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DiaA/HobA and DnaA: A Pair of Proteins Co-evolved to Cooperate During Bacterial Orisome Assembly

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Replication of the bacterial chromosome is initiated by binding the DnaA protein to *oriC*. Various factors control the ability of DnaA to bind and unwind DNA. Among them, *Escherichia coli* DiaA and *Helicobacter pylori* HobA have been characterized recently. They were found to interact with domain I of DnaA and stimulate DnaA binding to *oriC*. We examined HobA and DiaA functional homology and showed that, despite a high degree of structural similarity, they are not interchangeable because they are unable to interact with heterologous DnaA proteins. We revealed particular structural differences impeding formation of heterologous complexes and, consistently, we restored DiaA-enhanced *oriC* binding by the hybrid Ec^{I}-Hp^{II-IV}DnaA protein; i.e. *H. pylori* DnaA in which domain I was exchanged with that of *E. coli*. This proved that DiaA and HobA are functional homologs and upon binding to DnaA they exert a similar effect on orisome formation. Interestingly, we showed for the first time that the dynamics of DiaA- and HobA-stimulated orisome assembly are different. HobA enhances and accelerates HpDnaA binding to *oriC*, whereas DiaA increases but decelerates EcDnaA binding with *oriC*. We postulate that the different dynamics of orisome formation reflect the distinct strategies adopted by *E. coli* and *H. pylori* to regulate the frequency of the replication of their chromosomes. DiaA/HobA homolog have been identified in many proteobacteria and therefore might constitute a common, though species-specific, factor modulating bacterial orisome assembly.

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Abbreviations used: *oriC*, origin of chromosome replication; DUE, DNA unwinding element; ADLAS boxes, ATP-DnaA-preferential low-affinity sites; Ec, *E. coli*; Hp, *H. pylori*, $DnaA^{I-II}$, domains I-II of the DnaA protein; $Ec^{I}Hp^{II-IV}$DnaA, a hybrid protein composed of domain I of *E. coli* DnaA and domains II–IV of *H. pylori* DnaA; BTH, bacterial two-hybrid system; SPR, surface plasmon resonance; RU, resonance unit; X-gal, bromo-chloro-indolyl-galactopyranoside; dTMP, thymidylate.

Introduction

DNA synthesis is tightly controlled and strictly dependent on cell-cycle progression. It is primarily regulated at the first step; i.e. initiation.[1] Bacterial chromosome replication is initiated by cooperative binding of the DnaA–ATP protein to multiple sequences within *oriC* followed by DNA unwinding within the DUE region.[2-4] After DNA unwinding, a set of replication fork-forming proteins is loaded (e.g. DnaB helicase, DnaG primase and DNA III polymerase holoenzyme) and then DNA replication starts. Simultaneously, control mechanisms go into action and restrict replication to once per generation.[5,6] Among bacteria, the regulation of replication initiation is best understood in *Escherichia coli*. Some of the *E. coli* regulatory systems are present in other bacteria. However, numerous species, such as *Helicobacter pylori*, lack most of them[5] or encode species-specific factors such as *Bacillus subtilis* YabA.[7] In general, both *oriC* and DnaA are regulated by multiple mechanisms that control the timing of initiation complex formation and coordinate replication with the cell cycle.[5,6,8] These mechanisms control the assembly or disassembly of a multimeric protein–*oriC* complex (i.e. orisome) and regulate transitions between inactive orisome and an active initiation complex able to unwind DNA. Among bacterial initiation regulators, *E. coli* DiaA and *H. pylori* HobA have been characterized recently as factors that stimulate the formation of the initiation complex.[9-12] Both proteins interact with orisomes via domain I of the DnaA proteins and promote DnaA oligomerization around *oriC*. DiaA is not essential for *E. coli* cells but it is an important factor for synchronized initiations.[9] DiaA-stimulated multimerization of DnaA–ATP enables the protein to interact with ADLAS boxes at *oriC* shortly before initiation. This leads to subsequent DNA unwinding followed by DnaB helicase loading and further replication steps.[10,13] The role of HobA in *H. pylori* is less well understood. In contrast to *E. coli* DiaA, HobA is an essential protein for *H. pylori* survival. It presumably facilitates multimerization and appropriate spatial positioning of DnaA molecules on DnaA boxes within the Hp*oriC* region,[11] and, thus, similar to DiaA, triggers formation of the initiation complex competent in DNA unwinding. DiaA and HobA, despite the lack of sequence conservation (15% identity, 31% similarity), have been shown to be structural homologs. The recently determined crystal structure of HobA–HpDnaA$^{I\text{-}II}$ [12] and NMR analysis of DiaA–EcDnaA$^{I\text{-}II}$ [13] identified amino acids crucial for formation of complexes. The DnaA interaction surfaces involved in HobA–HpDnaA$^{I\text{-}II}$ interactions were similar to those identified in the DiaA–EcDnaA$^{I\text{-}II}$ complex, suggesting a conserved mechanism of binding of DiaA and HobA to DnaA.[12,13]

In this study, we asked whether DiaA and HobA are functional homologs. Using experimental methods and homology modeling, we demonstrated that species-specific binding between the proteins of the initiation complex preclude interchangeability of HobA and DiaA during orisome formation. Consistently, by circumventing species-specific interactions, we restored activation of a hybrid orisome assembly *in vitro*, which indicates that HobA and DiaA exert a similar influence on DnaA during binding to *oriC*. Interestingly, we discovered that the dynamics of HobA/DiaA-dependent *H. pylori* and *E. coli* orisome assembly are different, which can reflect differences in overall regulation strategies controlling the frequency of chromosome replication in the two bacteria. We show that HobA and DiaA are, in fact, phylogenetically related and we postulate that HobA/DiaA homologs are orisome-organizing factors ubiquitous in bacteria from different taxa.

Results

HobA and DiaA are not interchangeable *in vivo*

Despite the low level of sequence similarity, *H. pylori* HobA and *E. coli* DiaA were found to be structural homologs. The proteins form compact tetramers (dimer of dimers), which are the active forms of DiaA and HobA.[10,12,14] Moreover, we found that in a two-hybrid system[15] the two proteins are able to form heterocomplexes (Fig. 1a, sectors 2 and 3), indicating that the dimerization/tetramerization domains of DiaA/HobA are conserved. This supported our hypothesis about the functional homology between DiaA and HobA and aroused our interest to know whether *hobA* can be substituted by *diaA* in *H. pylori* cells and, *vice versa*, whether *hobA* can replace *diaA* in *E. coli*.

Gene replacement by allelic exchange was used to determine whether *diaA* can substitute for *hobA* (Fig. 1b). *H. pylori* 26695, J99 and N6 were transformed with pAZP01 and pAZP07-4 suicide plasmids in parallel (Table 1). pAZP01 encodes *diaA* and *aph-3*, cloned between fragments of *lysC* and *holB*, and should allow for direct replacement of *hobA* with *diaA* on the chromosome. pAZP07-4 served as a positive control of *H. pylori* transformation, site-specific recombination within the locus of interest (allows exchange of *hobA* for a fusion of *hobA* with *c-myc*) and the mutant selection process (introduces the *aph-3* cassette). After transformation with pAZP07-4, the correct transformants were selected for each strain, which was proved by PCR product digestion and sequencing (data not shown). In contrast, when using pAZP01 we did not obtain any mutant, which suggests that *hobA* replacement by *diaA* on the *H. pylori* chromosome is lethal for the

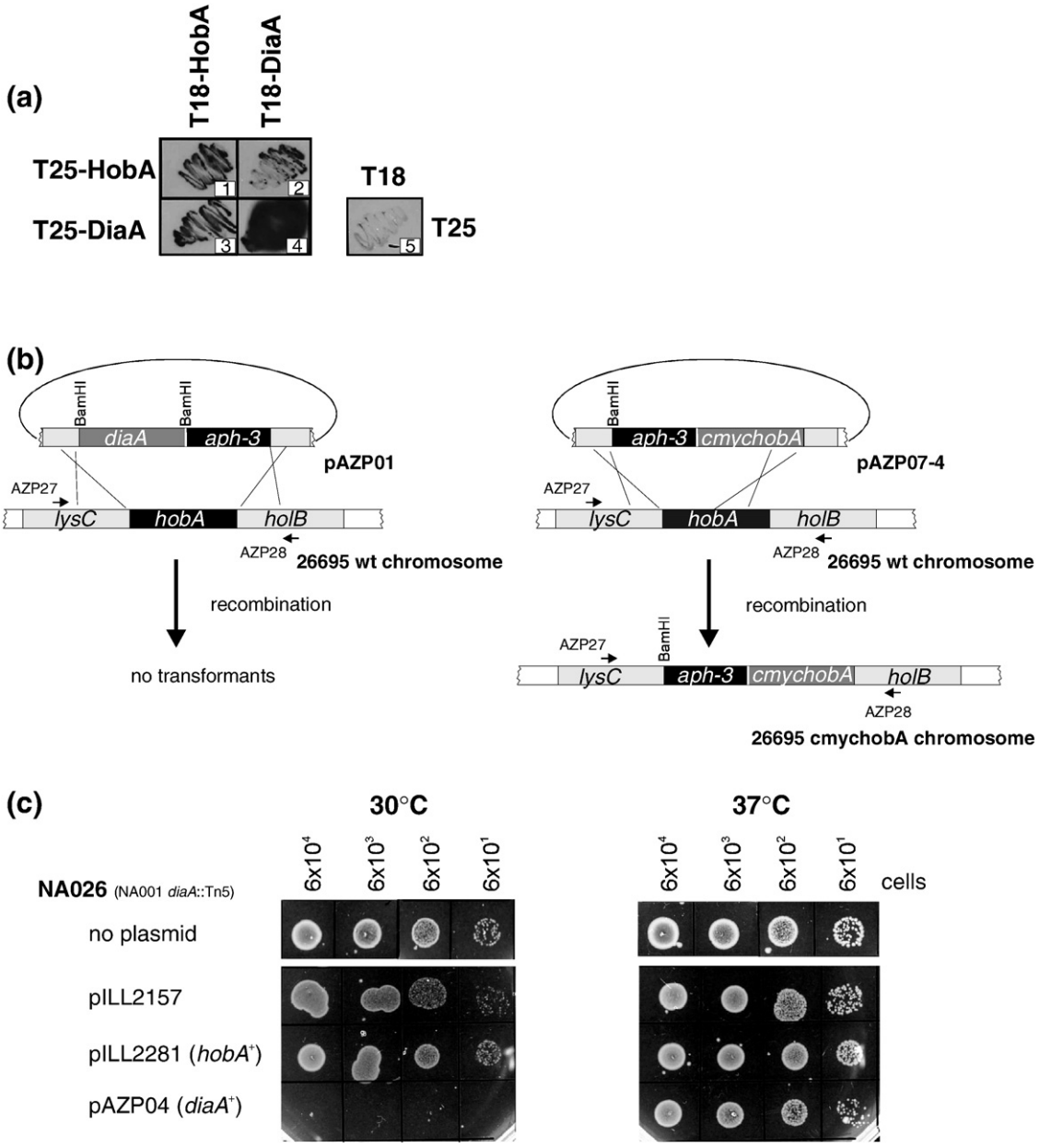

Fig. 1. Analysis of *diaA* and *hobA* ability to complement each other *in vivo*. (a) Bacterial two-hybrid analysis of HobA and DiaA interactions. The proteins were fused with the T25 or T18 domains of *Bordetella pertussis* CyaA (Table 1) and their interactions were analyzed in BTH101 cells grown on MacConkey/maltose agar plates at 30 °C for 2 days. Dark gray/black, interaction detected; pale gray/colorless, no interaction. (b) A representation of the strategy used to replace *hobA* with *diaA* directly in the chromosomal locus. The suicide vectors pAZP01 and pAZP07-4 were used as donors to introduce *diaA* or *c-mychobA* by recombination between homologous fragments of *lysC* and *holB* or *lysC* and 5′ *hobA*, respectively. Successful recombination should lead to the introduction of *diaA* or *c-mychobA* together with the *aph-3* cassette, allowing for mutant selection. Transformation with pAZP01 was lethal for *H. pylori* (no transformant obtained), whereas transformation with pAZP07-4 gave colonies which, after PCR and sequencing analysis, were shown to be the designed mutants (data not shown). (c) Complementation of *E. coli* NA026 by DiaA and HobA. *E. coli* NA026 derivative strains were grown overnight at 37 °C in liquid cultures and then serial dilutions of the cultures were spotted onto agar plates and incubated overnight at the temperature indicated. Cells with successful complementation of *diaA* grew only at 37 °C, whereas those not complemented grew at both 30 °C and 37 °C.

cells. The results were confirmed by the alternative two-step mutagenesis strategy[11] in which first plasmid-borne *diaA* was introduced into the *H. pylori* N6 strain and then *hobA* was subjected to deletion from its chromosomal locus by allelic gene replacement (Supplementary Materials and Methods and Fig. S1a). We were not able to obtain the desired *hobA*-deletion mutants, which proved that *hobA* cannot be complemented by its *E. coli* counterpart *in vivo*.

To analyze whether *hobA* can substitute for *diaA* in *E. coli* we used a derivative of the NA001 cold-sensitive strain NA026, in which *diaA* has been disrupted by the Tn5 transposon.[9] Loss of DiaA function enables NA026 to grow at low temperatures (30 °C), while plasmid complementation of

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

Strain/plasmid/ protein	Relevant genotype/feature	Reference/source
A. *E. coli*		
DH5alpha	F2, Φ80 d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsd* R17, (r_k^-,m_k^+), *sup*E44, *rel*A1, *deo*R, Δ(*lacZYAargF*)U169	Laboratory stock
NA026	KH5402-1 *dnaAcos diaA26*::Tn*5*	9
MC1061	F *araD139* (*ara-leu*)*7696 galE15 galK16*, (*lacX74*) *rpsL hsdR2 mcrA mcrB1*	29
BTH101	F-, *cya-99*, *araD139*, *galE15*, *galK16*, *rpsL1 (Str r)*, *hsdR2*, *mcrA1*, *mcrB1*	25
BL21	B F- dcm ompT hsdS(rB- mB)gal	GE Healthcare
WM2305	*W3110+pLD92 (pBAD18-QQL)* ΔftsK::kan	30
B. *H. pylori*		
26695	*Parental strain*	31
N6	*Parental strain*	32
J99	*Parental strain*	33
26695 *cmychobA*	*hobAΩc-mychobA*	This work
J99 *cmychobA*	*hobAΩc-mychobA*	This work
N6 *cmychobA*	*hobAΩc-mychobA*	This work
C. *Plasmids*		
pUC18	Cloning vector, Amp^R	MBI Fermentas
pILL2157	*E. coli* and *H. pylori* expression vector, IPTG-inducible, allows for relatively high but leaky gene expression in *H. pylori* and low-level expression in *E. coli*, Chl^R	34
pILL2150	*E. coli* and *H. pylori* expression vector, IPTG inducible, allows for relatively low but tightly controlled gene expression in *H. pylori* and high-level expression in *E. coli*, Chl^R	34
pILL2281	pILL2157 derivative, expressing HobA	11
pUT18C	BACTH expression vector, Amp^R	6
pKT25	BACTH expression vector, Kan^R	6
pAZP01	pILL2283 derivative, containing *diaA*, *aph-3* cassette and 5′ and 3′ *hobA* flanking regions, used for allelic exchange of *hobA* into *diaA*, Amp^R, Kan^R	This work
pAZP04	pILL2157 derivative, expressing *diaA*	This work
pAZP07-4	pUC18 derivative, containing *hobA* 5′ and 3′ flanking regions, *cmyc* fused with 5′ *hobA* (1–338 bp), and *aphA-3* cassette; used for allelic exchange of *hobA* into *cmychobA*, Amp^R, Kan^R	This work
pAZP16	pUT18C derivative, expressing HobA with an N-terminal T18 (T18-HobA)	This work
pAZP18	pKT25 derivative, expressing HpDnaA with an N-terminal T25 (T25-HpDnaA)	This work
pAZP19	pKT25 derivative, expressing HobA with an N-terminal T25 (T25-HobA)	This work
pAZP23	pUT18 derivative, expressing N-terminal domain of HpDnaA (residues 1–112) with a C-terminal T18 (HpDnaA (I-II)-T18)	This work
pAZP25	pUT18C derivative, expressing DiaA with an N-terminal T18 (T18-DiaA)	This work
pAZP26	pKT25 derivative, expressing EcDnaA with an N-terminal T25 (T25-EcDnaA)	This work
pAZP27	pKT25 derivative, expressing DiaA with an N-terminal T25 (T25-DiaA)	This work
pAZP28	pILL2150 derivative, expressing fusion Ec^IHp^{II-IV}DnaA	This work
pAZP30	pUT18C derivative, expressing Ec^IHp^{II-IV}DnaA with an N-terminal T18 (T18- Ec^IHp^{II-IV}DnaA)	This work
D. *Proteins*		
HpDnaA	Recombinant, untagged *H. pylori* DnaA protein	13 and this work
HobA	Recombinant, *H. pylori* HobA protein, released from hexahistidine tag with TEV protease	This work
EcDnaA	Recombinant, untagged *E. coli* DnaA protein	35
DiaA	Recombinant, *E. coli* DiaA protein, released from hexahistidine tag with TEV protease	This work
Ec^IHp^{II-IV}DnaA	Recombinant, untagged fusion DnaA protein composed of domain I of *E. coli* DnaA (1–81) and domains II–IV of *H. pylori* DnaA (93–457)	This work

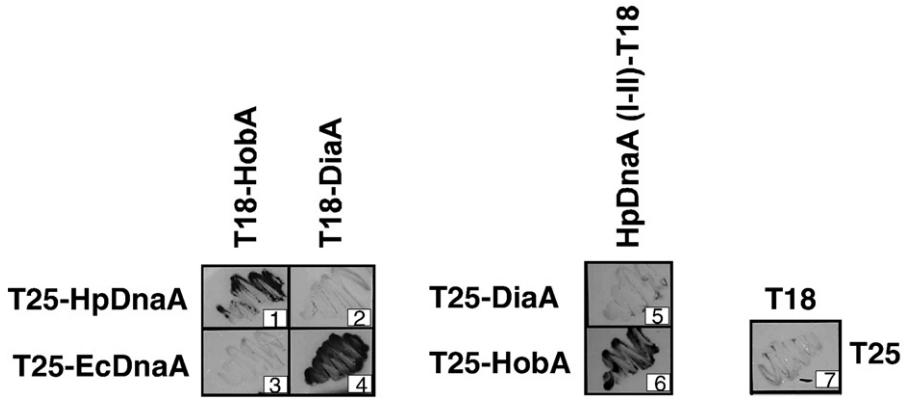

Fig. 2. Analysis of HobA and DiaA interactions with DnaA. Bacterial two-hybrid analysis of interactions between DiaA, HobA, and *H. pylori* and *E. coli* DnaA. The proteins were fused with T25 or T18 domains of *Bordetella pertussis* CyaA (Table 1) and their interactions were analyzed in BTH101 cells grown on MacConkey/maltose agar plates at 30 °C for 2–3 days. Dark gray/black, interaction detected; pale gray/colorless, no interaction.

diaA restores the cold-sensitive phenotype. Using pILL2157, pAZP04 and pILL2281 plasmids (Table 1), we analyzed the *diaA* and *hobA* complementation ability in *E. coli* NA026. We observed that expression of *diaA* inhibited growth of NA026 at 30 °C (Fig. 1c), which is consistent with the results of earlier studies.[9] Expression of *hobA* did not affect growth of NA026 at 30 °C or 37 °C, suggesting that HobA cannot complement for the lack of DiaA in *E. coli* NA026 (Fig. 1c).

HobA and DiaA modulate orisome assembly directly by enhancing DnaA affinity towards *oriC*.[11,13] We found that neither DiaA nor HobA could enhance the heterologous DnaA binding with DNA (surface plasmon resonance (SPR) data not shown). Because DiaA and HobA interact with orisomes via domain I of DnaA proteins, we speculated that the inability of DiaA and HobA to stimulate assembly of heterologous orisomes might originate from the lack of DiaA–HpDnaA and HobA–EcDnaA complex formation. Using the bacterial two-hybrid system, we demonstrated the absence of interaction between EcDnaA and HobA and between HpDnaA and DiaA (Fig. 2, sectors 3 and 2), whereas HpDnaA–HobA and EcDnaA–DiaA binding was confirmed (Fig. 2, sectors 1 and 4, respectively). Additionally, we could not detect binding of DiaA to the N-terminus of HpDnaA (domains I-II, $HpDnaA^{I\text{-}II}$), which interacts strongly with HobA (Fig. 2, sectors 5 and 6, respectively).[16]

We conclude that the absence of protein interaction between HobA and EcDnaA and between DiaA and HpDnaA is a key explanation for the inability to replace one protein with its structural counterpart during orisome assembly. It is important to add that, using gene replacement, it has been shown recently that PBPRA3229 of the Gram-negative, deep-sea *Photobacterium profundum* is a functional homolog of DiaA.[17] However, first, the proteins were highly similar (75% identity and 85% similarity) and second, nothing is known about initiation of *P. profundum* chromosome replication. Thus, it is difficult to discuss the role of PBPRA3229 in *P. profundum* orisome assembly and to compare it to the known initiation systems.

HobA and DiaA are adjusted to domain I of their cognate DnaA

Earlier studies suggested that HobA and DiaA residues involved in interactions with DnaA are similar[12] and indicated a common mode of binding DiaA/HobA to DnaA. Our analysis excluded HobA–EcDnaA and DiaA–HpDnaA interactions. Therefore, we searched for structural differences that might preclude binding between these heterologous proteins. The crystal structures of the HobA/HpDnaA complex (PDB code 2WP0), DiaA (PDB code 2YVA) and the NMR structure of $EcDnaA^{I}$ (PDB code 2EOG) were used to compare the properties of the DnaA binding mechanisms of HobA and DiaA. Both complexes seemed to be formed by binding helices α2 and α3 of DnaA to two cavities located at the dimer–dimer interface and involved a conserved loop (the ERP loop) of DiaA and HobA.[12,13] The structure of DiaA was superimposed on that of HobA and the structure of $EcDnaA^{I}$ was superimposed on that of $HpDnaA^{I}$. We observed that the resulting DiaA/EcDnaA complex was not entirely satisfactory (there were steric clashes) and that EcDnaA had to be adjusted manually. This was done by moving EcDnaA slightly so that the interactions identified by NMR by Keyamura and co-workers,[13] i.e. DiaA residues Arg71, Pro72, Leu190 and Phe191 interacting with EcDnaA residues Glu21, Trp50, Phe46 and Trp25, were respected (Fig. S2). This step resulted in a complex where structural elements of EcDnaA (α2

and α3) fit well into the surface cavities of DiaA (Supplementary Data Fig. S2). We also noticed that for one particular interaction (between DiaA Arg71 and EcDnaA Glu21), a rotation of the Arg71 side chain could lead to a salt bridge between the two residues.

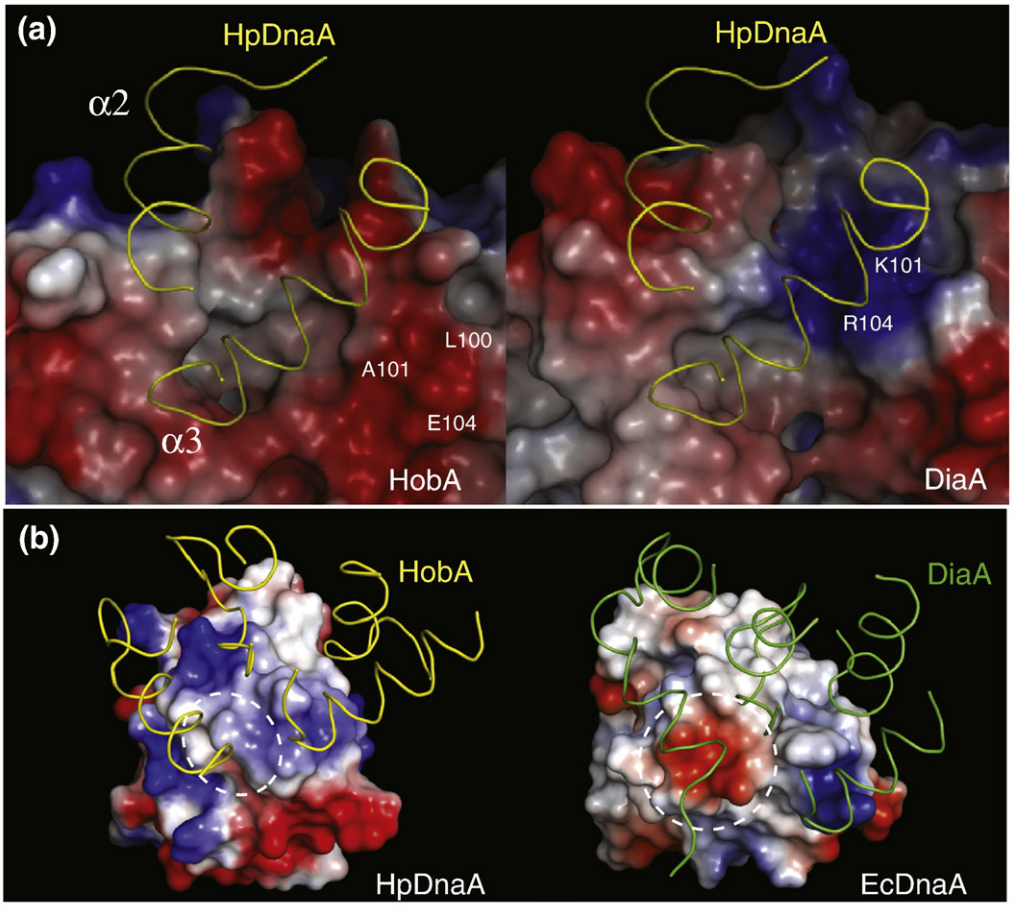

Fig. 3 (*legend on next page*)

By comparing the two complexes, we found that the cavities of the DnaA-binding sites of HobA and DiaA are in fact different (Fig. 3). The cavities are markedly different in shape, and this particularly concerns the DnaA α2 binding area (Fig. 3a). More importantly, the surfaces display completely opposite distributions of charge (Fig. 3a). In HobA, the cavity that binds to DnaA α3 is predominantly hydrophobic and negative (Leu100, Ala101, Glu104), whereas the same area of DiaA is positively charged with residues K101 and R104 (Fig. 3a). Interestingly, the HpDnaA and EcDnaA charge distribution of the surfaces involved in the binding are also opposite (Fig. 3b). This is due to the different properties of some key residues of the $DnaA^{I}$ α3 helix involved in the interface Arg45, Asp49, Asp53 for EcDnaA that correspond to Gln52, Thr56, Ala60 in HpDnaA. In addition, the size of the side chains of some HpDnaA residues involved in the binding is different in EcDnaA. In the HobA/$DnaA^{I-II}$ crystal structure, Val53 of HpDnaA is inserted into a small hydrophobic cavity, and the equivalent residue is Phe46, which is crucial for DnaA interaction with DiaA.[13] Analysis of the putative complex EcDnaA/HobA shows that Phe46 cannot be inserted into the same cavity as Val53 without steric clashes. Moreover, an additional clash would occur in the same model with Trp50 (Thr57 in HpDnaA). Altogether, these observations suggest that the DnaA-binding sites of HobA and DiaA are located in the same area and involve similar secondary structure elements, but the molecular properties of the binding residues are different. The differences presented provide a convincing structural explanation for *in vivo* and *in vitro* observations; i.e. that HobA and DiaA are not interchangeable.

HobA and DiaA are functional homologs, but assist orisome assembly in a species-specific manner

We showed that HobA and DiaA do not bind heterologous DnaA proteins and, therefore, they cannot stimulate assembly of the foreign orisomes. The question arose of whether, apart from the inability to physically interact with the foreign DnaA protein, the functions of DiaA and HobA in orisome assembly are similar. In an attempt to answer that question, we first analyzed *H. pylori* and *E. coli* orisome formation and then compared the roles of DiaA and HobA by establishing a model of a hybrid orisome (for details, see below). We chose SPR as the best method to qualitatively and quantitatively measure interactions between analyzed molecules (here both protein–protein and protein–DNA). Moreover, SPR enabled us to monitor the DiaA- and HobA-assisted orisome formation in real time, which allowed visualization of the dynamics of the process for the first time.

HpDnaA and EcDnaA interacted specifically with their *oriC* (see Fig. 4a and c);[18] DiaA and HobA did not bind DNA (data not shown). We chose a non-saturating concentration of DnaA (32 nM) in order to observe the influence of HobA/DiaA on DnaA binding in orisomes of the homologs. Both HobA and DiaA stimulated binding of their cognate DnaAs (Fig. 4b and d; Table 2). However, we observed particular differences in HobA and DiaA action during the assembly of their cognate orisomes, which might reflect the distinctions in orisome formation *in vivo*.

HobA, starting from an equimolar HobA/DnaA ratio, accelerated and increased binding to *oriC*. It also stabilized the nucleoprotein complex by reducing the dissociation of DnaA from *oriC* (Fig. 4b). Assuming that the stoichiometry of HpDnaA:HobA in the orisome complex is 1:1, similar to that of $(HpDnaA^{I-II})_4$–$HobA_4$ observed by Natrajan *et al.*,[12] the maximal observed RU increment implied 4.5-fold increased HpDnaA binding to *oriC* (Table 2). This confirmed that HobA enhances HpDnaA binding to *oriC* and stimulates orisome assembly. The mode of DnaA association with *oriC* in the presence of HobA might confirm *in vivo* observations concerning the necessity of HobA in initiation of *H. pylori* chromosome replication.

Fig. 3. Structural analysis of HobA/DnaA and DiaA/DnaA complexes. (a) Comparison of the HpDnaA binding site of HobA (left-hand panel) and the same area in DiaA (right-hand panel). The surfaces are colored according to their electrostatic potential (blue, positive; red, negative). In the HobA/DnaA complex, DnaA binds via its helices α2 and α3 (yellow ribbon) to two cavities that are very different in size/shape and potential in DiaA. In particular, the N-terminal portion of helix α3 of DnaA binds to a hydrophobic/negatively charged area in HobA, whereas the corresponding surface is positively charged in DiaA. (b) Surface charge comparison of HpDnaA (left-hand panel) and EcDnaA (right-hand panel) with the structure of HobA colored yellow and DiaA colored green. The surfaces of the two DnaAs are very different in shape and charge distribution. In particular, in the area corresponding to the N-terminal portion of α3 they have completely opposite charges (broken-line white circles), which correspond to the differences noticed in the HobA/DiaA structures. (c) Structure-based sequence alignment of HpDnaA (residues 1 – 70) and EcDnaA (residues 1 – 63) (top panel), and DiaA and HobA (bottom panel). Identical residues are shaded in black, and residues that participate in the interaction are indicated by a gray dot.[10,12,13] Residues mutated in other studies and shown to be essential for the interaction are indicated by a star. Negatively and positively charged residues are shaded in red and blue, respectively. Residues that participate in the interaction and have side chains of different sizes are shaded in green. Secondary structure elements are shown for HpDnaA (based on the HpDnaA crystal structure) and the conserved ERP loop is indicated by a broken-line box.

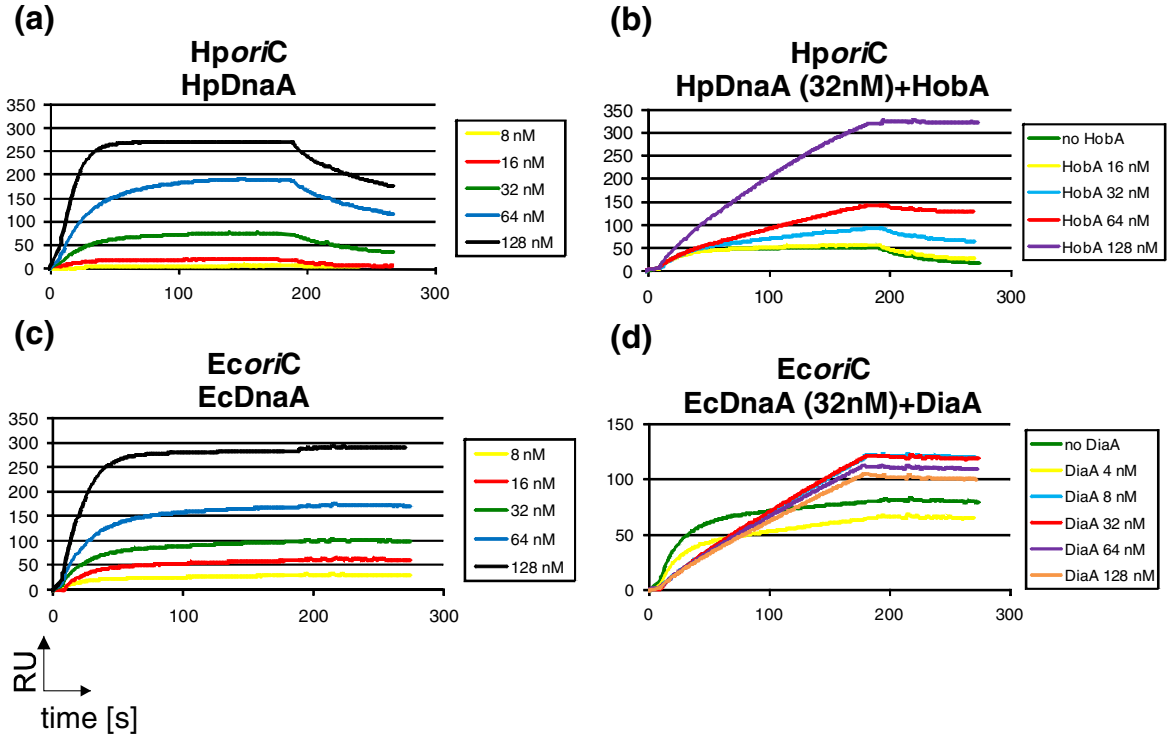

Fig. 4. Influence of DiaA and HobA on orisome formation. SPR analysis presenting (a and b) *H. pylori* and (c and d) *E. coli* orisome formation and the influence of HobA and DiaA on orisome assembly. The protein compositions, concentrations and the type of DNA fragments are given in the legends of the sensograms.

The DiaA protein, though increasing the amount of DnaA binding to *oriC* by 20%~30% (assuming the EcDnaA to DiaA ratio in the orisome complex to be 1.6:1[10] to 2.5:1[13]), simultaneously decelerated DnaA binding to DNA (Fig. 4d; Table 2). Within the association time (180 s) we did not reach a saturated level of DiaA-assisted DnaA binding to E*coriC* (i.e. equilibrium phase). However, the slope of the sensogram representing binding of EcDnaA in the presence of DiaA indicated that prolonged incubation would result in an overall two- to threefold increase in binding of EcDnaA to its *oriC*. This is similar to what had been observed by affinity chromatography.[10] Unlike HobA, DiaA stimulated EcDnaA binding only within an optimal EcDnaA: DiaA ratio of 4:1 – 1:1 (8–32 nM DiaA), similar to earlier results;[10] a further increase in the concentration of DiaA slightly decreased DnaA binding (compare Fig. 4d and b). The mode of *E. coli* orisome formation assisted by DiaA might confirm that the

Table 2. Analysis of DnaA binding to *oriC* in the presence of DiaA/HobA

Protein/concentration (nM)	DnaA:HobA or DnaA:DiaA	Δ*M* of a complex[a]	RU_{max}[b]	ΔRU[c]	DnaA binding[d]
HpDnaA/32	—	—	50	—	1
HpDnaA/32, HobA/128	1:1[12]	1.40	318	6.36	4.54
EcDnaA/32 nM	—	—	79	—	1
EcDnaA/32, DiaA/32	1.6:1[10] – 2.5:1[13]	1.28 - 1.18	121	1.51	1.20 - 1.30
$Ec^{I}HpII\text{-}^{IV}$DnaA/32	—	—	85	—	1
$Ec^{I}Hp^{II\text{-}IV}$DnaA/32, DiaA/128	Assumed as in EcDnaA+DiaA	1.29	294	3.46	2.68

In all calculations the values of the SPR analysis, which reflect the maximal increment of protein–DNA interactions in the presence of HobA/DiaA, were used.

Molecular mass of the proteins (in kDa): EcDnaA, 52; DiaA, 24; HpDnaA, 51.5; and EcHpDnaA, 50.8.

[a] Δ*M* of a complex, an increment of the molecular mass of the DnaA–HobA/DiaA complex bound to DNA, calculated for one DnaA molecule.

[b] RU_{max}, RU at the end of the association phase.

[c] ΔRU, increment of RU between DnaA and DnaA-HobA/DiaA interactions with *oriC*.

[d] DnaA binding without HobA/DiaA was set as 1.

function of DiaA consists of modulation and remodeling of DnaA binding with *oriC* in order to direct DnaA molecules to low-affinity DnaA sites (i.e. ADLAS boxes), rather than activation of robust and highly increased DnaA binding with *oriC*. This could explain why DiaA, though it is important for the synchrony and timing of the initiation process, is not absolutely required *in vivo*.

In order to determine whether DiaA and HobA are functional homologs we created a hybrid orisome consisting of DiaA, Hp*oriC* and the fusion protein $Ec^{I}Hp^{II-IV}$DnaA (domain I of *E. coli* DnaA fused to domains II–IV of *H. pylori* DnaA). Due to domain swapping, $Ec^{I}Hp^{II-IV}$DnaA gained the ability to interact with DiaA (Fig. 5a and c) and did not bind HobA (Fig. 5a). The interactions of HpDnaA and $Ec^{I}Hp^{II-IV}$DnaA with Hp*oriC* binding were very similar (Figs. 4a and 5b), which indicates that the overall *oriC*-binding properties were retained by the fusion protein and were not changed significantly by domain I of EcDnaA. As expected, $Ec^{I}Hp^{II-IV}$DnaA interaction with Hp*oriC* was stimulated by DiaA (Figs. 4b and 5c) and insensitive to HobA (data not shown). DiaA stimulation resulted in both faster association of the hybrid protein and an approximately 2.5-fold increase of $Ec^{I}Hp^{II-IV}$DnaA binding to *oriC* (Fig. 5c; Table 2). Thus, the kinetics of DiaA-assisted interaction between $Ec^{I}Hp^{II-IV}$DnaA and Hp*oriC* were similar to those of HobA-HpDnaA-Hp*oriC* (Figs. 4b and 5c). However, $Ec^{I}Hp^{II-IV}$DnaA was more sensitive to DiaA stimulation than HpDnaA to HobA: less DiaA was required to obtain maximal binding of the fusion DnaA to *oriC* than for maximal stimulation of HpDnaA–*oriC* interactions by HobA (equimolar DiaA–$Ec^{I}Hp^{II-IV}$DnaA and 4:1 HobA:HpDnaA). This resembles the sensitivity of EcDnaA to DiaA stimulation (similar stoichiometry and saturation of DnaA–*oriC* binding). The effect was probably a result of the exchanged domain I of the fusion protein. Domain I of EcDnaA and DiaA are optimally adjusted for interaction; thus, the stoichiometry and the sensitivity of their interaction applies also to the fusion protein $Ec^{I}Hp^{II-IV}$DnaA. It is possible that the self-interacting properties of the N-terminal domain from EcDnaA increased the oligomerization potential of the fusion $Ec^{I}Hp^{II-IV}$DnaA when

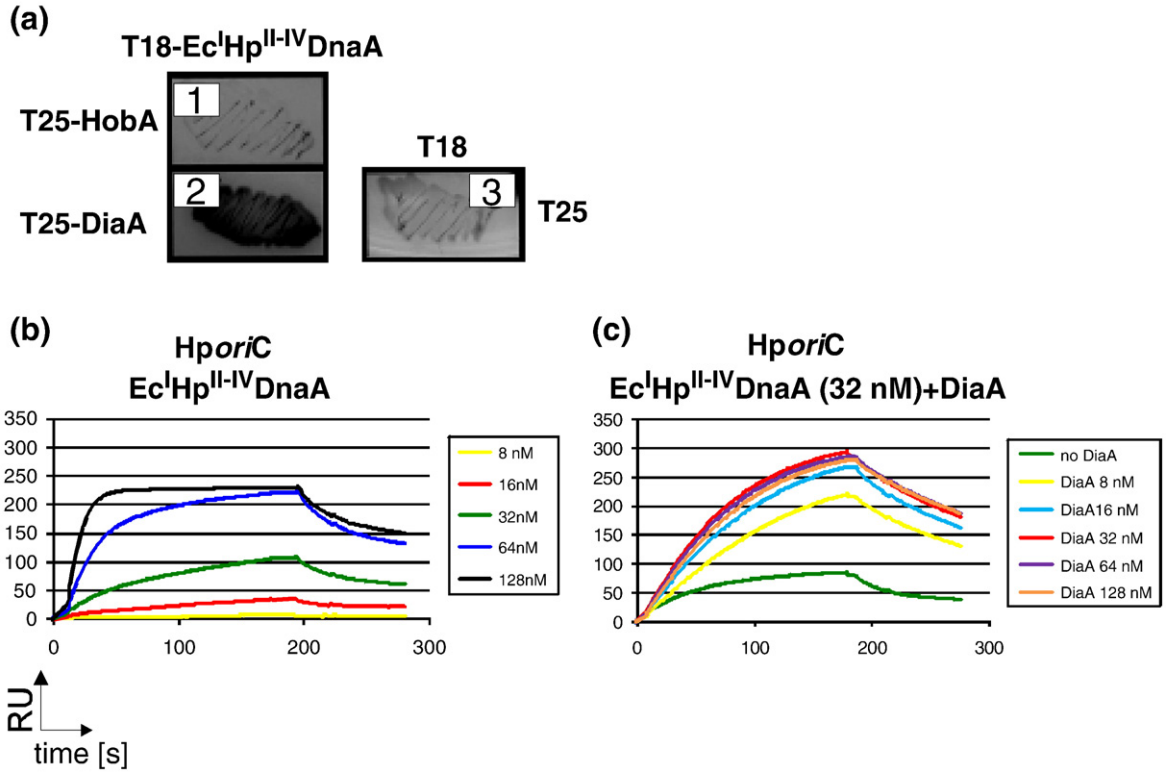

Fig. 5. Influence of DiaA on hybrid $Ec^{I}Hp^{II-IV}$DnaA-Hp*oriC* orisome formation. (a) Bacterial two-hybrid analysis of $Ec^{I}Hp^{II-IV}$DnaA and DiaA interactions. The proteins were fused with T25 or T18 domains of *Bordetella pertussis* CyaA (Table 1) and their interactions were analyzed in BTH101 cells grown on MacConkey/maltose agar plates at 30 °C for 2 days. Dark gray/black, interaction detected; pale gray/colorless, no interaction. (b and c) SPR analysis presenting (b) hybrid $Ec^{I}Hp^{II-IV}$DnaA-Hp*oriC* orisome formation and (c) the influence of DiaA on its assembly. The protein compositions and concentrations are given in the legends of the sensograms.

compared to HpDnaA, for which no dimerization of domain I has been shown.[12] Therefore, for maximal stimulation of DnaA binding, fewer DiaA molecules per $Ec^{I}Hp^{II-IV}$DnaA or EcDnaA molecule were required than HobA molecules per HpDnaA molecule.

The domain swapping experiment showed clearly that DiaA and HobA are functional homologs and upon binding to DnaA they exert the same effect on the replication initiator protein (Figs. 4b and d and 5c). Further, the experiment confirmed a modular function of DnaA; i.e. the fact that the N-terminus is responsible for species-specific interactions with other proteins including DnaA itself and DiaA/HobA, while domain IV is responsible for interactions with the cognate *oriC* and it emphasized that the crosstalk between the domains of the initiator protein is required for its proper function during orisome formation.

Discussion

The analysis of the function and structure of DiaA and HobA suggests that the two proteins play a similar role during initiation of chromosome replication in *E. coli* and *H. pylori*, respectively. Therefore, it became very interesting to ask whether DiaA and HobA can substitute for each other during orisome assembly and to compare the formation of the initiation complex in the two distantly related bacteria.

We found that DiaA and HobA cannot replace each other *in vivo* (Fig. 1; Supplementary Data Fig. S1). This indicated that they are specific only to their own bacteria and suggested either a different function of each protein or particular structural differences precluding the substitution of DiaA and HobA in the formation of heterologous orisomes. Published data together with our SPR results (Fig. 4) demonstrated that both proteins, in spite of the particular differences discussed below, enhance binding of their own DnaA with *oriC*. We found, however, that neither of the proteins can interact with their foreign DnaA partner (Fig. 2) and thus neither can stimulate assembly of heterologous orisomes. Indeed, the DiaA–EcDnaA and HobA–HpDnaA interaction surfaces are adjusted to each other (Fig. 3), which clearly explains why DiaA and HobA cannot substitute for each other *in vitro* and *in vivo*. When domain I of *H. pylori* DnaA was exchanged for domain I of *E. coli* DnaA and enabled DiaA to interact with hybrid $Ec^{I}Hp^{II-IV}$DnaA, DiaA stimulated *H. pylori* orisome assembly in a manner very similar to HobA (Fig. 4). Therefore, we concluded that both proteins are in fact functional homologs, but the low-level sequence conservation (15% identity and 31% similarity) and co-evolution with domain I of the cognate DnaA preclude interchangeability between DiaA and HobA.

Orisome components are adjusted to cooperate

DiaA and HobA organize orisome formation through enhancing DnaA binding. However, the dynamics of the stimulation were found to be different for each orisome (Fig. 4b and d). Addition of HobA rapidly increases HpDnaA interaction with Hp*oriC*, which suggests that binding of HobA triggers formation of an active orisome and allows replication to start. This would explain why HobA is absolutely required for initiation of *H. pylori* chromosome replication.[11,12] DiaA, in contrast to HobA, slows DnaA binding to *oriC* (Fig. 4d) but probably makes the orisome structure more organized. Thus, *diaA* inactivation, though causing perturbations in the initiation of *E. coli* chromosome replication, is not lethal for *E. coli* cells. In a hybrid orisome system, the DiaA-stimulated binding of the $Ec^{I}Hp^{II-IV}$DnaA protein (faster and enhanced) resembles the HobA-assisted HpDnaA interactions with Hp*oriC*, which confirms that the mode of DiaA and HobA action during orisome assembly is similar. However, it suggests also that the DiaA/HobA modulatory role in DnaA multimerization on *oriC* and orisome architecture depends on both DnaA and *oriC*. DnaAs and *oriC*s, though sharing similar structures and function, are not interchangeable between different bacterial species unless they are very closely related.[19] *oriC* provides an oligomerization scaffold formed by DnaA boxes whose sequence, number and arrangement are unique for each bacterium. DnaA proteins exhibit different sequence specificities for DnaA boxes,[20] require a particular number and arrangement of DnaA boxes within *oriC* for efficient unwinding,[21] and possibly interact with various, often species-specific proteins such as DiaA/HobA. DiaA and HobA, and their homologs abundant in bacterial species, have to fit into the overall orisome architecture to effectively arrange the initiation complex. Therefore, all three analyzed orisome components, *oriC*, DnaA and HobA/DiaA, presumably act in concert to form an active orisome and therefore are not interchangeable. It is important to add that we could not exchange HobA *in vivo* even for its closest sequence homolog Cj0545 from *Campylobacter jejuni* (27% identity and 52% similarity). Therefore, the species specificity of DnaA-HobA/DiaA interaction might be especially interesting as a potential target for antimicrobial drug design. In bacteria in which DiaA/HobA proteins are essential, as in *H. pylori*, such compounds could specifically affect orisome formation and hence prevent chromosome replication and bacterial growth.

DiaA and HobA are phylogenetically related and abundant in bacterial species

Because DiaA and HobA show a high degree of structural similarity and both influence orisome in

the same way (by interaction with DnaA domain I and promotion of DnaA oligomerization around *oriC*), we examined their phylogenetic relationship. All the phylogenetic analyses indicated that these functional and structural homologs, DiaA and HobA, are close relatives and show a common origin among phosphoheptose isomerases (GmhA) (all details are included in the Supplementary Data). We have identified 916 sequences representing DiaA/GmhA proteins and 48 sequences similar to that of HobA in the non-redundant GenBank database (Supplementary Data Table SI). HobA homologs are found exclusively in the ε-Proteobacteria and they appear as a much diverged GmhA lineage with deeply eroded sequence similarity to potential homologs, but retain their structural similarity[12,14] and phylogenetic relationship (Supplementary Data Fig. S4). In contrast to HobA, DiaA proteins show both clear structural and clear sequence similarity to their GmhA homologs. However, because of the lack of characteristic sequence features discriminating DiaA and GmhA, it is difficult to distinguish between these two proteins and unequivocally identify which gene encodes the DiaA-like protein involved in initiation of chromosome replication, but not the GmhA-like protein engaged in phosphosugar metabolism. Although it is possible to recognize some DiaA sequences, mainly in the γ- and β-Proteobacteria, using searches for the DiaA domain (Fig. S3), it should be noted that the statistical DiaA domain characteristic is likely to be too specific and is not able to recognize more distant DiaA proteins from other taxa. Additionally, it is possible that, because of the low level of sequence homology, some of the DiaA-like proteins escaped from the sequence analyses, as happened with HobA. In fact, there could be many more DiaA/HobA-like homologs than we were able to identify. However, the taxonomic distribution of the identified DiaA/HobA-like proteins, especially typical of DiaA with the widespread Leu-Phe motif[10] (Supplementary Data Table SI), suggests that DiaA/HobA-like proteins may be orisome-organizing factors common to many bacterial species.

Stimulate or inhibit: a lifestyle-dependent decision?

We postulate that the kinetic differences observed in modulation of orisome formation by DiaA and HobA, together with the presence or absence of other regulators of the process, might reflect the different modes of initiation regulation at *oriC* connected with accommodation to ecological niches inhabited by *H. pylori* and *E. coli*. *H. pylori* inhabits the human gastrointestinal tract—an invariable, protected ecological niche. *H. pylori* is a fastidious bacterium *in vitro* and is characterized by a relatively long generation time (3–4 h), rare initiation events (*ori/ter* ratio 2.7 ± 0.6) and slow DNA replication (120 bp/s), which is limited by the availability of the essential DNA precursor dTMP synthesized by ThyX thymidylate synthetase.[22] Therefore, HobA might be sufficient, but essential, to control chromosome replication in *H. pylori*. Indeed, despite many attempts to identify other proteins regulating the initiation of *H. pylori* chromosome replication (data not shown), HobA is the only regulator of the process identified so far. *E. coli*, in contrast to *H. pylori*, inhabits many environments, can divide every 20 mins, and can contain up to eight origins per single cell.[23] Additionally, its DNA replication is not limited by DNA synthesis, which reaches 1870 bp/s thanks to dTMP synthesized by the efficient ThyA thymidylate synthetase.[22] This explains why *E. coli* requires many regulatory factors, which should limit rather than induce initiation events. *E. coli* consistently possesses only one, so far identified, stimulator (DiaA) and many negative regulators (SeqA, Hda and *datA)* of the initiation process, which act in concert to perfectly adjust initiation events for the *E. coli* cell cycle.

DiaA and HobA are activators of chromosome replication. It has been found that DiaA is co-localized with *oriC* only at a specific moment in a cell cycle.[10,13] This raises the question of what regulates DiaA and HobA so that they act at a precise time point. It is especially interesting in the case of HobA, which licenses initiation of chromosome replication in *H. pylori*. Keyamura *et al.* suggested that activity of DiaA might be regulated by phosphosugar binding and/or other proteins,[10,13] but no regulatory factor has been recognized yet. The search for proteins interacting with HobA has been unsuccessful so far (our unpublished results and [16]). Further investigation is needed to identify a, so far unknown, cellular factor that regulates the activity of HobA and DiaA.

In summary, our analyses showed that HobA and DiaA originate from a common ancestor and are functional homologs that stimulate and organize binding of the DnaA protein to *oriC*. However, each regulator has co-evolved with its DnaA counterpart to maintain the ability to interact with the cognate initiator protein and modulate orisome assembly. The dynamics of DiaA- and HobA-stimulated orisome formation were found to be different in the two bacteria, which might explain why HobA, in contrast to DiaA, is indispensable for orisome assembly *in vivo*. More generally, it might reflect the overall regulation strategy adopted by each of the bacteria to control the initiation frequency. HobA/DiaA-related proteins are abundant in bacteria and might constitute an initiation regulatory system common to a wide variety of distantly related species. The species specificity of the interactions between HobA/DiaA and DnaA might be

an interesting target for a drug against Gram-negative pathogenic bacteria in which HobA/DiaA are essential cellular proteins.

Materials and Methods

Materials, strains and culture conditions

The plasmids and bacterial strains used in this work are given in Table 1 and Supplementary Data Table SIII. The oligonucleotide sequences are given in Supplementary Data Table SII. *H. pylori* 26695 and *E. coli* K12 genomic DNAs were used as templates to amplify DNA fragments for cloning. *E. coli*, unless stated otherwise, was grown at 37 °C on solid or liquid Luria-Bertani medium, supplemented with antibiotics when necessary (100 μg/ml ampicillin, 25 μg/ml kanamycin, 34 μg/ml chloramphenicol). *H. pylori* was cultivated and transformed as described.[11]

Construction of *H. pylori hobAΩdiaA* replacement mutants *diaA* in the *hobA* locus

diaA was amplified by PCR with a pair of primers (AZP03-AZP04, Supplementary Data Table SII) and cloned into the BamHI site of pILL2283,[11] resulting in plasmid pAZP01 (Table 1). *H. pylori* strains 26695, J99 and N6 were transformed with pAZP01 and mutant selection was done on BA plates supplemented with kanamycin.

C-mychobA in the *hobA* locus

pAZP07-4, the plasmid used for allelic exchange of *hobA* for fusion gene *c-mychobA*, was constructed in four steps. First, the downstream region flanking *hobA* (basepairs 820–1218 of *hp1229*), amplified by PCR with primers AZP06-AZP07, was cloned into pUC18 digested with HindIII/BamHI, giving pAZP07-1. The *aphA-3* non-polar cassette,[24] amplified by PCR with primers AZP08-AZP09 and digested with BamHI, was cloned into pAZP07-1 restricted with BamHI/SmaI, giving pAZP07-2. The annealed AZP10 and AZP11 oligonucleotides were subsequently cloned into KpnI/PstI sites of pAZP07-2, resulting in pAZP07-3. The pAZP07-3, digested with KpnI/EcoRI, was used as a vector to clone the 5′ fragment of *hobA* (basepairs 1–301) amplified by primers AZP12 and AZP13. The obtained pAZP07-4 was further used for allelic exchange of *hobA* into the fusion gene *c-mychobA*.

E. coli diaA complementation test

E. coli NA026 transformed with pILL2157 and its derivatives pAZP03 (expressing *diaA*) and pILL2281 (expressing *hobA*) were selected on LB/thy/chl agar plates (LB plates supplemented with 25 μg/ml thymine and 34 μg/ml chloramphenicol) at 37 °C. Liquid overnight cultures from selected colonies were used to prepare serial dilutions containing 1.2×10^4 to 1.2×10^7 cells/ml and 5 μl of the diluted cultures was spotted onto LB/thy/chl agar plates additionally supplemented with 1 mM IPTG and incubated for 24 h at 30 °C and at 37 °C.

Bacterial two-hybrid analysis (BTH)

A bacterial two-hybrid system [15,25] was used to study protein–protein interactions in *E. coli* BTH101. The system is based on the interaction-mediated reconstitution of the adenylate cyclase (CyaA) activity in *E. coli*, which can be observed by color development on selective indicator plates. (construction of all the BTH vectors is described in the Supplementary Data). Co-transformants of each pair of plasmids were selected at 30 °C on MacConkey/1% maltose plates supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin. The interaction was verified by re-streaking colonies onto fresh MacConkey/maltose or MacConkey/maltose/IPTG and LB/Xgal/IPTG (40 μg/ml X-Gal, 0.5 mM IPTG) selective plates and incubation at 30 °C for 48 h.

Construction of a chimeric *ec^{I}hp^{II-IV}*dnaA gene

5′ *E. coli dnaA* (basepairs 1–243), amplified by PCR with primers AZP36-AZP37, was cloned into pILL2150 restricted with NdeI/BamHI. The obtained plasmid was used to clone *H. pylori dnaA* (basepairs 277-1374), amplified by PCR with primers AZP38-AZP39, into SphI/BamHI restriction sites. pAZP28 obtained allows for overproduction of the untagged fusion $Ec^{I}Hp^{II-IV}$DnaA, representing domain I of *E. coli* DnaA (residues 1–81) fused to domains II–IV of *H. pylori* DnaA (residues 93–457). The fusion protein $Ec^{I}Hp^{II-IV}$DnaA was purified as described (Supplementary Data).[26]

Surface plasmon resonance (SPR)

The BIAcore system (BIAcore 3000, GE Healthcare) was used to study DNA–protein and protein–protein interactions by SPR.[27,28] The SPR response values are expressed in resonance units (RU); for most proteins, 1 RU represents binding of 1 pg of the protein/mm^2 of the surface chip. The biotinylated double-stranded DNA fragments were obtained by PCR with the pairs of primers AZP29-AZP30 (Ec*oriC*, 249 bp) and AZP31-AZP32 (Hp*oriC*, 191 bp) (Supplementary Data Table SII), and immobilized on a Sensor Chip SA (90~140 RU) according to the supplier's instructions (GE Healthcare). The measurements were done in KAC buffer (25 mM Hepes pH 7.6, 100 potassium acetate, 10 mM magnesium acetate, 2 mM ATP, 0.05% (v/v) Tween 20) at a continuous flow-rate of 15 μl min^{-1}. For measurements *of E. coli* DnaA, the protein sample was supplemented with DNA competitor Poly(dA-dC)•Poly (dG-dT) (Sigma, P0307) at a final concentration of 50 μg/ml. DnaA was mixed with DiaA/HobA shortly before injection. At the end of each cycle (180 s association followed by 90 s dissociation), the bound proteins were removed by washing with 0.05% (w/v) SDS for 20 s and the flow channels were equilibrated with KAC buffer until the baseline was stable. The program BIAevaluation 4.1 was used for data analysis.

Bioinformatic analyses

Collection of sequences, construction of alignments, CLANS clustering and phylogenetic analyses are described in detail in the Supplementary Data.

Protein purification

Protein purification methods are described in the Supplementary Data.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2011.02.045

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