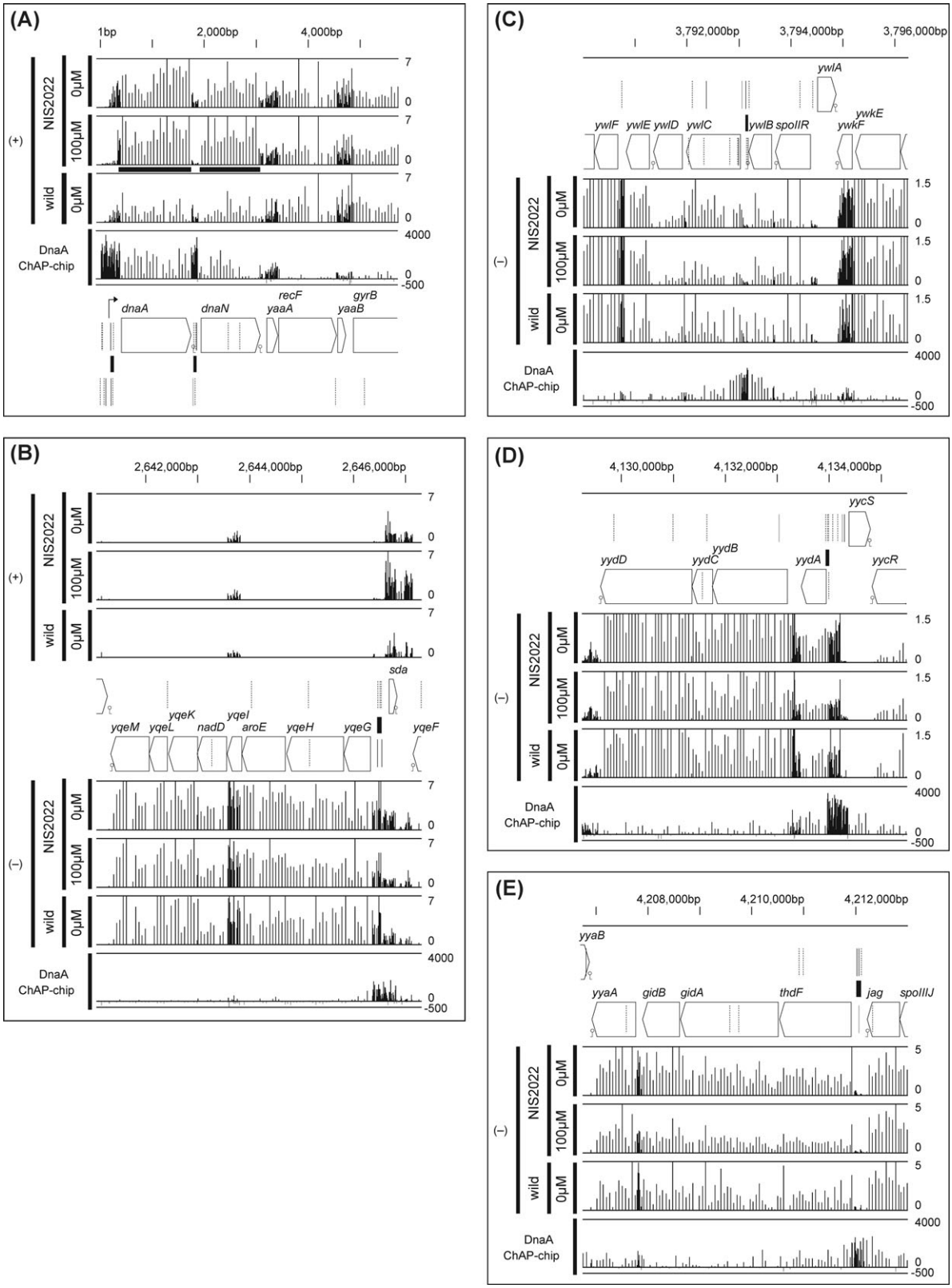

Figure 4. Transcriptional changes around the DnaA-binding regions in *dnaA*-induced and repressed cells. Transcriptional activity of NIS2022 cells in the absence and presence of 100 μM IPTG and wild-type cells were analyzed using tiling chip. Transcriptional signals for each probe were calculated by dividing hybridization intensities of cDNA synthesized from total RNA by those of total genome DNA, and shown by vertical blue bars at their corresponding genome coordinates, for regions surrounding the DnaA-binding sites. Signal intensities of probes on Watson (+) and Crick (−) strands were shown separately to indicate the direction of transcripts. The DnaA-binding signals (DnaA ChAP-chip) and location of genes and DnaA boxes are also shown, as in Fig. 2B. The transcriptional start sites, *dnaA* and *sda*, determined previously, are highlighted in the genetic maps with arrows. Possible termination signals of transcription are additionally specified. Signals marked by a bold underline in panel A are due to the expression of *dnaA–dnaN* placed at the *purA* locus in NIS2022 cells.

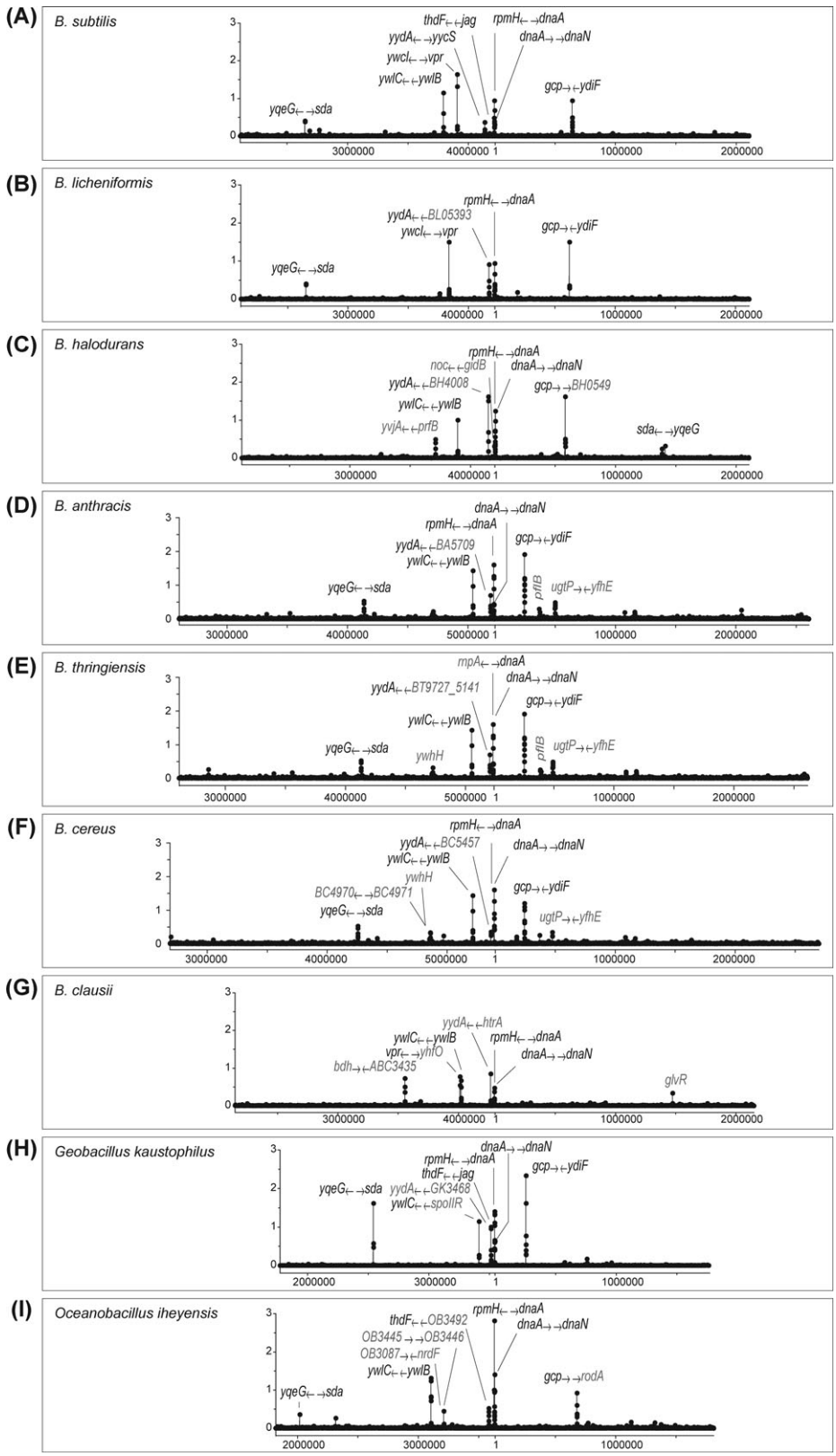

Figure 5. Estimation of the distribution of the possible DnaA-binding regions in genome sequences of the *Bacillus* species. The A value [(sum of affinities of four successive DnaA boxes)/d (average distance of adjacent DnaA boxes)] was calculated for all DnaA boxes, and plotted (closed circles joined by line) at corresponding genome coordinates in *B. subtilis* (**A**), *B. licheniformis* (**B**), *B. halodurans* (**C**), *B. anthracis* (**D**), *B. thuringiensis* (**E**), *B. cereus* (**F**), *B. clausii* (**G**), *Geobacillus kaustophilus* (H), and *Oceanobacillus iheyensis* (**I**). Genome sequences, except that of *B. subtilis*, were retrieved from the NCBI GeneBank database. The genes adjacent to the possible DnaA-binding regions are specified, and " → " indicates their orientations (5′ to 3′ direction) relative to DnaA-binding site located between them. Genes or possible DnaA-binding regions that are not conserved in *B. subtilis* are colored gray.

Table 2. Sequence characteristics of the DnaA-binding regions identified by ChAP-chip analysis

Region[a]			d[b]	Sum of affinity	A[c]	Number of *DnaA* boxes[d]	Number of perfect matches[e]
Surrounding genes	Start	End					
rpmH–dnaA	193	257	10.7	10	0.94	8	2 (2)
dnaA–dnaN	1797	1860	10.3	7	0.68	5	1 (1)
gcp–ydiF	643 951	643 999	5.3	5	0.94	8	4 (3)
yqeG–sda	2 646 452	2 646 536	17.3	7	0.40	5	2 (2)
ywlC–ywlB	3 793 128	3 793 181	7.0	8	1.14	5	2 (1)
ywcI–vpr	3 906 700	3 906 743	3.7	6	1.64	5	3 (0)
yydA–yycS	4 133 932	4 133 997	11.0	4	0.36	4	0 (0)
thdF–jag	4 212 013	4 212 108	21.0	10	0.48	4	3 (2)

[a]Regions with A value >0.3 are shown. When DnaA box clusters overlap, those with the highest A value are indicated.
[b]Average distance between four adjacent DnaA boxes.
[c]A = (sum of affinities of four successive DnaA boxes)/d. Only the highest value in each DnaA box cluster is shown. See text for detail.
[d]Number of DnaA boxes allowing one base mismatch of the consensus DnaA box, TTATNCACA, in successive DnaA box clusters with an A value >0.3.
[e]The number of perfectly matched sequences to the consensus DnaA box, TTATNCACA. The number of DnaA boxes with highest affinity (TTATCCACA) is indicated in parentheses.

supporting the biological significance of DnaA-mediated regulation of these genes. Interestingly, a DnaA box cluster downstream of *gcp* is highly conserved, although its biological role is not clear at present. On the other hand, DnaA box cluster upstream of the *thdF-gidA-gidB-noc* operon is not conserved, although those genes are conserved in all bacteria examined (Table 3), thus the DnaA-dependent expression of the operon appears not to be general. Similarly, DnaA box clusters upstream of *vpr* are present in limited bacteria. In addition, other possible DnaA-binding regions (colored red in Fig. 5B–I) that are absent in *B. subtilis* have been identified. Interestingly, DnaA-binding regions with a high A value are generally located near *oriC* in all the bacteria examined.

4. Discussion

In this study, we developed a ChAP-chip method, based on our original protocol for the purification of protein complexes.[30] The binding sites of Spo0J determined using this technique were consistent with those reported earlier,[31,37] clearly indicating that the ChAP-chip methods are sufficiently sensitive for the genome-wide determination of specific binding sites of DNA-binding proteins. Using this method, we have successfully visualized the distribution of RNA polymerase core enzyme and associated factors, SigA, NusA, and GreA (unpublished results). Thus, the ChAP-chip assay should be effective for proteins that are both stably and dynamically associated with genome DNA.

Table 3. Conservation of DnaA-binding sequences identified in *B. subtilis* and adjacent genes in genome sequences of *Bacillus* spices

Genus	Species	*rpmH*←	→*dnaA*→	→*dnaN*	*yydA*←	→*yycS*	*gcp*→	←*ydiF*	*thdF*←	←*jag*	*ywlC*←	←*ywlB*	*yqeG*←	→*sda*	*ywcI*←	→*vpr*
Bacillus	*B. anthracis*	+	+	+	+	+	+	+	(+)	(+)	+	+	+	+		△
	B. cereus	+	+	+	+		+	+	△	△	+	+	+	+		△
	B. thuringiensis		+	+	+	+	+	+	△	△	+	+	+	+		△
	B. clausii	+	+	+	+		△	△	△	△	+	+	△	△		+
	B. halodurans	+	+	+	+		+	+	△	△	+	+	+	+		△
	B. licheniformis	+	+	△	+		+	△	(+)	(+)	(+)	(+)	+	+	+	+
	B. subtilis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Geobacillus	*G. kaustophilus*	+	+	+	+		+	+	+	+	+		+	+		
Oceanobacillus	*O. iheyensis*	+	+	+	△	△	+	(+)	+	+	+		△			△

Symbols indicate conservation of genes. When DnaA box clusters with $A \geq 0.3$ and $A < 0.3$ exist in the similar arrangement with that in *B. subtilis*, cells are indicated as "+" and "(+)", respectively. When DnaA box clusters do not exit, cells are indicated as "(Δ)". "→" indicates orientation of genes (5′ to 3′) relative to DnaA-binding site located between them.

The initiator protein of chromosomal replication, DnaA, forms stable complexes at eight intergenic regions on the *B. subtilis* genome, including the *oriC* sequences, *oriC*1 and *oriC*2. *In vitro* assays demonstrate that DnaA protein binds to a single DnaA box.[39,40] However, characterization of the DNA sequences bound to DnaA *in vivo* reveals that the presence of densely clustered DnaA boxes and affinity of these boxes for DnaA protein are critical factors for stable DNA–protein interactions *in vivo*. The recent crystal structure of *Aquifex aeolicus* DnaA bound to the non-hydrolyzable ATP analog, AMP-PCP, disclosed that ATP-DnaA forms a right-handed filament defined by specific protein–ATP interactions.[3,32] This finding strongly suggests that through filament formation of multiple ATP-DnaA bound to *oriC*, a right-handed *oriC* DNA wrap is formed around the initiation nucleoprotein complex. It is plausible that this type of higher-order structure of the DnaA–DNA complex is formed at the stable DnaA-binding sites detected by our group.

Notably, genes related to basic cellular functions are over-represented in neighboring genes. These include chromosome replication (*dnaA* and *dnaN*), translation (*yqeI, yqeH*: ribosome assembly; *gidA, thdF*: modification of tRNA; *gidB*: modification of 16S rRNA), and unknown essential functions (*gcp, ywlC*). In combination with transcriptome analysis of cells containing increased or decreased amounts of DnaA, we demonstrated that in addition to autoregulation, DnaA proteins stably associated with DNA directly regulate *sda* expression in a positive and *ywlC* and *yydA* expression in a negative manner. Furthermore, DnaA-dependent regulation of these genes was suggested to be maintained in other *Bacillus* species, indicating their biological significance. The DnaA-dependent control of *dnaA* and *sda* is well documented. In contrast, the functions of *ywlC* and *yydA* and the rationale for their DnaA-dependent regulation await further investigation. We additionally show that although *thdF-gidA-gidB-noc* expression is under control of DnaA in *B. subtilis*, this regulation is not universal in the *Bacillus* species.

DnaA generally acts as a repressor of transcription in *B. subtilis*, probably through blocking the formation of initiation complex of transcription and/or a transcriptional roadblock. However, the location of DnaA boxes and transcriptional organization of the *yqeG–sda* region suggests an alternative mechanism of transcriptional regulation by DnaA. Interestingly, *sda* transcription is positively regulated by DnaA. It is possible that DnaA interacts with RNA polymerase as a transcriptional activator, although experimental evidence of these interactions is yet to be obtained. We identified a novel transcript negatively regulated by DnaA, which traverses the *sda* gene in the opposite orientation. RNA polymerase collisions would occur if both transcriptions were active simultaneously. If these collisions negatively regulate *sda* transcription, negative regulation of the opposite reaction would have a positive effect on *sda* expression. Additionally, upregulation of the *sda* promoter may be mediated through changes in superhelicity of the DNA template. If DnaA forms helical filaments and bound DNA is wrapped around the DnaA helix, as discussed earlier, positive superhelicity will be stabilized, as demonstrated by Erzberger *et al.*[32] Superhelicity of template DNA is an important factor in determining transcriptional activity.[44,45]

Estimation of stable DnaA-binding sequences using the *A* value in genome sequences of *Bacillus* species suggests that in addition to the upstream regions of *dnaA, dnaN, sda*, *yydA*, and *ywlC*, DnaA binds downstream of *gcp* in most bacteria examined. In addition, specific DnaA-binding sequences for each bacterium may be present in close proximity to *oriC*. These observations suggest that DnaA plays several additional roles, such as control of the amount of effective initiator, ATP-DnaA, as proposed for *E. coli*,[46] and/or stabilization of domain structure of genome around *oriC* for the proper initiation of chromosome replication.

However, although the importance of orientation of adjacent DnaA boxes for stable binding of DnaA has been investigated in *E. coli*,[47] we did not take into account this factor to evaluate the DnaA-binding regions. Further improvements of bioinformatic methods to estimate the DnaA-binding regions are necessary.

In conclusion, we have analyzed the distribution of the DnaA protein on the *B. subtilis* genome in actively growing cells, and confirmed its role as a direct regulator of several genes. Moreover, we propose that DnaA has additional functions related to chromosome replication. Thus, genome-wide analysis of DnaA-binding sites should facilitate our understanding of the role of *B subtilis* DnaA as a coordinator of bacterial cell growth.

Supplementry Data: Supplementry data are available online at www.dnaresearch.oxfordjournals.org.

Funding: This work was supported by KAKENHI (Grant-in-Aid for Scientific Research) on Priority Areas Systems Genomics from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Leonard, A. C. and Grimwade, J. E. 2005, Building a bacterial orisome: emergence of new regulatory features for replication origin unwinding, *Mol. Microbiol.*, **55**, 978–985.
2. Kaguni, J. M. 2006, DnaA: controlling the initiation of bacterial DNA replication and more, *Annu. Rev. Microbiol.*, **60**, 351–375.
3. Mott, M. L. and Berger, J. M. 2007, DNA replication initiation: mechanisms and regulation in bacteria, *Nat. Rev. Microbiol.*, **5**, 343–354.

4. Zakrzewska-Czerwinska, J., Jakimowicz, D., Zawilak-Pawlik, A. and Messer, W. 2007, Regulation of the initiation of chromosomal replication in bacteria, *FEMS Microbiol. Rev.*, **31**, 378–387.
5. Braun, R. E., O'Day, K. and Wright, A. 1985, Autoregulation of the DNA replication gene *dnaA* in *E. coli* K-12, *Cell*, **40**, 159–169.
6. Ogura, Y., Imai, Y., Ogasawara, N. and Moriya, S. 2001, Autoregulation of the *dnaA-dnaN* operon and effects of DnaA protein levels on replication initiation in *Bacillus subtilis*, *J. Bacteriol.*, **183**, 3833–3841.
7. Jakimowicz, D., Majka, J., Lis, B., Konopa, G., Wegrzyn, G., Messer, W., Schrempf, H. and Zakrzewska-Czerwinska, J. 2000, Structure and regulation of the *dnaA* promoter region in three *Streptomyces* species, *Mol. Gen. Genet.*, **262**, 1093–1102.
8. Salazar, L., Guerrero, E., Casart, Y., Turcios, L. and Bartoli, F. 2003, Transcription analysis of the *dnaA* gene and *oriC* region of the chromosome of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG, and its regulation by the DnaA protein, *Microbiology*, **149**, 773–784.
9. Greendyke, R., Rajagopalan, M., Parish, T. and Madiraju, M. V. 2002, Conditional expression of *Mycobacterium smegmatis dnaA*, an essential DNA replication gene, *Microbiology*, **148**, 3887–3900.
10. Messer, W. and Weigel, C. 1997, DnaA initiator—also a transcription factor, *Mol. Microbiol.*, **24**, 1–6.
11. Nozaki, N., Okazaki, T. and Ogawa, T. 1988, In vitro transcription of the origin region of replication of the *Escherichia coli* chromosome, *J. Biol. Chem.*, **263**, 14176–14183.
12. Wang, Q. P. and Kaguni, J. M. 1989, *dnaA* protein regulates transcriptions of the *rpoH* gene of *Escherichia coli*, *J. Biol. Chem.*, **264**, 7338–7344.
13. Quinones, A., Wandt, G., Kleinstauber, S. and Messer, W. 1997, DnaA protein stimulates *polA* gene expression in *Escherichia coli*, *Mol. Microbiol.*, **23**, 1193–1202.
14. Theisen, P. W., Grimwade, J. E., Leonard, A. C., Bogan, J. A. and Helmstetter, C. E. 1993, Correlation of gene transcription with the time of initiation of chromosome replication in *Escherichia coli*, *Mol. Microbiol.*, **10**, 575–584.
15. Ogawa, T. and Okazaki, T. 1994, Cell cycle-dependent transcription from the *gid* and *mioC* promoters of *Escherichia coli*, *J. Bacteriol.*, **176**, 1609–1615.
16. Gon, S., Camara, J. E., Klungsoyr, H. K., Crooke, E., Skarstad, K. and Beckwith, J. 2006, A novel regulatory mechanism couples deoxyribonucleotide synthesis and DNA replication in *Escherichia coli*, *EMBO J.*, **25**, 1137–1147.
17. Augustin, L. B., Jacobson, B. A. and Fuchs, J. A. 1994, *Escherichia coli* Fis and DnaA proteins bind specifically to the *nrd* promoter region and affect expression of an *nrd-lac* fusion, *J. Bacteriol.*, **176**, 378–387.
18. Herrick, J. and Sclavi, B. 2007, Ribonucleotide reductase and the regulation of DNA replication: an old story and an ancient heritage, *Mol. Microbiol.*, **63**, 22–34.
19. Ogawa, T., Yamada, Y., Kuroda, T., Kishi, T. and Moriya, S. 2002, The *datA* locus predominantly contributes to the initiator titration mechanism in the control of replication initiation in *Escherichia coli*, *Mol. Microbiol.*, **44**, 1367–1375.
20. Morigen, , Molina, F. and Skarstad, K. 2005, Deletion of the *datA* site does not affect once-per-cell-cycle timing but induces rifampin-resistant replication, *Bacteriol.*, **187**, 3913–3920.
21. Smulczyk-Krawczyszyn, A., Jakimowicz, D., Ruban-Osmialowska, B., Zawilak-Pawlik, A., Majka, J., Chater, K. and Zakrzewska-Czerwinska, J. 2006, Cluster of DnaA boxes involved in regulation of *Streptomyces* chromosome replication: from in silico to in vivo studies, *J. Bacteriol.*, **188**, 6184–6194.
22. Burkholder, W. F., Kurtser, I. and Grossman, A. D. 2001, Replication initiation proteins regulate a developmental checkpoint in *Bacillus subtilis*, *Cell*, **104**, 269–279.
23. Goranov, A. I., Katz, L., Breier, A. M., Burge, C. B. and Grossman, A. D. 2005, A transcriptional response to replication status mediated by the conserved bacterial replication protein DnaA, *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 12932–12937.
24. Hottes, A. K., Shapiro, L. and McAdams, H. H. 2005, DnaA coordinates replication initiation and cell cycle transcription in *Caulobacter crescentus*, *Mol. Microbiol.*, **58**, 1340–1353.
25. Collier, J., Murray, S. R. and Shapiro, L. 2006, DnaA couples DNA replication and the expression of two cell cycle master regulators, *EMBO J.*, **25**, 346–356.
26. Katou, Y., Kaneshiro, K., Aburatani, H. and Shirahige, K. 2006, Genomic approach for the understanding of dynamic aspect of chromosome behavior, *Methods Enzymol.*, **409**, 389–410.
27. Oshima, T., Ishikawa, S., Kurokawa, K., Aiba, H. and Ogasawara, N. 2006, *Escherichia coli* histone-like protein H-NS preferentially binds to horizontally acquired DNA in association with RNA polymerase, *DNA Res.*, **13**, 141–153.
28. Navarre, W. W., Porwollik, S., Wang, Y., McClelland, M., Rosen, H., Libby, S. J. and Fang, F. C. 2006, Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*, *Science*, **313**, 236–238.
29. Lucchini, S., Rowley, G., Goldberg, M. D., Hurd, D., Harrison, M. and Hinton, J.C. 2006, H-NS mediates the silencing of laterally acquired genes in bacteria, *PLoS Pathogens.*, **2**, 746–752.
30. Ishikawa, S., Kawai, Y., Hiramatsu, K., Kuwano, M. and Ogasawara, N. 2006, A new FtsZ-interacting protein, YlmF, complements the activity of FtsA during progression of cell division in *Bacillus subtilis*, *Mol. Microbiol.*, **60**, 1364–1380.
31. Lin, D. C. and Grossman, A. D. 1998, Identification and characterization of a bacterial chromosome partitioning site, *Cell*, **92**, 675–685.
32. Erzberger, J. P., Mott, M. L. and Berger, J. M. 2006, Structural basis for ATP-dependent DnaA assembly and replication-origin remodeling, *Nat. Struct. Mol. Biol.*, **13**, 676–683.
33. Kunst, F., Ogasawara, N., Moszer, I., *et al.* 1997, The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*, *Nature*, **390**, 249–256.
34. Moriya, S., Imai, Y., Hassan, A. K. and Ogasawara, N. 1999, Regulation of initiation of *Bacillus subtilis* chromosome replication, *Plasmid*, **41**, 17–29.
35. Morimoto, T., Loh, P. C., Hirai, T., Asai, K., Kobayashi, K., Moriya, S. and Ogasawara, N. 2002, Six GTP-binding proteins of the Era/Obg family are essential for cell growth in *Bacillus subtilis*, *Microbiology*, **148**, 3539–3552.

36. Ishikawa, S., Yamane, K. and Sekiguchi, J. 1998, Regulation and characterization of a newly deduced cell wall hydrolase gene (*cwlJ*) which affects germination of *Bacillus subtilis* spores, *J. Bacteriol.*, **180**, 1375–1380.
37. Murray, H., Ferreira, H. and Errington, J. 2006, The bacterial chromosome segregation protein Spo0J spreads along DNA from *parS* nucleation sites, *Mol. Microbiol.*, **61**, 1352–1361.
38. Yoshikawa, H. and Ogasawara, N. 1991, Structure and function of DnaA and the DnaA-box in eubacteria: evolutionary relationships of bacterial replication origins, *Mol. Microbiol.*, **5**, 2589–2597.
39. Fukuoka, T., Moriya, S., Yoshikawa, H. and Ogasawara, N. 1990, Purification and characterization of an initiation protein for chromosomal replication, DnaA, in *Bacillus subtilis*, *J. Biochem.*, **107**, 732–739.
40. Messer, W. 2002, The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode to initiate DNA replication, *FEMS Microbiol. Rev.*, **26**, 355–374.
41. Fujikawa, N., Kurumizaka, H., Nureki, O., Terada, T., Shirouzu, M., Katayama, T. and Yokoyama, S. 2003, Structural basis of replication origin recognition by the DnaA protein, *Nucleic Acids Res.*, **31**, 2077–2086.
42. Mackiewicz, P., Zakrzewska-Czerwinska, J., Zawilak, A., Dudek, M. R. and Cebrat, S. 2004, Where does bacterial replication start? Rules for predicting the *oriC* region, *Nucleic Acids Res.*, **32**, 3781–3791.
43. Hansen, F. G., Christensen, B. B., Nielsen, C. B. and Atlung, T. 2006, Insights into the quality of DnaA boxes and their cooperativity, *J. Mol. Biol.*, **355**, 85–95.
44. Peter, B. J., Arsuaga, J., Breier, A. M., Khodursky, A.B., Brown, P. O. and Cozzarelli, N. R. 2004, Genomic transcriptional response to loss of chromosomal supercoiling in *Escherichia coli*, *Genome Biol.*, **5**, R87.
45. Blot, N., Mavathur, R., Geertz, M., Travers, A. and Muskhelishvili, G. 2006, Homeostatic regulation of supercoiling sensitivity coordinates transcription of the bacterial genome, *EMBO Rep.*, **7**, 710–715.
46. Hansen, F. G., Christensen, B. B. and Atlung, T. 1991, The Initiator titration model: computer simulation of chromosome and minichromosome control, *Res. Microbiol.*, **142**, 161–167.
47. Hansen, F. G., Christensen, B. B. and Atlung, T. 2007, Sequence characteristics required for cooperative binding and efficient in vivo titration of the replication initiator protein DnaA in *E. coli*, *J. Mol. Biol.*, **367**, 942–952.