The Interplay between Different Stability Systems Contributes to Faithful Segregation: *Streptococcus pyogenes* pSM19035 as a Model

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ABSTRACT The *Streptococcus pyogenes* pSM19035 low-copy-number θ-replicating plasmid encodes five segregation (seg) loci that contribute to plasmid maintenance. These loci map outside of the minimal replicon. The segA locus comprises β2 recombinase and two six sites, and segC includes segA and also the γ topoisomerase and two ssiA sites. Recombinase β2 plays a role both in maximizing random segregation by resolving plasmid dimers (segA) and in catalyzing inversion between two inversely oriented six sites. segA, in concert with segC, facilitates replication fork pausing at ssiA sites and overcomes the accumulation of “toxic” replication intermediates. The segB1 locus encodes ω, ε, and ζ genes. The short-lived ε2 antitoxin and the long-lived ζ toxin form an inactive ζε2ζ complex. Free ζ toxin halts cell proliferation upon decay of the ε2 antitoxin and enhances survival. If ε2 expression is not recovered, by loss of the plasmid, the toxin raises lethality. The segB2 locus comprises δ and ω genes and six parS sites. Proteins δ2 and ω2, by forming complexes with parS and chromosomal DNA, pair the plasmid copies at the nucleoid, leading to the formation of a dynamic δ2 gradient that separates the plasmids to ensure roughly equal distribution to daughter cells at cell division. The segD locus, which comprises ω2 (or ω2 plus ω2ζ) and parS sites, coordinates expression of genes that control copy number, better-than-random segregation, faithful partition, and antibiotic resistance. The interplay of the seg loci and with the rep locus facilitates almost absolute plasmid stability.

INTRODUCTION

The long-term maintenance of low-copy plasmids cannot rely on random distribution to ensure their proper propagation to daughter cells at cell division. It relies largely on plasmid-encoded functions that promote their replication and ensure their faithful vertical transmission to daughter cells. Low-copy plasmids make use of several different genetic loci, which usually map outside of the minimal replicon, to ensure almost absolute plasmid stability (1, 2). A subset of plasmid-encoded functions ensures one or two rounds of replication per cell cycle and the distribution of the replicated copies to nascent daughter cells. Others halt proliferation of cells that do...
not receive a plasmid copy or regulate the interplay between the segregation (seg) loci among them and with the replication locus. To study this interplay pSM19035, a Streptococcus pyogenes low-copy number plasmid (2 ± 1 copies/cell) widely distributed in bacteria of the Firmicutes phylum, was used as a model system (Fig. 1) (3). This plasmid as well as Streptococcus agalactiae pIP501 (4), Enterococcus faecalis pAMB1, pW9-2, and pRE25 (5, 6, 7), Enterococcus faecium pLP186, and pVEF series (pVEF1, pVEF2, and pVEF3) (8, 9) belong to the inc18 incompatibility group (Fig. 2) (10, 11). With few exceptions, the nucleotide sequences of the minimal backbone region required for plasmid replication and stable segregation are highly conserved (>92% identity). The inc18 plasmids, which are ubiquitous among erythromycin/lincomycin-, vancomycin-, or methicillin-resistant bacteria, can be divided in three large groups: (i) non-self-transmissible plasmids, with duplicated and inverted replication (IR) and stable maintenance regions, separated by long nonrepeated (NR) segments, represented by pSM19035 (Fig. 1A), pSM22095 and pSM10419 (3, 12); (ii) self-transmissible or non-self-transmissible, with directly repeated (DR) regions separated by long NR segments, represented by pRE25 self-transmissible or pLP186 and the plasmids of the pVEF series (pVEF1, pVEF2, or pVEF3) non-self-transmissible (Fig. 2) (6, 9, 13); and (iii) self-transmissible, that contain neither IR nor DR segments, represented by pAMB1 and pIP501 (Fig. 2) (4, 5, 14). These plasmids, which replicate “unidirectionally” via the θ or circle-to-circle replication mode (15, 16, 17, 18, 19), encode a sophisticated control to warrant that a sufficient number of copies populates daughter cells.

A minimal replicon is defined as the smallest segment of a plasmid necessary and sufficient for autonomous replication with wild-type copy number. The smallest autonomous replicating segment of inc18 plasmids does not qualify as a minimal replicon. This segment contains a bipartite replication origin (oriS and ssiA), the structural gene (rep) that codes for a replication initiation protein (e.g., RepS in pSM19035), and genetic information for control of rep gene expression (CopS, a countertranscribed antisense RNA [RNAIII] and a cis-acting element) (Fig. 1B) (10, 15, 17, 18, 20, 21, 106). In plasmids containing long IRs, functions other than the rep locus are essential for plasmid replication (Fig. 1A) (22). In contrast, in plasmids lacking IR regions, this minimal replicon requires these functions to keep the copy number of the parental plasmid. It is likely that functions mapping outside of the minimal replicon are necessary to overcome any potential roadblock, which might reduce the overall replication rate, or remove any remaining catenates (22). In the present report, the plasmid replication machinery will be discussed in the context of the interplay between stabilization and replication functions.

pSM19035 has evolved different stability systems that minimize plasmid loss at cell division, coordinate replication, segregation, and cell growth making plasmid maintenance extremely stable. Sequence analysis has revealed six different loci (rep, segA, segB1, segB2, segC, and segD) in the IR regions that are required for the structural and segregational stability of a derivative of pSM19035 (i.e., pDB101) in Bacillus subtilis (Fig. 1A) (21, 23). pSM19035-derivatives lacking IR regions (e.g., pBT233 and pBT234; Fig. 1A) replicate autonomously and are >105-fold more stable than expected for random segregation (21), suggesting the presence of discrete regions outside the minimal replicon that may contribute to the stabilization of the plasmid (seg loci). As we will describe in more detail later in this review, there are three systems that directly contribute to stable maintenance of low-copy-number plasmids (defined by functions encoded by the segA, segB1, and segB2 loci) and an avenue involved in facilitating the processing of replication intermediates (segC) (22). There is also a system that contributes to coordinate copy number control and stable inheritance (segD). The interplay between the different seg loci facilitates the separation of sister plasmids and, in coordination with the replication machinery, allows bona fide replication and stable inheritance. Most of the studies described here have been performed using B. subtilis as a host, with derivatives of pSM19035 (pDB101, pBT233, etc.) (Fig. 1A).

THE segA LOCUS

An oligomeric plasmid molecule, generated by recombination-dependent crossover or by replication of oligomers, has to be resolved to monomers by conservative site-specific reciprocal recombination between two short directly oriented cognate DNA sequences (24, 25, 107). A segA-encoded function, which catalyzes the resolution of plasmid dimers (or higher-order oligomers) into monomers, maximizes the distribution of plasmid copies to nascent daughter cells and stabilizes the plasmids ∼5-fold (21). The segA locus encodes α and β genes, and two inversely oriented six sites (Fig. 1A). Protein β, which exists as a dimer (β2) (26), regulates the expression of the locus (27). The orfa, which has different names in other members of the inc18 family (orfG, orfS, etc.; see Fig. 2), shows relatively low levels of identity (<45%) at the
nucleotide level, when compared with the β gene or with the upstream untranslated region (which contains the lagging strand replication origin [ssiA] and the six site) that share >91% identity among the different members of inc18 (22). The β2 resolvoinvertase binds to a 90-bp six site, which can be divided in two adjacent subsites,
one of them named synaptic subsite I where recombination takes place, and an accessory subsite II (24, 26, 28, 29, 30, 31). Two $\beta_2$ resolvoinvertases bind to each subsite; $\beta_2$ bound to IR sequences at subsite I enhances its binding to subsite II that contains only a half binding site (one arm of the IR region present in subsite I) (24, 26, 29, 30, 32, 33). Resolvoinvertase $\beta_2$ requires a sequence-independent DNA-bending protein, such as Hbsu histone-like protein, to stabilize a synaptic complex with a relative geometry of the six sites and for catalyzing both resolution and inversion activities at its cognate sites (29, 32, 34, 35). While the eukaryotic HMGB1 histone-like protein can efficiently replace Hbsu as $\beta_2$-accessory protein (36, 37, 38, 39, 40, 41, 42), this is not the case for numerous other bacterial DNA-bending proteins. For example, *Escherichia coli* encodes for at least six histone-like proteins: HU, IHF, Fis, H-NS, Hha, and Lrp. HU efficiently stimulated $\beta_2$-mediated recombination, the effect of IHF was partial, whereas the stimulatory effect of the chromatin-associated proteins Fis, H-NS, Hha, and *B. subtilis* LrpC was undetectable (35, 43).

Hbsu mainly binds between both subsites and stabilizes a synaptic complex with a relative topology of the six sites. When $\beta_2$ binds the subsite I it limits Hbsu binding. As a consequence, $\beta_2$ mediates resolution between two directly oriented six sites on a supercoiled dimeric DNA substrate (32; see below). DNA supercoiling helps to overcome the energetic barrier; resolution does not occur in relaxed DNA (32, 34, 35, 44).

A segA locus, contributes to genetic isolation by impairing horizontal gene transfer via plasmid transformation or plasmid transduction (43, 46, 47). Plasmid DNA, released from bacterial populations that harbored plasmids consisting of monomers-only due to segA activity, cannot transform natural competent cells.
(B. subtilis) or do so with very low efficiency (Streptococcus pneumoniae) (48, 49), whereas plasmid DNA, released from bacterial populations that harbor even a low proportion of plasmids dimers (∼5%), can efficiently transform naturally competent cells (43). In Firmicutes, bacteriophage-mediated transduction is one of the main forms of plasmid horizontal gene transfer (45, 46, 47). A plasmid-transducing particle, which consists of an oligomeric head-to-tail molecule, can be generated either via viral-directed plasmid replication or by multimeric plasmid replication, as observed in the absence of end-processing functions (e.g., in the addAB [counterpart recBCD] context) (50, 51, 52). If the segA locus catalyzes the resolution of the plasmid oligomers on the donor strain, the concatemer should not have the required length for efficient packaging into a viral prohead of a generalizing transducing phage, and indirectly reduces plasmid transduction. However, following introduction of the transducing plasmid DNA, and after circularization, the segA locus positively contributes to resolve the concatemeric plasmid molecule (53, 54).

**THE segC LOCUS**

The segC locus comprises three trans-acting products (orf7, the resolvoinvertase β2 [described as part of the segA locus], and the type I topoisomerase γ), and two inversely oriented cis-acting regions: ssiA and six (Fig. 1A). The mode of action of the segC locus is briefly described in this section. For a more detailed description, the reader is referred to a recent review (22).

Plasmids pSM19035 and pDB101 have two perfect long IR segments, which encompass ∼80 and 68% of the molecule, respectively, separated by two NR sequences (NR1 and NR2) of different lengths (Fig. 1A). Both plasmids lack genuine dedicated replication termination (ter) regions (18, 55). The essential role of the segC locus in these plasmids with IR regions is illustrated by mutations in the β gene, or a deletion of the six site, which abolish the intramolecular recombination event that causes the flipping of the NR2 region, leads to the accumulation of knotted replication intermediates, and causes gross genome rearrangements (27, 30, 55, 56). Replication of pSM19035 or pDB101 may proceed "bidirectionally" from each of the inversely oriented replication origins (a ∼50-bp oriS region where leading strand synthesis begins, and the ∼150-bp lagging strand ssiA site) located downstream of the rep gene and immediately upstream of the segC locus (15, 16, 17, 18). Upon firing unidirectional replication from one of the origins, β2-mediated inversion between the two inversely oriented six sites flips the orientation of this replication fork with respect to the still unfired second origin (see reference 22). Firing of the second origin should lead to unidirectional replication with both forks traveling in the same direction around a circular monomer template (see reference 22). After one full round of replication, the forks undergo β2-mediated inversion, and replication is terminated at the site where the two forks meet (see reference 22). The flip back should lead to two equi-molar monomeric forms that differ from each other in the orientation of the two unique halves (the NR sequences; Fig. 1A) as detected in plasmid pDB101 purified from B. subtilis cells (18, 55).

The segC locus in plasmids lacking IR regions is partially dispensable and works as a helper of the rep locus (Fig. 2) (15, 16, 55, 57). In these plasmids, the segC locus modulates the synchronization of leading- and lagging-strand DNA synthesis, controls the termination of replication, and stabilizes by ∼25-fold plasmid segregation by reducing a fitness cost (55). Indeed, deletion of the ssiA site, which lies ∼50 bp downstream from oriS (15, 58), leads to the accumulation of linear high-molecular-weight DNA and high plasmid structural and segregational instability (20).

**THE segB LOCI**

Low-copy plasmids rely on segregation systems to ensure that each daughter cell harbors newly replicated plasmids or to induce the proliferation stop of those cells that lost all plasmid copies or did not receive at least a plasmid copy at cell division (1, 2, 59, 60, 108, 109). Accurate distribution of a newly replicated genome to daughter cells at cell division is a precise process, but subject to occasional error or inactivation by spontaneous mutations (expected to be 0.5 × 10⁻⁸ to 5 × 10⁻⁸). pDB101 is lost at a frequency <1 × 10⁻⁷ per generation, suggesting that it has highly efficient segregation functions to warrant stable (better-than-random) segregation at cell division (21). The segB region, whose expression is under the control of the ω2-transcriptional regulator, is divided in two discrete loci, segB1 and segB2 (Fig. 1A and 1B). The segB1 locus encodes the ω2 repressor, the dimeric ε (ε2) antitoxin and the monomeric ζ toxin (21, 61, 62). When pBT233 or pBT234 variants were analyzed, the toxin-antitoxin [TA] system (Fig. 1A) stabilizes the plasmid >10,000-fold by inhibiting the proliferation of plasmid-free cells (21, 61). The segB2 locus comprises two trans-acting proteins (δ2-ATPase, and ω2 acting as a centromeric-
binding protein [CBP]) and six parS sites (21, 63, 64). The segB2 locus is responsible for the partition of plasmid copies to daughter cells at cell division (Fig. 1), increasing by ~50-fold the stability of pBT233 mini-derivatives (63). Some members of the inc18 family encode a second ParAB-like system (segE locus) (Fig. 2). The analysis of this segE locus, the mechanisms that underlie the segregation of plasmids with two genuine partition systems (segB2 and segE), and the interplay between them remain to be characterized.

The segB1 Locus

Stabilization of low-copy-number plasmids in the bacterial population can be enhanced by the action of TA systems that contribute to plasmid stability through the postsegregational proliferation halt of the cell that has lost the plasmid. Five different types of TA systems have been described to date (reviewed in references 59, 66, 67, 68, and 109).

The TA system of inc18 plasmids is genetically linked to antibiotic resistance genes. Indeed, the spread of the resistance to vancomycin, methicillin, gentamycin, erythromycin, linezolid, glycopeptide resistance, and multiresistant cfr among enterococci and staphylococci (2, 9, 13, 69, 70, 71) could be mainly attributed to their coexistence with the plasmid-borne ζ-ε TA module (22). Unlike the majority of TA systems that are autogenously controlled, the expression of the ζ-ε TA module is regulated by the δ2-transcriptional regulator, which regulates its own synthesis and also controls plasmid copy number (see “The segD Locus” below). Conditions that facilitate antitoxin ε2 degradation, by the LonA and/or ClpXP protease, permit ζ toxin to act freely to block cell proliferation (61, 62, 72). Therefore, the potential threat that is posed by the accumulation of multiple resistance genes on plasmids with a TA module is of great concern.

The Firmicutes ζ2ζ2 TA module consists of two monomeric long-living 287-amino-acid ζ toxins (half-life > 60 min) separated by a short-lived 90-amino-acid-long ε2 antitoxin (half-life < 20 min) (Fig. 3A) (72, 73, 74). The superfamily of ζ toxins, which are bacteriostatic by nature (61, 75), are among the most abundant in bacteria of the Firmicutes phylum (76). Massive ζ-toxin overexpression leads to cell death (77, 78).

The TA system is the ultimate stabilization function of pSM19035 (21, 23) and many other plasmids of the inc18 family. This TA system was also found in the chromosome of S. pneumoniae (known as PezAT [79]) as well as in the chromosome of many bacteria of the Enterococcus genus. A minimal replicon carrying the segB1 locus will be stably maintained by the contribution of a long-living plasmid-encoded toxin that inhibits the proliferation of plasmid-free daughter cells. However, this maintenance is not absolute. The rate of cured cells should be between the subpopulation of non-inheritable toxin-resistant cells (1 × 10^{-3} to 5 × 10^{-5} toxin tolerants) and toxin-inactive mutants (0.5 × 10^{-8} to 5 × 10^{-8} spontaneous mutants) (61, 75).

Toxin ζ inhibits the first step of peptidoglycan biosynthesis by phosphorylating the 3'-OH group (3P) of the amino sugar moiety of uridine diphosphate-N-acetylgalactosamine (UNAG), leading to the accumulation of a fraction of unreactive UNAG-3P in vitro (78). Within the first 15 min of toxin expression, physiological levels of free wild-type (wt) ζ or short-living ζ variant (ζY83C) alter the expression of about 2% of total genes (reduces the expression of essential genes involved in cell membrane synthesis and increases the expression of RelA), increases synthesis of (p)pGpp with subsequent decrease of the GTP pool, and reversible induces dormancy (61, 62). Within the 30 to 90 min of toxin expression, ζY83C decreases macromolecule synthesis (DNA replication, RNA transcription, and protein translation), the intracellular pool of both ATP and GTP, and inhibits cell wall biosynthesis followed by the death of a small fraction (20 to 30%) of the cell population (22, 75). Expression of ε2 antitoxin then reverses ζ-induced dormancy (75).

The segB2 Locus

The plasmid or bacterial chromosomal partition machinery is functionally equivalent to a simplified mitotic apparatus and consists of a motor ATPase, a “centromeric” binding protein, and a cis-acting site considered to be analogue to the eukaryotic centromere (reviewed in references 2, 22, 80, 81, and 108). Most of the low-copy-number plasmids encode a partition system of the ParA type (reviewed in references 2, 80, 82, and 108). Plasmids of the inc18 family can be classified into three discrete groups on the basis of their partition system: (i) those that have traces of a segB2 partition system in their genome, as pIP501 (6, 9, 13, 23, 65, 83); (ii) those that encode a single ParAB system (segB2) as pSM19035, pAMB1, and pVEF4 plasmids; and (iii) those that encode two different ParAB (segB2 and segE) loci as pRE25, pIP816, and pVEF2. In pSM19035, the segB2 locus includes two trans-acting proteins (the δ2-ATPase, and the δ2-CBP) and a variable number of cis-acting parS sites located outside of the minimal replicon (Fig. 1B and 3B through 3E). Unlike other known partition systems, in this case, the two genes are separately
transcribed; however, they are coregulated by the action of the CBP ω2-transcriptional regulator (63). Unlike the large type Ia ATPases (P1-ParA and F-SopA), which have an N-terminal extension needed for autoregulated expression, the small type Ib ATPases, as δ2, do not function as transcriptional regulators of the par locus. The δ2-ATPase contains an N-terminal deviant Walker A box domain, a C-terminal nonspecific DNA binding domain, and a poorly defined domain involved in the interaction with its regulator (ω2) (Fig. 3B) (65, 84). δ2, upon binding to ATP, binds DNA, and ATP hydrolysis is not required for this reaction (65, 84). Indeed, δ2D60A-ATP·Mg2+, which binds but fails to hydrolyze ATP, binds DNA with high efficiency, whereas δ2K36A, a δ2 variant, which neither binds nor hydrolyzes ATP, fails to bind DNA (84, 85).

The monomer of ω is a 71-residue polypeptide with an unstructured N-terminal domain (residues 1 to 19) and a ribbon-helix-helix (RHH) fold (residues 20 to 71) (Fig. 3D) (86, 87, 88, 89). Protein ω2 or its variant ω2ΔN19, which lacks the first 19 residues, transiently binds, with high affinity and cooperativity (apparent dissociation constant [KD] 5 ± 1 nM) to its cognate sites (forming partition complex 1, PC1) (Fig. 3E and 3F) (63, 64, 84, 89, 90). However, the interaction of the unfolded N-terminal end of ω2 with δ2, even in the apo form, significantly increases the binding affinity of ω2 for parS DNA (KDapp 0.7 nM) leading to the long-living partition complex 2 (PC2) (Fig. 3F, condition 2) (84). In pSM19035, the ω2-target sites are six parS sites: two parS1/parS1’ sites, located in the promoter (P) region of δ gene (Pδ), two parS2/parS2’ in Pα, and two parS3/parS3’ in Pcory, featuring, respectively, 9, 7, and 10 contiguous heptads (in direct → or inverse → orientation) with the sequence 5‘-WATCACW-3’ (where W is an A or a T) (Fig. 1A and 4A) (64). In other members of the family, the number and relative orientation of the highly conserved repeats varies (Fig. 4A). The minimal protein binding site consists of two contiguous heptads in direct (→→) or inverted (←←) orientations, to which two ω2 molecules bind, and the complex is stabilized by cooperative interdimer contacts (64, 89). However, the binding site to two contiguous heptads in (→→) orientations was poor (64, 89). Protein ω2 (ω22, Fig. 4B) belongs to the family of proteins with a RHH2 fold (87, 88, 91). The half DNA binding determinant is located at residues 28 to 32, which constitutes a β sheet, and is followed by two α helices (α1 [residues 34–46] and α2 [residues 51–66]) involved in the interaction with the phosphate backbone, with itself and with another dimer (88, 90, 92, 93). In ω2 the two β strands are antiparallel and contact its cognate sequence in the major groove of DNA with high specificity and affinity, and the two α1 regions make nonspecific contacts with the DNA phosphate backbone (88, 89, 90, 93). The structure of ω2 bound to a mini parS DNA (→→ or ←←) was solved (Fig. 3E) (89). This structure shows that ω2 binds symmetrically to this mini parS DNA with a 0.3 Å deviation with respect to the central C-G pair of each repetition (89). Residues Thr29 and Arg31 from the antiparallel β strands are essential for specific binding (Fig. 3C) (89, 90, 93).

The structure of pSM19035-δ2, in the nucleotide-bound form, is U-shaped with each arm of the U representing one monomer, and the ATP facing the cleft of the U (65, 84). The surface charge of δ2 is negative near the bottom of the U, and positive at the tips of the arms of the U (65, 84). The positively charged residues are necessary to form small blobs on DNA (Fig. 3G) rather than filaments (65, 84). Upon binding to ATP, δ2 undergoes conformational changes that make it proficient for DNA binding (Fig. 3G) (84). Like type Ia P1-ParA (94), δ2 upon binding to DNA, in a sequence-independent manner, forms a transient δ2-chromosomal DNA complex. In this binary complex, 10 to 20 δ2 molecules are clustered on the DNA (63, 84, 85). Protein δ2, upon interaction with ω2, bound to parS (a cluster of up to 10 ω2 and ~15 δ2 molecules), as part of two discrete segregosomes (parS-ω2-δ2) complexes (SC), lead to plasmid pairing or bridging complex formation (parS-ω2-δ2-ω2-parS) (BC) (Fig. 3F, conditions 3 and 4).

Several studies revealed that pSM19035 partition is a multistep process similar to the diffusion ratchet model proposed by the Mizuuchi and Funnell groups for P1-ParAB- and F-SopAB-mediated partition (82, 94, 95, 96, 108). In the pSM19035, P1 and F partition systems the motive force for plasmid positioning does not directly rely on the polymerization of the motor protein ParA, but, instead, it is directed by a dynamic gradient of ParA in the cell. The uneven distribution of ParA molecules in the cell depends on the affinity of ParA for nonspecific host nucleoid DNA when bound to ATP, and the ability of the CBP ParB bound to the plasmid DNA to release ParA from the nucleoid by stimulating its ATPase activity.

THE segD LOCUS

In B. subtilis, the ratio between pBD101 and the oriC (origin of chromosomal replication) is approximately 2:1. The smallest possible increase in plasmid copy
number, i.e., from 2 to 4, corresponds to a dramatic 100% change in concentration. Thus, the feedback control of low concentrations is notoriously difficult and easily leads to random oscillations. Furthermore, slight variations in the feedback control scheme result in markedly different efficiencies of noise suppression. The segD locus, which comprises the $\omega_2$ transcriptional repressor and its cognate sites upstream the promoter regions of the $\omega_1$-like repressors: $\omega_2$ (a 71-residue-long peptide) and $\omega_2'$ (a 79-residue-long peptide). Protein $\omega_2$, which also belongs to the family of proteins with a RHH2 fold (Fig. 3C), shares with $\omega_1$ the first unstructured 25 residues, the DNA binding specificity determinants located in the $\alpha_1$ helix (residues 34 to 46) (Fig. 4B). Both proteins repress the levels of CopS, and indirectly increase the supply of the $\text{rep}$ mRNA (63; A. Volante, unpublished results). Once $\omega_2$ or $\omega_2'$ bind the upstream $P_{\text{copS}}$ and $P_{\text{copS}'}$ regions, they inhibit copS gene expression and indirectly correct any downward copy number fluctuation (63, 102, 103, 106). Indeed, repression of CopS synthesis by $\omega_2$ or $\omega_2'$ correlates with an increase in plasmid copy number and indirectly ensures stable plasmid maintenance. It is likely that the interplay of CopS and $\omega_2$ is part of a negative-feedback control system of the minimal replicon of inc18.

Protein $\omega_2$ also couples the expression of genes of the segB1 and segB2 loci by repressing the utilization of $P_\delta$ and $P_{\omega_0}$ (Fig. 4A). After plasmid establishment or upon downward fluctuations in plasmid copy number, the low levels of $\omega_2$ result in increased ParAB and TA and decreased CopS-RepS ratios with increased copy number. At high-copy-number conditions, $\omega_2$ concentrations increase, which results in a rapid reduction in the levels of $\omega_2$ and $\omega_0$ (ParAB system) and of $\epsilon_2$ and $\zeta$ (TA system) proteins required for active partitioning and control of cell proliferation, respectively (61, 63, 65, 72, 74, 85).

Some plasmids of the inc18 family encode two $\omega_1$-like repressors: $\omega_2$ (a 71-residue-long peptide) and $\omega_2'$ (a 79-residue-long peptide). Protein $\omega_2$, which also belongs to the family of proteins with a RHH2 fold (Fig. 3C), shares with $\omega_1$ the first unstructured 25 residues, the DNA binding specificity determinants located in the $\alpha_1$ helix (residues 34 to 46) (Fig. 4B). Both proteins repress the levels of CopS, and indirectly increase the supply of the $\text{rep}$ mRNA (63; A. Volante, unpublished results). Once $\omega_2$ or $\omega_2'$ bind the upstream $P_{\text{copS}}$ and $P_{\text{copS}'}$ regions, they inhibit copS gene expression and indirectly correct any downward copy number fluctuation (63, 102, 103, 106). Indeed, repression of CopS synthesis by $\omega_2$ or $\omega_2'$ correlates with an increase in plasmid copy number and indirectly ensures stable plasmid maintenance. It is likely that the interplay of CopS and $\omega_2$ is part of a negative-feedback control system of the minimal replicon of inc18.

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**FIGURE 3** Protein structures. (A) Structure of the $\omega_2$ inactive heterotetramer. The atomic coordinates of the inactive $\omega_2$ heterotetramer (74) were obtained from the 1GVN Protein Data Bank (PDB) entry. (B) Structure of the $\omega_2$ heterotetramer. The atomic coordinates of $\omega_2$ were derived from the 2OZE PDB entry (65). (C) Model of the three-dimensional structure of $\omega_2$ complexed with DNA. (D) The three-dimensional structure of the $\omega_2$ protein. The atomic coordinates of $\omega_2$ were derived from the 1R0Q PDB entry (68). (E) Three-dimensional structure model of $\omega_2$ bound to $\parS$ was derived from the 2BNW, 2BNZ, and 2CAX PDB entries (89). $\parS$ DNA is space filling, $\omega_2$ is orange/red ribbons, and the repeats are indicated below. The modeled structures were prepared with DeepView/Swiss-Pdb-Viewer 3.7. (F) Complexes formed by $\omega_2$ and $\delta_2$ upon binding to $\parS$ DNA. Protein $\omega_2$ bound to $\parS$ DNA led to the formation of a short-living partition complex 1 (PC1) (condition 1); and $\omega_2$ bound to $\parS$ DNA in the presence of $\delta_2$ (out of scale), in the apo form, led to the formation of a long-living partition complex 2 (PC2) (2). $\delta_2$ bound to PC1 led to segosome complex (SC) formation (3); and the interaction of two SCs led to bridging complex (BC) formation (4). (G) Complexes formed by $\omega_2$ and $\delta_2$ upon binding to non-$\parS$ DNA. Protein $\delta_2$ bound to DNA leading to dynamic complex (DC) formation (1); $\omega_2$ binding to DC led to a transient complex (TC) (2); the interaction of one TC and one DC led to pseudo-bridging complex 2 (BC2) formation (3); and the interaction of one DC and one TC led to bridging complex 1 (BC1) formation (4). doi: 10.1128/microbiolspec.PLAS-0007-2013.f3.
Any stochastic decrease of the ωεζ mRNA decreases the concentration of ω2 and of the short half-living ε2 antitoxin with relative increase of the long-living ζ toxin. Then, free ζ toxin halts cell proliferation (61, 72, 75). To overcome these disadvantages, ω2 promotes the synthesis of the ωεζ operon to exit ζ-mediated dormancy (61, 63).

Plasmids of the inc18 family with IR encode two copies of ω gene (e.g., pSM19035, pRE25, pVEF3 with 100% identity among them), and plasmids that encode ermB also code for ω2 gene (e.g., pIP501, pAMβ1, pRE25) (Fig. 2). The ω2 gene is invariably located upstream of the ermB gene (Fig. 2 and 4C). A truncated ω2 gene was detected upstream of the ermB gene also in pSM19035 (Fig. 2 and 4C; C. E. César, personal communication). The genetic linkage between ω2 and the erythromycin resistance cassette remains to be unraveled, but suggests that both genes should be present in a common ancestor. The role of the segD locus in the spread of the ermB cassette is poorly understood.

The segD locus may participate also in the formation of a plasmid cointegrate. It has been proposed that ω2 bound to parS DNA might constitute a barrier to DNA replication (22). This natural impediment, albeit with low efficiency, might lead to one-ended double-strand break, so that the PC1 complex may constitute a recombination hotspot upon plasmids pairing (see Fig. 2) (22). Then, homologous recombination functions in concert with ω2-mediated site-specific BC formation might lead to the formation of cointegrates. Indeed, it was reported that a Lactococcus lactis recipient strain received a transconjugate, in a cointegrate form, and such transfer was blocked if RecA or the segD locus was absent in the donor strain (104, 105), suggesting that the PC1 complex, in concert with RecA, works as a pseudo mob region. Plasmid pairing is enhanced >20-fold in the presence of δ2 (BC formation, Fig. 3F) (84, 85), but the contribution of δ2 in this mobilization avenue remains to be characterized.

CONCLUDING REMARKS

Low-copy plasmids commonly contain several distinct loci to enhance their maintenance. In plasmids of the inc18 family, there is an active interplay of the rep locus with the segB1, segB2, and segD loci to warrant plasmid segregational stability, and with the segA and segC loci to warrant plasmid structural stability without disadvantage to their host. The assessment of the significance of a particular locus in plasmid stabilization revealed that the segB1 locus stabilizes the plasmid ~10,000-fold by impairing growth of plasmid-free segregants, and segB2 stabilizes the plasmid ~50-fold by active partition (21, 61, 63, 65). The segA locus, which optimizes plasmid random segregation, and the segA and segC loci in concert, which correct errors in the shift from DNA polymerase I to the DNA replicase (PolC and DnaE) to replicate the DNA, stabilize the plasmid ~25-fold (17, 21, 24). The segC locus, which maps outside the minimal replicon, is essential for replication of plasmids with IR regions. The segD locus, which is responsible for reducing plasmid copy number fluctuations to less than two copies and for regulating expression of the segB1 and segB2 loci, stabilizes the plasmid ~10-fold (63).

**FIGURE 4** Conserved organization of the segD locus. (A) The _P_copS_, _P_β, and _P_ω regions of plasmid pSM19035, _P_coph_ or _P_copf_ of pIP501 or pAMβ1, _P_ω2 of pIP501 or pAMβ1, _P_β of pAMβ1, _P_ε25, _P_re25_ and _P_vef3_ are indicated. The variable number of contiguous 7-bp heptad repeats (iterons) and their relative orientations (← or →) are shown. The _P_ω region is highly conserved among plasmids of the inc18 family. (B) Sequence alignment of the transcriptional repressors ω2 and their highly relative ω2. The alignment was done by using Clustal W2 and visualized with default coloring of the different residues by using Jalview v 14.0. (C) Conserved genetic organization of the ermB locus. The open reading frames and their relative orientations are represented by arrows. The _erm_ gene coding for the leader peptide and _ermB_ are denoted in dark gray (arrowhead and arrow, respectively), and the conserved downstream gene in light gray. The genes of the segB1, segB2, and segC loci are indicated. The ω2 or ω2_δ_ proteins are denoted by red and pink arrowheads, respectively, and the _Ps_ sensitive to ω2 or ω2_δ_ repression are denoted as red boxes. Traces of a given gene are denoted by broken-line squares with the name of the gene indicated (e.g., pSM19035 containing traces of ω2). Fusions (e.g., pLM300 where ω2 was fused to an upstream gene) or deletions (e.g., pIP501 contains a deletion in the _ζ_ gene) are also indicated. Similar organization was observed in pAM77 and pUL631. doi: 10.1128/microbiolspec.PLAS-0007-2013.f4
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REFERENCES

14. Berryman DJ, Rood JI. 1995. The closely related ermB-ermAM genes from Clostridium perfringens, Enterococcus faecalis (pAM11), and Streptococcus agalactiae (pIP501) are flanked by variants of a directly repeated sequence. Antimicrob Agents Chemother 39:1830–1834.
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