

The Sporulation Protein SirA Inhibits the Binding of DnaA to the Origin of Replication by Contacting a Patch of Clustered Amino Acids[∇]

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Bacteria regulate the frequency and timing of DNA replication initiation by controlling the activity of the replication initiator protein DnaA. SirA is a recently discovered regulator of DnaA in *Bacillus subtilis* whose synthesis is turned on at the start of sporulation. Here, we demonstrate that SirA contacts DnaA at a patch of 3 residues located on the surface of domain I of the replication initiator protein, corresponding to the binding site used by two unrelated regulators of DnaA found in other bacteria. We show that the interaction of SirA with domain I inhibits the ability of DnaA to bind to the origin of replication. DnaA mutants containing amino acid substitutions of the 3 residues are functional in replication initiation but are immune to inhibition by SirA.

Fundamental to an organism's survival is the ability to create one complete and accurate copy of its genome for every cell division. Underreplication leaves some cells without any genetic information, and overreplication wastes resources and could lead to aneuploidy. For these reasons, controlling the frequency of DNA replication initiation is critical to the cell cycle. Bacteria accomplish this by regulating the availability and activity of the replication initiator protein DnaA. An AAA⁺ ATPase, DnaA binds cooperatively to the origin of replication when in the ATP-bound state and distorts and melts the double helix. This then allows recruitment and loading of the replicative helicase onto the single-stranded DNA.

The DnaA protein consists of four domains (23). The most N-terminal of these, domain I, dimerizes with other domain I monomers (1) and interacts with other proteins that can modulate DnaA function (13). Domain II is unstructured and flexible and is poorly conserved. Domain III contains the AAA⁺ ATPase motif, multimerizes when bound to ATP, and forms contacts with the single-stranded DNA of the open complex (24). The most C-terminal domain, domain IV, binds double-stranded DNA via a helix-turn-helix DNA binding motif, recruiting DnaA to the consensus DnaA boxes at the origin of replication (5).

The mechanisms of DnaA function and the regulators of its activity are well studied in *Escherichia coli*. In this and other Gram-negative bacteria, the activity of DnaA is controlled by several mechanisms. These include regulation of the nucleotide binding state of DnaA (6, 11, 12, 28), regulation of the availability of the origin region (sequestration) to interact with DnaA (21, 26), binding to high-affinity DnaA-binding sites (*cis*-acting elements) that reduce the levels of DnaA that are

free to interact with origin DNA (15, 16), and regulation of DnaA's affinity for DNA by interaction with other proteins (10, 13, 14). In Gram-positive bacteria, however, there are no homologs of the proteins or *cis*-acting elements responsible for these well-understood regulatory mechanisms in *E. coli*. In the Gram-positive model organism *Bacillus subtilis*, only two regulators of DnaA function have been identified, YabA (3, 8, 22) and Soj (17, 18), and the mechanisms by which these proteins control DnaA are not yet known.

In response to nutrient starvation, cells of *B. subtilis* undergo a regulated and complex developmental program to create a robust and dormant spore. Recently, we discovered a gene, *sirA*, that is induced at the start of sporulation and whose product is an inhibitor of DNA replication (25). Cells lacking *sirA* have more chromosomes than wild-type cells when challenged to sporulate under conditions that promote replication initiation. When synthesis of SirA is artificially induced during growth, replication is impaired, resulting in cells that lack a chromosome (25). Wagner et al. have shown that SirA acts through DnaA (29). Cells that initiate replication through a plasmid origin, *oriN*, that uses an initiator protein other than DnaA are unaffected by *sirA* expression. Additionally, SirA and DnaA interact in a yeast two-hybrid assay (29), suggesting that SirA affects DnaA function directly.

To investigate further the interaction between SirA and DnaA and the mechanism by which SirA regulates replication initiation, we selected for mutations in *dnaA* that rendered initiation of replication immune to SirA. We discovered four such mutations, which altered three codons for amino acids located in domain I of DnaA. Using a yeast two-hybrid assay, we demonstrate that these residues are required for the interaction of DnaA with SirA and that domain I alone is sufficient for interaction with SirA. Finally, taking advantage of one of the mutant proteins, we demonstrate that at the start of sporulation SirA reduces the amount of wild-type DnaA bound at the origin of replication and does so in a manner that depends on its interaction with DnaA.

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MATERIALS AND METHODS

Strains, plasmids, and primers. Strains were derived from *B. subtilis* PY79. All strains, plasmids, and primers used in this work are described in Table 1.

Mutagenic PCR. PCR was performed with AcuStart PCR supermix (Quanta Biosciences) on chromosomal DNA from strain LR166 with primers LRL96 and LRL174. Reaction mixtures (400 μ l) were run in 12- μ l aliquots to increase the diversity of mutations and then pooled. Reaction mixtures were cycled at 94°C for 2 min, followed by 10 cycles of 94°C for 1 min, 65°C – 1°C per cycle for 1 min, and 72°C for 5 min. Finally, 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 5 min were run. Pooled PCR products were transformed directly into strain LR158 as described previously (30).

Identifying mutations in *dnaA*. Chromosomal DNA was isolated from colonies able to grow on plates containing LB medium with chloramphenicol and IPTG (isopropyl- β -D-thiogalactopyranoside). PCRs were run on these templates using primers LRL171 and LRL83. The reactions were set up with 1 μ l of template DNA, 100 pM of each primer, 20 μ l of 10 \times Platinum Pfx polymerase buffer (Invitrogen), 1 μ l of 25 mM deoxynucleoside triphosphates, 2 μ l of 50 mM MgSO₄, and 1 μ l of platinum Pfx polymerase (Invitrogen) in a 100- μ l volume, and mixtures were thermocycled under standard conditions. PCR products were purified with a PCR purification kit (Qiagen) and sequenced with primers LRL171, LRL99, LRL100, LRL101, and LRL83.

Yeast two-hybrid assays. Yeast two-hybrid experiments were performed using a Matchmaker Gold yeast two-hybrid system (Clontech). *sirA* was cloned into the EcoRI/BamHI sites of the prey plasmid pGADT7, and *dnaA*, the two truncated *dnaA* genes, and *E. coli dnaA* were cloned into the NdeI/BamHI sites of the bait plasmid pGBKT7, as described in Table 1. To make the *dnaA* point mutations, pLR48 and pLR57 were mutated with a QuikChange site-directed mutagenesis kit (Stratagene) as described in Table 1. The prey plasmids (pLR55 and empty pGADT7) were transformed into *Saccharomyces cerevisiae* strain Y187 as per the manufacturer's directions, and transformants were selected on SD-Leu plates. The bait plasmids (pLR48 to pLR54, pLR57, pLR58, and empty pGBKT7) were transformed into yeast strain Y2HGGold, and transformants were selected on SD-Trp plates. Prey and bait strains were mated by being streaked together on yeast extract-peptone-dextrose (YPD) agar and then replica plated onto an minimal synthetic defined (SD)-Trp-Leu plate to select for both bait and prey plasmids. To assay for prey-bait interactions, the mated strains were restreaked onto SD-Trp-Leu-His-Ade plates, grown at 30°C for 3 days, and then photographed.

Chromatin immunoprecipitation. Cultures were grown in 25% LB to an optical density at 600 nm (OD₆₀₀) of 0.25. *kinA* expression was induced with 100 mM IPTG. After 1 h, 50-ml cultures were cross-linked in 1% formaldehyde for 20 min and then quenched with 5 ml of 2.5 M glycine. Cell pellets were washed once in cold phosphate-buffered saline (PBS) and then frozen at –80°C. Pellets were resuspended in 3 ml of 10 mM Tris, pH 8, 20% sucrose, 50 mM NaCl, 10 mM EDTA, 1 mg/ml lysozyme, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and then incubated at 37°C for 30 min. Three milliliters of 2 \times immunoprecipitation (IP) buffer (100 mM Tris, pH 7, 300 mM NaCl, 2% Triton X-100) was added, and the mixture was sonicated for 1 min at 15% power output with a microtip. Cell debris was pelleted and 40 μ l of the lysate set aside in 40 μ l TE (10 mM Tris, pH 8, 0.1 mM EDTA) and 9 μ l of 10% sodium dodecyl sulfate (SDS) as the total DNA sample. One-milliliter aliquots of the lysate were used for each chromatin immunoprecipitation (ChIP) pulldown. Two microliters polyclonal rabbit antiserum for DnaA, which behaves similarly to the previously described chicken antibodies against DnaA (27), was added, and the samples were incubated at 4°C with gentle agitation overnight. Thirty microliters 50% protein A-Sepharose slurry was added, and the samples were incubated for an additional hour at room temperature. The lysate was removed, and the beads were washed six times with 1 ml of 1 \times IP buffer, followed by a 1-ml wash in TE. The DNA-protein complexes were eluted from the beads by incubation with 250 μ l TE with 0.67% SDS at 65°C for 10 min. Immunoprecipitate and total samples were incubated overnight at 65°C to reverse cross-linking. Proteinase K (20 mg/ml; Qiagen) was added to 250 μ l of TE, pH 7.6, and the reaction mixtures were incubated at 37°C for 2 h. LiCl was added to 400 mM, and the DNA was purified with phenol chloroform extraction. Glycogen (20 μ g) was added, and the DNA was precipitated in 75% ethanol at –80°C for 20 min. DNA pellets were resuspended in 75 μ l TE.

Quantitative PCR. Quantitative PCRs (qPCRs) (20- μ l mixtures) were set up with LightCycler 480 SYBR green Master mix (Roche) and 2 μ l immunoprecipitated DNA without dilution or total DNA diluted 1:500. To amplify the origin, primers WKS139 and WKS140 were used. To amplify *yhaX*, a site not bound by DnaA (2), primers WKS145 and WKS146 were used. The reactions were run on a LightCycler 480 II instrument (Roche). Signals were analyzed

using LightCycler 480 SW 1.5 software (Roche), according to the manufacturer (Advanced RelQuant; second derivative of Max, using Median Cp for calculation). Reactions were run in duplicate, and the average crossing point (Cp) value was used. Standard curves of total DNA samples from strain LR227 were used to find the concentration of the template DNA in arbitrary units. To find the fold enrichment, the concentration of the origin in each IP sample normalized to the concentration of the origin in the total for that sample was divided by the concentration of *yhaX* in that sample normalized to the concentration of *yhaX* in the total.

RESULTS

Isolation of DnaA mutants that are resistant to the growth defect caused by engineered expression of *sirA*. We sought to isolate mutants of DnaA that were immune to the action of SirA. To achieve this, we took advantage of the severe growth defect caused by the addition of inducer to cells engineered to produce SirA during growth in response to IPTG. *dnaA* was mutagenized using error-prone PCR. As a template, we used chromosomal DNA from a strain in which a gene conferring resistance to chloramphenicol had been inserted just upstream of the origin of replication (LR166). Primers LRL96 and LRL174 resulted in a PCR product that contained the antibiotic resistance gene, the origin of replication, *dnaA*, and flanking regions on either side for homologous recombination (Fig. 1).

This PCR product was transformed into strain LR158, which was engineered to express *sirA* in response to IPTG. The cells were allowed to grow on LB plates containing chloramphenicol and IPTG at 22°C for 3 days. Transformants that were chloramphenicol resistant and able to grow in the presence of IPTG were colony purified and subjected to further analysis as follows. The origin of replication, including *dnaA*, was amplified from each transformant and subjected to sequence analysis. Four different mutants were isolated, and all contained single nucleotide substitutions in the coding sequence of *dnaA* (Table 2). The resulting amino acid substitutions, N47D, F49Y, A50V, and A50T, were clustered in the N-terminal region of DnaA domain I. The same mutation causing the N47D substitution was isolated independently from two PCRs.

Next, we analyzed the locations of the amino acid substitutions in the structure of domain I, as inferred from the nuclear magnetic resonance (NMR) structure of *Escherichia coli* DnaA domain I (1). As shown in Fig. 2, all of the residues are clustered in a patch on the surface of the protein.

Strains separately containing each of the four *dnaA* point mutations showed no obvious growth defect and had a doubling time in liquid culture indistinguishable from that of strains containing a wild-type allele of *dnaA*, with the possible exception of the strain containing the F49Y substitution, whose doubling time appeared to be slightly slower than that of the wild type (data not shown).

The DnaA mutants are defective in interacting with SirA. Wild-type DnaA interacts with SirA as judged by a yeast two-hybrid assay (29). We sought to determine if our DnaA mutants were defective in interacting with SirA. We used SirA as the prey, fusing the coding sequence in yeast to that for the activation domain of the GAL4 transcription factor. Wild-type DnaA and the four point mutants were used as bait, with their coding sequences fused to that for the DNA-binding domain of the GAL4 transcription factor. As expected, wild-type DnaA

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence (reference or source)
Bacillus strains	
LR158.....	<i>thrC</i> ::P _{hyperspank} - <i>sirA</i> (25)
LR166.....	<i>cat</i> inserted 406 bp upstream from <i>dnaA</i> translational start by LFH ^a with primers LRL96, LRL170, LRL171, and LRL100 (9)
LR227.....	<i>amyE</i> ::P _{hyperspank} - <i>kinA</i> from MF2250 (lab strain), <i>cat</i> upstream of <i>dnaA</i> (LR166)
LR228.....	<i>amyE</i> ::P _{hyperspank} - <i>kinA</i> , <i>cat</i> upstream of <i>dnaA</i> (A50V)
LR229.....	<i>amyE</i> ::P _{hyperspank} - <i>kinA</i> , <i>cat</i> upstream of <i>dnaA</i> , <i>sirA</i> :: <i>tet</i> from LR74 (25)
LR230.....	<i>amyE</i> ::P _{hyperspank} - <i>kinA</i> , <i>cat</i> upstream of <i>dnaA</i> (A50V), <i>sirA</i> :: <i>tet</i>
S. cerevisiae strains	
LR192.....	Y2HGold with pGBKT7 (Clontech)
LR193.....	Y187 with pGADT7 (Clontech)
LR197.....	Y187 with pLR55
LR198.....	Y2HGold with pLR48
LR199.....	Y2HGold with pLR49
LR200.....	Y2HGold with pLR50
LR201.....	Y2HGold with pLR51
LR202.....	Y2HGold with pLR52
LR203.....	Y2HGold with pLR53
LR204.....	Y2HGold with pLR54
LR205.....	Y2HGold with pLR57
LR206.....	Y2HGold with pLR58
Plasmids	
pLR48.....	pGBKT7 (Clontech) with <i>B. subtilis dnaA</i> cloned into NdeI/BamHI with primers LRL195 and LRL196
pLR49.....	pGBKT7 with <i>B. subtilis dnaA</i> domain I cloned into NdeI/BamHI with primers LRL195 and LRL198
pLR50.....	pGBKT7 with <i>B. subtilis dnaA</i> domains I and II cloned into NdeI/BamHI with primers LRL195 and LRL197
pLR51.....	pLR48 with A50V point mutation made using primers LRL205 and LRL206
pLR52.....	pLR48 with N47D point mutation made using primers LRL207 and LRL208
pLR53.....	pLR48 with F49Y point mutation made using primers LRL209 and LRL210
pLR54.....	pLR48 with A50T point mutation made using primers LRL211 and LRL212
pLR55.....	pGADT7 (Clontech) with <i>sirA</i> cloned into EcoRI/BamHI with primers LRL193 and LRL194
pLR57.....	pGBKT7 with <i>E. coli dnaA</i> cloned into NdeI/BamHI with primers LRL224 and LRL225
pLR58.....	pLR57 with V47A point mutation made using primers LRL230 and LRL231
Primers	
LRL83.....	5'-ATGGTACCACTCCCTGTAAAGGATACTC-3'
LRL96.....	5'-CATTTCGGCTGTAAAGCCTGCA-3'
LRL99.....	5'-CTAAGCCGACGCCCCATAGAT-3'
LRL100.....	5'-CTAAAATTGCAATTCTCGTTTCTAG-3'
LRL101.....	5'-ATTTTTTCATGCGCATGAATAACGGT-3'
LRL170.....	5'-CAATTCGCCCTATAGTGAGTCGTGATATCCACATGCGTGATTTTAGG-3'
LRL171.....	5'-CCAGCTTTTGTCCCTTTAGTGAGTTTTTCGGCTTTTTTTAGTATCCACAG-3'
LRL174.....	5'-GATGCACGATCAATGGCCTGAAG-3'
LRL193.....	5'-GCCGAATTCATGGAACGTCACTACTATAACG-3'
LRL194.....	5'-ATGAGGGATCCGGTTTTAGACAAAATTCTTTC-3'
LRL195.....	5'-CGGCATATGGAAAATATATTAGACCTGTGG-3'
LRL196.....	5'-AGACTGGATCCTATTTAAGCTGTTCTTTAATTC-3'
LRL197.....	5'-ATGCAGGATCCTTATTTTGGAGAAAATCAGATGTATCTTC-3'
LRL198.....	5'-AGCAGGATCCTTTAATGACAACTTAATGCTCAATTCCTC-3'
LRL205.....	5'-CACGGCTCCCAATGAATTTGTGTCAGAGACTGG-3'
LRL206.....	5'-CCAGTCTCTGACAAATTCATTGGGAGCCGTG-3'
LRL207.....	5'-CACGGCTCCCGATGAATTTGCCAGAGACTGG-3'
LRL208.....	5'-CCAGTCTCTGGCAAATTCATCGGGAGCCGTG-3'
LRL209.....	5'-CACGGCTCCCAATGAATATGCCAGAGACTGG-3'
LRL210.....	5'-CCAGTCTCTGGCATATTCATTGGGAGCCGTG-3'
LRL211.....	5'-CACGGCTCCCAATGAATTTACCAGAGACTGG-3'
LRL212.....	5'-CCAGTCTCTGGTAAATTCATTGGGAGCCGTG-3'
LRL224.....	5'-CGGCATATGGTGTCACTTTTCGCTTTGGCAGC-3'
LRL225.....	5'-AGACTGGATCCGTTTACGATGACAATGTTCTGATTAA-3'
LRL230.....	5'-CCATATTGCATGGTATGTAGCTATGGATCAATTATGGTACATTGGAGATC-3'
LRL231.....	5'-GATCTCCAATGTACCATAATTGATCCATAGCTACATACCATGCAATATGG-3'
WKS139.....	5'-GGAGGACGTGATCATACGA-3'
WKS140.....	5'-TAGGGCCTGTGGATTTGTG-3'
WKS145.....	5'-CGAGCAAGGTGTCGCTTA-3'
WKS146.....	5'-GCAGGCGTTCATCATGTA-3'

^a LFH, long flanking homology.

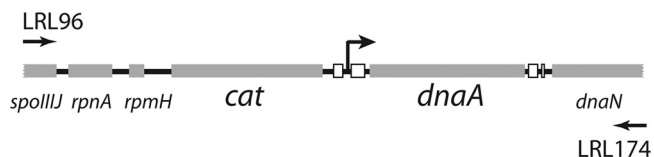


FIG. 1. Replication origin region of the *B. subtilis* chromosome. Shown is the site of insertion of the gene for resistance to chloramphenicol (*cat*) in strain LR166. The promoter for *dnaA* is marked by an arrow. The essential elements of the origin of replication are shown as white boxes. Primers LRL96 and LRL174 were used to amplify this region, which also includes the genes *rpnA* and *rpmH* and portions of the genes *spoIIIJ* and *dnaN*.

was able to interact with SirA, activating expression of *HIS3* and *ADE2* as judged by the ability of auxotrophic yeast cells to grow on medium lacking histidine and adenine (Table 3 and Fig. 3A). In contrast, none of the four yeast strains in which mutant DnaA proteins had been used as bait were able to grow on the selective media (Table 3 and Fig. 3C). These results suggest that the amino acid substitutions in DnaA that allowed for growth in the presence of SirA were defective in the interaction between DnaA and SirA and likely form part of the site where DnaA contacts SirA.

Domain I of DnaA is sufficient for interaction with SirA. Because the amino acid substitutions in DnaA were clustered near each other in domain I of the protein, we wondered whether domain I was sufficient for interaction with SirA. If this is the case, then SirA should be able to interact with a truncated version of DnaA that contains only domain I. We tested two truncations in our yeast two-hybrid assay, one that retained domain II, the flexible linker between the N-terminal domain and the rest of the protein, and a more severe truncation that retained only domain I. In both cases, SirA was able to interact with the truncated protein (Table 3 and Fig. 3B), supporting the idea that domain I contains the entire binding site for SirA.

DnaA A50 is coconserved with SirA. Of the three residues that underwent substitution in the DnaA mutants, two (N47 and F49) are widely conserved. Residue 50, however, which is an alanine in *B. subtilis*, is often a valine or a threonine in other species. In fact, as shown in Fig. 4, an alanine at this position correlates with the presence of a SirA homolog. In species without SirA, valine or threonine is found at this position. The coconservation of A50 and SirA is consistent with the view that DnaA A50 is important for SirA function.

A single amino acid substitution enables *E. coli* DnaA to interact with SirA. As just indicated, species that lack a SirA homolog contain valine or threonine at the residue corresponding to A50. In *Escherichia coli*, the corresponding residue (residue 47) is a valine. Thus, *E. coli* DnaA is naturally equiv-

TABLE 2. Mutations in the isolated DnaA mutants

Nucleotide position	Nucleotide substitution	Amino acid substitution
139	A to G	N47D
146	T to A	F49Y
148	G to A	A50T
149	C to T	A50V

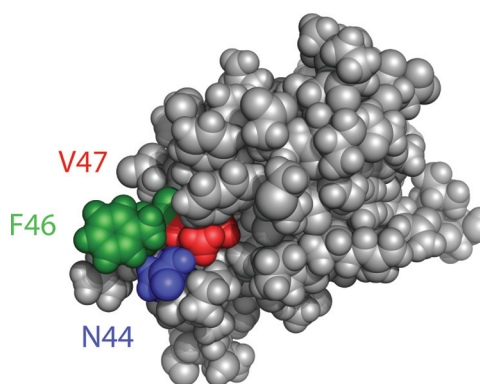


FIG. 2. SirA contacts a patch of residues on the surface of domain I of DnaA. The structure of *E. coli* DnaA domain I is shown from Abe et al. (1). N44 (*B. subtilis* N47) is shown in blue, V47 (*B. subtilis* A50) is shown in red, and F46 (*B. subtilis* F49) is shown in green.

alent to our *B. subtilis* A50V mutant. Indeed, as shown in Table 3, we could detect little or no interaction between *E. coli* DnaA and *B. subtilis* SirA in the yeast two-hybrid assay. We therefore wondered whether we could convert *E. coli* DnaA into a SirA-interacting protein simply by switching the valine at residue 47 (corresponding to residue 50 in *B. subtilis*) to an alanine. Strikingly, simply switching V47 to an alanine in *E. coli* DnaA was sufficient to confer on the heterologous protein the ability to interact with SirA (Table 3 and Fig. 3D). This finding reinforces the view that A50 in *B. subtilis* DnaA forms part of the DnaA-SirA binding site. It would be interesting in future work to engineer an *E. coli* strain that produces the V47A substitution and ask whether engineered synthesis of SirA blocks DNA replication in the heterologous host.

SirA inhibits the binding of wild-type but not mutant DnaA to the origin of replication. Evidence based on the use of a DnaA-green fluorescent protein (GFP) fusion indicated that SirA interferes with the association of DnaA with the origin region of the chromosome (29). We wondered whether we could observe this effect more directly and at higher resolution by use of chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) and, if so, whether DnaA harboring the A50V substitution would be refractory to the effect of SirA. To measure DnaA binding to the origin, we performed ChIPs

TABLE 3. Summary of yeast two-hybrid assay results^a

GAL BD ^b fusion	Interaction with GAL AD ^c -SirA
<i>B. subtilis</i>	
DnaA.....	Yes
DnaA residues 1-109.....	Yes
DnaA residues 1-80.....	Yes
DnaA(N47D).....	No
DnaA(F49Y).....	No
DnaA(A50T).....	No
DnaA(A50V).....	No
<i>E. coli</i>	
DnaA.....	No
DnaA(V47A).....	Yes

^a Results from Fig. 3 and data not shown.

^b GAL BD, DNA-binding domain of the GAL4 transcription factor.

^c GAL AD, activation domain of the GAL4 transcription factor.

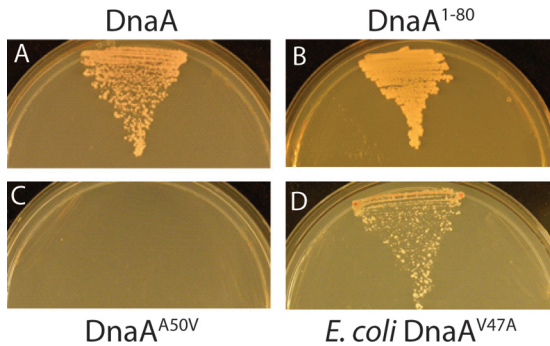


FIG. 3. A yeast two-hybrid assay identifies residues required for the binding of SirA to DnaA. Shown are streaks of yeast cells in which SirA was used as prey and the following forms of DnaA were used as bait: wild-type *B. subtilis* DnaA, *B. subtilis* DnaA residues 1 to 80, *B. subtilis* DnaA(A50V), and *E. coli* DnaA(V47A). For each plate, streaked on the left as a control is yeast harboring the experimental DnaA vector and the empty prey vector, streaked in the center is yeast harboring the experimental DnaA bait vector and the SirA prey vector, and streaked on the right as a control is yeast harboring the empty bait vector and the SirA prey vector.

using anti-DnaA antibodies followed by qPCR and measured the amount of origin DNA that had been cross-linked to DnaA compared to that for a control locus unrelated to DnaA.

To carry out the experiment, we artificially induced sporulation using cells that were engineered to produce KinA in response to an inducer. KinA is one of several histidine kinases that phosphorylate the master regulator for sporulation Spo0A via a phosphorelay. Overexpression of KinA efficiently triggers sporulation even in rich medium in which cells are growing rapidly and would otherwise sporulate poorly (7). We had previously shown that, under these conditions, cells lacking *sirA* overreplicate their chromosomes (25). Indeed, as shown in Fig. 5A, about 3-fold more DnaA was observed at the origin of replication in cells of a strain lacking *sirA* (LR229) than in cells of the wild type (LR227). In a separate experiment, Western blotting showed that expression of *sirA* had little or no effect on the total amount of DnaA protein present in the cell (data not shown). Thus, the results from ChIP analysis support the idea that SirA interferes with the binding of DnaA to the origin or removes it from the origin once bound.

Next, we performed the same experiment with strains that

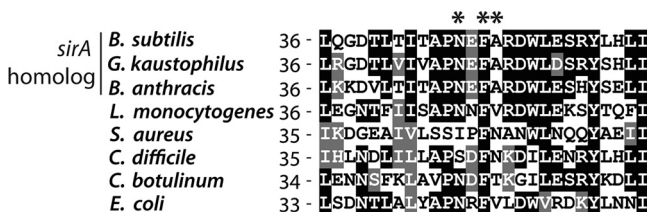


FIG. 4. Alanine 50 of DnaA is conserved among DnaA homologs in species that contain a SirA homolog. Shown is a portion of the amino acid sequence of DnaA domain I for the indicated species (*L. monocytogenes*, *Listeria monocytogenes*; *S. aureus*, *Staphylococcus aureus*; *C. difficile*, *Clostridium difficile*; *C. botulinum*, *Clostridium botulinum*). Residues at which substitutions conferred immunity to SirA are marked with asterisks. *B. subtilis*, *Geobacillus kaustophilus*, and *Bacillus anthracis* possess SirA homologs. Sequences were aligned with ClustalW.

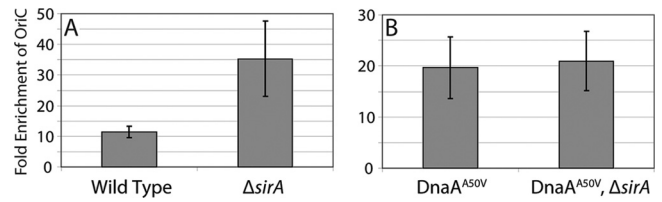


FIG. 5. SirA reduces the amount of wild-type but not mutant DnaA bound at the origin of replication. Cells were induced to sporulate in rich medium. The graphs show the results of ChIP experiments, expressed as fold enrichment of the origin of replication over that for an unrelated locus in the pulldown of DnaA from cell lysates of strains with or without *sirA*. (A) Wild-type DnaA (strains LR227 and LR229). (B) DnaA(A50V) (strains LR228 and LR230). Error bars represent the standard errors from six replicates.

produced DnaA(A50V) instead of wild-type DnaA (LR228 and LR230). In this case, the presence or absence of *sirA* had no measurable effect on the amount of the mutant protein observed at the origin of replication (Fig. 5B).

Interestingly, and independently of the presence or absence of *sirA*, the A50V substitution mutation seemed to alter the amount of protein at the origin. We observed twice as much DnaA(A50V) at the origin of replication (20-fold enrichment) (Fig. 5B) as we did with wild-type DnaA (10-fold enrichment) (Fig. 5A). Evidently, the A50V substitution increased the affinity of DnaA for origin DNA or, conceivably, its affinity for our antibody.

Taken together, these data support a model in which SirA binds to DnaA via a cluster of residues located in domain I. Moreover, our findings together with those of Wagner et al. (29) indicate that the effect of SirA is to antagonize the ability of DnaA to associate with the origin and hence to initiate replication. Thus, the induction of SirA synthesis at the start of sporulation helps to ensure that further rounds of DNA replication are suppressed so that the sporangium will contain no more than two chromosomes.

DISCUSSION

The principal contribution of the present investigation is the demonstration that SirA interacts with DnaA by contacting a small patch of amino acids that sit at the surface of domain I of the initiator protein and that this interaction inhibits the binding of DnaA to the origin of replication or acts to remove DnaA that is already bound at the origin. For comparison, YabA, a negative regulator of DnaA function, is thought to interact with domain III of the protein (3), but it is not known how this interaction interferes with DnaA function. Soj, which is also a negative regulator, is known to interact with DnaA, but it is not known which part of the protein it contacts (18).

Domain I is also the contact site for two regulators of DnaA in Gram-negative bacteria that promote replication initiation. These are DiaA from *E. coli* (13) and HobA from *Helicobacter pylori* (20). DiaA interacts with DnaA in a way that increases the initiator's affinity for DnaA boxes, resulting in more DnaA binding to the origin and faster initiation (14). HobA, which is essential in *H. pylori*, dimerizes DnaA domain I monomers (20). *H. pylori* DnaA lacks the W6 residue that is essential for

dimerization of this domain in *E. coli* (1), and it is thought that HobA is required to replace this function.

Interestingly, DiaA and HobA interact with DnaA at residues corresponding to those we have identified as the binding site for SirA (13, 20). This is the case even though these three proteins alter DnaA function in different ways. Both *E. coli* and *B. subtilis* DnaA have the W6 residue for which HobA compensates in *H. pylori*. Like DiaA, SirA influences DnaA's interaction with the origin of replication, but DiaA promotes this interaction and SirA antagonizes it. Thus, the residues on the surface of domain I seem to have evolved as a common interaction site that different species have exploited for diverse purposes.

HobA and DiaA have been called "structural homologs," because they share no primary sequence homology but have the same three-dimensional shape (19). SirA does not share sequence similarity with either of these proteins, but it is similar to them in size (18 kDa for SirA, versus 21 kDa for both HobA and DiaA) and binds to the same site on DnaA. Conceivably, SirA is a homolog of HobA and DiaA whose relatedness has been preserved at the structural level. Initial attempts to model the structure of SirA by protein threading (4) through HobA and DiaA were unsuccessful. If SirA proves to be structurally related, then all three proteins may have a common evolutionary origin even though their functions have diverged.

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