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

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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 *yulC* 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 lividans,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 Kaguni⁹), *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...
Distribution of DnaA on the Bacillus subtilis genome

polA expression. 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. 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, although DnaA has been demonstrated previously to regulate positively nrdAB promoter. DnaA might have a dual role in modulating nrdAB expression; low levels of DnaA-ATP stimulate the transcription whereas high levels repress it. In addition, a high-affinity DnaA-binding sequence containing five DnaA boxes, dat (DnaA Tiration), has been identified. Multicopy plasmids harboring the dat sequence interfere with the normal replication cycle, although its exact role is unclear. A similar high-affinity DnaA-binding region is involved in regulating the initiation of chromosome replication in S. coelicolor. 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. The sequence upstream of the sda promoter includes five DnaA boxes, and DnaA binding to these is essential for sda expression. Transcripont analysis of replication-inhibited cells and chromatin immunoprecipitation assays reveal that yhlB (flsL), dnaB, ywlC, and yydA are negatively regulated by DnaA binding to their promoter regions, 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. 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. 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. 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. 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. Clearly, DnaA forms stable complexes at eight intergenic regions on the B. subtilis genome, including oriC, oriC1, and oriC2. 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 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. Apart from the rRNA operon, rRNA genes were excluded from probe design. In addition, skin element, two prophage (PNSX and sp), and seven prophage-like sequences 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-Δspo0J 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 EcoRI–XhoI 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 HindIII and BamHI 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 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 0.5 μg/ml erythromycin and 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 cytometry (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 OD₆₀₀ 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 OD₆₀₀ 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 locus4 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 OD₆₀₀ value of 0.01, followed by culture at 30°C.
At OD\textsubscript{600} of 0.4, cells were harvested from 40 mL of culture, and total RNA extracted, as described previously.\textsuperscript{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.\textsuperscript{6} Synthesis of cDNA, terminal labeling, and hybridization to the oligonucleotide chip were performed using established methods.\textsuperscript{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 x histidine-tag under denatured conditions.\textsuperscript{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. 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\textsuperscript{2+}/C\textsubscript{24} 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 were 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. 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. 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, ygcG–sda, ywLB–ywL, jag–thdF, ygcS–yylA, ywcL–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 (oriC1), and dnaA–dnaN (oriC2) comprise cis-acting sequences essential for the initiation of chromosome replication, and the upstream sequence contains the dnaA promoter that is repressed by the corresponding translated protein. Expression of sda is dependent on DnaA binding to the upstream sequence, and promoters of yulC and yydA were reported to be negatively regulated by DnaA. Multiple DnaA boxes upstream of thdF were identified 17 years ago, but with no evidence of actual DnaA binding. Our results additionally suggest a possibility that DnaA regulates the expression of vpr and/or ywcL–secT. Binding to the gcp–ydiF region is characteristic 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. 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. 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. 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. 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. 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 yulC, 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...
conditions, thus those genes may still be repressed by DnaA. The suppression of \textit{ywlC} and \textit{yydA} expression by DnaA is in accordance with reported microarray data.\textsuperscript{23} We have demonstrated DnaA-regulated expression of \textit{thdF-gidA-gidB-noc} by northern blot analysis using NIS2022 cells (Ogura \textit{et al.}, unpublished result). These transcriptional alterations were reproducible in two independent experiments (Supplementary Fig. S3). Additionally, the DnaA level affected \textit{rocA}, \textit{rocD}, and \textit{yvdCD} expression positively, and \textit{ykuNOP} and \textit{dbhA} 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
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*. Recently, the crystal structure of the DNA-binding domain of *E. coli* DnaA complexed with a DnaA box (TTATCCACA) was resolved. 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. Initially, we evaluated the density of DnaA boxes along the genome, using a modified method of Mackiewicz et al. 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: $C > A = G > T$. Accordingly, we...
assigned the binding affinities as 4, 2, 1.5, 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 $A = \frac{\text{sum of affinities of four successive DnaA boxes}}{d}$. 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, ywIC, and ygdA are also present in most bacteria.

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

| Direction | Gene | Essential | Function |
|-----------|------|----------|----------|
|           | rpsA | 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 |
|           | rnaD | yes      | Nicotinate-nucleotide adenyltransferase |
|           | 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 |
|           | ywiD | no       | Function-unknown protein with an amino acid translocator motif (PF01810) |
|           | ywiC | yes      | Escherichia coli homolog, YrdC, binds to dsRNA |
|           | ywiB | no       | Function-unknown protein with an acetyltransferase domain (Pfam PF00583) |
|           | spoHR| no       | Sporulation protein transcribed by sporulation specific sigma factor, sF |
|           | sacT | no       | Transcriptional antiterminator involved in positive regulation of sacA and sacP |
|           | yucI | no       | Function-unknown and unique protein with one transmembrane domain |
|           | vpr  | no       | Extracellular serine protease expressed transiently at $T_d$ in a phoR-dependent manner |
|           | yggA | no       | Conserved protein with a domain of unknown function (Pfam PF02590) |
|           | yggS | no       | Function-unknown and unique protein with one transmembrane domain |
|           | roc  | 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 RNA |
|           | gidA | no       | Escherichia coli homolog involved in tRNA modification. The disruptant shows slow growth |
|           | thdF | no       | Escherichia coli homolog, MmmE, 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, ygdG, shows lethal phenotype |

*The location of DnaA-binding site is indicated by circle and direction of the adjacent transcriptional units is indicated by arrow.
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) / 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.
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. The binding sites of Spo0J determined using this technique were consistent with those reported earlier, 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 2. Sequence characteristics of the DnaA-binding regions identified by ChAP-chip analysis

| Regiona | Surrounding genes | Start | End | d^b | Sum of affinity | A^c | Number of DnaA boxesd | Number of perfect matches^e |
|---------|------------------|-------|-----|-----|----------------|-----|-----------------------|-----------------------------|
| rpmH–dnaA | 193–257 | 193–257 | 10.7 | 10 | 0.94 | 8 | 2 (2) |
| dnaA–dnaN | 1797–1860 | 1797–1860 | 10.3 | 7 | 0.68 | 5 | 1 (1) |
| gcp–ydiF | 643 951–643 999 | 643 951–643 999 | 5.3 | 5 | 0.94 | 8 | 4 (3) |
| yqeG–sda | 2 646 452–2 646 536 | 2 646 452–2 646 536 | 17.3 | 7 | 0.40 | 5 | 2 (2) |
| ywiC–ywiB | 3 793 128–3 793 181 | 3 793 128–3 793 181 | 7.0 | 8 | 1.14 | 5 | 2 (1) |
| yqeG–sda | 3 906 700–3 906 743 | 3 906 700–3 906 743 | 3.7 | 6 | 1.64 | 5 | 3 (0) |
| gcp–ydiF | 4 133 932–4 133 997 | 4 133 932–4 133 997 | 11.0 | 4 | 0.36 | 4 | 0 (0) |
| thdF–yag | 4 212 013–4 212 108 | 4 212 013–4 212 108 | 21.0 | 10 | 0.48 | 4 | 3 (2) |

aRegions with A value > 0.3 are shown. When DnaA box clusters overlap, those with the highest A value are indicated.
bAverage distance between four adjacent DnaA boxes.
cA = (sum of affinities of four successive DnaA boxes)/4. Only the highest value in each DnaA box cluster is shown. See text for detail.
dNumber of DnaA boxes allowing one base mismatch of the consensus DnaA box, TTATNCACA, in successive DnaA box clusters with an A value > 0.3.
eThe number of perfectly matched sequences to the consensus DnaA box, TTATCCACA. The number of DnaA boxes with highest affinity (TTATCCACA) is indicated in parentheses.

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

| Genus | Species | rpmH→ → dnaA→ → dnaN yqeG→ → gcp→ → ydiF thdF→ → yag→ → ywiC→ → ywiB yqeG→ → sda yqeI→ → vpr |
|-------|--------|-------------------------------------------------|
| Bacillus | B. anthracis | + + + + + + + + (+) (+) + + + + △ |
| | B. cereus | + + + + + + △ △ + + + + △ |
| | B. thuringiensis | + + + + + + △ △ + + + + △ |
| | B. clausii | + + + + △ △ △ △ + + △ △ + |
| | B. halodurans | + + + + + + △ △ + + + + △ |
| | B. licheniformis | + + △ + + △ (+) (+) (+) + + + + |
| | B. subtilis | + + + △ + + △ △ △ △ + + + + |
| Geobacillus | G. kaustophilus | + + △ △ △ △ △ + + + + |
| Oceanobacillus | O. hevensis | + + + + △ △ △ △ △ △ △ △ △ |

Symbols indicate conservation of genes. When DnaA box clusters with A ≥ 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 exist, cells are indicated as “(△)”. “→” 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, oriCl and oriC2. *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*,\(^{37}\) 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.

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

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