Omega (ParB) binding sites together with the RNA polymerase-recognized sequence are essential for centromeric functions of the $P_\omega$ region in the partition system of pSM19035

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INTRODUCTION

The stable inheritance of low-copy-number plasmids in bacteria cannot be achieved via the random diffusion of plasmid molecules to daughter cells, and thus depends on mechanisms that ensure better-than-random segregation, i.e. active partition (PAR) (Dmowski & Jagura-Burdzy, 2013a; Sengupta & Austin, 2011) and postsegregational killing (PSK) or toxin–antidote (TA) systems (Dmowski & Jagura-Burdzy, 2013b; Sengupta & Austin, 2011). PAR systems precisely distribute plasmids to future daughter cells, whereas PSK/TA systems function after cellular division to eliminate from the population those cells that have lost the plasmid.

PAR systems are composed of two proteins and a cis-acting DNA sequence. In the archetypical PAR system of the P1 plasmid, the ParB protein binds the centromeric region $parS$, whereas ParA, a deviant Walker-type ATPase (Koonin, 1993), provides the driving force for plasmid segregation to daughter cells. Components with similar functions have also been identified in other partition systems from both Gram-negative and Gram-positive bacteria (Dmowski & Jagura-Burdzy, 2013a; Schumacher, 2012). The $parA$-like and $parB$-like genes are usually organized into a single operon regulated by their protein products, ensuring the tight regulation of $par$ gene expression and the proper balance of Par proteins. Plasmid partition system classification is based on the type of Par proteins and the location of the centromeric region (Gerdes et al., 2000; Möller-Jensen & Gerdes, 2007). Type I systems encode Walker-type ATPases that in subtype Ia, but not Ib, systems also bind to DNA through an N-terminal helix–turn–helix (HTH) motif to regulate $par$ operon expression. Type II systems encode actin-like ATPases, whereas type III systems encode tubulin-like GTPases. The DNA-binding proteins of type Ia partition systems are large, highly conserved proteins (ParB family) with
HTH motifs; in contrast, type Ib, II and III proteins are variable but possess domains structurally related to the ribbon–helix–helix (RHH) class. In all partition systems, a specific DNA sequence, designated the centromere-like sequence (parS), plays an important role involving formation of a nucleoprotein complex designated the segrosome. These regions are located either downstream of the par operon (most type Ia systems) or in its promoter region (type Ib, II and III systems) (Gerdes et al., 2010). In general, there is a unique parS sequence in the DNA molecule to be partitioned. However, plasmids, such as RK2 (Williams et al., 1998) or RA3 (Kulinska et al., 2008, 2011), exist in which ParB recognizes multiple binding sites in the plasmid genome, among which only one site acts as the centromeric sequence. Conversely, in the linear phage N15, any of the centromeric sites (parS1–parS4) located at a distance from the par operon can act as the centromeric sequence for plasmids carrying the parAB cassette albeit at least two parS sequences are necessary for almost complete stability of a test plasmid (Grigoriev & Lobocka, 2001). Multiple centromeric sequences can be beneficial because the probability of the simultaneous loss of all sites is low, especially if one of them is situated in the minimal replicon (Grigoriev & Lobocka, 2001).

Multiple centromeric sequences are also characteristic of the partition systems present in bacterial chromosomes. In general, chromosomal parAparB loci are located close to the origin of replication, whereas the centromeric sequences parS are dispersed throughout the chromosome. Nonetheless, the majority of parS sites are clustered within the 20% of the genome that is origin-proximal (Bartosik & Jagura-Burdzy, 2005; Livny et al., 2007). It was recently shown in *Pseudomonas aeruginosa* that four origin-proximal parS sites (parS1–4) are efficient in partitioning and cannot be substituted by any of the remaining six parS (parS5–10) sites present in the

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**Fig. 1.** Omega-binding sites in pSM19035. Genes from the duplicated and unrepeated regions are shown in black and grey, respectively. The Omega protein specifically binds to DNA in the promoter regions $P_{\text{copS}}$ (of the copS copy number regulator), $P_1$ (of the gene encoding the partition ATPase) and $P_\omega$ (ω operon) to regulate its own expression (gene ω) and the expression of genes ε and ζ (postsegregational killing system). The organization of repeated WATCACW heptads in each promoter region is shown. The −10 sequences recognized by RNA polymerase are highlighted in grey. Arrowheads correspond to transcription start sites. The start triplets are underlined.
genome. Moreover, a single intact origin-proximal parS site (when all other sites were mutated) was sufficient to preserve the wild-type phenotype of P. aeruginosa, with regard to the process of chromosome partitioning (Jecz et al., 2015).

In this work, we analysed the centromeric sites of the partition system of plasmid pSM19035 from Streptococcus pyogenes, which is representative of broad host range inc18-family plasmids known to carry multiple resistance genes. Plasmids of this family are found in enterococci, lactococci, staphylococci and streptococci (Liu et al., 2007; O’Connor et al., 2007; Zha et al., 2010). The partition system of plasmid pSM19035 is encoded by genes δ and ω, which encode a Walker-type ATPase (ParA-like) and an RHH DNA-binding protein (ParB-like), respectively. In contrast to other partition systems, the δ and ω genes are organized into separate transcriptional units (Dmowski et al., 2006), and ω forms an operon together with the ε and ζ genes, which encode the postsegregational killing system of plasmid pSM19035 (Ceglowski et al., 1993; Zielenkiewicz & Ceglowski, 2005). Moreover, the product of gene ω, the ParB-like protein Omega, acts as a repressor that binds to repeated WAT-CACW heptads that differ in number and organization and are located in the promoter region of the partition gene δ (Pδ→→→→→→→→), the operon ωεζ (Pω→→→→→→→→) as well as the promoter region of the copy number control gene copS (PcopS→→→→→→→→) (de la Hoz et al., 2000) (Fig. 1). Based on bioinformatic analyses, the Delta protein (a Walker-type ATPase with no DNA-binding region at its N-terminus) was initially classified as a component of the type Ib partition system (Gerdes et al., 2000), a classification that led to the supposition that the centromeric sequence of this system may be located upstream of the parA-like gene δ – in its promoter region Pδ (Fig. 1). However, we later showed that substitution of the Pδ region with either Pω or PcopS has no effect on the replicon-stabilizing ability of the δ-ω-encoding partition system in Bacillus subtilis (Dmowski et al., 2006), indicating that the Pδ region is exchangeable. Furthermore, a partition-mediated incompatibility assay demonstrated that any of the three Omega-binding regions (PcopS Pδ and Pω) constitutes an incompatibility determinant: when provided in trans, they destabilize a replicon carrying a functional δ-ω-encoded partition system (Dmowski et al., 2006). This finding led to the conclusion that each region containing repeated heptads may constitute a centromeric sequence. Alternatively, it can be speculated that the presence of a supplementary Omega-binding site in trans may result in titration of this ParB-like protein, thus impairing the partition system. Additional support for the hypothesis of multiple centromeric sequences in pSM19035 was obtained by in vitro observation demonstrating that provision of more than four heptads does not increase Omega’s affinity for DNA and that there is no difference in Omega binding to the three promoters (de la Hoz et al., 2000, 2004). Further studies of Omega-binding regions have been largely limited to detailed in vitro analyses of protein–DNA interactions, whereas functional studies of the centromeric function of Pδ, PcopS and Pω are scarce. Because demonstrating function is complicated by the ability of Omega to bind upstream of both the δ and ω genes, we constructed a synthetic δω operon, which provided an excellent tool for further analysis of the role of individual Omega-binding sites in the partition process of inc18 plasmids.

METHODS

**Bacterial strains, growth and transformation.** The bacterial strains used in this work were Escherichia coli DH5α (F gyrA96 recA1 relA1 endA1 thi hsdR17 supE44 deoR Δ[lacZAM15]) (Hanahan & Harbor, 1983) and B. subtilis YB1015 (amyE metB5 trpC2 xin-1 attSP6 ΔrecA4) (Friedman & Yasbin, 1983). The E. coli and B. subtilis strains were grown routinely at 37 °C in L broth, liquid or solidified with 1.5 % agar. For plasmid stability tests, B. subtilis cells were grown at 30 °C in GM1 minimal medium (Rotthuender & Trautner, 1970). When necessary, antibiotics were added at the following concentrations: ampicillin 100 µg ml⁻¹ (E. coli), streptomycin 20 µg ml⁻¹ (E. coli) and chloramphenicol 5 µg ml⁻¹ (B. subtilis). B. subtilis and E. coli were transformed according to standard procedures (Rotthuender & Trautner, 1970; Sambrook & Russell, 2001).

**DNA manipulations.** Plasmid DNA was isolated using Plasmid Mini, Plasmid MiniAX or Plasmid MidiAX kits (A&A Biotechnology). PCRs were performed according to Sambrook & Russell (2001). Polymerases, restriction endonucleases and modifying enzymes were supplied by MBI Fermentas (Thermo Scientific), EURLx or New England Biolabs and used as recommended by the suppliers. DNA fragments obtained upon enzymatic reactions or PCR amplification were purified using Clean-up or Gel-out extraction kits (A&A Biotechnology).

**Plasmids.** The plasmids used in this study are presented in Table 1, Table S1 (available in the online Supplementary Material) and Fig 2. PCR primers are listed in Table S2. Details of plasmid construction are presented in the supplementary file.

**RNA isolation and RT-PCR.** Total RNA from B. subtilis was isolated using the modified method of Chomczynski (1993). A 12 ml culture of a selected B. subtilis strain grown overnight in GM1 medium at 30 °C was centrifuged. The pellet was resuspended in 250 µl DEPC-diethylpyrocarbonate (DEPC)-treated water with lysozyme (10 mg ml⁻¹) and incubated for 5 min at room temperature, followed by three cycles of freezing and thawing. One millilitre of TRI Reagent (Molecular Research Center, Inc.) was added, and the mixture was incubated for 5 min at room temperature. After the addition of 200 µl chloroform, the samples were vortexed for 15 s, incubated at room temperature for 10 min and centrifuged at 12 000 g for 15 min at 4 °C. The aqueous phase was transferred to a new tube, and RNA was precipitated with 500 µl isopropanol for 10 min at room temperature. After centrifugation at 12 000 g for 10 min, the pelleted RNA was washed with 1 ml 70% ethanol and dissolved in 100 µl DEPC-treated water by heating for 10 min at 56 °C.

cDNA synthesis was performed as follows. First, a mixture of RNA, dNTPs and reverse primers RBamHI or OmegaER was heated to 65 °C for 5 min and incubated on ice for 1 min. The buffer for Superscript Reverse Transcriptase and DTT (Invitrogen) were then added, and the mixture was transferred to 42 °C for 2 min. After the addition of Superscript Reverse Transcriptase, the mixture was incubated at 42 °C for 50 min and finally at 70 °C for 15 min. The cDNA was used as the template in standard PCR (30 cycles of amplification) with primer pairs that allowed the separate amplification of δ (FPthr/RBamHI) and ω.
Plasmid stability tests. Stability experiments were performed as previously described (Dmowski et al., 2006). B. subtilis cells containing the plasmids under analysis were cultured with aeration overnight at 30°C in GM medium supplemented with chloramphenicol (time = 0). The cultures were diluted 1:1000 with fresh GM medium and aerated at 30°C for 24h, which is equivalent to 10 generations. An identical dilution procedure was performed every 24h. To obtain single colonies, dilutions were plated in parallel on L agar without antibiotics, and 100 of these colonies were replica-plated on L agar containing chloramphenicol. The number of colonies able to grow on the antibiotic-containing medium represented the percentage of plasmid-carrying cells in the bacterial population. The rates of plasmid loss (the percentage of plasmids lost per generation) were calculated according to the equation \((1-\alpha^n) \times 100\), where \(n\) is the fraction of plasmid-containing cells after \(n\) generations relative to the fraction of plasmid-containing cells at time ‘0’ (Ravin & Lane, 1999). The stability of each plasmid was analysed in triplicate.

Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant features/cloned fragment</th>
<th>Source/reference</th>
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<tr>
<td>pUC18 derivatives</td>
<td></td>
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<tr>
<td>pDO1000</td>
<td>(\delta)–(\omega) genes under native promoters</td>
<td>Dmowski et al. (2006)</td>
</tr>
<tr>
<td>pDO1910</td>
<td>(\delta\omega) operon under the (P_{\beta}) promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl13 derivatives</td>
<td></td>
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<tr>
<td>pUHCl13</td>
<td>Shuttle vector for (E. coli/B. subtilis)</td>
<td>Dmowski et al. (2006)</td>
</tr>
<tr>
<td>pUHCl1100</td>
<td>(\delta)–(\omega) genes, (P_{\beta}) containing a reduced number of Omega-binding heptads</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl1910</td>
<td>(\delta\omega) operon under the (P_{\beta}) promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl1912</td>
<td>(\delta\omega) operon under the (P_{\beta}) promoter, two Omega-binding heptads downstream of the operon</td>
<td>This work</td>
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<tr>
<td>pUHCl1914</td>
<td>(\delta\omega) operon under the (P_{\beta}) promoter, four Omega-binding heptads downstream of the operon</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl1922</td>
<td>(\delta\omega) operon under the (P_{\beta}) promoter, (P_p) downstream of the operon</td>
<td>This work</td>
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<tr>
<td>pUHCl1982</td>
<td>(\delta\omega) operon under the (P_p) promoter, (P) downstream of the operon</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl1994</td>
<td>(\delta\omega) operon under the (P_p) promoter, (P) and (P_{\omega}) downstream of the operon</td>
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<tr>
<td>pUHCl3910</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl3000</td>
<td>(\delta)–(\omega) genes, (\delta) under the (P_{\omega}) promoter</td>
<td>This work</td>
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<tr>
<td>pUHCl3012</td>
<td>(\delta)–(\omega) genes, (\delta) under the (P_{\omega}) promoter, (P_p) downstream</td>
<td>This work</td>
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<tr>
<td>pUHCl3910</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl3922</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, (P_p) downstream of the operon</td>
<td>This work</td>
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<td>pUHCl3942</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, (P_{\omega}) downstream of the operon</td>
<td>This work</td>
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<tr>
<td>pUHCl3982</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, (P) downstream of the operon</td>
<td>This work</td>
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<tr>
<td>pUHCl3983</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, seven Omega-binding heptads from (P_{\omega}) ((P_{\omega})–26bp) downstream of the operon</td>
<td>This work</td>
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<tr>
<td>pUHCl3984</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, (P_{\omega})–18bp downstream of the operon</td>
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<tr>
<td>pUHCl3985</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, (P_{\omega})–10bp (contains the sequence) downstream of the operon</td>
<td>This work</td>
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<tr>
<td>pUHCl3986</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, truncated (P) with four Omega-binding heptads downstream of the operon</td>
<td>This work</td>
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<tr>
<td>pUHCl3987</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, synthetic (P) downstream of the operon</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl7910</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl7922</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, (P_p) downstream of the operon</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl7942</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, (P_{\omega}) downstream of the operon</td>
<td>This work</td>
</tr>
<tr>
<td>pUHCl7982</td>
<td>(\delta\omega) operon under the (P_{\omega}) promoter, (P) downstream of the operon</td>
<td>This work</td>
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Analysis of plasmid copy-number. Total DNA was isolated from \(R. subtilis\) cells using Genomic Micro AX Bacteria + Gravity (A&A Biotechnology) according to the manufacturer’s instructions. Next, DNA was fragmented by digestion with EcoRV and diluted. Quantitative PCR was performed using a LightCycler 480 instrument (Roche) and Real-Time 2× HS-PCR Master Mix SYBR A (A&A Biotechnology) with primer pairs IB16SaF and IB16SaR, and repA131F and repA285R.

RESULTS

The role of \(P_{\beta}\) in partitioning

In our previous studies, \(P_{\beta}\) was replaced by other Omega-regulated promoters with no loss of partition function. In the present study, we attempted to modify the Omega-binding sequence in \(P_{\beta}\) or to replace it with an Omega-independent promoter. First, the number of WATCACW repeats in \(P_{\beta}\) was reduced from nine to four, a number that ensures
Fig. 2. Schematic representations of the plasmids analysed in this work and their loss rates. The promoter regions of $P_d$, $P_a$, $P_{copS}$ and $P_x$ are shown in green, blue, purple and orange, respectively. Modified $P_d$ or $P_a$ sites are shown as triangles, and heptads are shown as white arrowheads. (a) Replacement of $P_d$ by its truncated form $P_d^*$ (a reduced number of Omega-binding heptads) or $P_a$ (a promoter not regulated by Omega). (b) The artificial $\delta\omega$ operon under the control of the $P_d$ promoter, also with additional Omega-binding sequences located downstream: $P_d$, $P_a$, $P_{copS}$, two or four WATCACW heptads. (c) The artificial $\delta\omega$ operon under the control of $P_d$, $P_a$, $P_{copS}$ or $P_a$. (d) The $\delta\omega$ artificial operon under the control of the $P_a$ promoter with...
an Omega-binding affinity similar to the full-length sites (de la Hoz et al., 2004), to generate plasmid pUHC1110. The plasmid was maintained in 70% of the bacterial population after 40 generations of growth (loss rate 0.75) (Figs 2a and S1a), demonstrating reduced stability when compared to the pUHC1000 plasmid with an unmodified partition region (loss rate 0.15). This indicates that either δ gene regulation is affected in pUHC1110 or that more than four heptads are necessary for the centromeric sequence to be functional.

Next, Pδ was replaced by the Pa region – the Omega-independent promoter of gene α on plasmid pSM19035 (plasmid pUHC5000). This plasmid with Pa–δ–Pω (pUHC5000) was maintained in more than 80% of the population (loss rate 0.25), demonstrating relatively high stability, albeit lower than that conferred by Pδ–δ–Pω (pUHC1000). Moreover, reinsertion of the Pa region downstream of the ω gene (plasmid pUHC5012) did not restore the stability observed for the native partition system (Figs 2a and S1a). These results suggest that Pδ is not the main centromeric sequence and that Pa ensures an almost physiological level of δ gene expression, with only slightly reduced partition efficiency.

**Construction of the δω operon**

Previous studies have suggested that each of the Omega-binding DNA regions, Pcaps, Pδ and Pω, can act as a centromeric site because they have similar Omega-binding affinities (de la Hoz et al., 2004) and exert partition-mediated incompatibility, and because Pδ can be replaced by either Pcaps or Pω (Dmowski et al., 2006). Therefore, to examine the role of a single set of Omega-binding heptads in the partition process we constructed a synthetic δω operon under the control of the δ gene promoter Pδ (Fig. 3). The putative terminator of gene δ as well as the −35 and −10 sequences of gene ω were removed from pDO1000 (via deletion of the BamHI–KpnI fragment) without affecting the putative Shine–Dalgarno sequence of gene ω (Vellanoorth & Rabinowitz, 1992), creating plasmid pDO1910 (Fig. 3). Then, the Pa–δω operon from pDO1910 was transferred into the pUHC13 shuttle vector, creating the pUHC1910 plasmid (Table 1 and supplementary methods).

To verify the correct construction of the δω operon, δ and ω gene transcription was analysed by RT-PCR. Total RNA from *B. subtilis* carrying the pUHC1910 plasmid was used to produce cDNA, which was used as a template in PCR with FPsI and RBamHI, FPsI and OmegaER, and FKPnl and OmegaER primer pairs for the amplification of δ, δω and ω sequences, respectively (Fig. S2). The appropriate PCR products were generated using these primer pairs (Fig. S2), proving the functionality of the engineered operon.

**Stabilizing function mediated by the δω operon**

The ability of the newly constructed partition δω operon to stabilize an unstable replicon was tested in *B. subtilis*; over 45% of the cells maintained the Pδ–δω plasmid (pUHC1910) after 40 generations (loss rate 1.40) (Fig. 2b and S1b). This result indicates that the δω operon with a single set of Omega-binding sites (Pδ) is sufficient for plasmid stabilization, though at an efficiency lower than that of the natural δ–ω partitioning cassette with both promoters (Pδ and Pω; loss rate 0.15). The loss of partition efficiency of Pδ–δω was caused either by the inappropriate expression of δ and/or ω genes or by the absence of a second set of Omega-binding sites. To investigate the latter possibility, the Pa region was reinserted downstream of the Pδ–δω operon (plasmid pUHC1982), which resulted in increased stability, to over 80% after 40 generations (loss rate 0.39) (Figs 2b and S1b). This shows that the lower retention of plasmid pUHC1910 is caused by the absence of the second centromeric region rather than by the inappropriate expression of partition genes.

**Requirements for the second centromeric region**

Because the reinsertion of Pa downstream of the Pδ–δω operon enhanced its stabilizing activity, we decided to analyse whether other Omega-binding regions at this location have a similar effect on plasmid stability. First, we inserted Pa downstream of the Pδ–δω operon (plasmid pUHC1922); after 40 generations, the Pδ–ω–Pω plasmid was retained in 70% of *B. subtilis* cells, for a loss rate of 0.65 (Figs 2b and S1b). This indicates that a second Pδ region increases the stabilizing property of the Pδ–δω operon, albeit less efficiently than an additional Pa sequence. Next, we constructed a pUHC1982 derivative with a third set of Omega-binding sites, the caps gene promoter Pcaps, located downstream of Pω (plasmid pUHC1994). Surprisingly, the plasmid containing Pδ–ω–Pω–Pcaps (pUHC1994) was lost at a rate of 0.42, being retained in approximately 80% of *B. subtilis* cells after 40 generations (Figs 2b and S1b). This result is similar to that obtained for the Pa–ω–Pω plasmid (pUHC1982) and shows that the third Omega-binding region cannot further increase plasmid stability.

**In vitro studies of DNA binding by Omega have shown** that two adjacent WATCACW heptads are sufficient for this interaction and that Omega binds to DNA containing three or more adjacent heptads without changes in affinity (de la Hoz et al., 2004). For a detailed assessment of the requirements for the second Omega-binding site, we therefore
constructed pUHC1910 derivatives with two or four heptads located downstream of the \( P_\delta \omega \) operon, plasmids pUHC1912 and pUHC1914, respectively, and analysed their stability in \( B. subtilis \) cells. With loss rates of approximately 1.4 (Figs 2b and S1b), the two plasmids were retained in less than 50% of the cells, results that are highly similar to those obtained for plasmid pUHC1910 (loss rate 1.4), demonstrating that the ability of protein Omega for two or four heptads is insufficient to constitute a centromeric site. It is possible that DNA sequences other than Omega-binding sites acting solely as centromeric sites.

**Regulation of operon expression and the centromeric function of Omega-binding promoters**

Previous studies have shown that in the \( \delta - \omega \) partition cassette, substitution of the \( \delta \) gene promoter with either of the other Omega-regulated promoters has no effect on the stabilizing activity of the system (Dmowski et al., 2006). These results suggest that the three Omega-regulated promoters can support gene expression of the operon under the partition operon and act as a centromeric site, the \( P_\delta \omega \) operon cannot fully stabilize the plasmid, similarly to that of the native partition cassette. In contrast, when under \( P_{\omega} \) control, the \( \delta - \omega \) operon cannot fully stabilize the plasmid, similarly to the operon under \( P_\delta \) control. Therefore, a single \( P_\delta \) can simultaneously properly regulate the expression of the \( \delta - \omega \) operon and act as the centromeric site to promote high segregational plasmid stability. In contrast, \( P_{\omega} \) and \( P_\delta \) are not fully functional centromeric regions, or fail to properly regulate the expression of the \( \delta - \omega \) operon. Finally, as expected, the \( \delta - \omega \) operon under \( P_\delta \) regulation failed to stabilize the plasmid efficiently. However, when compared to the control plasmid (pUHC13), the \( P_{\omega} - \delta - \omega \) operon (pUHC3910) did confer residual stability, even though no Omega-binding region was present in the plasmid. Importantly, the copy number of pUHC3910 relative to pUHC13 is 0.77, as measured using real-time PCR. It should be noted that in \( B. subtilis \), the ParB-like protein Spo0J binds non-\( parS \) DNA 10-fold less effectively than specific DNA (Breier & Grossman, 2007); however, such interactions are sufficient for the bridging of DNA molecules (Taylor et al., 2015). Moreover, it has been demonstrated that for plasmid P1, ParA-ParB complexes are formed in vitro in the absence of \( parS \) DNA (Havéy et al., 2012). Therefore, it can be speculated that in our experiments, the lack of centromeric sites was compensated for by low-affinity, non-specific DNA binding by partition proteins, resulting in residual plasmid stabilization.

**Omega-binding sites acting solely as centromeric sites**

Our results obtained for plasmids that carry the \( \delta \) gene or the \( \delta - \omega \) operon under the control of \( P_\delta \), a promoter not
regulated by Omega, encouraged us to further analyse the centromeric function of each Omega-binding site independently of their role in regulating gene expression. Therefore, we cloned \( P_\beta \), \( P_\gamma \), and \( P_{\text{ops}} \) downstream of the pUHC910-encoded \( P_\alpha - \delta \omega \) operon to obtain plasmids pUHC922, pUHC9582 and pUHC9584, respectively, and then analysed their stability in \( B. subtilis \) cells after 40 generations. When compared to \( P_\alpha - \delta \omega \) (pUHC910), \( P_\alpha - \delta \omega - P_\gamma \) (pUHC922) conferred only an insignificant increase in stability: it was present in 29% of cells, with a loss rate of 2.8 (Figs 2d and S1d). In contrast, plasmids with \( P_\alpha - \delta \omega - P_{\text{ops}} \) (pUHC9582) and \( P_\alpha - \delta \omega - P_{\text{ops}} \) (pUHC9584) were lost at rates of 0.44 and 0.45, respectively, being retained in approximately 80% of the bacterial population (Figs 2d and S1d). Together, our results demonstrate that (i) \( P_\beta \) has no centromeric function, (ii) although \( P_{\text{ops}} \) cannot support appropriate expression of the \( \delta \omega \) operon, it constitutes a functional centromeric region and (iii) \( P_\gamma \) is proficient in both these functions.

To analyse in more detail the centromeric function of the \( P_\alpha \) region, we constructed pUHC910 derivatives with synthetic \( P_\alpha \) variants cloned downstream of the \( P_\alpha - \delta \omega \) operon (Fig. 4). Plasmids pUHC983, pUHC984 and pUHC985 contain \( P_\alpha \) lacking 26, 18 and 10 nucleotides, respectively, but harbour all seven Omega-binding heptads. The fourth plasmid, pUHC986, contains four heptads sufficient for Omega binding, together with the −35 and −10 sequences and the gene \( \omega \) transcription start site (Fig. 4). The results of plasmid stability tests revealed that plasmids pUHC983, pUHC984 and pUHC986 were only moderately stabilized: after 40 generations, they were lost at rates of 2.10, 1.48 and 1.59, respectively, being retained in less than 50% of \( B. subtilis \) cells (Figs 2e and S1e). In contrast, plasmid pUHC985, the only one that contains all seven heptads and the −10 region (Fig. 4), was lost at a rate of 0.37 and was retained in over 80% of the cells after 40 generations (Figs 2e and S1e). Thus, neither the seven heptads from \( P_\alpha \) nor the promoter sequences with four heptads from \( P_\alpha \) are sufficient to form a fully functional centromere. In a control experiment, full synthetic \( P_\alpha \) was cloned downstream of the \( P_\alpha - \delta \omega \) operon, and the resulting plasmid, pUHC987, recovered a stability similar to that of pUHC982 and pUHC985 (loss rate 0.41) (Figs 2e and S1e). Moreover, a spontaneous C–A mutation at position 6 in the fourth heptad of \( P_\alpha \) in the \( P_\alpha - \delta \omega - P_\gamma \) plasmid had no effect on plasmid stability (data not shown). We also compared the copy number of plasmid pUHC910 and its derivatives using real-time PCR. When compared to pUHC910, the relative copy number of pUHC983, pUHC984, pUHC985, pUHC986 and pUHC987 was 1.35, 1.37, 1.26, 1.07 and 1.13, respectively, demonstrating no changes in copy number that could influence plasmid stability. Altogether, these results show that heptameric sequences in promoter regions are not the only elements essential for centromeric functions. It can be speculated that the non-heptameric portions of \( P_\beta \), \( P_\gamma \) and \( P_{\text{ops}} \) (18 nucleotides downstream of WATCACW heptads in \( P_\gamma \)) that encompass the −10 sequence recognized by RNA polymerase are the elements that make centromeric sites more or less ‘efficient’.

**Improved plasmid stabilization efficiency**

As shown above, the \( \delta \omega \) operon under \( P_\alpha \) control (plasmid pUHC910) confers stabilization that is almost identical to that of the natural bicistronic \( P_\beta - \delta \omega - P_\omega \) partition system. As we sought to verify whether it is possible to improve the efficiency of the partition system, we cloned each of the Omega-binding promoter regions downstream of the \( P_\alpha - \delta \omega \) operon to generate plasmids pUHC9722 (\( P_\alpha - \delta \omega - P_\beta \)), pUHC9742 (\( P_\alpha - \delta \omega - P_{\text{ops}} \)) and pUHC9782 (\( P_\alpha - \delta \omega - P_\gamma \)). The stability test in \( B. subtilis \) revealed a loss rate for the \( P_\alpha - \delta \omega - P_\beta \) plasmid pUHC9722 of 0.34, indicating that insertion of \( P_\gamma \) downstream of the \( P_\alpha - \delta \omega \) operon did not influence stability improving effect. In contrast, the loss rate for a plasmid with \( P_\alpha - \delta \omega - P_\omega \) or \( P_\alpha - \delta \omega - P_{\text{ops}} \) (pUHC9782 and pUHC9742) was 0.05 and 0.03, respectively (Figs 2f and S1f). Therefore, \( P_\alpha - \delta \omega \) with \( P_\gamma \) or \( P_{\text{ops}} \) downstream showed improved stabilizing activity when compared with the native \( P_\beta - \delta \omega - P_\omega \) partition cassette (pUHC1000, loss rate 0.15) or the \( P_\alpha - \delta \omega \) operon (pUHC9710, loss rate 0.22). These results, together with those obtained from a plasmid with \( P_\alpha - \delta \omega - P_\gamma \) (pUHC9582) or \( P_\alpha - \delta \omega - P_{\text{ops}} \) (pUHC9584), demonstrate that the \( P_\gamma \) and \( P_{\text{ops}} \) regions are the most ‘efficient’ centromeric sites.

**DISCUSSION**

The results obtained in the presented study provide details of the biological function of the Omega-binding promoter/centromeric regions \( P_\beta \), \( P_\gamma \) and \( P_{\text{ops}} \) and do not fully confirm previous biochemical analyses with regard to their centromeric function. A summary of relative plasmid loss rates (Fig. 5a) shows that plasmids analysed in this work can be classified into stability groups and it clearly appears that the most stable plasmids that which are lost by less than 0.5% of cells per generation (relative loss rate below 3) contain \( P_\gamma \). Therefore, we conclude that high plasmid partition efficiency necessitates the presence of \( P_\gamma \) whereas \( P_\beta \) or \( P_{\text{ops}} \) are dispensable. Moreover, the essential role of \( P_\gamma \) in partition is the centromeric function whereas expression of partition genes can be regulated by other promoters (Fig. 5a). Therefore, our results also reject the hypothesis (suggested by the genetic organization of \( \delta \) and \( \omega \) genes) that \( P_\beta \) is the main centromeric sequence as well as the hypothesis that each Omega-binding region can act as centromeric sites.
Fig. 5. (a) Summary of segregational stability of plasmids described in this work. Plasmid loss rates were normalized to the loss rate of the plasmid with native \( P_d - d - P_a \) partition cassette. Plasmid retention in the population (after 40 generations of growth) is shown. Plasmids were grouped according to their relative loss rates. \( P_d - 10 \) bp, \( P_d - 18 \) bp and \( P_d - 26 \) bp denote \( P_d \) derivatives with all seven Omega-binding heptads and deletions of 10, 18 or 26 bp, respectively. \( P_d - 4h \) and \( P_d - 2h \) denote the \( P_d \) derivatives with only four Omega-binding heptads, respectively. 2h and 4h denote two and four Omega-binding heptads, respectively. (b) Alignment of the \( P_d \), \( P_a \) and \( P_{copS} \) regions from inc18 plasmids. WATCACW heptads in direct or inverted orientations are shown as black and grey arrows, respectively. Black shading corresponds to non-matching bases, and start codons are shown in grey type.
sequence. This conclusion reinforces the central role of the \( P_\omega \) promoter in pSM19035 plasmid stabilization: not only does this promoter regulate the expression of partition and postsegregational killing genes (the \( \omega \zeta \) operon), but it is also the main centromere-like region. Finally, \( P_{\text{cops}} \) located at a distance from partition genes \( \delta \) and \( \omega \) in pSM19035 and other inc18 plasmids also supported a high level of stability when cloned downstream of the \( P_\alpha-\delta \omega \) operon, suggesting that \( P_{\text{cops}} \) might constitute a secondary centromeric sequence. One can speculate that this redundancy may be beneficial when one of these sequences is lost, or in specific bacterial hosts or growth conditions. Alternatively, the second centromeric sequence may facilitate plasmid condensation or the formation of a larger partition complex which segregates plasmids to daughter cells more efficiently, as observed for plasmids with \( P_\omega \) or \( P_{\text{cops}} \) inserted downstream of the \( P_\alpha-\delta \omega \) operon (Fig. 5a). These processes may involve intramolecular interactions between centromeric sequences.

Comparison of \( P_\omega \) regions from various inc18 plasmids (Fig. 5b) demonstrates that the \( \cdots \) organization of the heptads in \( P_\omega \), and the \( 42 \)-nucleotide sequence between the heptads and the \( \omega \) GTG start triplet are highly conserved. In contrast, heptad organization in the \( P_\delta \) region of pSM19035 differs from that found in other inc18 plasmids; the heptad organization in the \( P_\delta \) region of other inc18 plasmids is similar to that in the \( P_\omega \) regions of both pSM19035 and other inc18 plasmids (Fig. 5b). Therefore, the possibility that the \( P_\delta \) regions from other inc18 plasmids can act as efficient centromeric sites cannot be excluded and requires further investigation. Heptad organization in the \( P_{\text{cops}} \) regions of inc18 plasmids contains \( \cdots \) stretches of heptads, also similar to those found in \( P_\omega \). Therefore, because \( P_{\text{cops}} \) from pSM19035, as opposed to \( P_\delta \), is able to support a centromeric function, one can speculate that \( \cdots \) heptad organization is essential for this centromeric function.

Although in vitro studies have shown that providing more than four WATCACW heptads does not significantly increase Omega-binding affinity (de la Hoz et al., 2004), our in vivo studies show that the complete set of heptads is necessary for the proper regulation of gene expression, as observed for the modified \( P_\delta \) promoter regulating expression of the \( \delta \) gene (Fig. 2a). Similarly, the complete number of heptads is necessary for full stabilization by the single centromeric sequence \( P_\omega \) (Fig. 2c). This suggests that the DNA–protein complex formed in vitro does not reflect the complexity of the biologically active complex formed in vivo, which is necessary to regulate gene expression and plasmid partition.

With regard to \( P_\omega \), the nucleotide sequence located between the heptads and the start codon of the \( \omega \) gene is necessary for the full functioning of the \( P_\omega \) centromeric region (Figs 2c, 4 and 5a). This finding suggests that sequences other than Omega-binding heptads, especially the \( -10 \) sequence recognized by RNA polymerase (RNAP) (Fig. 4), are involved in formation of the nucleoprotein complex; indeed, deletion of the \( -10 \) sequence from \( P_\omega \) results in the impairment of centromeric function of this region. Therefore, our results indicate that the recently reported (Volante et al., 2015) cooperative binding of Omega and RNA polymerase is essential not only for regulating gene transcription but also for the partition process itself. The importance of RNA polymerase and transcription for segregation of bacterial chromosomes has already been reported (Dworkin & Losick, 2002; Kjos & Veening, 2014; Stracy et al., 2015; Woldringh, 2002). Transcription of many genes distributed throughout the genome might provide driving force for chromosome segregation, maintain appropriate chromosomal structure with supercoils separated by expressed regions, or reorganize the nucleoid within the cell (Kjos & Veening, 2014; Le et al., 2013; Stracy et al., 2015; Woldringh, 2002). Moreover, transference (co-transcriptional translational and protein translocation) of membrane proteins might generate DNA movement resulting from competition of DNA–RNAP–mRNA–ribosome complexes for membrane surface (Matsumoto et al., 2015; Woldringh, 2002). However, to the best of our knowledge the involvement of RNA polymerase in the process of plasmid segregation has not been demonstrated so far. To explain our observations that RNA polymerase binding to the promoter/centromere-like region aside from its role in transcription initiation is important for efficient plasmid partitioning, several models can be proposed: (i) fine tuning of specific Omega binding to repeated heptads in the centromere; (ii) changing the local topology of DNA and shaping the DNA–Omega–Delta nucleoprotein complex; (iii) locating the centromere-like sequence at the periphery of the nucleoid where DNA density is lowest to facilitate plasmid migration toward cell poles. These models need to be tested in future studies. Moreover, elucidation of the mechanism of concerted action of multiple centromeric sequences needs further molecular analyses.

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