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Helicobacter pylori oriC—the first bipartite origin of chromosome replication in Gram-negative bacteria

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ABSTRACT

Binding of the DnaA protein to *oriC* leads to DNA melting within the DNA unwinding element (DUE) and initiates replication of the bacterial chromosome. *Helicobacter pylori oriC* was previously identified as a region localized upstream of *dnaA* and containing a cluster of DnaA boxes bound by DnaA protein with a high affinity. However, no unwinding within the *oriC* sequence has been detected. Comprehensive *in silico* analysis presented in this work allowed us to identify an additional region (*oriC2*), separated from the original one (*oriC1*) by the *dnaA* gene. DnaA specifically binds both regions, but DnaA-dependent DNA unwinding occurs only within *oriC2*. Surprisingly, *oriC2* is bound exclusively as supercoiled DNA, which directly shows the importance of the DNA topology in DnaA-*oriC* interactions, similarly as previously presented only for initiator-origin interactions in Archaea and some Eukaryota. We conclude that *H. pylori oriC* exhibits bipartite structure, being the first such origin discovered in a Gram-negative bacterium. The *H. pylori* mode of initiator-*oriC* interactions, with the loop formation between the subcomplexes of the discontinuous origin, resembles those discovered in *Bacillus subtilis* chromosome and in many plasmids, which might suggest a similar way of controlling initiation of replication.

INTRODUCTION

Initiation is the first and strictly regulated step in chromosome replication (1,2). The basic mechanism of initiation is conserved in bacteria, Archaea and Eukaryota: a multiprotein complex (i.e. initiator) recognizes and binds a specific chromosomal region (or multiple regions in Archaea and Eukaryota) known as the origin of replication (*ori*) (3–5). The formation of an initiator-*ori* complex leads to DNA unwinding within the helically unstable AT-rich region. In bacteria, Archaea and lower Eukaryota, the *ori* regions are characterized by the presence of specific initiator binding sequences (5). However, no conserved initiator binding sequences have been identified in higher eukaryotes (metazoans), whose *ori* regions are featured by less-specific markers including CpG islands, DNA topology (especially negative supercoiling, loop formation) or nucleosome-free regions (5,6).

Most of the information about bacterial chromosome replication comes from studies on *Escherichia coli*, whose key initiation elements, *oriC* and DnaA, have been thoroughly characterized (reviewed in (7–9)). DnaA is composed of four functional domains, which are specialized in DnaA oligomerization, interaction with other proteins (e.g. DnaB, DiaA, Hda, HU) or cofactors (ATP/ADP), and DNA binding (9). The *E. coli oriC* consists of high- and low-affinity DnaA binding sites (DnaA boxes), an AT-rich region with a DNA unwinding element (DUE), and the binding sites for regulatory proteins IHF, Fis, IciA and SeqA (7,8). Sequential, cell-cycle coordinated DnaA binding to the DnaA boxes leads to formation of a highly ordered nucleoprotein complex (orisome) resulting in DNA unwinding at the DUE (7,8). Once the open complex is formed, DnaB, DnaG and finally DNA Pol III are loaded, forming replication forks which bi-directionally synthesize nascent DNA strands.

The initiation of chromosome replication is much less understood in bacteria other than *E. coli*. Almost all bacteria encode DnaA homologs (10,11). In all Gram-negative and in many Gram-positive bacteria, *oriC* is composed of a single DnaA-box cluster (DBC) and the DUE, which are localized in the intergenic

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region, usually, with the notable exception of *E. coli,* upstream or downstream of *dnaA* (12). However, in a few bacteria such as Gram-positive *Bacillus subtilis* and mollicute *Mycoplasma pulmonis*, the *oriC* is composed of two or three clusters indispensible for *oriC* activity (13–15). In such cases, DnaA binds and oligomerizes onto individual clusters, but usually also interacts with DnaA molecules bound to neighboring clusters, often forming a DNA loop (16,17). The AT-rich region with a DUE, a second conserved modular element of *oriC*s, is composed of a few tandem AT-rich repeats (e.g. 13-mers in *E. coli*) or is a stretch of an AT-rich sequence (27-mer in *B. subtilis* or 19-mer in *Mycobacterium tuberculosis* (16,18)).

Advanced *in silico* genome analyses have allowed many putative bacterial *oriC* regions to be identified. The methods are mainly based on the cumulative analysis of the genome skews, localization of DBC and an AT-rich region in the vicinity of the *dnaA* gene (10,19,20). However, the reliability of the *in silico* identification is limited and the results need to be confirmed experimentally. The DnaA binding sites might be localized outside *oriC* and serve as negative regulators of chromosome replication (*datA* in *E. coli* (21), D78 cluster in *Streptomyces coelicolor* (22) and DBCs in *B. subtilis* (23)) or regulatory sites in the promoters of genes controlled by DnaA, including *dnaA* autoregulation (24–26). Similarly, the *in silico* predicted helically unstable DNA sequences might be connected with gene transcription; thus their melting upon initiator binding should be experimentally proved. Indeed, out of many predicted bacterial *oriC* regions only a small number have been shown to be functional *in vivo*, whereas the DUE regions have been precisely localized only in a few of them (20).

Helicobacter pylori oriC has been identified *in silico* and, subsequently, DnaA-*oriC* interactions were characterized by a number of *in vitro* experiments (27–29). It is localized upstream of *dnaA* and contains five DnaA boxes bound with different affinities by DnaA (28,29). The binding of DnaA to *oriC* is enhanced in the presence of HobA—a protein interacting with DnaA, which is a structural homolog of DiaA from *E. coli* (30,31). The *oriC* region does not contain any other sequences related to known protein binding sites such as IHF or Fis, and genes encoding proteins homologous to known *oriC*-interacting proteins are not present on the *H. pylori* chromosome. Despite many attempts, no unwinding of the *oriC* region has been detected *in vitro* so far.

In this study, we used combined computational and experimental analyses to identify and characterize the DUE site within *H. pylori oriC*. In contradiction to our previous assumptions, this region was predicted downstream of the *dnaA* gene (here called *oriC2*). However, both upstream (*oriC1*) and downstream (*oriC2*) *dnaA* regions are required *in vivo* for the initiation of *H. pylori* chromosome replication, which indicates a bipartite structure of *H. pylori oriC*. Interestingly, *oriC2* is bound by DnaA exclusively as supercoiled, resembling some archaeal and eukaryotic initiators, whose DNA binding activity also relies on local DNA topology.

MATERIALS AND METHODS

Materials, strains and culture conditions

The plasmids, proteins and bacterial strains used in this work are listed in Table 1. The oligonucleotide sequences are presented in Table 2. *H. pylori* 26 695 genomic DNA was used as a template to amplify DNA fragments for electron microscopy (EM), surface plasmon resonance (SPR) and cloning; *H. pylori* N6 $\Delta dnaA_{H(L)}$ was used to prepare pTZ57R/TX plasmids. *E. coli* was grown at 37°C on solid or in liquid Luria-Bertani medium, supplemented with 100 μg/ml ampicillin when necessary. *H. pylori* was cultivated as described previously (32).

In silico methods

WebSIDD (33) was used for the prediction of putative DUE(s) (34) in the *H. pylori* 26 695 *dnaA* region. The *dnaA* (HP1529)—*punB* (HP1530) upstream intergenic region (pos. 1 608 816–1 609 315) and the *dnaA* (HP1529)—HP1527 downstream intergenic region (pos. 1 607 325–1 607 824) were subjected to WebSIDD predictions as 2.5 kb DNA fragments with the intergenic regions located approximately in the middle (http://benham.genomecenter.ucdavis.edu/sibz/). Default values (37°C, 0.1 M salt, circular DNA, copolymeric) were chosen for the predictions, and negative superhelicity values were tested in the range of $\sigma = -0.040$ (low) to $\sigma = -0.060$ (high) in increments of 0.005 (33). The prediction output data were obtained as raw text files and further processed with Microsoft Excel v97SR-1 and Corel Draw v.11.

Construction of *H. pylori* N6 $\Delta dnaA_H$ mutant

H. pylori N6 $\Delta dnaA_H$ mutant was constructed exactly as described for *H. pylori* N6 $\Delta dnaA_L$ (*32*) using pILL2157 instead of pILL2150 (*35*).

Surface plasmon resonance

SPR analysis was done as previously described (30). Biotinylated DNA fragments were obtained by polymerase chain reaction (PCR) with the following primer pairs: *oriC1* (P-6 and P-11; 191 bp), *oriC2* (P-8 and P-12; 296 bp), non-box DNA (P-13 and P-14; 191 bp).

P1 nuclease assay and PE analysis

P1 nuclease assay was performed as described (36) with several modifications. The reaction mixture (15 μl) contained 25 mM Hepes-KOH (pH 7.6), 12% (v/v) glycerol, 1 mM $CaCl_2$, 0.2 mM EDTA, 5 mM ATP, 0.1 mg/ml BSA, 200 ng of pori1ori2, pori2 or pori1 (50, 80 or 76 fmol, respectively) and *H. pylori* DnaA (50, 100, 200 ng; 1, 2, 4 pmol). After incubation at 30°C for 15 min, P1 nuclease (Sigma) was added (0.75 unit in 0.01 M sodium acetate, pH 7.6) and the reaction was continued for 5 min at 30°C. The P1 digestion was stopped by the addition of 85 μl of water and 300 μl of QG buffer (Qiagen) followed by immediate DNA purification using QIAquick spin columns (Qiagen). The P1 digestion was monitored either by restriction analysis or primer extension (PE) analyses. In the case of agarose gels analyses, the whole

Table 1. Strains, plasmids and proteins used in this work

Strain/plasmid/protein	Relevant genotype/feature	Reference/source
H. pylori		
26695	Parental strain	(68)
N6 Δ*dnaA*$_L$	N6 Δ*dnaA*Ω*aphA-3*, pILL2282, underproducing DnaA when compared with wild type 26695.	(32)
N6 Δ*dnaA*$_H$	N6 Δ*dnaA*Ω*aphA-3*, pILL2157*dnaA*, overproducing DnaA when compared with wild type 26695.	This work
Plasmids		
GHPAQ41	DNA fragment of *H. pylori* 26695 genome (1607422-1609162 bp) cloned into SmaII site of pUC18, Amp^R. The *H. pylori* DNA encodes *dnaA* flanked by 164 bp upstream (*oriC1*) and 202 bp downstream (*oriC2*) of the gene.	TIGR/ATCC microbial genome special collection
pOC170	Plasmid carrying the *E. coli oriC* sequence, the replication origin of pBR322 on the NotI cassette and the *bla* gene of pBR322.	(69)
pILL2157	IPTG-inducible *E. coli* and *H. pylori* expression vector, Chl^R	(35)
pTZ57R/T	Cloning vector, Amp^R	Fermentas
pori1ori2	A pOC170 derivative, lacking *E. coli oriC*, containing DNA fragment of *H. pylori* 26695 genome (1607422–1609162 bp), restricted from GHPAQ41 with EcoRI and PstI.	This work
pori1	A pOC170 derivative, lacking *E. coli oriC*, containing *oriC1* region amplified with primers P-1 and P-2 and cloned into EcoRI site.	This work
pori2	A pOC170 derivative, lacking *E. coli oriC*, containing *oriC2* region amplified with primers P-3 and P-4 and cloned between EcoRI and PstI sites.	This work
pILL2157*dnaA*	pILL2157 derivative containing *dnaA*, constructed similarly as pILL2282 (32)	This work
pTZ57R/T1	pTZ57R/T derivative carrying gentamicin resistance cassette used for homologous recombination with *H. pylori* N6 Δ*dnaA*$_L$ or N6 Δ*dnaA*$_H$ in order to delete *oriC1*.	This work
pTZ57R/T2	pTZ57R/T derivative carrying gentamicin resistance cassette used for homologous recombination with *H. pylori* N6 Δ*dnaA*$_L$ or N6 Δ*dnaA*$_H$ in order to delete *oriC2*.	This work
pTZ57R/T3	pTZ57R/T derivative carrying gentamicin resistance cassette used for homologous recombination with *H. pylori* N6 Δ*dnaA*$_L$ or N6 Δ*dnaA*$_H$ in order to delete both *oriC1–oriC2* subregions.	This work
pTZ57R/T4	pTZ57R/T derivative carrying gentamicin resistance cassette used for homologous recombination with *H. pylori* N6 Δ*dnaA*$_L$ or N6 Δ*dnaA*$_H$ in order to exchange *aph-3* for gentamicin cassette.	This work
Proteins		
DnaA	Recombinant, untagged *H. pylori* DnaA protein	(28,30)

purified DNA was digested by ScaI restriction enzyme, loaded on a 1% agarose gel and separated. After ethidium bromide staining, the gels were analyzed with the Typhoon 8600 Variable Mode Imager (GE Healthcare). For a single PE reaction (37), 0.3 unit of Taq DNA polymerase (Fermentas), 20 fmol of digested DNA and 350 fmol ^{32}P-labeled primer were used. After PE (30 s at 95°C, 30 s at 55°C and 60 s at 72°C, 30 cycles) samples were separated on an 8% polyacrylamide gel under denaturing conditions and analyzed with the Typhoon 8600 Variable Mode Imager (GE Healthcare).

RIP mapping

Replication initiation point (RIP) mapping was performed similarly as previously described (38–40). *H. pylori* cells were grown in 400 ml of brain heart infusion (32) to OD_{600} = 1.0 and pelleted. The bacterial pellet was resuspended in 30 ml of TEN buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl) and disrupted by the addition of sodium dodecyl sulphate (SDS) and sodium sarkosyl to 1% concentration of each. After one-step extraction with phenol:chloroform (1:1), 1.1 g/ml CsCl and 100 μg/ml Hoechst-33 258 were added to the aqueous phase and the refractive index was adjusted to 1.400 with 5 M CsCl. Then genomic DNA was purified by CsCl gradient ultracentrifugation. To enrich for replicating intermediates, total isolated DNA (400 μg) was passed through a BND-cellulose column pre-equilibrated with NET buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 M NaCl). After washing with 5 column volumes of NET buffer, DNA was eluted at 50°C with NET buffer containing 1.8% caffeine. In order to remove nicked DNA, the recovered DNA (ca 40 μg) was subjected to phosphorylation by T4 kinase (Fermentas) followed by λ-exonuclease (Fermentas) digestion. For PE reaction 1 unit of vent (exo-) DNA polymerase (New England Biolabs), 0.3 or 1.2 μg of prepared DNA and 350 fmol of ^{32}P-labeled primer were used. After 35 cycles of reaction (30 s at 95°C, 30 s at 55°C and 60 s at 72°C) amplification products were separated on an 8% polyacrylamide gel under denaturing conditions and analyzed with the Typhoon 8600 Variable Mode Imager (GE Healthcare).

Table 2. Oligonucleotides used in this work

	5′ – 3′ sequence
P-1	GCGAATTCCGCAAAGCAGCATGAAAATCC
P-2	GCGAATTCTTCAATATTGTTGTTGGTATCCAT
P-3	GCCTGCAGGGTTTAGTAAAAGTCATAAATA
P-4	GCGAATTCCCACAACCCCCCTAAAAAC
P-5	CCAGCGCAAAGCAGCATGAAAATC
P-6	CAATATTGTTGTTGGTATCCATGG
P-7	CCCGCTTTCAATTCAAGTGAATG
P-8	AAAGGGATTTTTTCATGCTTATT
P-9	CCACAACCCCCCTAAAAACG
P-10	CATGTTTGACAGCTTATCATCG
P-11	Bio-CCAGCGCAAAGCAGCATGAAAATC
P-12	Bio-CCGCTTGAACGAATTGAACGAC
P-13	Bio-CTCTATTTTGAAAACCCCTATTTC
P-14	CTATATTTTTTCAATGGTTTAGTGC
P-15	GATGAGTTCCCTGAATTCCC
P-16	CCCATCAATGAGTTTGGTG
P-17	GGCTAACACATTAGAGAGC
P-18	CGCTACCCCCCTATCGTCATT
P-19	CAGTTTCTATGTGAATGAAATTAT
P-20	TTTTGCTGATGGAGCTGCACGCTTTATAATAAGCTAATGGATG
P-21	TCGCCAGTCGATTGGCTGAGGATCTGGTACCCGGGTG
P-22	GCAAAGGACGCCATCGGC
P-23	CCATTTAAAGATCCGCGCGA
P-24	TTTTGCTGATGGAGCTGCACCACATTATTCCCTCCAGGTA
P-25	TCGCCAGTCGATTGGCTGACTATAACCTATTTATGACTTTTAC
P-26	GTTGTTTCTAAAGAAAGTTTTTCA
P-27	GCTCCCTATAAAAATAAGGCTT
P-28	TTTTGCTGATGGAGCTGCACCACCCGGGTACCAGATCC
P-29	TCGCCAGTCGATTGGCTGACCTGGAGGGAATAATGTGAA
P-30	GCTCGATCACAAGGGCTTG
P-31	GTGCAGCTCCATCAGCAAAA
P-32	TCAGCCAATCGACTGGCGA
P-33	CTTATTTTGCTAGGAATTGCTAAAG
P-34	CGCACCCTTTCAAAAAGAGCC
P-35	CGCAAAGCAGCATGAAAATCC

Bio - biotin.

Electron microscopy

60 ng of plasmid DNA was mixed with 30 ng of the *H. pylori* DnaA protein in 20 µl of buffer containing 25 mM Hepes-KOH (pH 7.6), 12% (v/v) glycerol, 1 mM $CaCl_2$, 0.2 mM EDTA and 5 mM ATP. Samples were incubated for 15 min at 30°C followed by fixation in 0.2% glutaraldehyde for 10 min at 30°C. DNA was purified by gel filtration using Sephacryl S500 (GE Healthcare) and spin columns in buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ (41). The purified nucleoprotein complexes were adsorbed to mica as described (42). In the case of samples subjected to restriction enzyme digestion, glutaraldehyde was titrated by the addition of 50 mM glycylglycine (10 min at 30°C) and subsequently the sample volume was increased to 60 µl by the addition of 24.5 µl of water, 5 µl of $MgCl_2$ (0.1 M), 5 µl of NaCl (1 M) and the appropriate restriction enzyme (5 units, 0.5 µl). Digestion was carried out for 1 h at 37°C. DNA was treated afterwards as described for undigested samples. The formed complexes were analyzed using a Philips CM100 electron microscope (FEI, Hillsboro, USA) with a Fastscan CCD camera (TVIPS, Gauting, Germany). The positions of the proteins bound to the DNA were measured on 35 mm negatives using an LM4 digitizer (Brühl, Nuremberg, Germany).

Immunoprecipitation assay

The immunoprecipitation assay was done as described elsewhere (43). *H. pylori* cells were grown in 30 ml of brain heart infusion (32) to $OD_{600} = 1.0$. The affinity-purified anti-DnaA rabbit antibody was used to precipitate DnaA–DNA nucleoprotein complexes. The following primers were used to amplify regions of interest: P-5 and P-6—*oriC1*, P-7 and P-8—*oriC2*, P-15 and P-16—*dnaN* gene fragment, P-17 and P-18—*dnaB* gene fragment. The PCR fragments were resolved in 1% agarose gel and analyzed on a Pharos FX Plus imager (Bio Rad).

Deletion of *oriC1* and *oriC2* regions *in vivo*

pTZ57R/TX (X: 1, 2, 3, 4) plasmids (Table 1 and Figure 8), bearing DNA fragments which allowed for homologous recombination with desired *H. pylori* chromosomal regions and the resistance cassette cloned into pTZ57R/T plasmid (Fermentas) were used to delete the *H. pylori oriC* (sub)regions. The fusion amplicons of *H. pylori* DNA and gentamicin cassette were prepared by the three-fragment PCR fusion method (44). N6ΔdnaA$_L$ was used as a template to amplify the *H. pylori* DNA. The gentamicin cassette was amplified from pUC1813Gm (45) using primers P-31 and P-32. Primer pairs P-19, P-20 and

P-21, P-22 were used to amplify ca 300 bp regions upstream and downstream of *oriC1*, while the external P-19 and P-22 primers were used to amplify the final fused DNA used to delete *oriC1*. The same approach was used to construct cassettes to delete *oriC2* (primers P-23, P-24 and P-25, P-26), the whole *oriC* region (P-19, P-20 and P-25, P-26) or to exchange *aph-3* for gentamicin (P-27, P-28 and P-29, P-30). Resulting fusion PCRs were cloned into pTZ57R/T plasmid. The obtained plasmid constructs were used to transform *H. pylori* N6 $\Delta dnaA_H$ or N6$\Delta dnaA_L$ strains. Mutant selection was done on BA plates supplemented with 20 μg/ml apramycin and 1 mM IPTG.

RESULTS

In silico prediction of the DUE in the *H. pylori* replication origin

In previous experiments we showed that *in silico* predicted *H. pylori oriC* was specifically bound by DnaA (27,28), but we could not show any DNA unwinding within the identified sequence (data not shown). Therefore we aimed for *in silico* predictions and employed the WebSIDD tool to localize the DNA-unwinding site within *H. pylori oriC*. WebSIDD was developed by Bi and Benham to predict SIDD (stress-induced DNA duplex destabilization) sites, i.e. short DNA sequences that are prone to strands opening under negative superhelical stress (33). Although designed for the analysis of superhelicity-responsive promoters in prokaryotes (46), we thought it likely that an origin-specific DUE (34) might be detectable by this prediction method.

The results of the WebSIDD predictions are shown in Figure 1 (upper 2 panels, see also size-adjustable Supplementary Figure S1). A single strong SIDD site is predicted for the *dnaA* downstream region (*oriC2*) with strand-opening at high negative superhelicity in a particularly AT-rich stretch of 64 bp (AT: 81% versus 61% of overall AT content) (Figure 1, see also Figure 4). No significant prediction for an SIDD site was obtained for the *dnaA* upstream region (*oriC1*). To address the question whether the predicted SIDD site in *oriC2* coincides with the DUE of *H. pylori oriC*, we performed WebSIDD prediction analyses for *oriC* of *E. coli* and the *incC* (47) region of *B. subtilis oriC*. The predicted strong SIDD sites in both cases agree well with the sites of DNA unwinding *in vitro* (16,48) (Figure 1, lower 2 panels). This supports the notion that the WebSIDD prediction is meaningful also for *H. pylori oriC*2 and suggests that the method can be used to predict the DUE sites of other bacterial chromosomes (CW, manuscript in preparation).

DnaA-dependent unwinding takes place at *oriC2 in vitro* and *in vivo*

In order to experimentally verify the *in silico* identified *H. pylori* DUE, two experimental methods were applied: P1 nuclease assay, and RIP mapping. The first allows localization of the DNA sequences unwound upon DnaA binding *in vitro*, whereas the second maps the start site for chromosome replication at the nucleotide level directly in the cell.

For the P1 nuclease assay we used plasmids containing single (pori1 or pori2) or the two *oriC* regions separated by the *dnaA* gene, exactly as on *H. pylori* 26 695 chromosome (pori1ori2). The supercoiled plasmids were incubated with the increasing amounts of *H. pylori* DnaA protein and subsequently treated with P1 nuclease, which hydrolyzes single-stranded DNA at the opened helix and hence linearizes the unwound plasmid. The further digestion by ScaI or BglII (pori1ori2 and pori1) and ScaI or DraI (pori2), which cut the plasmids once, excised the DNA fragment from the plasmid and allowed us to approximately determine the region unwound by DnaA (Figure 2). In the case of pori2 and pori1ori2, a DNA fragment of about 0.7 kb was excised by P1 and ScaI, whereas 0.8 and 1.7 kb was excised by P1-DraI and P1-BglII, respectively. The excision was observed only in the presence of DnaA and never

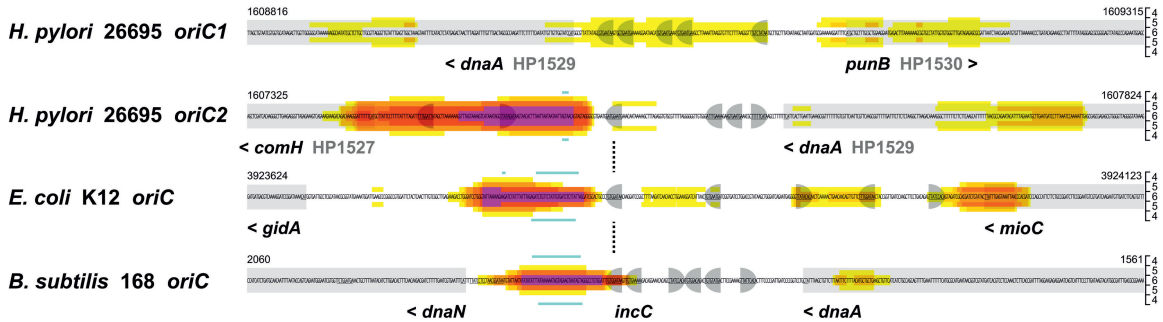


Figure 1. *In silico* prediction of the DUE in *H. pylori oriC*. The heatmaps visualize the WebSIDD predictions for the central 500 bp of the 2.5 kb DNA sequences analyzed. Energy input values (kcal•mol^{-1}) required for strand separation as predicted basewise are shown by the following color code: no color >5, yellow <5, light orange <4, orange <3, dark orange <2, red <1, pink <0 above and below the sequence. Superhelicity values tested from σ = –0.040 (4) to σ = –0.060 (6) in increments of 0.005 are shown as the *y*-axis on the right of the heatmaps, mirrored on the sequence. Genome position numbering is according to GenBank entries for *H. pylori* 26695 [AE000511], *E. coli* K12 W3110 [AP009048] and *B. subtilis* 168 [AL009126]. Open reading frames are shown as light gray boxes with the assigned gene names (and/or IDs); arrowheads indicate the direction of transcription. DnaA-binding sites are shown within the DNA sequences as gray half-circles, rightward-bound for the consensus TTWTNCACA and leftward-bound for the reverse orientation TGTGNAWAA, respectively, according to Schaper and Messer (1995) (51). Light blue lines above and below the heatmaps indicate the experimentally determined unwound regions; data are from this study for *H. pylori oriC2* and taken from Krause *et al.* (1997) for *E. coli oriC* and *B. subtilis oriC* (*incC*) (16).

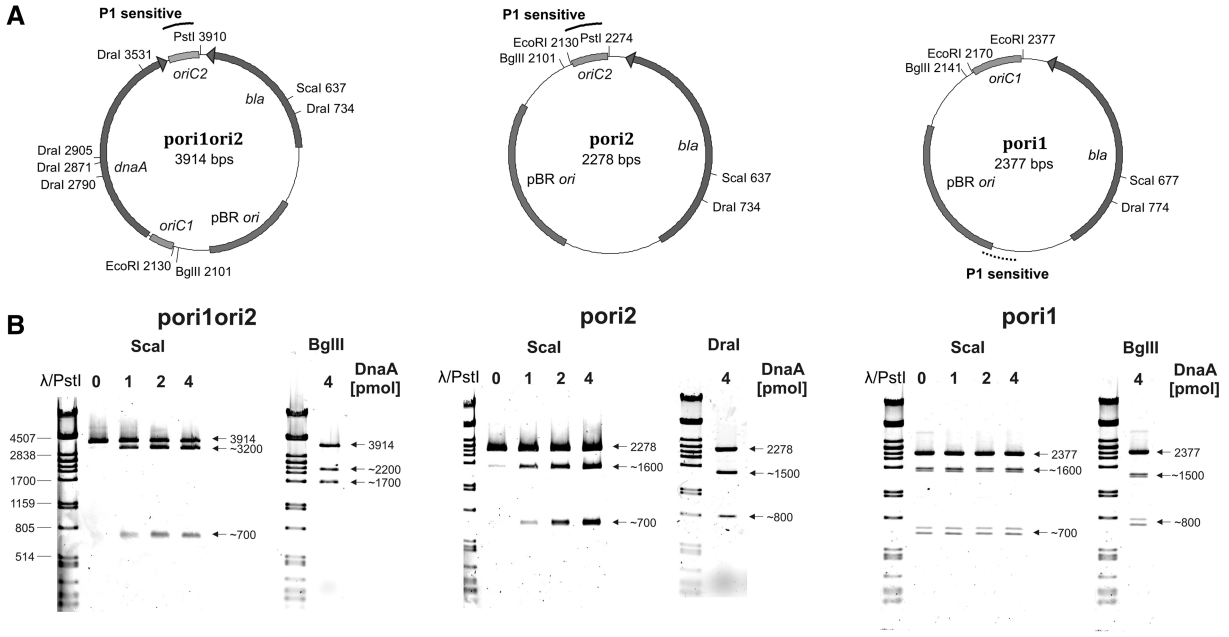


Figure 2. *In vitro* identification of the DUE in *H. pylori oriC*. (**A**) Maps of the plasmids used in the P1 nuclease assay. The *oriC* regions, *dnaA*, *bla,* plasmid origin of replication and the positions of the most important restriction sites are marked. The P1 sensitive sites are indicated; the DnaA-dependent unwinding is distinguished from DnaA-independent by solid and dotted lines, respectively. (**B**) P1 nuclease assay localizing the region unwound by *H. pylori* DnaA. Supercoiled plasmids were incubated with the indicated amounts of the HpDnaA protein, treated with P1 nuclease and restricted by ScaI. The unwinding site was additionally verified by BglII or DraI digestion. The DNA fragments were analyzed by separation in 1% agarose gel and ethidium bromide staining.

occurred outside of *oriC2* (Figure 2B). The presence of the two regions, *oriC1* and *oriC2*, probably favors the orisome formation and unwinding at *oriC2*, because pori1ori2 is melted and the reaction reaches the saturation point at a lower DnaA concentration than pori2 (see also Figure 3). In contrast to *oriC2*, we could not detect any unwinding within *oriC1*, in the case of either pori1 or pori1ori2. The size of ScaI- or BglII-excised DNA fragments from pori1 indicated that melting was DnaA-independent and took place within a vector sequence mapping around the plasmid origin (i.e. helically unstable region, Fig. 2). This is consistent with the known phenomena that plasmids may contain helically unstable regions, usually related to origins of replication or transcription units, which, when negatively supercoiled, might undergo strand separation under certain conditions (49).

The above experiments proved that *H. pylori* DnaA unwinds DNA exclusively within *oriC2*. To precisely determine the unwound region, the P1-digested plasmids were subjected to PE using ^{32}P labeled primers P-9, P-10 and P-35 (Figure 3, Supplementary Figures S2–S4 and Table 2). The primers, which hybridize to the template DNA 90 bp (P-9 on pori1ori2 and pori2, P-10 on pori2) and 130 bp (P-10 on pori1ori2) away from the *in silico* predicted DUE region or 57 bp from box1 of *oriC1* (P-35 on pori1ori2), are extended by Taq polymerase until they reach P1 digested DNA. The observed extension products (Figure 3 and Supplementary Figure S3) confirm that DNA unwinding occurs at *oriC2,* which corresponds well with the predicted DUE sequence and allow one to estimate the unwound region of pori1ori2 and pori2 for about 20 bp (Figures 3 and 4). In the case of pori1ori2, the unwound region is broader, extending in the 3′ direction and reaching up to 52 bp. The fewer unwound base pairs in the case of pori2 might suggest that the interaction with the two *oriC* subregions, when compared with the single *oriC2* origin binding, changes the oligomerization mode of DnaA, and leads to formation of a larger open complex possibly meeting the requirements for loading of other initiation proteins. The increased intensity of the extension products corresponds with the increasing concentration of DnaA and confirms that the reaction is strictly DnaA dependent. Moreover, the intensity of the extension products on pori1ori2 is higher than that on pori2, suggesting that the presence of the two *oriC* sites on one plasmid favors the orisome formation and the unwinding reaction.

To prove that the unwinding *in vivo* occurs at the identified DUE, we performed RIP analysis, which allows determination of the initiation sites in non-synchronized cultures (50). The chromosomal DNA was extracted from exponentially growing *H. pylori* cells and the replication intermediates were enriched on BND-cellulose (see Materials and Methods). Series of subsequent enzymatic reactions selectively separated nascent strands from nicked DNA and prepared the template for the PE reaction. The shortest extension product should represent the DNA fragment amplified on a leading strand starting at the unwinding site. The comparison of the lengths of the obtained PE products with the control sequencing reactions indicated that the replication initiation starts within *oriC2* and co-localizes with the identified *in vitro* DUE region (Figure 5, see also Figure 4 and Supplementary Figure S4).

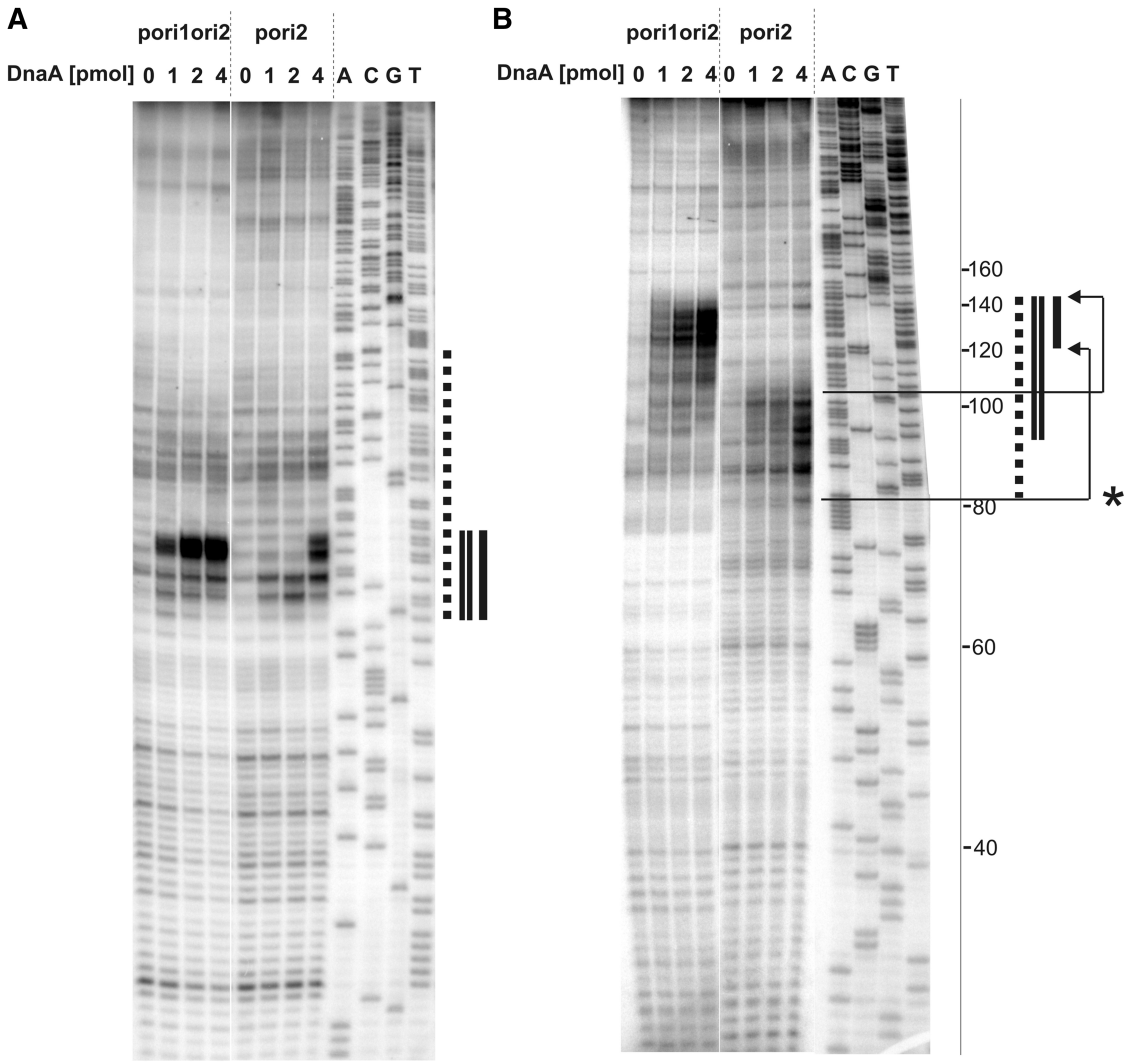


Figure 3. Determination of the *H. pylori oriC* sequence unwound by DnaA *in vitro*. Plasmid DNA, after incubation with the indicated amounts of the DnaA protein and P1 nuclease treatment, was used as a substrate for PE analysis. ^{32}P labeled primers P-9 and P-10 were complementary to the coding strand (with respect to the *dnaA* gene) (**A**) and non-coding strand (**B**), respectively. Dotted line corresponds to the AT-rich region identified *in silico* as a DUE. Single and double lines refer to the areas susceptible to P1 nuclease digestion in pori2 and pori1ori2 plasmids, respectively. A, C, G, T sequencing reactions were carried out with ^{32}P labeled primers P-9 (A), P-10 (B) and the pori1ori2 plasmid DNA. The ladder on the right side of the figure corresponds to the distance from P-10 primer annealing site on pori1ori2. The P-10 PE product on pori2 is 40 bps shorter than that on pori1ori2 (Supplementary Figure S4). Thus to determine the position of the unwound region on pori2 it is necessary to add 40 bp to the observed PE bands positions; * - arrows correspond to the actual position of the pori2 unwound region within *oriC2*.

The results obtained by independent experimental methods clearly demonstrated that the DNA is unwound in the predicted downstream *dnaA* region; thus we postulate that the *H. pylori* chromosome replication starts in *oriC2*.

DnaA binds only supercoiled *oriC2*

It has been shown previously that HpDnaA exhibits the highest affinity for 'strong' TCATTCACA DnaA box and especially for 'twin' DnaA boxes in *oriC1* (boxes 2–3 and 4–5, Figure 4) (27–29). The *oriC2* region is characterized by the presence of a few putative DnaA boxes matching the consensus *E. coli* TTWTNCACA sequence (51) (Figures 1 and 4); however, only one TCATTCACT resembles a strong *H. pylori* DnaA box (28). In order to analyze DnaA binding to *oriC2* and compare the results with DnaA-*oriC1* interactions, we performed SPR, gel shift and EM analyses.

The SPR excluded interactions between DnaA and *oriC2*, while it confirmed previously characterized DnaA binding to *oriC1* (Figure 6A and (30)). Similar results were obtained by gel shifts (data not shown). Moreover, no *oriC2* binding was observed with the *E. coli* DnaA protein, which interacted with *H. pylori oriC1* region (data not shown and (29)). The results were contradictory to the unwinding experiments in which we observed DnaA-dependent DNA melting. However, it has to be noted that, in contrast to SPR and gel shift, in all unwinding experiments supercoiled *oriC* plasmids were used. Therefore, we applied EM to visualize the interaction

Figure 4. *H. pylori oriC1* and *oriC2* regions. Coding strand sequence (with respect to the *dnaA* gene) is presented. The most important features are indicated as follows: genes are distinguished by open boxes, DnaA boxes are shaded in gray, *in silico* identified DUE is indicated by a dotted line, and the position of the RIP (Figure 5) is marked by a thick triangle. Single and double lines refer to the areas unwound by DnaA *in vitro* and susceptible to P1 digestion (as determined by comparison of PE results on primers P-9 and P-10, Figure 3) on pori2 and pori1ori2 plasmids, respectively. The position of the region of the highest P1 nuclease sensitivity is similar for both tested plasmids and is indicated as a major cut region.

between supercoiled *oriC2* and DnaA. The supercoiled pori1 and pori2 plasmids (Table 1) were incubated with DnaA under similar conditions as in the unwinding assay. The complexes were fixed by glutaraldehyde and then visualized by EM. The analysis revealed the interactions between DnaA and each of the plasmids (Figure 6B). Only one DnaA complex was observed on one supercoiled plasmid molecule. The binding of DnaA to *oriC1* and *oriC2* was further confirmed by ScaI digestion and measuring the distance between the bound protein and one of the plasmid ends, similarly as described by Krause *et al.* (16) (data not shown, see also below). The complexes were visible at about 500 nm from the proximal plasmid end which corresponds to the loci of *oriC1* or *oriC2* sites on the respective plasmids (700 bp from ScaI site, see Figure 2). When similar analyses were performed with linear DNA (PCR product or linearized plasmids) the HpDnaA binding to *oriC1* was confirmed while no complexes were visible on *oriC2* (data not shown and Figure 7C). This clearly shows that *oriC2* is bound only when supercoiled, while DnaA binds to linear and supercoiled *oriC1*. Interestingly, the affinity of DnaA for supercoiled *oriC1* was lower than for *oriC2*, since about 16% of pori1 and 41% of pori2 molecules were bound by DnaA (Figure 6C). Thus, these experiments show that superhelicity is the crucial factor enabling specific and high-affinity DnaA-*oriC2* interactions (Figure 6C) leading to DNA unwinding (Supplementary Figure S2).

oriC1 and *oriC2* are both important for initiation

The presented results revealed that *H. pylori* DnaA binds and unwinds the DNA within supercoiled *oriC2*. DnaA also interacts with linear and supercoiled *oriC1* (Figures 6A and 6B and (29)); however, the presence of *oriC1* is not required for DNA unwinding within *oriC2* (Figure 2B). Additionally, the incidence of DnaA binding to supercoiled pori1 is lower compared with pori2 (Figure 6C), suggesting higher DnaA affinity for supercoiled *oriC2* than for supercoiled *oriC1*. The question arose if and why in *H. pylori* both regions are required and what is the exact structure of *H. pylori* origin.

To analyze DnaA binding to *oriC1* and *oriC2*, EM and immunoprecipitation were performed. As observed by EM, the incubation of DnaA with supercoiled pori1ori2 led to formation of a single-nucleoprotein complex per plasmid molecule (Figure 7A). The distance measurements between the plasmid ends and the protein core on ScaI digested nucleoprotein complexes confirmed the binding of DnaA to *oriC1* and/or *oriC2* (Figures 7B and 7E). By P1 digestion of the pori1ori2-DnaA complex we additionally showed, that the protein core of the DnaA-*oriC2* subcomplex is localized upstream from the unwound DUE site (Figure 7D). The pori1ori2 was bound 1.4 and 3.5 times more frequently than pori2 and pori1 plasmids, respectively (Figures 6C and 7E), which shows that the presence of both regions enhanced DnaA binding to plasmid molecules. 65% of the complexes were formed only at one of the sites: 3% at *oriC1* and 62% at *oriC2*, which confirmed higher DnaA affinity for supercoiled *oriC2* than for *oriC1*. 30% of the complexes were visible as protein cores joining the two DNA strands (Figure 7A and B). The looped DNA, preserved after ScaI digestion, indicated that the two strands of a plasmid were indeed joined via DnaA-*oriC1* and DnaA-*oriC2* sub-complexes, similarly as in *B. subtilis* (16). However, unlike in *B. subtilis*, the loop formation was observed only when

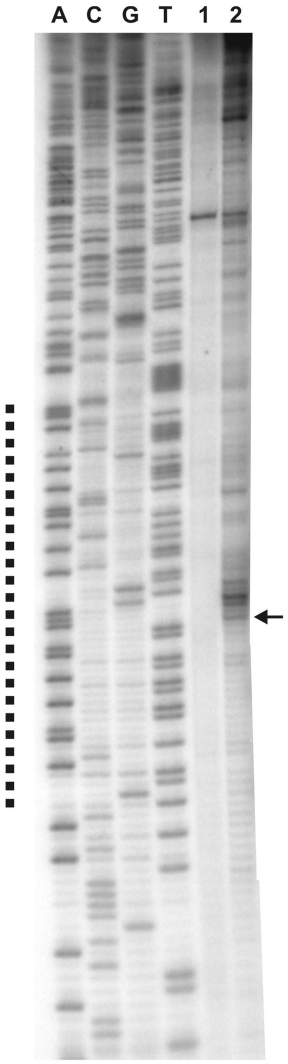
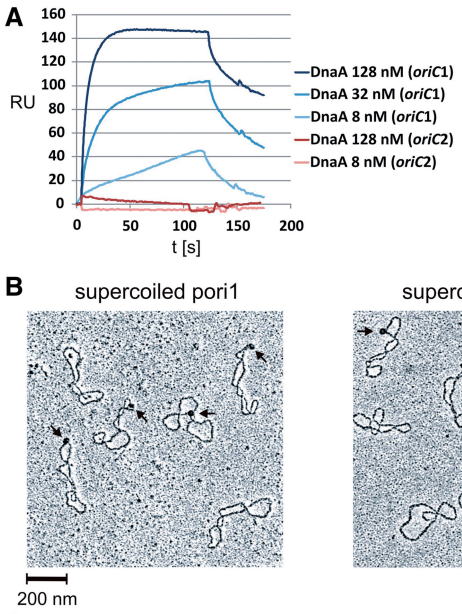


Figure 5. Mapping of the RIP *in vivo*. PE was performed with enriched replication intermediates and ^{32}P labeled oligonucleotide P-9 complementary to the coding strand (with respect to the *dnaA* gene). Dotted line corresponds to the AT-rich region, identified *in silico* as a DUE. Arrow indicates the transition point between continuous and discontinuous DNA synthesis. 0.3 µg (lane 1) and 1.2 µg (lane 2) of a template DNA was used; A, C, G, T sequencing reactions were carried out with ^{32}P labeled primer P-9 and the pori1ori2 plasmid DNA.



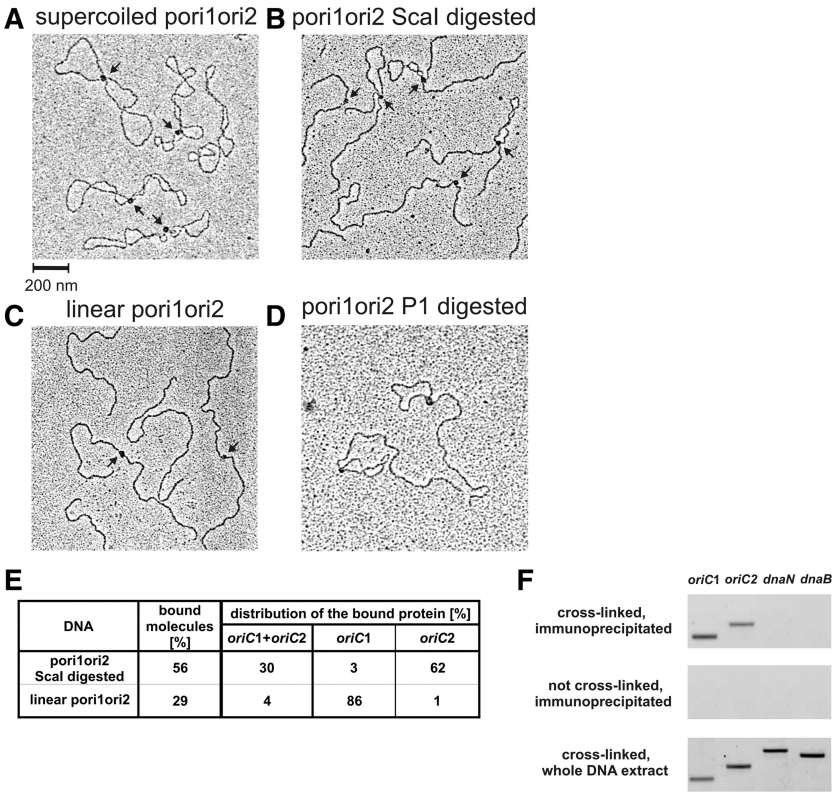
C

DNA	bound molecules [%]	distribution of the bound protein [%]	
		*oriC*1	*oriC*2
pori1	16	91	-
pori2	41	-	94

Figure 6. Comparative analysis of the DnaA interaction with *oriC1* and *oriC2* regions. (**A**) SPR analysis. Protein concentrations and the types of DNA fragments used in the analysis are given in the legend of the sensogram. (**B**) EM study of the DnaA binding to the supercoiled pori1 and pori2 plasmids. (**C**) Statistical distribution and the level of DnaA binding to DNA molecules used in EM. The percentage of bound molecules was calculated on the basis of 250 molecules analyzed. Distribution of complexes on the DNA was evaluated by measuring 120 bound DNA molecules for each plasmid; data refer only to the molecules bound within *oriC1* and/or *oriC2*.

oriC1 and *oriC2* were located on a supercoiled plasmid (Figure 7).

In order to analyze whether the two regions were also bound by DnaA in *H. pylori* cells, immunoprecipitation was performed. Exponentially growing 26 695 *H. pylori* cells were crosslinked with formaldehyde, sonicated and the DnaA–DNA complexes were immunoprecipitated with purified anti-DnaA antibodies. The immunoprecipitated DNA was identified by PCR reactions with pairs of primers amplifying *oriC1*, *oriC2* and the intergenic regions of *dnaB* and *dnaN* genes as negative controls (Figure 7F). The PCR products indicated that *oriC1* and *oriC2* were specifically bound by DnaA, while comparable intensity of the *oriC1* and *oriC2* amplicons suggested equal binding by the initiator protein. Since the *oriC1* DnaA box cluster is located in the promoter region of *dnaA*, the question arose whether *oriC1* is indeed necessary for replication or is used rather for autoregulation of *dnaA* expression. To analyze whether both *oriC*s are important for initiation, a comprehensive *oriC* deletion analysis was performed. To distinguish the role of *oriC1* in initiation and/or in transcription regulation, we used *H. pylori* N6 $\Delta dnaA_H$ and N6 $\Delta dnaA_L$ strains (Table 1), in which DnaA was ectopically synthesized at different expression levels (L, low, H, high) from the pILL2157 or pILL2150 vectors, respectively, and therefore independent from its own promoter (35,32). The pTZ57R/TX (X: 1, 2, 3) plasmids were constructed (Table 1) to allow deletion of *oriC1*, *oriC2* or both regions simultaneously. In three independent transformations we were not able to delete any of the *oriC* regions (Figure 8). The control transformations with pTZ57R/T4 plasmid allowed exchange of kanamycin resistance cassette for gentamicin resistance cassette located between the two *oriC* sites, which confirmed that the inability to delete *oriC1*, *oriC2* or *oriC1–oriC2* regions was not due to transformation problems but was connected with the *oriC* function in *H. pylori* cells.



DNA	bound molecules [%]	distribution of the bound protein [%]		
		oriC1+*oriC2*	*oriC1*	*oriC2*
pori1ori2 ScaI digested	56	30	3	62
linear pori1ori2	29	4	86	1

Figure 7. *In vitro* and *in vivo* analyses of DnaA binding to *oriC1–oriC2* region. (**A**) DnaA interaction with supercoiled pori1ori2. The putative interactions between DnaA-*oriC1* and DnaA-*oriC2* subcomplexes are visible as DNA loops. (**B**) The localization of the complexes at *oriC1* and *oriC2* as well as the interactions between DnaA bound to *oriC1* and *oriC2* were confirmed by ScaI digestion and subsequent measurements. (**C**) DnaA interaction with linear pori1ori2. No loop structures were visible and the DnaA interacted almost exclusively with *oriC1*. (**D**) DnaA interaction with *oriC1* and *oriC2* regions on pori1ori2 and subsequent P1 nuclease digestion. The DnaA-*oriC2* complex is formed between *dnaA* and the unwound DUE region, thus the P1 digestion allows to maintain the loop between *oriC1*-DnaA and *oriC2*-DnaA subcomplexes. (**E**) Statistical distribution and the level of DnaA binding to DNA molecules observed by EM. The percentage of bound molecules was calculated on the basis of 250 molecules analyzed. Distribution of complexes on the DNA was evaluated by measuring 120 bound DNA molecules for each plasmid; data refer only to the molecules bound within *oriC1* and/or *oriC2*. (**F**) *In vivo* immunoprecipitation of *H. pylori* genomic DNA with a purified anti-DnaA antibody followed by PCR analysis. Amplified regions (*dnaN* and *dnaB* served as negative controls) and DNA templates used are indicated on the picture.

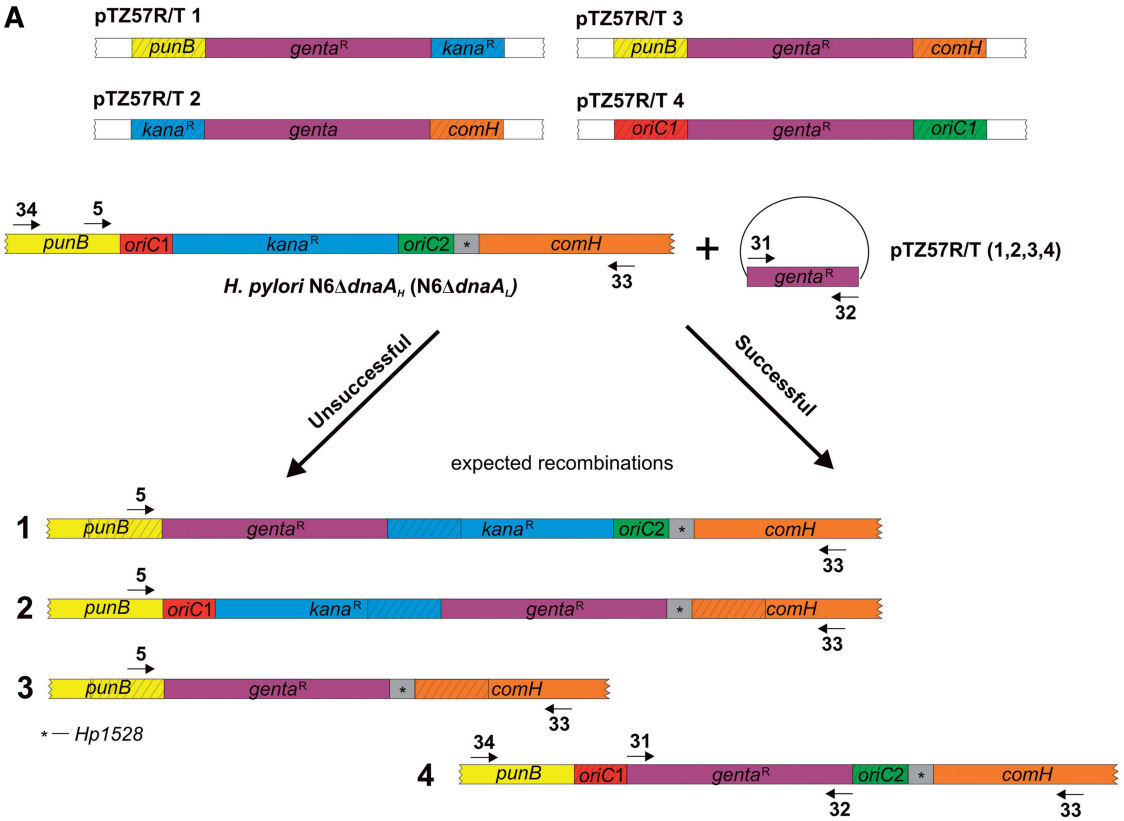
The performed experiments allowed us to conclude that DnaA binds *oriC1* and *oriC2 in vitro* and *in vivo*, and that both regions are important for initiation of *H. pylori* chromosome replication.

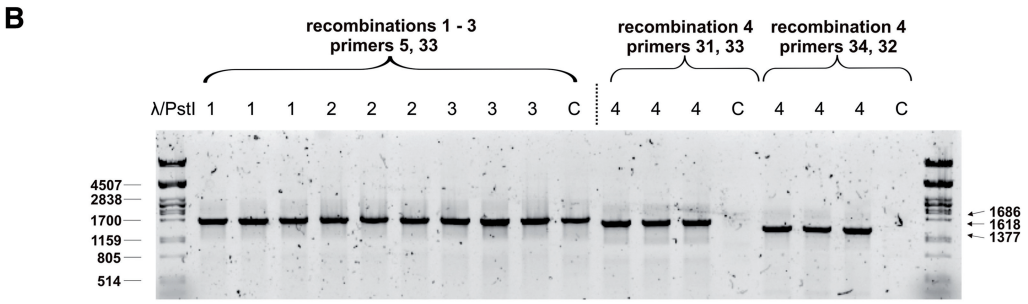
DISCUSSION

The replication of a bacterial chromosome starts at a single chromosomal region (*oriC*) and is initiated by DnaA protein. Though the principle of initiation is similar in almost all bacteria, the *oriC* structure as well as the composition and architecture of nucleoprotein initiation complexes usually differ between unrelated species. In this work, we present new data concerning the initiation of *H. pylori* chromosome replication. Our experiments revealed the bipartite structure of *H. pylori oriC*; besides previously characterized *oriC1*, localized in the upstream region of *dnaA*, we identified the second *oriC2,* situated downstream of *dnaA*, which is subjected to DnaA-dependent unwinding *in vitro* (Figures 2B and 3) and was proven to be the replication initiation site *in vivo* (Figure 5). Neither *oriC1* nor *oriC2* can be deleted from *H. pylori* cells and both of them are bound by DnaA *in vivo*, which presumably leads to the loop formation (Figure 7B). Surprisingly, *oriC2* is bound exclusively as a supercoiled DNA, indicating the importance of the DNA topology in the replication initiation. The recognition of DNA with respect to the particular sequence and topology revealed a new, so far hardly characterized feature of DnaA protein.

oriC2 supercoiling determines DnaA binding and subsequent unwinding

Our previous analysis identified the DnaA box cluster localized on the *H. pylori* chromosome, which, on the basis of the *in vitro* and *in silico* analyses, was assumed to be the *H. pylori oriC* (here named *oriC1*) (28,27). Despite many attempts, we were unable to detect DNA unwinding within *oriC1*. Here we showed that the DNA is in fact unwound within the neighboring region *oriC2*. Surprisingly, we discovered that *oriC2* was bound by DnaA exclusively when present on a supercoiled plasmid, which proved that direct interaction between supercoiled *oriC2* and DnaA leads to the DNA unwinding. In contrast, the *E. coli* DnaA binds *oriC* regardless of its topology (52). The recent data indicate that the *E. coli* initiator can unwind not only supercoiled but also the linear *oriC* (53). Thus, our studies for the first time





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H. pylori N6 Δ*dnaA*+ pILL2150(57)*dnaA*	genta resistance	total number of recombinants	PCR analysis (*oriC* region)		successful
no modification	-	-	1686 bp		-
+ pTZ57R/T 1	+	16	expected 2374 bp observed 1686 bp		0
+ pTZ57R/T 2	+	11	expected 2505 bp observed 1686 bp		0
+ pTZ57R/T 3	+	10	expected 1451 bp observed 1686 bp		0
+ pTZ57R/T 4	+	78	primers 31,33	expected 1618 bp observed 1618 bp	78
			primers 34,32	expected 1377 bp observed 1377 bp	

Figure 8. Comparative analysis of the DnaA interaction with *oriC1* and *oriC2* regions. (**A**) Schematic representation of the strategy used to delete chromosomal *oriC1* and/or *oriC2* regions. Fully viable *H. pylori* N6 Δ$dnaA_H$ and N6 Δ$dnaA_L$ strains with a plasmid-borne *dnaA* gene (Table 1) were used to ensure that *dnaA* expression was not changed in case of *oriC1* deletion. *H. pylori* cells were transformed with pTZ57R/TX (X: 1, 2, 3, 4) suicide vectors in order to introduce the gentamicin resistance gene by recombination between homologous fragments. Successful recombinations should lead to deletion of *oriC1* (pTZ57R/T 1), *oriC2* (pTZ57R/T 2), *oriC1* and *oriC2* (pTZ57R/T 3) or to exchange of the *aph-3* cassette for the gentamicin cassette (pTZ57R/T 4). The first three attempts were lethal for the cells, however, in a small number of transformants the insertion of the gentamicin resistance gene into an unknown chromosomal locus was observed leading to acquisition of the antibiotic resistance without detrimental changes within *oriC* (sub)regions. Control transformation with pTZ57R/T 4 resulted in desired recombination in case of all obtained clones. The primers used PCR analysis are indicated by arrows. (**B**) PCR analysis of a representative set of acquired recombinants. Primers used: P-5, P-33 for pTZ57R/TX (X: 1, 2, 3) transformations; P-31, P-33 and P-34, P-32 for pTZ57R/T 4 transformation; primers P33 and P34 hybridized to the chromosomal regions flanking the designed 3′ and 5′ homology arms, respectively. Symbols above lanes correspond to a recombination type (numbers 1–4) and control reactions with a DNA template of the N6 Δ$dnaA_H$ unmodified strain (C letter). (**C**) Table summary of data relevant to this experiment.

directly showed that eubacterial DnaA is dependent on DNA topology for binding to *oriC*—a phenomenon reported previously only for initiator-*ori* binding in Archaea and higher eukaryotes (metazoans). In Archaea it was recently shown that apart from the ORB sequence, the Orc1/Cdc6 initiator also recognizes the local DNA structure (54). Metazoan ORC exhibit higher affinity for the supercoiled origin than for similar DNA sequences in linear form, and the clear lack of sequence conservation between known origins was already demonstrated (55,56). The *H. pylori* DnaA-*oriC2* binding resembles that of archaeal Orc1/Cdc6-*ori* binding—it is both sequence- (i.e. localized within *oriC2*) and topology-specific. Thus, we conclude that topology-dependent recognition of DNA is not only restricted to archaeal and metazoan initiators, as suggested by Dueber *et al.* (54), but might be common in all three domains of life. All the initiators share the AAA+ domain with the Initiator-Specific Motif (ISM), which was suggested to participate in DNA structure recognition (7). In bacteria the ISM is engaged in ssDNA binding (57,53), but it cannot be excluded that it also recognizes dsDNA and its structure. In the future, we plan to identify a motif in the *H. pylori* DnaA that is responsible for topology-dependent recognition of DNA sequences within *oriC2*. More generally, our study demonstrated the need to reevaluate DnaA sensitivity toward DNA topology for orisome formation and function in other bacteria.

Bipartite structure of *H. pylori oriC*

Bacterial *oriC* regions, characterized by the presence of the DBC and the DUE, are often situated at the 3′ or 5′ regions of *dnaA*. However, in some Gram-positive bacteria, the two DBCs flank the *dnaA* gene. Both clusters can be indispensible for *oriC* activity (*B. subtilis* (13,14) and *Mycoplasma pulmonis* (15)) or one, the 5′ box cluster, is involved in autoregulation of *dnaA* expression whereas the second, the 3′ box cluster, serves as the *oriC* region (*Streptomyces* (58), *Spiroplasma citri* (13), *Mycobacterium* (18)). The two DnaA-box clusters were also reported to act as origins in Gram-negative *Pseudomonas* sp., however no mutual relationship between them have been established and only one of the origins (*oriC1*) was finally shown to be indispensible *in vivo* (59,60). We identified the two DnaA interaction regions on the *H. pylori* chromosome, *oriC1* and *oriC2*, located in the 5′ and 3′ regions of the *dnaA* gene, respectively. Both are bound by DnaA *in vitro* and *in vivo* and neither of them can be deleted from the *H. pylori* chromosome, which suggested the discontinuous structure of the *H. pylori oriC*—the first bipartite origin discovered in a Gram-negative bacterium. The DUE is located within *oriC2* and *in vitro* it does not require *oriC1* for DnaA-dependent unwinding. The question arose then why *H. pylori* requires *oriC1*. Since the *oriC1* DnaA cluster is located in the *dnaA* promoter region, it could be involved in regulation of *dnaA* expression. However, first, the DnaA level is invariant in growing and not growing *H. pylori*, and second, in contrast to some other bacteria (61,62), the alterations in cellular level of the DnaA protein have no effect on frequency of initiation replication in *H. pylori* strains expressing *dnaA* from inducible plasmids ((35,32), Table 1 and data not shown). Moreover, the *oriC1* region cannot be deleted in N6 $\Delta dnaA_H$ strain ectopically expressing *dnaA* from a plasmid (Figure 8, see also Table 1). Thus, we postulate that the *oriC1* region exerts presumably only a marginal influence on *dnaA* expression but is indispensable for the origin activity and that both regions, *oriC1* and *oriC2*, constitute the integral origin of *H. pylori* chromosome replication. The reason why some of the bacterial and plasmid origins are split into two parts is still not fully understood, but it is probably a way to control the initiation events, possibly through loop formation. Loop formation is important for many cellular processes, including initiation of DNA replication (63,64). However, the significance of this phenomenon is still unclear. It may generate cooperativity in the binding of the initiator protein, help to stabilize the nucleoprotein complex or provide the entry site for proteins regulating initiation of DNA replication (64). In *Streptomyces lividans* the observed loop formation between the two DBCs separated by a non-coding 134 bp region was suggested to be important for proper orisome formation (65). In *B. subtilis* the DNA looping between *incA*/*B* and *incC* DBCs was suggested either to be used for a coupled control for *dnaA* expression and the initiation of replication or, similarly as in P1, F factor and R6K plasmid or Epstein–Barr virus (EBV) viral origins constitute regulatory structures for initiation control by association of normally physically separated DnaA box regions ((16,14) and references herein). The initiator-mediated interaction between the two clusters of the initiator binding sites (DnaA boxes or iterons), either by looping (if positioned in *cis*) or by handcuffing (when placed in *trans*), are suggested to be the mechanism of negative regulation of chromosomal and plasmid replication. Interestingly, we have previously shown that HobA, the DiaA-related protein (30), can bridge the interaction between the two DnaA-*oriC1* complexes (32). Particular χ structures have been observed in EM in which HobA mediated the cross-interaction. Our preliminary results suggest that HobA also increases the bridging between DnaA-*oriC1* and DnaA-*oriC2* subcomplexes, but we did not observe any significant influence on DNA unwinding (data not shown). On the other hand, HobA stimulates DnaA-*oriC1* binding, and was suggested to be an activator of the initiation process (30). It is possible that HobA rearranges the orisome structure, but further studies are necessary to elucidate its role in the initiation of *H. pylori* chromosome replication.

The bipartite structure of the bacterial *oriC* region raises an intriguing question of whether and how the self-assembly of DnaA into filaments (66,57) is required for the formation of a functional orisome. Recent studies indicated that in *B. subtilis* inhibition of DnaA helix formation by Soj (an ortholog of ParA) stalls initiation of replication (67). Whether at the certain stage(s) of replication initiation the *H. pylori* DnaA protein forms filament, and more generally, what is the topology of such

complexes with the bipartite *oriC,* is still not known and need to be elucidated in future.

In summary, our analyses showed that *H. pylori oriC* exhibits bipartite structure, being the first such origin discovered in a Gram-negative bacterium. Together with previously identified bipartite *oriC*s in Gram-positive bacteria and plasmids, it might represent a larger group of origins controlled by similar regulatory strategies. Our work also showed for the first time the direct relationship between DnaA initiator and the topology of its target sequence. This suggests that the DNA structure might be an important factor controlling DNA replication in three domains of life.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–4.

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Conflict of interest statement. None declared.

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