

Mapping of the interactions between partition proteins Delta and Omega of plasmid pSM19035 from *Streptococcus pyogenes*

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Formation of the segrosome, a nucleoprotein complex crucial for proper functioning of plasmid partition systems, involves interactions between specific partition proteins (ParA-like and ParB-like), ATP and specific DNA sequences (the centromeric sites). Although partition systems have been studied for many years, details of the segrosome formation are not yet clear. Organization of the pSM19035-encoded partition system is unique; in contrast with other known *par* systems, here, the δ and ω genes do not constitute an operon. Moreover, Omega [a ParB-like protein which has a Ribbon-Helix-Helix (RHH) structure] recognizes multiple centromeric sequences located in the promoters of δ , ω and *copS* (copy-number control gene). The ParA-like protein Delta is a Walker-type ATPase. In this work, we identify the interaction domains and requirements for dimerization and hetero-interactions of the Delta and Omega proteins of pSM19035 plasmid. The RHH structures are involved in Omega dimerization *in vivo* and its N-terminal unstructured part is indispensable for association with Delta, both *in vivo* and *in vitro*. Omega does not need to form dimers to interact with Delta. ATP binding is not required for Delta dimerization but is important for interaction with Omega *in vivo*. The *in vitro* interaction between Delta and Omega depends on ATP but does not require the presence of specific DNA segments (the centromere) recognized by Omega. The C-terminal part of the Delta protein (aa 198–284) is indispensable for interaction with Omega. Delta most probably interacts with Omega as a dimer since two amino acid substitutions in a conserved region between the A' and B motifs abolish both the dimerization of Delta and its interaction with Omega.

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INTRODUCTION

Plasmid active partition systems that ensure stable inheritance of low copy number plasmids in dividing cells are composed of three elements: (i) a *cis*-acting DNA sequence (also called centromere-like region), (ii) a DNA-binding protein (B component) and (iii) an NTPase (A component). The two genes *parA* and *parB* usually form an operon regulated by ParA and/or ParB proteins (Gerdes *et al.*, 2000, 2004). Similar systems are engaged in bacterial chromosome partitioning; genome sequencing has confirmed the occurrence of *parA*–*parB* operons in close proximity to *oriC* in the majority of bacterial chromosomes (Bartosik & Jagura-Burdzy, 2005). However, our understanding of partition processes comes mainly from studies on plasmid-encoded systems.

Segregation of plasmids by a partition system requires segrosome assembly, which implies interactions between participants. Models of this nucleoprotein complex formation are based on observations of homo- and heterodimerization of both ParA- and ParB-type proteins (Hayes & Barillà, 2006). The well-studied ParB-like proteins (ParB of P1, SopB of F, KorB of RK2 and ParG of TP228) have been shown to form dimers (Barillà & Hayes, 2003; Funnell, 1991; Hanai *et al.*, 1996; Williams *et al.*, 1993). Some of them, classified as ParBs of type Ia [large, containing Helix-Turn-Helix (HTH) motifs] oligomerize and spread on DNA causing transcriptional silencing. The ParB and SopB proteins of P1 and F plasmids, respectively, have been studied extensively, and their interaction domains have been mapped: the dimerization domain is located in the C-terminal part of the protein, whereas interactions with their partner proteins (ParA and SopA) are ensured by the N-terminal parts (Ravin *et al.*, 2003; Surtees & Funnell, 2001). The dimerization domain of KorB protein of RK2 is also located in its C-terminal part whereas the domain of interaction with IncC (ParA homologue) has been mapped to the central part (Lukaszewicz *et al.*, 2002).

Abbreviations: DBD, DNA-binding domain; HTH, Helix-Turn-Helix; RHH, Ribbon-Helix-Helix.

Two supplementary figures, a supplementary table of primer sequences and supplementary methods describing plasmid construction are available with the online version of this paper.

Partition ParA-like proteins are either Walker-type ATPases, actin-like ATPases (Gerdes *et al.*, 2000) or recently described tubulin-type GTPases (Larsen *et al.*, 2007; Møller-Jensen & Gerdes, 2007). Walker-type ATPases from partition systems can be divided into two subgroups (Bignell & Thomas, 2001). The difference between them is the presence of an N-terminal region which contains the HTH motif involved in DNA binding. Longer ParA proteins are components of type Ia plasmid partition systems and are usually involved in regulation of expression of partition genes. However, there are type Ia partition ATPases which do not contain a DNA-binding motif in the N terminus (IncC1 of RK2) or exist only in a shorter form like IncC of RA3 (IncU) (Kulinska *et al.*, 2008) and chromosomally encoded ParA proteins (Lasocki *et al.*, 2007; Leonard *et al.*, 2005; Quisel & Grossman, 2000). In type Ib partition systems, ParA-like proteins are usually not transcriptional regulators and do not contain N-terminal DNA binding regions. The ParA-like proteins, regardless of their type, can dimerize, polymerize and form filaments (Akhtar *et al.*, 2009; Barillà *et al.*, 2005; Bouet *et al.*, 2007; Lim *et al.*, 2005; van den Ent *et al.*, 2002).

Detailed studies of the dimerization of ParA-like proteins have been conducted for some representative proteins of the ParA-family, mainly of type Ia. It has been shown that dimer formation by ParA from P1 plasmid is induced by ATP binding and that ADP, as well as non-hydrolysable analogues, can promote DNA binding (Davey & Funnell, 1994). Studies of K122E mutant ParA from P1 showed that this substitution did not alter the protein configuration, but reduced the ATPase activity. Moreover, this protein was defective in DNA binding regardless of the presence of ATP and thus could not regulate the *par* operon (Davis *et al.*, 1996; Li *et al.*, 2004). Later, Fung *et al.* (2001) suggested that the lysine substitution altered the ability of ParA to bind ATP. The observation that ATP binding, but not hydrolysis, reinforces ParA repressor activity has strengthened this suggestion. All these findings support Bouet & Funnell (1999), who concluded that both ADP and ATP mediate dimerization of P1 ParA and the two nucleotides act as a switch; ADP-bound ParA acts as a repressor, whereas ATP-bound ParA is involved in partition activity. Moreover, a K16E derivative of ParA from the *Vibrio cholerae* chromosome (position equivalent to K122 of ParA of P1; see Supplementary Fig. S1, available with the online version of this paper) was unable to ensure partition, and visualization by fluorescence microscopy demonstrated its homogeneous distribution and a lack of foci (Fogel & Waldor, 2006). Those observations probably reflected the fact that ATP binding stimulates ParA-like protein polymerization, as observed for MinD (Suefuji *et al.*, 2002), ParF (Barillà *et al.*, 2005) and SopA (Bouet *et al.*, 2007; Lim *et al.*, 2005). A study of ParA-like protein Soj from *Thermus thermophilus* confirmed that a K20A substitution (in a position equivalent to K122 of ParA of P1; see Supplementary Fig. S1) abolished ATP hydrolysis due to the inability to bind the nucleotide (Leonard *et al.*,

2005). The same study also showed that Soj needs ATP for dimerization and that ATP hydrolysis promotes the dissociation of dimers. These observations explain why wild-type and K20A Soj did not dimerize (the former hydrolysed ATP whereas the latter did not bind it). Another Walker-type ATPase, the UvrA ATPase, a subunit of the DNA repair enzyme ABC, has also been shown to be defective when carrying a K37A substitution (Myles *et al.*, 1991).

The interactions of ParA-like proteins with ParB-like proteins have been studied in some partition systems only. The few detailed mutational studies of partition protein interactions concerned mainly type Ia systems (Walker-type ATPases with a DNA-binding function and ParB-like proteins with HTH DNA-binding motifs). ParB of Ia stimulates both the ATPase activity of ParA (Davey & Funnell, 1997) and the polymerization of ParA-like proteins (Barillà *et al.*, 2007; Bouet *et al.*, 2007; Machón *et al.*, 2007) crucial for separation of plasmid molecules. In the systems studied, it has been demonstrated that both proteins are able to interact with each other only in the dimeric form (Bouet & Funnell, 1999).

We analysed Delta and Omega proteins, components of the partition system of pSM19035 plasmid, a low-copy-number plasmid isolated from *Streptococcus pyogenes* (Behnke *et al.*, 1979). The two genes, δ and ω , are transcribed separately, which is unique among the partition systems studied so far (Dmowski *et al.*, 2006). Nevertheless, the tight co-expression of the two partition genes is ensured by their common regulator, the Omega protein. This transcriptional repressor binds to repeated heptameric sequences WATCACW situated in promoter regions in various arrangements. Besides plasmid partitioning, Omega also regulates the plasmid copy number (*copS* gene) and expression of the ω - ϵ - ζ operon that encodes the toxin-antidote system of pSM19035 (de la Hoz *et al.*, 2000; Zielenkiewicz & Ceglowski, 2005). Any of the three Omega-binding regions (*PcopS*, *P δ* and *P ω*) may act as a centromeric sequence (Dmowski *et al.*, 2006).

Crystal structures of Delta (Murayama *et al.*, 2001) and Omega (Pratto *et al.*, 2008) proteins have been solved. The Delta protein is a Walker-type ATPase and its activity is stimulated by Omega protein. In the presence of Omega and ATP, Delta polymerizes. Sitkiewicz (2002) detected Delta dimerization in the yeast two-hybrid system, but failed to demonstrate its associations with Omega as well as Omega dimerization. The Omega protein of the pSM19035 plasmid does not share sequence similarity with ParB-like proteins. It is a small, 71 aa protein (~8 kDa) whose crystal structure shows similarity to the Arc/MetJ family of repressors. Proteins from this family bind DNA and form a Ribbon-Helix-Helix (RHH) structure (reviewed by Schreiter & Drennan, 2007). Such proteins have also been found to act as ParB-like components of type Ib bacterial partition systems, as in plasmids TP228 (Golovanov *et al.*, 2003) and pB171 (Ringgaard *et al.*, 2007), and type II

partition systems from pSK41 (Schumacher *et al.*, 2007) and pB171 (Møller-Jensen *et al.*, 2007) (Supplementary Fig. S2, available with the online version of this paper).

In this work we analysed *in vivo* and *in vitro* associations of Delta and Omega proteins related to those found in type Ib systems. Regions involved in dimerization of each protein and interaction with their partners were defined.

METHODS

Bacterial strains. The bacterial strains used in this work were *Escherichia coli* DH5 α {F⁻, *gyrA*96, *recA*1, *relA*1, *endA*1, *thi*1, *hsdR*17, *supE*44, *deoR*, Δ (*lacZYA-argF*)U169, [Φ 80 Δ (*lacZ*)M15]} (Hanahan, 1983), *E. coli* GS1129 [MC4100 *pheA*905 λ (*sulA*::*lacZ*); kindly provided by G. V. Stauffer] and *E. coli* BL21(DE3) [F⁻ *ompT* *hsdS*_B(r_B⁻ m_B⁻) *gal dcm* BL21(DE3); Studier & Moffatt, 1986].

Plasmid construction. The plasmids and primers used in this work are listed in Table 1 and Supplementary Table S1 (available with the online version of the paper), respectively. If not indicated otherwise, plasmid pDO1000 was used as the template for amplification of studied genes by PCR. Standard plasmid DNA isolation, modification and PCR amplification, as well as PCR mutagenesis by overlap extension and site-directed mutagenesis, were used as described by Sambrook & Russell (2001). Details of the plasmid construction are available online as Supplementary Methods.

Bacterial growth and transformation. *E. coli* strains were routinely grown in L broth (liquid or solidified with 1.5% agar) at 37 °C (Sambrook & Russell, 2001). When necessary, antibiotics were added at the following concentrations: 100 μ g ampicillin ml⁻¹, 20 μ g tetracycline ml⁻¹, 20 μ g kanamycin ml⁻¹. *E. coli* strains were transformed according to standard procedures (Sambrook & Russell, 2001) and plated on L agar plates supplemented with an appropriate antibiotic.

Determination of β -galactosidase activity in *E. coli* cells. The β -galactosidase activity of *E. coli* GS1129 strain containing appropriate plasmids was determined as described by Miller (1992). Each plasmid pair was analysed at least in triplicate.

Protein overexpression and purification. An overnight culture of *E. coli* BL21(DE3) transformant was diluted 1:100 in 50 ml fresh L broth supplemented with appropriate antibiotic. This new culture was aerated at 30 °C until OD₆₀₀ 0.6–0.8. Then, IPTG was added to 1 mM final concentration and the culture was aerated for another 2 h at 30 °C. The 6 \times His-tagged proteins were purified under native conditions using the Protino Ni-TED 1000 system (Macherey-Nagel) according to the manufacturer's instructions, except the elution step which was done using LEW buffer (50 mM Na₂HPO₄, 300 mM NaCl) with increasing amounts of imidazole (25, 50, 100, 200, 250 mM). Protein concentration was determined by using the Bradford assay (Bradford, 1976).

Co-purification of Delta and Omega proteins. Purified 6 \times His-tagged Omega protein eluted with 2 ml LEW buffer containing 25 mM imidazole was used for copurification of its partner using the Protino Ni-IDA 150 system (Macherey-Nagel). The 6 \times His-tagged Omega (~0.15 mg) was mixed with the soluble fraction of proteins from bacterial cells overproducing the Delta protein (~1 mg). When indicated, P δ DNA amplified using primers FHindIII and RPstI (~10 ng) and/or ATP (final concentration 1.25 mM) was added. As a control, the supernatant was also mixed with purified 6 \times His-tag. After 15 min incubation at room temperature, mixtures were loaded on Protino Ni-IDA 150 columns. The flow-through was collected, the

column washed with 2 \times 250 μ l LEW buffer and then treated with LEW buffer supplemented with increasing amounts of imidazole (25, 50, 100, 150, 200 and 250 mM).

Protein electrophoresis. Proteins were separated by using SDS-PAGE (Laemmli, 1970) with modifications allowing separation of small proteins in 15% polyacrylamide gels as described by Schägger & von Jagow (1987). Gels were silver stained as described by Morrissey (1981).

Western blot analysis. Proteins were electro blotted onto nitrocellulose (Optiran BA-S 83, Schleicher & Schuell) using the semi-dry transfer procedure. Immunological detection was performed using the SNAP i.d. protein detection system (Millipore) according to the manufacturer's recommendations. As primary antibody, anti-Delta serum (Kucharczyk TE) was used. ImmunoPure alkaline-phosphatase-conjugated goat anti-rabbit IgG (Pierce Biotechnology) was used as the secondary antibody and detected with CDP-Star (Roche).

RESULTS

Delta and Omega interactions *in vivo* were analysed in a bacterial two-hybrid system based on the LexA repressor (Dimitrova *et al.*, 1998). The N-terminal part (70 aa) of protein LexA constitutes the DNA-binding domain (DBD) while the C-terminal part is responsible for LexA dimerization, a step essential for LexA repressor activity. One of the proteins analysed in this system is fused to the N-terminal part of the wild-type version of the LexA protein (LexA^{WT}), whereas the other is fused to a modified version of the N terminus of LexA (LexA⁴⁰⁸). LexA^{WT} recognizes the wild-type half of the operator sequence, while LexA⁴⁰⁸ binds to the modified half of the LexA operator inserted in the *PlacZ* upstream of the β -galactosidase gene in *E. coli* GS1129. The LexA repressor can be reconstituted only when there is interaction between proteins to which LexA^{WT} and LexA⁴⁰⁸ are fused. Repression of the *lacZ* expression by LexA is indicative of an interaction of the proteins studied. Protein interaction was analysed by measuring the β -galactosidase activity in *E. coli* GS1129 harbouring pDP804 and pMS604 plasmid derivatives carrying genes encoding proteins of interest fused to the two forms of LexA. The level of β -galactosidase activity (in Miller units) was measured and calculated as the percentage of the activity obtained for the GS1129 strain carrying two empty vectors, pMH723 and pMH724 (100%), where the *lacZ* gene was unrepressed. The analysis of Delta and Omega variants gave similar results regardless of the variant of LexA they were fused to.

Delta protein dimerization

For Delta dimerization analysis in the bacterial two-hybrid system, plasmids pMS2000 (LexA^{WT}Delta) and pDP2000 (LexA⁴⁰⁸Delta) were simultaneously introduced into the *E. coli* GS1129 strain. As a control, strains carrying pairs of plasmids – pMH723 + pMH724, pMH723 + pMS2000 or pDP2000 + pMH724 – were used. The enzyme activity in strains carrying an empty vector combined with δ -encoding plasmid was the same as that of the strain with two empty vectors, whereas it dropped to 50% in a strain containing the two LexA–Delta fusion-encoding plasmids

Table 1. Plasmids used in this work

Details of plasmid construction are given in the text and in Supplementary Methods, available with the online version of this paper.

Plasmid	Relevant feature	Source/reference
pDP804 derivatives		
pDP804	Bacterial two-hybrid system vector, carries translational fusion of LexA ₁₋₈₇ 408-Jun zipper (triple substitution P40A-N41S-A42S), p15A replicon, Ap ^R	Dimitrova <i>et al.</i> (1998)
pDP2000	LexA ₁₋₈₇ 408-Delta translational fusion (δ gene cloned as <i>Bss</i> HIII- <i>Bgl</i> II fragment)	This work
pDP4000	LexA ₁₋₈₇ 408-Omega translational fusion (ω gene cloned as <i>Bss</i> HIII- <i>Bgl</i> II fragment)	This work
pDP4030A	LexA ₁₋₈₇ 408-Omega V30A, V32A translational fusion	This work
pDP4039A	LexA ₁₋₈₇ 408-Omega I39A, I40A translational fusion	This work
pDP4059A	LexA ₁₋₈₇ 408-Omega L59A, I63A translational fusion	This work
pMH723	pDP804 derivative with 408-Jun zipper gene deleted by digestion with <i>Bss</i> HIII and <i>Bgl</i> II	Lochowska <i>et al.</i> (2004)
pET28a+ derivatives		
pET28a+	<i>ori</i> _{ColE1} , Km ^R , T7p	Novagen
pET δ	T7p- δ	This work
pET δ 36A	T7p- δ K36A	This work
pET δ 60A	T7p- δ D60A	This work
pET ω H	T7p- ω , C-terminal 6 \times His tag	This work
pET ω 5NH	T7p- ω Δ 2-5, C-terminal 6 \times His tag	This work
pET ω 39AH	T7p- ω I39A, I40A, C-terminal 6 \times His tag	This work
pET ω 59AH	T7p- ω L59A, I63A, C-terminal 6 \times His tag	This work
pMS604 derivatives		
pMS604	Bacterial two-hybrid system vector, carries translational fusion of LexA ₁₋₈₇ WT-Fos zipper, <i>ori</i> _{ColE1} , Tc ^R	Dimitrova <i>et al.</i> (1998)
pMS2000	LexA ₁₋₈₇ WT-Delta translational fusion (δ gene cloned as <i>Bst</i> EII- <i>Xho</i> I fragment)	This work
pMS2023	LexA ₁₋₈₇ WT-Delta Δ 24-39 translational fusion	This work
pMS2036A	LexA ₁₋₈₇ WT-Delta K36A translational fusion	This work
pMS2036E	LexA ₁₋₈₇ WT-Delta K36E translational fusion	This work
pMS2060A	LexA ₁₋₈₇ WT-Delta D60A translational fusion	This work
pMS2104A	LexA ₁₋₈₇ WT-Delta L104A, I105A translational fusion	This work
pMS2198	LexA ₁₋₈₇ WT-Delta Δ 198-284 translational fusion	This work
pMS4000	LexA ₁₋₈₇ WT-Omega translational fusion (ω gene cloned as <i>Bst</i> EII- <i>Xho</i> I fragment)	This work
pMS4001A	LexA ₁₋₈₇ WT-Omega V1A translational fusion	This work
pMS4002A	LexA ₁₋₈₇ WT-Omega I2A translational fusion	This work
pMS4005	LexA ₁₋₈₇ WT-Omega Δ 2-5 translational fusion	This work
pMS4010	LexA ₁₋₈₇ WT-Omega Δ 2-10 translational fusion	This work
pMS4018	LexA ₁₋₈₇ WT-Omega Δ 2-18 translational fusion	This work
pMS4023A	LexA ₁₋₈₇ WT-Omega I2A, V3A translational fusion	This work
pMH724	pMS604 derivative with WT-Fos zipper gene deleted by digestion with <i>Bst</i> EII and <i>Xho</i> I	Lochowska <i>et al.</i> (2004)
pUC18 derivatives		
pUC18	<i>E. coli</i> general cloning vector <i>ori</i> _{ColE1} , Ap ^R	Yanisch-Perron <i>et al.</i> (1985)
pDO1000	δ and ω genes cloned into <i>Hind</i> III- <i>Eco</i> RI restriction sites	Dmowski <i>et al.</i> (2006)
pDO4000	<i>Hind</i> III- <i>Eco</i> RI DNA fragment from pMS4000 (LexA ₁₋₈₇ WT-Omega translational fusion)	This work

(Fig. 1). These results demonstrate that Delta is capable of dimerization *in vivo*.

Analysis of other A-type proteins involved in plasmid partitioning has demonstrated a role for ATP binding and/or hydrolysis in protein dimerization (Davey & Funnell, 1994; Fung *et al.*, 2001; Leonard *et al.*, 2005). To elucidate the role of the Walker A and A' motifs in Delta dimerization we have chosen the most conserved residues in the motif A and A' in ParA-like ATPases. Plasmids pMS2036A and pMS2036E encode Delta with the Lys36 residue substituted by alanine (K36A) or glutamic acid (K36E), respectively.

Another pMS2000 plasmid derivative, pMS2023, encoding Delta lacking aa 24–39 (the Walker A motif of ATPase) was also constructed. The fourth δ allele had the conserved Asp60 residue in the A' motif substituted by alanine (plasmid pMS2060A producing Delta D60A). The four mutant plasmids, paired with pDP2000, were used for transformation of *E. coli* GS1129 and the β -galactosidase activity in the transformants was analysed. None of the Lys36 substitutions studied abolished Delta dimerization; the relative *lacZ* activities were 36% (K36A) and 37% (K36E) (Fig. 2a, i). In contrast, deletion of the Walker A motif (pMS2023) abolished Delta protein dimerization. The possibility that

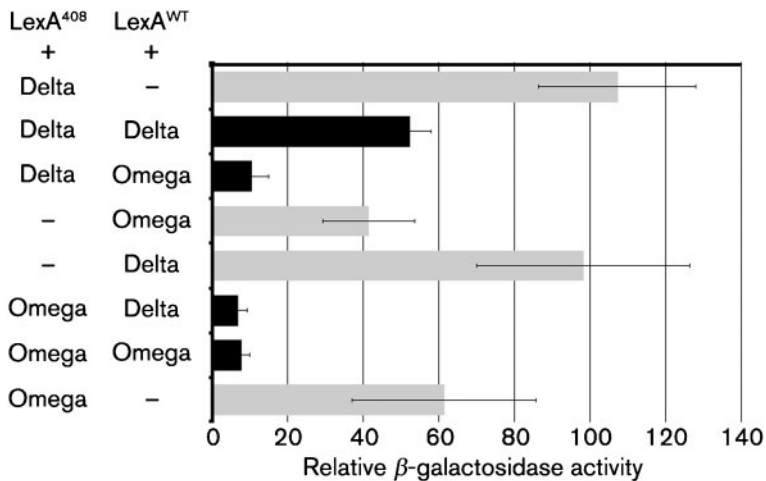


Fig. 1. Delta and Omega protein interactions in the bacterial two-hybrid system. β -Galactosidase activity was assayed in strains harbouring pairs of plasmids encoding fusions of LexA DBDs with Delta or Omega. The activity is presented relative to the *lacZ* activity measured in *E. coli* GS1129 with pMH723 and pMH724 (100%). LexA⁴⁰⁸Delta^{WT} fusion was expressed from pDP2000, LexA^{WT}Delta^{WT} fusion from pMS2000, LexA^{WT}Omega^{WT} fusion from pMS4000 and LexA⁴⁰⁸Omega^{WT} fusion from pDP4000. Error bars show SD.

such a deletion causes an extensive conformational change of Delta cannot be excluded. Delta dimerization was also unaffected by the D60A mutant (plasmid pMS2060A); the relative β -galactosidase activity was 49% (Fig. 2a, i).

In a study of *Pseudomonas aeruginosa* partition proteins, a region of ParA_{P.a} protein involved in both dimerization and interaction with its partner ParB_{P.a} was identified (K. Lasocki and G. Jagura-Burdzy, unpublished data). Alignment of the ParA_{P.a} and Delta amino acid sequence indicated the homologous region of Delta (Supplementary Fig. S1). For further analysis, two residues from this region (Leu104 and Ile105) were selected for substitution. Plasmid pMS2104A (Delta L104A, I105A) was constructed and the relative β -galactosidase activity was measured in the *E. coli* GS1129 derivative carrying this plasmid together with pDP2000 (Fig. 2a, i). The unrepressed level of β -galactosidase activity (106%) indicated that this double substitution abolished Delta dimerization. The soluble fractions of extracts from strains tested in the bacterial two hybrid system were screened by Western blotting with anti-Delta antibodies (Supplementary Fig. S3, available with the online version of this paper). The levels of LexA–Delta and LexA–Delta L104A I105A hybrid proteins were comparable, excluding the possibility that lack of self-interactions is due to the instability of such a Delta variant.

In order to analyse the role of the C terminus of Delta protein in its dimerization, plasmid pMS2198 (Delta lacking the 86 C-terminal residues) was analysed. Its analysis in the bacterial two-hybrid system revealed that the C terminus does not play an important role in Delta dimerization: the relative β -galactosidase activity in the *E. coli* GS1129 strain carrying pMS2198 and pDP2000 plasmids constituted 62% of the control strain carrying empty vectors (intact Delta gives repression to 55%) (Fig. 2a, i).

Omega protein dimerization

To analyse the ability of Omega protein to dimerize *in vivo*, plasmids pMS4000 (LexA^{WT}Omega) and pDP4000

(LexA⁴⁰⁸Omega) were used for transformation of *E. coli* GS1129. Interestingly, strains with a combination of an empty vector and one LexA–Omega fusion showed some *lacZ* repression; this was 42–62% of the *lacZ* activity detected in the strain with empty vectors. The relative β -galactosidase activity of the strain containing Omega fused with two LexA DBDs was six- to ninefold lower than that observed in the strain with a single LexA–Omega fusion (Fig. 1). These results indicate that the assay can be used to demonstrate Omega–Omega interaction *in vivo*.

First, the role of the flexible N terminus of Omega in its dimerization was studied. For this, plasmids pMS4005, pMS4010 and pMS4018 encoding Omega variants deprived of 4, 9 or 17 N-terminal amino acids, respectively, were prepared. The β -galactosidase activity of *E. coli* GS1129 strains carrying either of these plasmids coupled with the pDP4000 plasmid encoding the intact Omega protein was analysed (Fig. 2b, ii). The results demonstrated that the removal of 4, 9 or even 17 amino acid residues from the N terminus had no effect on Omega dimerization (10, 13 and 10% β -galactosidase activity, respectively, compared with intact Omega, 7%).

On the basis of the crystal structure of Omega protein it has been previously suggested that hydrophobic residues located in both ribbon and helix structures are involved in protein dimerization (Murayama *et al.*, 2001). In order to verify the role of the β , α 1 and α 2 structures in Omega dimerization, three Omega derivatives with pairs of hydrophobic amino acids from these regions converted into alanines were constructed (Supplementary Fig. S2). *E. coli* GS1129 was transformed with plasmid pMS4000 paired with one of the following plasmids: pDP4030A (Val30 and Val32 converted into alanines), pDP4039A (Ile39 and Ile40 converted into alanines) and pDP4059A (Leu59 and Ile63 converted into alanines). Analysis of β -galactosidase activity expressed in such strains showed that abrogation of potential hydrophobic interactions in any of the secondary structures investigated had a negative effect on Omega dimerization. The substitutions in the β , α 1 and α 2

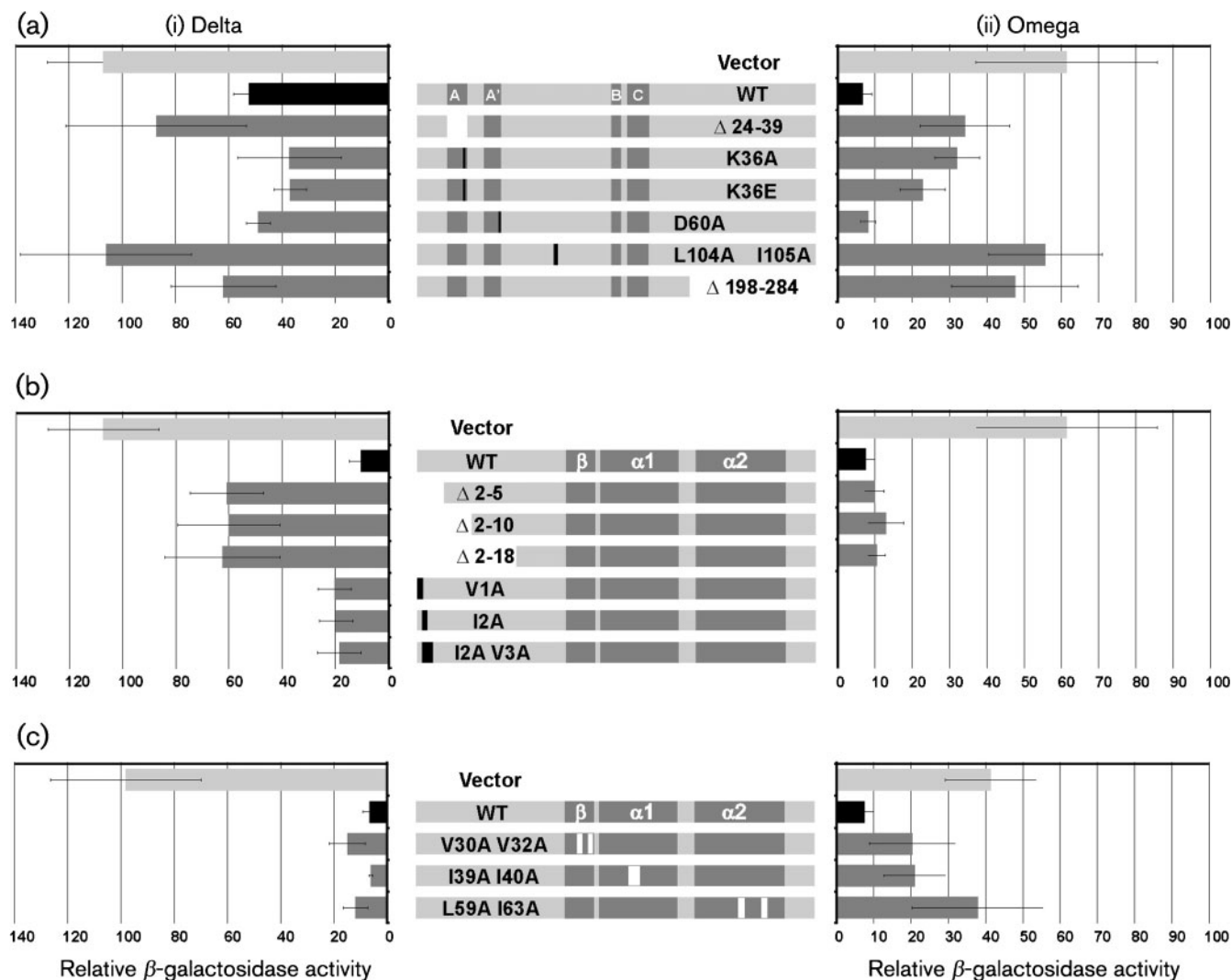


Fig. 2. Dimerization and hetero-interactions of Delta and Omega protein variants in bacterial two-hybrid system. β -Galactosidase activity was assayed in *E. coli* GS1129 harbouring pairs of plasmids carrying: (a) alleles encoding Delta variants fused with LexA^{WT} in combination with Delta or Omega fused with LexA⁴⁰⁸ (similar results were obtained when Delta variants were fused with LexA⁴⁰⁸); (b) alleles encoding Omega variants modified in the N-terminal part fused with LexA^{WT} in combination with Delta or Omega fused with LexA⁴⁰⁸ (similar results were obtained when Omega variants were fused with LexA⁴⁰⁸); (c) alleles encoding Omega variants modified in the RHH structures fused with LexA⁴⁰⁸ in combination with Delta or Omega fused with LexA^{WT} (similar results were obtained when Omega variants were fused with LexA^{WT}). β -Galactosidase activity is presented relative to that in *E. coli* GS1129 harbouring plasmids encoding LexA domain only (pMH723 and pMH724; 100%). Modifications introduced in Delta and Omega proteins are shown. Error bars show SD.

regions roughly halved the ability of Omega to dimerize (Fig. 2c, ii).

Delta and Omega protein interaction

Models of plasmid partition predict a direct interaction between the ParA-like and ParB-like proteins. To investigate the interaction between Delta and Omega proteins, pairs of plasmids pDP2000 + pMS4000 and pDP4000 + pMS2000 were introduced into *E. coli* GS1129 to use the β -galactosidase activity as a measure of the Delta–Omega interaction. The

lacZ activity in strains carrying the Delta and Omega fusions with LexA DBDs represented 7 and 10% of the β -galactosidase activity found in cells carrying empty vectors (Fig. 1), indicating a strong interaction between the two proteins. Thus, the LexA-based bacterial two-hybrid system proved very useful for monitoring the interaction of Delta and Omega proteins *in vivo*.

The association of Delta and Omega proteins was also analysed *in vitro*. Purified Omega protein with a 6 \times His-tag at the C-terminal end (produced from pET ω H) was coupled

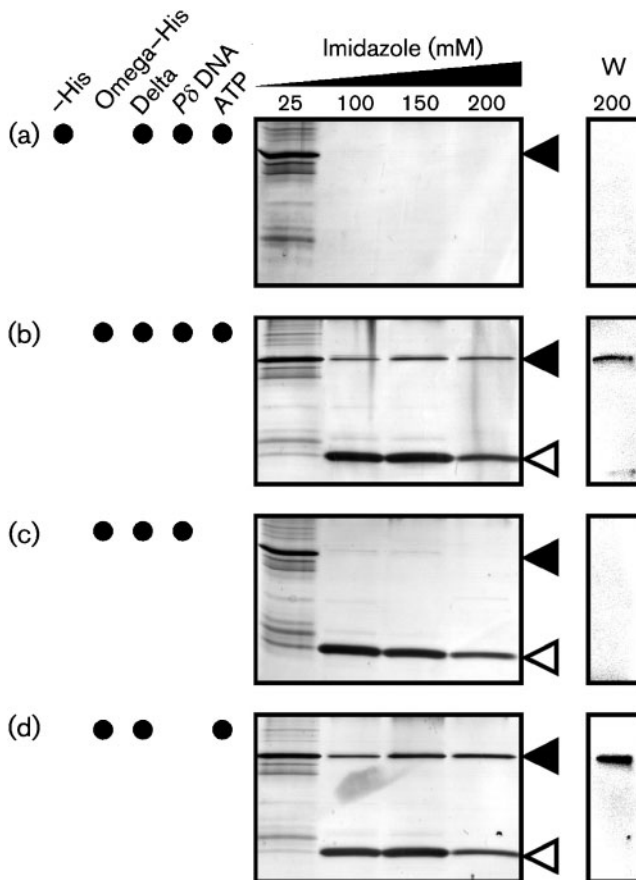


Fig. 3. Association of Delta and Omega proteins *in vitro*. Purified Omega-6×His protein was mixed with soluble fraction of proteins obtained from a Delta overproducing strain BL21(DE3) pET δ . Where indicated, ATP (1.25 mM) and DNA fragment with Omega-binding sites ($P\delta$) were added to the mixture before column loading. Eluates obtained with increasing imidazole concentrations in wash buffer (25, 100, 150 and 200 mM) were separated by SDS-PAGE and silver stained. The composition of the incubation mixtures (a–d) is indicated on the left. Black and white arrowheads indicate positions of Delta and Omega on the gel, respectively. W, Immunodetection of Delta with anti-Delta antibodies in eluate with 200 mM imidazole.

with Delta in a native form (produced from pET δ). In the control experiment, the purified Omega-6×His was replaced with a purified short polypeptide with a 6×His-tag (pET28a+). Each protein mixture was supplemented with ATP and Omega-binding DNA sequences ($P\delta$ region). In the presence of Omega-6×His, the binding of Delta to Ni²⁺-agarose was strong, as it remained attached to the column even in the presence of 150 mM imidazole (Fig. 3b). In the control experiment (Delta coupled with 6×His), Delta could be eluted at 25 mM imidazole (Fig. 3a), indicating that the interaction of Delta with the resin was dependent on the presence of Omega.

In order to examine whether exogenous ATP or Omega-binding DNA sequences are necessary for the Delta–Omega

interaction, mixtures deprived of one of these components were prepared and treated as above. This experiment showed that omitting ATP in the mixture resulted in a decreased Delta affinity to the column, similar to that observed when Delta was incubated with the short polypeptide with 6×His alone (Fig. 3c). In contrast, the absence of the $P\delta$ region had no negative influence on the Delta binding to Ni²⁺-agarose with immobilized Omega-6×His (Fig. 3d). These results indicate that ATP is required for Omega association with Delta *in vitro*, whereas Omega-binding DNA sequences are not.

To analyse the role of the Walker A and A' motifs in Delta interaction with Omega, plasmids pMS2036A, pMS2036E, pMS2023 and pMS2060A (which produce Delta variants able to dimerize) were used for co-transformation of *E. coli* GS1129 together with plasmid pDP4000 (LexA⁴⁰⁸Omega^{WT}). Both substitutions of Lys36 as well as the removal of the ATPase motif A substantially decreased the ability of Delta to interact with Omega. The relative *lacZ* activities were 32 % (DeltaK36A–Omega), 23 % (DeltaK36E–Omega) and 34 % (Delta Δ WA–Omega) compared with 7 % activity in the presence of DeltaWT–Omega (Fig. 2a, ii). Single substitution of Asp60 had no effect on the Delta interaction with Omega; the relative β -galactosidase activity was 8 % (DeltaD60A–Omega) (Fig. 2a, ii).

Delta variants carrying substitutions in the Walker A or A' motifs were also tested for their interaction with Omega *in vitro*. Two Delta derivatives were produced from plasmids pET δ 36A, pET δ 60A and mixed with Omega-6×His in the presence of ATP. DeltaK36A was unable to bind Omega (Fig. 4c). In contrast, DeltaD60A seemed to be unaffected in Omega protein binding (Fig. 4d).

We also analysed a Delta variant with substitutions in the region between motifs A' and B (Delta L104A, I105A), which compromise Delta dimerization. When the pMS2104A plasmid was tested in a pair with plasmid pDP4000 (LexA⁴⁰⁸Omega), the relative β -galactosidase activity was 56 % (Fig. 2a, ii), indicating that Delta loses the ability to interact with Omega at the same time as the dimerization defect appears.

Deletion of 86 C-terminal amino acids had no effect on Delta dimerization. The role of the C-terminal part of the Delta protein was analysed by using plasmid pMS2198 (Delta Δ 198–284) co-introduced with pDP4000. Removal of the 86 C-terminal residues from Delta had a negative effect on its ability to interact with Omega (β -galactosidase activity 48 % of control) (Fig. 2a, ii). This value is similar to that (61 %) obtained when an empty vector was coupled with pDP4000 (LexA⁴⁰⁸Omega). This clearly demonstrates that the C-terminal deletion analysed abolished the ability of Delta to interact with Omega.

Analysis of the crystal structure of ParG – a protein which folds into an RHH structure, similarly to Omega – has indicated that the unstructured N-terminal tail and α -helical regions may be involved in interactions with the

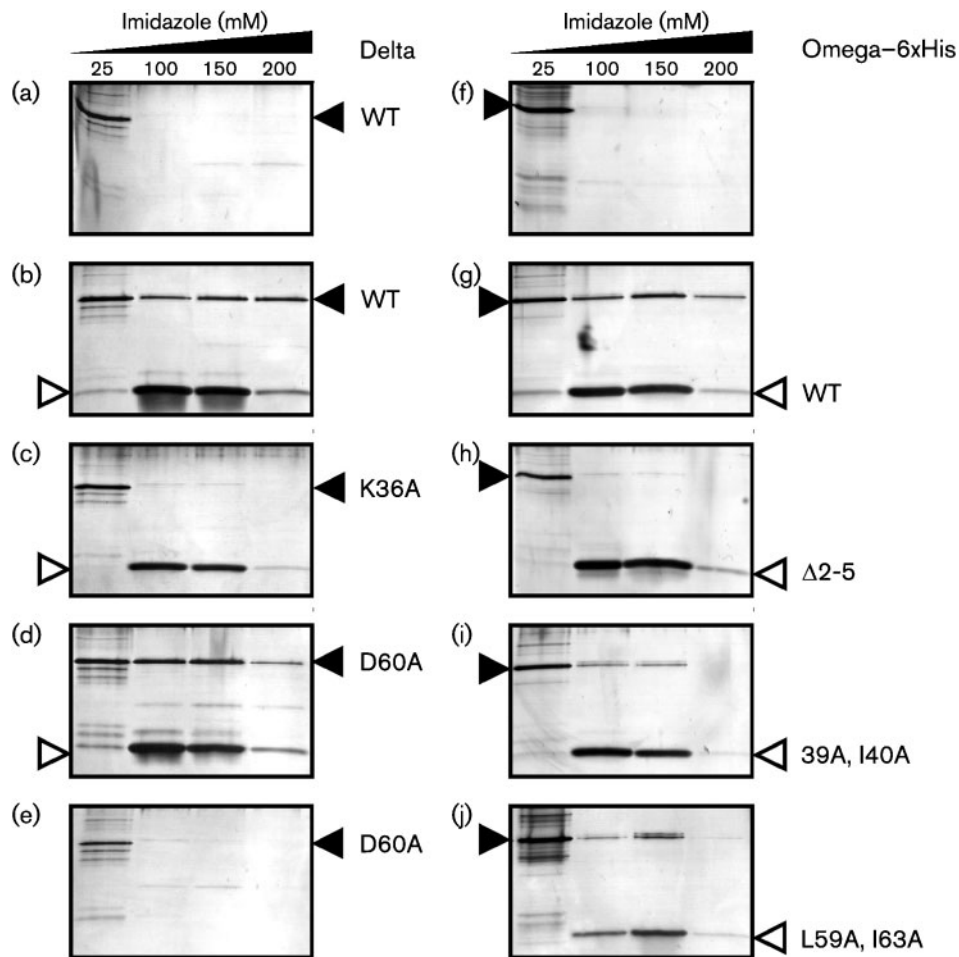


Fig. 4. Association of modified Delta and Omega proteins *in vitro*. Eluates obtained with increasing imidazole concentrations in wash buffer (25, 100, 150 and 200 mM) were analysed by SDS-PAGE and silver stained. (a–e) Purified Omega-6×His protein (white arrowhead) was mixed with the soluble fraction of proteins obtained from strains producing different untagged variants of Delta protein (black arrowhead) (b–d) in the presence of 1.25 mM ATP. In control experiments (a and e) purified His-tag was used instead of Omega-6×His. (f–j) Purified Omega-6×His protein or its variants (white arrowhead) were mixed in the presence of 1.25 mM ATP with the soluble fraction of proteins obtained from strains producing Delta protein (black arrowhead) (g–j). In the control (f) the soluble fraction of proteins containing Delta was mixed with purified His-tag instead of Omega-6×His.

ParF ATPase, the Delta homologue in this system (Golovanov *et al.*, 2003). In order to verify the role of the N-terminal part of Omega (not essential for Omega dimerization) in the interaction with Delta, Omega variants deprived of a different number of N-terminal amino acids (plasmids pMS4005, pMS4010 and pMS4018) were tested in the two-hybrid system together with Delta. Analysis of β -galactosidase activity in double transformants of *E. coli* GS1129 showed that removal of 17, 9 or even 4 amino acids located in the N terminus of Omega strongly affected Omega interaction with Delta: the *lacZ* activity was 60% of control (11% for WT Omega) (Fig. 2b, i).

The role of the N-terminal part of the Omega protein in its interaction with Delta was also tested *in vitro*. The Omega Δ 2-5–His variant was produced from plasmid

pET ω 5NH and used for Delta co-purification. Omega Δ 2-5 failed to associate with Delta *in vitro* (Fig. 4h).

A more detailed analysis of the Omega N terminus was then attempted. Each of the three N-terminal amino acids was substituted with alanine (plasmids pMS4001A, pMS4002A and pMS4023A). These constructs, paired with pDP2000 (LexA⁴⁰⁸Delta), were used for co-transformation of *E. coli* GS1129. Analysis of β -galactosidase activity showed that both the single and double amino acid substitutions in the N-terminal tip of Omega had a slight negative effect on the ability of Omega to interact with Delta (twofold higher β -galactosidase activity than that with WT Omega) (Fig. 2b, i).

The Omega variants deficient in dimerization carrying substitutions of hydrophobic amino acids in the β , α 1 or α 2

structures were also tested for their ability to associate with Delta (plasmid pMS2000 paired with pDP4030A, pDP4039A or pDP4059A). The analysed amino acid substitutions hardly affected the Omega–Delta interaction. The relative β -galactosidase activities in strains carrying plasmids with these mutations were 15 % (β), 6 % ($\alpha 1$) and 11 % ($\alpha 2$) compared with 7 % for the WT Omega (Fig. 2c, i).

Additionally, Omega variants I39A I40A and L59A I63A substitutions were produced from plasmids pET ω 39AH and pET59AH. The ability of these purified Omega–His variants to interact with Delta in the presence of ATP was analysed *in vitro*. Omega I39A I40A and L59A I63A were still able to associate with Delta, albeit somewhat weaker than WT Omega (Fig. 4i, j).

DISCUSSION

The first attempts in our laboratory to demonstrate Omega dimerization *in vivo* with the use of a yeast two-hybrid system were unsuccessful (Sitkiewicz, 2002). This was thought to be due to improper folding of the hybrid protein. The use of a bacterial two-hybrid system in the present study allowed demonstration of Omega–Omega interactions *in vivo*; the β -galactosidase activity of a strain carrying Omega fusions with two LexA DBDs was about 20-fold lower than that observed for a non-repressed negative control (expressing non-fused LexA DBDs) (Fig. 1). However, we also observed slight repression of *lacZ* in control strains carrying pairs of plasmids with Omega fused to only one LexA DBD. It is possible that Omega, a DNA-binding protein, represses the reporter gene by a non-specific interaction with its promoter region, or a dimer of the LexA_{DBD}–Omega hybrid protein is able to recognize both arms of the artificial LexA operator and interfere with the transcriptional machinery. Nevertheless, specific Omega dimerization has been clearly demonstrated using this system. Omega translational fusion proteins were also used to analyse the Delta–Omega interaction. The detected interactions were weaker than those seen in the Omega dimerization assay, although strong enough to allow analysis of the mutated alleles of both genes. The results of the Delta and Omega mutant analysis using a bacterial two-hybrid system supported by *in vitro* studies on purified proteins are summarized in Fig. 5.

The analysis of Omega structure performed by Murayama *et al.* (2001) has predicted that the dimerization depends on hydrophobic residues in the C-terminal helical structures of the protein. Similar observations based on structural studies were made for the ParG protein from TP228 (Golovanov *et al.*, 2003). Here, we directly showed that alanine substitution of two hydrophobic residues in the $\alpha 1$ (I39A and I40A) or $\alpha 2$ helix (L59A and I63A) (Supplementary Fig. S2) precluded Omega dimerization (Fig. 2c, ii). Interestingly, the substitution of two hydrophobic residues in the β -ribbon (V30A and V32A) also abolished dimerization of Omega *in vivo*. As Omega

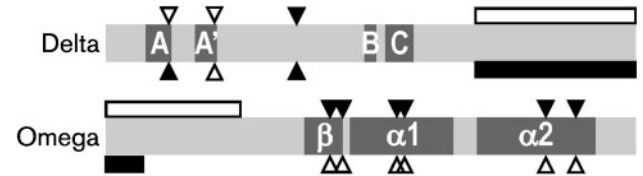


Fig. 5. Summary of mutant Delta and Omega protein features. Amino acids modified in Delta and Omega variants are indicated by arrowheads above and below the schematic protein representations. Black/white arrowheads indicate substantial/negligible effects, respectively, on homodimerization (symbols above bar) and interaction with the partner (below the bar). Deletions analysed are shown by rectangles.

binds specific DNA sequence as a dimer (de la Hoz *et al.*, 2004), our results correlate with the observation that the T29A substitution in the β -ribbon, which did not change the conformation of Omega, reduced its DNA binding and *P δ* promoter repression (Welfle *et al.*, 2005). A comparative analysis of RHH transcription regulators involved in plasmid partition showed that hydrophobic residues analysed in the present work are conserved in these positions of β , $\alpha 1$ and $\alpha 2$ structures (Supplementary Fig. S2) (Schreiter & Drennan, 2007).

It was previously shown that the repressor activity of Omega was unaffected when the protein was deprived of 7, 17 or 18 N-terminal amino acids (Welfle *et al.*, 2005). It was therefore postulated that the N terminus is not involved in Omega dimerization. In this work, we directly demonstrate that deletion of 5, 10 or 18 amino acids had no effect on Omega self-association (Fig. 2b, ii, and Supplementary Fig. S2). In the partition system of TP228, deletion of 9 or 19 aa from the N terminus of ParG did not abolish ParG dimerization (Carmelo *et al.*, 2005). Analysis of another RHH-type ParB protein (from pB171 plasmid) also confirms that its 19 N-terminal amino acids are not involved in dimerization. In contrast, this region was indispensable for formation of high-molecular-mass complexes, suggesting its involvement in the pairing of *parC* centromeres (Ringgaard *et al.*, 2007).

Golovanov *et al.* (2003) suggested that β -ribbons and α -helices of the RHH protein ParG are involved in its interaction with its partition partner ParF. Our *in vivo* analysis of Omega derivatives with amino acid substitutions in β , $\alpha 1$ and $\alpha 2$ structures did not confirm this hypothesis; all three Omega variants impaired in self-association (Omega V30A V32A, Omega I39A I40A, and Omega L59A I63A) efficiently formed heterodimers with Delta (Fig. 2c and Supplementary Fig. S2). The Omega I39A I40A and L59A I63A variants could interact with Delta *in vitro* as well (Fig. 4i, j). This clearly suggests that the ability of Omega to dimerize is not a prerequisite for interaction with Delta, in contrast to what has been observed for type Ia ParB protein from *P. aeruginosa* (Bartosik *et al.*, 2004).

Omega variants lacking 17, 9 or 4 residues were tested for association with Delta. The lack of an interaction between the N-terminally truncated Omega and its partner protein Delta (both *in vivo* and *in vitro*) has clearly demonstrated that the unstructured N terminus of Omega is involved in interaction with Delta (Figs 2b and 4h). An analysis of this region by single or double amino acid substitutions also supported the above conclusion: these modifications weakened the interaction of Omega with Delta, although none eliminated the interactions completely (Fig. 2b).

Our results clearly demonstrate that the C-terminal part of Omega is the dimerization domain, whereas the unstructured N-terminal part does not participate in dimerization but is indispensable for Omega–Delta interactions (Fig. 5). These results correlate with the previous observation that deletion of 19 N-terminal amino acids had a marginal stimulating effect on Delta ATPase activity and polymerization (Pratto *et al.*, 2008). As for other systems, an involvement of the ParG N terminus in interaction with ParF was confirmed by the observed ability of ParG to promote ParF polymerization (Barillà *et al.*, 2007). Machón *et al.* (2007) have shown that such ParF stimulation is not fully specific since it can also be brought about by heterologous ParG-like proteins from the pTAR and pB171 partition systems.

The mutational analysis of Delta Walker A motif (VILNNYFKGGVGVGKSKL) showed that substitution of the conserved lysine residue at position 36 with alanine or glutamic acid (K36A or K36E) had no effect on Delta dimerization (Fig. 2a and Supplementary Fig. S1), although such substitution abolished ATP binding by ParA of P1 (Davis *et al.*, 1996). These observations contradict the results obtained for other ParA-like proteins showing the necessity of ADP or ATP binding for dimerization (Davey & Funnell, 1994; Fung *et al.*, 2001; Leonard *et al.*, 2005). However, the crystal structure of Delta (Pratto *et al.*, 2008) demonstrates a cleft in the Delta dimer which allows free exchange of ATP and ADP. These authors also observed dimers of Delta in solution in the presence of ATP or ADP, but also in the absence of a nucleotide.

Delta protein derivatives with single amino acid substitutions (K36A or K36E) in the Walker A motif still capable of dimerization have lost their ability to associate with Omega (Figs 2a and 4c and Supplementary Fig. S1). The lack of interaction of these Delta mutants with Omega can indicate the necessity of ATP binding for such interactions. Also the results of the *in vitro* Delta–Omega co-purification assay clearly show that ATP is required for the Delta–Omega association (Fig. 3). These results explain the observed inability of Delta K36A to promote plasmid pairing (Pratto *et al.*, 2008).

Our study also demonstrated that substitution of a conserved aspartic acid in the A' motif with alanine (Delta D60A derivative) affected neither Delta dimerization nor its interaction with Omega (Figs 2a, 4d and 5). Pratto *et al.* (2009) found that a D60A substitution severely affected the disassembly of a complex formed *in vitro* by the Delta and

Omega proteins bound to the centromeric sequence. An equivalent substitution in Soj (D44A; Supplementary Fig. S1) abolished ATP hydrolysis but not ATP binding (Leonard *et al.*, 2005). It could be hypothesized that ATP hydrolysis provokes and/or is necessary for the disassembly of Delta and Omega proteins and, as a consequence, plasmid pair separation. The L104A I105A Delta variant, with modifications of two hydrophobic residues in the highly conserved region between the A' and B motifs was unable to dimerize (Fig. 5 and Supplementary Fig. S1). This region has previously been indicated as being responsible for homo-dimerization of the chromosomally encoded ParA protein of *P. aeruginosa* and interaction with its ParB partner (K. Lasocki and G. Jagura-Burdzy, unpublished data). The L104A and I105A amino acid substitutions resulted in the inability of Delta to both dimerize and interact with Omega (Fig. 2a). Since the level of hybrid protein LexA–Delta L104A I105A is comparable with LexA–Delta or LexA–Delta D60A (Supplementary Fig. S3) the instability of this hybrid cannot be responsible for the lack of interactions. These data seem to confirm the necessity of Delta dimerization for its interaction with Omega and indicate that the partition ATPases have a highly similar organization and function regardless of being encoded by plasmids or chromosomes.

An interesting observation is that the Delta variant deprived of the C terminus (Delta Δ 198–284) can dimerize but cannot associate with Omega. This would identify the C terminus of Delta as the region of interaction with Omega. It has been shown previously (Kim & Shim, 1999; Ravin *et al.*, 2003) that the C-terminal amino acids of F SopA protein are essential for its interaction with SopB.

In summary, our study not only complements the *in vitro* analyses of Delta and Omega proteins but also provides important new information. Delta dimerization does not require ATP binding. The conserved region with L104 and I105 seems to be important for the dimerization as well as for interactions with Omega. The ability to interact with Omega is conditioned by Delta binding of ATP and the presence of an intact C terminus. Omega dimerization involves not only its α -helices but also the β -sheet. Omega can interact with Delta as a monomer and the unstructured N-terminal part of Omega is involved in this interaction.

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