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## Isolation of a New Hemimethylated DNA Binding Protein Which Regulates *dnaA* Gene Expression

Emmanuelle d'Alençon,<sup>†</sup> Aziz Taghbalout, Claire Bristow, Renée Kern, Revital Aflalo, and Masamichi Kohiyama\*

*Institut J. Monod, Université Paris VI-VII, 75251 Paris Cedex 05, France*

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**In this report, we show that *yccV*, a gene of unknown function, encodes a protein having an affinity for a hemimethylated *oriC* DNA and that the protein negatively controls *dnaA* gene expression in vivo.**

The minimum replication origin of *Escherichia coli* (*oriC*) has an elevated number of DNA adenine methylation (Dam) sites (18) that become hemimethylated immediately after initiation of replication and stay in this state for one-quarter of the generation time; these sites differ from most Dam sites located elsewhere on the chromosome (6). The finding that the hemimethylated *oriC* interacts with an outer membrane preparation of *E. coli* (20) prompted a search for hemimethylated DNA binding proteins in the membrane fraction. The existence of such a category of proteins in the membrane has been reported previously; in fact, Southwestern blot analysis of the membrane preparation demonstrated the presence of 25- and 16-kDa peptides reacting with a hemimethylated *oriC* probe (9). These proteins might participate in the hemimethylated *oriC* membrane sequestration that prevents initiation of chromosome replication in *E. coli* (16).

Lu et al. (17) isolated the *seqA* gene that codes for a 21-kDa peptide and has an affinity for the hemimethylated *oriC* DNA (24). In a *seqA* mutant, the duration of the hemimethylation period of *oriC* is shortened, and the reinitiation of replication occurs at *oriC* repeatedly in a single replication cycle (17). These phenotypes may be explained by a lack of hemimethylated *oriC* sequestration by either SeqA or a SeqA membrane complex; the membrane obtained from the *seqA* mutant failed to bind the hemimethylated *oriC* DNA in vitro (2, 24). Although these results show the predominant role of the SeqA protein in *oriC* sequestration, the auxiliary role played by the membrane in the process should not be disregarded. Shakibai et al. (23) observed that the hemimethylated *oriC* binding activity of SeqA is stimulated by addition of a membrane protein preparation designated SeqB. It has also been found that the membrane obtained from a *seqA* mutant stimulates the protective activity of a His-tagged SeqA protein against DNase I attack of hemimethylated *oriC* DNA (7).

In order to isolate auxiliary factors of SeqA, we adopted a strategy of searching for factors among the *dnaA46* suppressor gene products. The rationale behind this approach was based

on the observation that the *seqA* mutation partially suppresses the temperature sensitivity of *dnaA46* (17). Likewise, mutation of the auxiliary factor gene should also result in suppression of *dnaA46*. Previously, this type of work has been undertaken by Katayama's group, who used a system involving random insertion of Tn10-Tet<sup>r</sup> into the chromosome. In this way, they found one new suppressor mutation for *dnaA46*, *hslU* (12).

**Isolation of *dnaA46* suppressors by mini-Tn10 insertion.** Briefly, random insertion of mini-Tn10 into the chromosome containing *dnaA46* (KA413ΔH) (Table 1) was accomplished by infection of the mutant with a lambda phage (λ1098) carrying mini-Tn10 (27), followed by plating of the culture on Luria-Bertani (LB) agar plates containing tetracycline and incubation at 40°C. Twenty-three colonies were isolated. To identify the mini-Tn10 insertion site on the chromosome, *PstI* chromosome fragments containing mini-Tn10 were cloned into the Bluescript plasmid (pSK+) or pUC18 (Table 1), and DNA sequences surrounding mini-Tn10 were determined and compared to the *E. coli* whole genome sequence (3) by using the BLAST program (Genetics Computer Group [GCG], University of Wisconsin, Madison, Wis.). To determine these sequences, DNA fragments surrounding mini-Tn10 were amplified by PCR with one primer, P15 (5' GATCATATGACAA GATGTGTATCCACC), homologous to IS10R and a second primer, P10 (5' ACGCAAACCGCTCTCCCCG) or P11 (5' GCGAAAGGGGGATGTGTCTGC), homologous to the vectors on either side of the polylinker. These fragments were sequenced with the same primers.

The locations were confirmed by Southern hybridization of chromosomal *PstI* digests with the corresponding probes.

At present, we have identified three sites of mini-Tn10 insertion that suppress the *dnaA46* thermosensitivity; they are *yccV* (in strain M2-24, with the corresponding *PstI* fragment cloned into pSK+, resulting in plasmid pSK24), *rpoN* (in strain M2-5, with the corresponding *PstI* fragment partially deleted after cloning into pUC18, resulting in plasmid pUC5), and *mutS* (in strain M2-11, with the corresponding *PstI* fragment also partially deleted after cloning into pSK+, resulting in pSK11), and they are located at 22, 74, and 69 min, respectively, on the genetic map.

The insertion mutants were further analyzed for genetic linkage between the tetracycline-resistant element (mini-Tn10) and the suppressor mutations. P1 phages prepared from M2-24, M2-5, or M2-11 were used for transduction of the tetracy-

\* Corresponding author. Mailing address: Institut J. Monod, Université Paris VI-VII, 2, Place Jussieu, 75251 Paris Cedex 05, France. Phone: 33 1 4427 6948. Fax: 33 1 4427 5716. E-mail: kohiyama@ccr.jussieu.fr.

<sup>†</sup> Present address: Laboratoire de Pathologie Comparée, UMR5087 INRA-CNRS-Université Montpellier II, 30380 Saint Christol les Ales, France.

TABLE 1. Bacterial strains, phage, and plasmids

Strain, phage, or plasmid	Description	Reference or source
<b>Strains</b>		
KH5402-1	<i>thyA thr trpE9829</i> (Am) <i>ilv tyrA</i> (Am) <i>metE deo supF6</i> (Ts)	T. Katayama 19
KA413	KH5402-1 <i>dnaA46 ilv</i> <sup>+</sup>	11
KA413Δ <i>H</i>	KA413 <i>aphA</i> ::Km	This study
M2-24	Temperature-resistant revertant of KA413Δ <i>H</i>	This study
M2-5	Temperature-resistant revertant of KA413Δ <i>H</i>	This study
M2-11	Temperature-resistant revertant of KA413Δ <i>H</i>	This study
JM109		28
C600		1
C600 <i>dam</i>	C600 <i>dam-13</i>	This study
C600 <i>yccV</i>	C600 <i>yccV</i> ::mini-Tn10	This study
C600 <i>dam yccV</i>	C600 <i>dam-13 yccV</i> ::mini-Tn10	This study
WM1032	<i>dnaA508 ilvB</i> or <i>ilvG lac supD thi trp</i>	W. Messer
WM1032 <i>yccV</i>	WM1032 <i>yccV</i> ::mini-Tn10	This study
WM2007	TC3145 <i>lac his</i> RB1 <i>proB902 rpsL supF81</i> (Ts) <i>thi trp</i> (Am) <i>tsX</i>	W. Messer (RB220 derivative <sup>c</sup> )
WM2007 <i>yccV</i>	WM2007 <i>yccV</i> ::mini-Tn10	This study
<b>Phage</b>		
λ1098	Pam80 <i>cI857</i> (Ts) <i>nin5</i> mini-Tn10	27
<b>Plasmids</b>		
pSK+	Bluescript, pBR322 derivative vector	Stratagene
pUC18	pBR322 derivative vector	28
pSK24	6.7-kbp <i>PstI</i> chromosomal fragment carrying <i>yccV</i> ::mini-Tn10 cloned on pSK+ <sup>a</sup>	This study
pSK11	Deletion part of a <i>PstI</i> chromosomal fragment carrying <i>mutS</i> ::mini-Tn10 cloned on pSK+	This study
pUC5	Deletion part of a <i>PstI</i> chromosomal fragment carrying <i>rpoN</i> ::mini-Tn10 cloned on pUC18	This study
pDK5	pBR322 derivative expression vector	13
pES6ΔHE	Derived from pDK5 by inserting between <i>EcoRI</i> and <i>HindIII</i> two complementary oligonucleotides, generating an <i>XbaI</i> site flanked by a hexahistidine coding sequence	This study
pES6ΔHEE1	RBS from P <sub>17</sub> and <i>YccV</i> ORF cloned between the <i>EcoRI</i> and <i>XbaI</i> sites of pES6ΔHE <sup>b</sup>	This study

<sup>a</sup> The fragment was from *PstI* position 8776 of GenBank sequence ae000198.gb\_ba2 to *PstI* position 1008 of GenBank sequence ae000199.gb\_ba2.

<sup>b</sup> RBS, ribosome binding site; ORF, open reading frame.

<sup>c</sup> See reference 5.

cline resistance marker in KA413Δ*H* at 30°C and then tested for growth at 40°C. The cotransduction frequencies between tetracycline resistance and growth capacity at 40°C were 100% for deletions of *yccV* and *rpoN* and 50% for the *mutS* deletant. Therefore, the mini-Tn10 insertion in either *yccV* or *rpoN* is responsible for suppression of *dnaA46* thermosensitivity, whereas the insertion in *mutS* is not sufficient for suppression, indicating that an unidentified mutation closely linked to *mutS* is necessary.

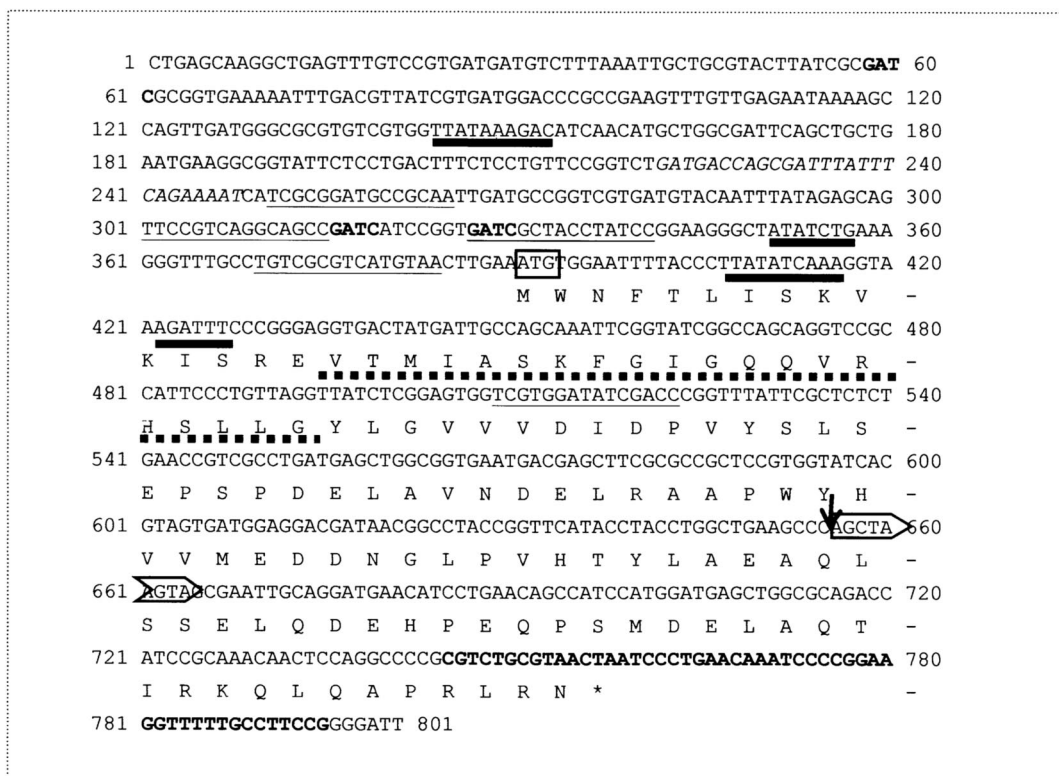
**Precise insertion site of mini-Tn10 and reading frame of *yccV*.** As shown in Fig. 1, mini-Tn10 was found at almost the middle of the *yccV* gene in the M2-24 suppressed strain according to our DNA sequencing data. The *yccV* gene, including its upstream region, has some interesting features; first, it has two possible translational starts, one at Met1-391nt (Fig. 1A) and the other at Met18-442nt, which produce 122- and 104-amino-acid peptides, respectively. Second, the upstream region of *yccV* contains two DnaA boxes, two ATP-DnaA boxes (22, 25), three Dam sites, and surprisingly, five FIS binding sites (10). A putative helix-turn-helix motif is located near the N-terminal region of *yccV*, which is partially deleted when the second translation start is considered. Except with *yccV* paralogs, no significant homology could be found in the GenBank-EMBL database with *yccV* coding sequence by a BLAST search when the GCG program was used.

We constructed a vector (pES6ΔHEE1) that produced *YccV* (from Met1-391nt) fused to hexahistidine at the C-ter-

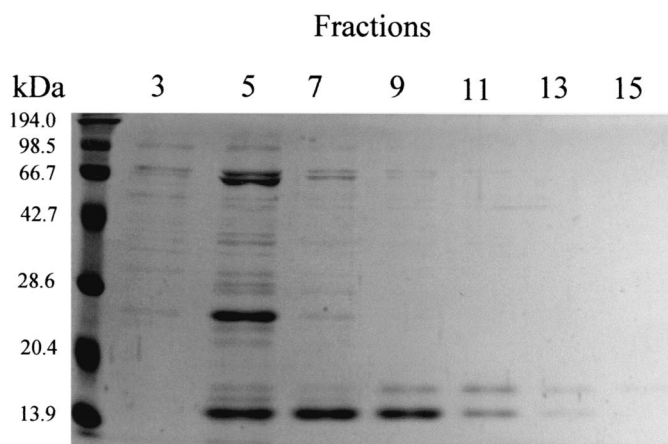
minal end. Western analysis of the purified protein with anti-His tag antibodies revealed that the two translation starts may have been active in our conditions, producing two peptides of the expected length, the more active of which was Met1-391nt (Fig. 1B and C). Construction of plasmid pES6ΔHEE1 was performed as follows. The polylinker of expression vector pDK5 (13) was modified in order to add a polyhistidine tail at the carboxy-terminal end of the cloned gene (at an *XbaI* site [underlined nucleotides]) by insertion of two annealed complementary oligonucleotides, P28 [5' AATTCCTCTAGA(CA T)<sub>6</sub>TGA] and P29 [5' AGCTTCA(ATG)<sub>6</sub>TCTAGAGG], downstream of the *Ptac* promoter between the *EcoRI* and *HindIII* restriction sites, resulting in plasmid pES6ΔHE. The *yccV* gene was then PCR amplified from the *E. coli* chromosome with primers P36 (5' GGAATTCGAAGGAGATATACATATGTGG AATTTTACCTTATA) and P31 (5' GCTCTAGAGTTACGCA GACGCGGGGC). The PCR fragment, containing a ribosome binding site (indicated by boldface type in the P36 sequence), was cut by *EcoRI* and *XbaI* (underlined nucleotides) and cloned into pES6ΔHE cut by *EcoRI* and *XbaI* to obtain pES6ΔHEE1.

**Overexpression of *dnaA* in *yccV* mutant.** *dnaA46 aphA yccV*::mini-Tn10 (KA413Δ*H yccV*) can form colonies on LB agar plates at 40°C but not at 42°C. Suppression does not depend on the *aphA* mutation (21) since *yccV*::mini-Tn10 can suppress the thermosensitivity of KA413. *yccV*::mini-Tn10 also suppresses another *dnaA* thermosensitive mutation, *dnaA508*

**A**



**B**



**C**

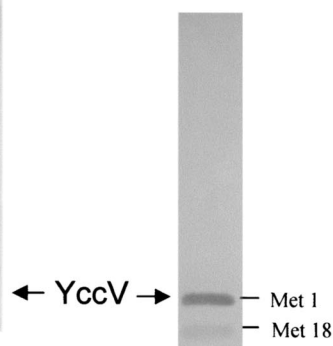



FIG. 1. *yccV* sequence and purification of the His-tagged YccV protein. (A) Coding region (122 amino acids) and flanking sequences. The start codon is enclosed in a box. A putative helix-turn-helix motif (according to the hthscan program in GCG sequence analysis software) is underlined with a dotted line. The vertical arrow indicates the location of the mini-Tn10 insertion in the nucleotide sequence. The horizontal arrow indicates the 9 bp duplicated upon transposon insertion. A putative transcription promoter sequence described by Blattner et al. (3) is indicated by italics. A putative transcription terminator (according to the GCG software) is indicated by boldface type. GATC sites are indicated by boldface type. Sequences that exhibit a reasonable match with suggested consensus binding sequences for DnaA protein (22, 25) and for FIS (10) are underlined with thick and thin lines, respectively. (B) Purification. Strain JM109 harboring plasmid pES6ΔHEE1 (Table 1) was grown in LB medium containing ampicillin (100 μg/ml). At an optical density at 600 nm of 0.8, 0.5 mM isopropyl-β-D-thiogalactopyranoside was added to induce synthesis of the fusion protein for 3 h at 37°C. The YccV protein was purified according to the manufacturer's instructions by using 5 ml of chelating Sepharose FF (Pharmacia) coupled with nickel ion. The results of polyacrylamide gel electrophoresis of the eluted fractions followed by Coomassie blue staining are shown. Fractions 7 to 9 containing His-tagged YccV (14.9 kDa) were pooled for further experiments. The protein was dialyzed against buffer containing 50 mM Tris-HCl, 5 mM EDTA, 1 mM dithiothreitol, and 1 M NaCl (pH 7.5) supplemented with 10% glycerol before freezing at -80°C. (C) Western blot analysis of the purified fractions with monoclonal anti-His tag antibodies.

Strains	C600 <i>wt</i>		<i>dam</i>		<i>yccV</i>		<i>dam</i> <i>yccV</i>	
Relative amount of DnaA	1		0.6		1.65		0.5	
Extract, $\mu$ l	5	15	5	15	5	15	5	15



Strains	KA413 $\Delta$ H ( <i>dnaA46</i> )		<i>dnaA46</i> <i>yccV</i>	
Relative amount of DnaA	1		1.8	
Extract, $\mu$ l	15	30	15	30

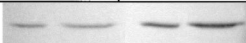


FIG. 2. Quantitation of DnaA protein. Overnight cultures were diluted 100-fold in LB medium and grown to an optical density at 650 nm of 0.8 at 37°C (C600 derivatives) or at 30°C (KA413 derivatives). Cells were collected by centrifugation and lysed in buffer containing 25 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 20% glycerol, and 50 mM  $\beta$ -mercaptoethanol. Total protein contents were estimated by the Bradford assay, and cell extracts were normalized on the basis of their protein concentrations. Extracts were serially diluted into sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer and boiled. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the samples were transferred to a polyvinylidene difluoride membrane for immunoblot analysis. DnaA protein was detected on the membrane by using an anti-DnaA antiserum with the aid of a peroxidase-conjugated secondary antibody in the presence of 1,4-dichloronaphthol. The signal on the membrane was scanned and quantified with the Imagequant program (Molecular Dynamics).

(strain WM1032) at 40°C. The main mechanism of suppression of temperature sensitivity of *dnaA*(Ts) is either induction of stable DNA replication (14) or overexpression of *dnaA*(Ts) (8).

We examined the possibility of overexpression by measuring cellular concentrations of the DnaA46 protein in both the mutant and revertant cultures by immunoblotting analysis followed by densitometric quantification. We found 1.8 times more DnaA46 protein in the revertant culture than in the parental strain (Fig. 2). This overexpression can explain the reversion of temperature sensitivity at 40°C.

Since the *dnaA* promoter region contains many Dam sites like *oriC* and its activity is affected by Dam methylation (5, 15), we tested the effect of introducing the *dam-13* mutation into the *yccV* single mutant. Again, quantitative immunoblot analysis (Fig. 2) demonstrated that introduction of the *dam-13* mutation resulted in reductions in the amount of the DnaA protein in both the wild-type and *yccV* strains to the same level, indicating that Dam methylation is required for overexpression of *dnaA* even in the presence of the *yccV* deletion.

In order to confirm that the overexpression of *dnaA46* in the *yccV* deletion mutant operates at the transcriptional level, we compared expression of *lacZ* under the control of *dnaAP1* and *-P2* (4) in strain WM2007 with and without *yccV* deletion and

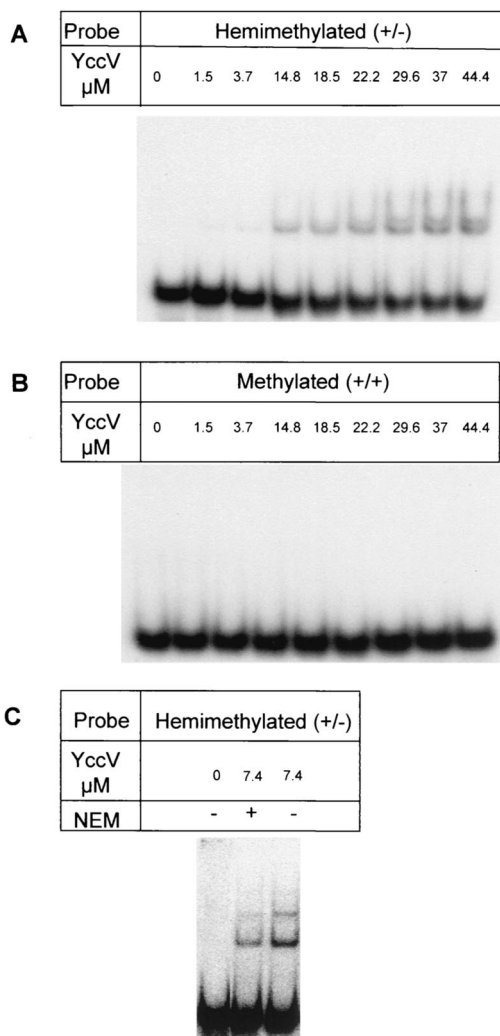


FIG. 3. Gel shift experiments. A  $^{32}$ P-labeled double-stranded oligonucleotide (75 bp) corresponding to *oriC* (nucleotide positions 74 to 148) was used as the probe for gel retardation experiments in the presence of different amounts of YccV protein. Hemimethylated (A and C) or fully methylated (B) probe was generated by annealing equal amounts of the complementary oligonucleotides, including (if necessary) *N*<sup>6</sup>-methyl deoxyadenosine, at the four GATC sites. After hybridization, the double-stranded oligonucleotide was purified by 12% polyacrylamide gel electrophoresis before labeling with [ $\gamma$ - $^{32}$ P]ATP (3,000 Ci/mmol; Amersham) for 1 h at 37°C in the presence of T4 polynucleotide kinase (Promega). The labeled oligonucleotide was then purified through a Micro Bio-Spin 30 chromatography column (Bio-Rad). Gel retardation assays were performed as described by Taghbalout et al. (26) by using 20- $\mu$ l reaction mixtures containing 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 5 mM MgCl<sub>2</sub>, and 0.1% bovine serum albumin, the radioactive probe (5,000 cpm/0.5 fmol), and poly(dI-dC) as a competitor added at a 1,000-fold excess. After 10 min of incubation at room temperature, the reaction mixtures were loaded onto native 5% polyacrylamide gel electrophoresis gels, and electrophoresis was carried out at 4°C with a constant voltage in 0.5 $\times$  Tris-borate-EDTA buffer. (A and B) YccV prepared as described in the legend to Fig. 1. (C) YccV preparation dialyzed against the final buffer without dithiothreitol was preincubated with 10 mM *N*-ethylmaleimide (NEM) (+) for 15 min at 37°C. -, no NEM treatment.

found that the beta-galactosidase activity was consistently 1.5 times higher in WM2007 *yccV* than in WM2007, indicating that there was a higher level of transcription of *dnaA* in the *yccV* deletion mutant (data not shown).

The effect of the *yccV* mutation is not specific for *dnaA* since it also increases *malE* expression.

**YccV as a hemimethylated DNA binding protein.** The observation that the *dam-13* mutation cancels *dnaA* overexpression provoked by the *yccV* deletion suggests that YccV can negatively regulate *dnaA* gene expression when its promoter region is either methylated or hemimethylated. This could occur through binding of YccV itself to fully or hemimethylated DNA.

We examined this possibility by performing gel retardation experiments using as a probe either fully methylated or hemimethylated DNA corresponding to a part of *oriC*, in the presence of His-tagged YccV. An *oriC* probe was used because it contains multiple Dam sites.

The results of this experiment demonstrated that in fact YccV bound preferentially to the hemimethylated DNA probe, forming two complexes, whereas it failed to bind to the fully methylated DNA (Fig. 3A and B). A 50% shift of the hemimethylated probe (50 pM) was obtained at a high concentration of YccV (40 μM), indicating that the affinity of YccV for the hemimethylated DNA was relatively low compared to the affinity of SeqA. However, this shift was not due to contamination of SeqA during preparation of the His-tagged YccV since pretreatment of the preparation with *N*-ethylmaleimide (10 mM) for 10 min at 37°C, which inactivates SeqA (2), did not affect the binding activity (Fig. 3C).

We examined the sequence specificity of YccV by performing DNase I footprinting with an *oriC* probe and did not find any protection even at a high concentration of YccV. Therefore, we cannot tell the sequence of YccV binding sites.

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#### ADDENDUM

After our oral presentation concerning the *yccV* mutant at an EMBO workshop in 2000, T. Katayama communicated to us that he and coworkers isolated a *yccV* deletion as a suppressor of *dnaA46* a few years ago.

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