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Coordination between replication, segregation and cell division in multi-chromosomal bacteria: lessons from *Vibrio cholerae*

Elena Espinosa[1], François-Xavier Barre[1,*] and Elisa Galli[1,*]

[1] Institute for Integrative Biology of the Cell (I2BC), Université Paris-Saclay, CEA, CNRS, Université Paris Sud, 1 avenue de la Terrasse, 91198 Gif sur Yvette, France

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Summary. Bacteria display a highly flexible cell cycle in which cell division can be temporally disconnected from the replication/segregation cycle of their genome. The accuracy of genetic transmission is enforced by restricting the assembly of the cell division apparatus to the low DNA-density zones that develop between the regularly spaced nucleoids originating from the concurrent replication and segregation of genomic DNA. In most bacteria, the process is simplified because the genome is encoded on a single chromosome. This is notably the case in *Escherichia coli*, the most well studied bacterial model organism. However, ~10% of bacteria have domesticated horizontally acquired mega-plasmids into extra-numerous chromosomes. Most of our current knowledge on the cell cycle regulation of multi-chromosomal species derives from the study of replication, segregation and cell division in *Vibrio cholerae*, the agent of the deadly epidemic human diarrheal disease cholera. A nicety of this model is that it is closely related to *E. coli* in the phylogenetic tree of bacteria. Here, we review recent findings on the *V. cholerae* cell cycle in the context of what was previously known on the *E. coli* cell cycle.

Keywords: *Vibrio cholerae* · DNA replication · chromosome segregation · cell division

Introduction

During vegetative proliferation, cell division must be coordinated with the duplication of genomic DNA and its equal repartition in opposite cell halves to avoid the formation of non-viable cells. In eukaryotes, it is achieved by coupling the formation of the division apparatus, the divisome, to the activity of the segregation machinery, the mitotic spindle, whose assembly is itself delayed to the end of replication by a checkpoint mechanism. In contrast, cell division can be disconnected from replication and segregation in bacteria, which can multiply faster than the time it takes to replicate their genome by running multiple replication cycles in parallel and can live as and/or transiently form polyploid filamentous cells as an adaptation to their environment. How such flexibility is achieved without putting in jeopardy the accuracy of genetic transmission is linked to two features of the bacterial cell cycle. First, segregation of newly replicated DNA is progressive and concurrent with replication. Bacterial chromosomes carry a single origin of bidirectional replication, which defines two replication arms. As replication progresses along the two arms, newly replicated loci rapidly segregate to opposite cell halves [6]. Second, the cellular arrangement of genomic DNA directly controls cell division. The genome of bacteria forms a nucleus-like region within cells, the nucleoid. A process termed nucleoid occlusion impedes divisome assembly over the bulk of the nucleoid, thus restricting cell division to the low DNA-density zone that develops between newly forming nucleoids during each replication/segregation round [1].

As most bacteria harbour a single chromosome, nucleoids normally correspond to the territory occupied by individual chromosomes, as illustrated by studies in the 3 major bacterial models, *Escherichia coli*, *Bacillus subtilis* and *Caulobacter crescentus*. However, the genome of ~10% of sequenced bacteria is divided on multiple chromosomes, raising questions on

* To whom correspondence should be sent:
francois-xavier.barre@i2bc.paris-saclay.fr

the mechanism coordinating cell division to the replication/segregation cycle of each of their chromosomes [18]. Several multi-chromosomal species are under scrutiny, including *Burkholderia*, *Rhyzobium*, *Rhodobacter* and *Brucella*. However, most of our current knowledge on the cell cycle regulation of multi-chromosomal species mainly derives from the study of replication, segregation and cell division in *Vibrio cholerae*, the agent of the deadly epidemic human diarrheal disease cholera.

The *V. cholerae* model

V. cholerae belongs to the Vibrionaceae family, a large family of fresh and salt water γ-proteobacteria, which includes most of the bioluminescent bacteria, many sea animal symbionts, and many human and sea animal pathogens [10]. All of the species within the family carry two circular chromosomes of uneven size [30,47,63]. The largest of these is called primary chromosome because it carries almost all of the essential genes of the cell [26] and because its replication origin and partition machinery group with the replication origin and partition machinery of mono-chromosomal γ-proteobacteria [64]. On the contrary, the smallest chromosome is called secondary chromosome because it only carries a few essential genes [26] and because its replication origin and partition machinery group with those of plasmids [64]. In other bacterial families, extra-numerous bacterial chromosomes also harbour plasmid features and it is now largely admitted that they derive from the domestication of horizontally acquired mega-plasmids. One such domestication event might have participated to the evolutionary separation of the Vibrionaceae from the Enterobacteriales and to their expansion in aquatic environments.

V. cholerae first attracted the attention of research scientists because of its worldwide importance as a human pathogen. However, it soon became a reference model for basic research on multi-chromosomal management because its 2.96 Mbp primary chromosome, Chr1, carries homologues of most (if not all) of the genes implicated in replication, chromosome organization and cell division in *E. coli* (Figure 1). In particular, it encodes homologues of the *E. coli* DNA adenosine methylation (*dam*) restriction-modification system, SeqA and MatP proteins, which together contribute to the regulation of replication initiation and the organization and segregation of *E. coli* chromosome (Figure 1, [7,36]). A notable difference is the presence of a polar organizing factor, HubP, which directs the action of a partition machinery, ParAB1 [61](Figure 1). In addition, *V. cholerae* Chr1 carries a gene encoding for DciA, the primordial loader/activity regulator of the replication helicase, which was replaced by DnaC in *E. coli* [8] (Figure 1). In agreement with its plasmid origin, the 1.07 Mbp secondary chromosome of *V. cholerae*, Chr2, carries genes dedicated to its sole replication and segregation, *rctB* and *parAB2*, respectively (Figure 1).

Coordination of Chr1 and Chr2 replication

Both Chr1 and Chr2 carry a single origin of replication, *oriC1* and *oriC2*, respectively. *oriC1* is very similar in sequence to the origin of replication of the *E. coli* chromosome, *oriC* [19]. It contains an AT rich region flanked by five putative high affinity binding sites for DnaA (Figure 2A). *E. coli* DnaA is a weak ATPase. DnaA-ATP promotes the unwinding of the *oriC* AT-rich region by binding to lower affinity sites within *oriC* [15,16]. Like *oriC*, *oriC1* also harbours a putative binding site for IHF, which stimulates the action of DnaA-ATP [15,16]. In *E. coli*, several mechanisms prevent over-initiation by regulating the quantity and/or availability of DnaA-ATP (Figure 2A): (i) DnaA represses its expression; (ii) there are >10-fold more DnaA binding sites in the *E. coli* chromosome than in the origin region, which titrate DnaA away from it; (iii) a protein that specifically binds to hemi-methylated GATC sites, SeqA, sequesters the low affinity DnaA binding sites present on *oriC* and in the *dnaA* promoter; (iv) a cluster of DnaA binding sites, *datA*, catalyses the conversion of active DnaA-ATP to inactive DnaA-ADP; (v) the ATPase activity of DnaA is stimulated when it encounters the replisome, which prevents DnaA activity during the elongation phase of DNA replication (RIDA for regulatory inactivation of DnaA); (vi) two chromosomal loci termed DnaA reactivating sequences (DARS) help to recharge DnaA with ATP, which permits to trigger overlapping rounds of replication in rapidly growing cells [43]. *oriC1* can functionally replace *oriC*, suggesting that similar regulatory circuits probably operate on Chr1 [14], as confirmed by studies on the role of *dam* and SeqA [14,19].

In contrast, *oriC2* is similar in structure to the iteron-based replication origin of large low copy number plasmids such as

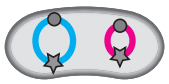
		Replication				Segregation					Division	
E. coli		*dnaA*	*dnaC*	*dam*	*seqA*	-	-	*matP*	*xerCD*	*ftsK*	*minCDE*	*slmA*
V. cholerae		*dnaA*	*dciA*	*dam*	*seqA*	*hubP*	*parAB1*	*matP*	*xerCD*	*ftsK*	*minCDE*	*slmA*
		rctB	-	-	-	-	*parAB2*	-	-	-	-	-

Fig. 1. Cell cycle effectors. The main effectors of DNA replication, chromosome segregation and cell division in *E. coli* and *V. cholerae*.

F and P1 (Figure 2B). It harbours a single DnaA binding site. Unwinding of its AT-rich region results from the binding of its own replication initiator, RctB, to short 11-12mers motifs (Figure 2B). RctB presents structural similarities to plasmid initiators [40]. Like iteron-based origins, it is inactivated by dimerization, which is counteracted by the action of chaperones [28,29]. However, several features distinguish the regulation of Chr2 replication initiation from plasmids. First, *oriC2* harbours structurally different RctB binding sites to activate or repress initiation, 11-12mers and 29-39mers, respectively (Figure 2B). RctB dimers mask the 11-12mers by directly bridging them to three 29-39mers [52]. RctB represses its production by binding to a 29-39mer within its gene promoter [53]. The action of the 29-39mer on the other end of *oriC2* is inhibited by transcription from the promoter of a small RNA, *rctA*, and by the binding of ParB2 to an adjacent *parS2* site [51,54]. ParB2 can also inhibit the action of the central 29-39mers by directly binding to it [51]. Second, *dam* is essential in *V. cholerae* because of its role in Chr2 replication [14,50]. The 11-12mers of *oriC2* contain a *dam* methylation site (Figure 2A) and need to be fully methylated for efficient RctB binding [14]. In contrast, RctB binds to the 29-39mers repressors independently of *dam*. Thus, *dam* methylation serves to prevent Chr2 over-initiation in a similar way to how *dam*/SeqA prevents Chr1 over-initiation. Third, Chr2 replication initiation is coupled to the cell cycle, unlike F and P1 replication. It occurs when ⅔ of Chr1 have been replicated [41]. As Chr2 is only ⅓ of Chr1 in length, it leads to synchronous termination of replication of the 2 chromosomes [41]. This is due to a short intergenic sequence located on one arm of Chr1, *crtS*, whose duplication acts as a timer for Chr2 replication initiation (Figure 2A). Thus, *crtS* couples Chr2 replication initiation to the progress of Chr1 replication in a similar

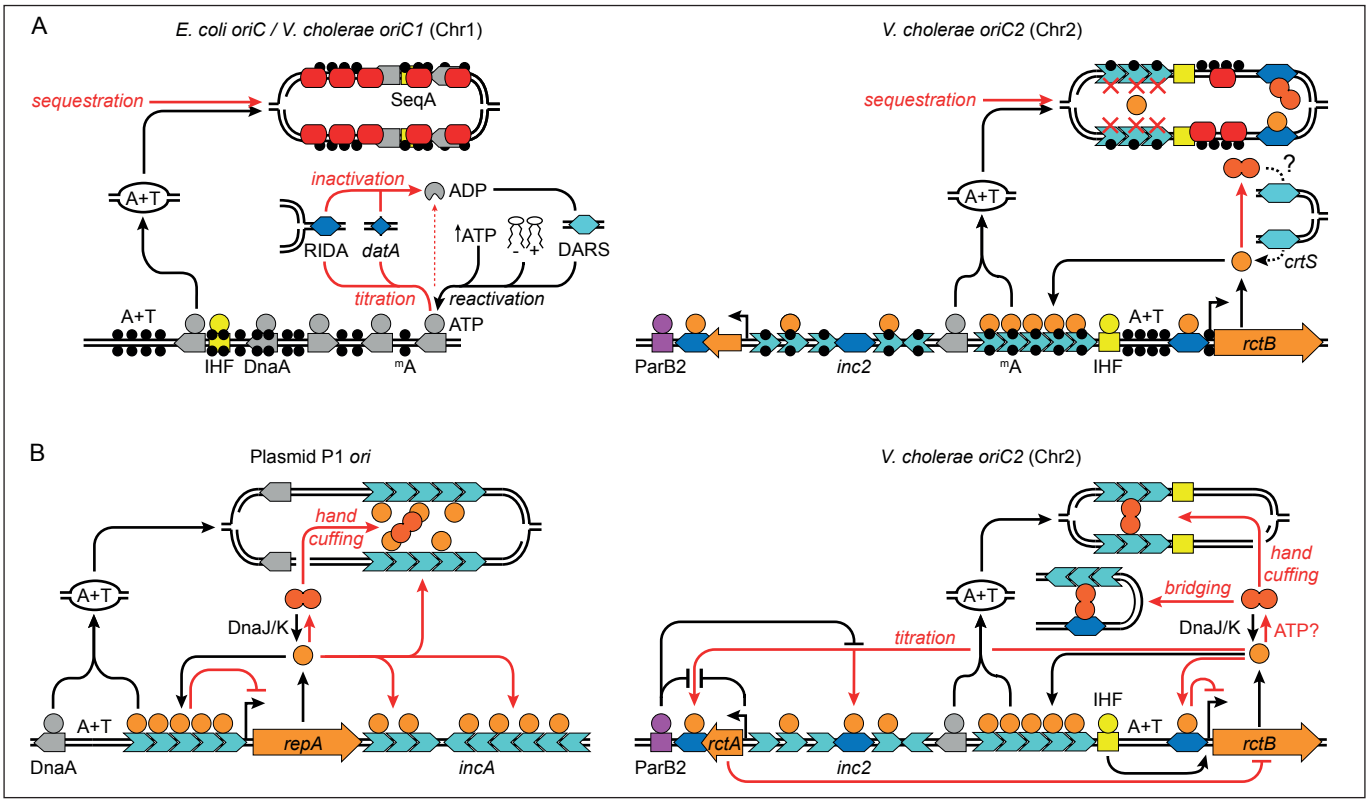


Fig. 2. Control of Chr1 and Chr2 replication initiation. A. Chromosome-like regulatory mechanisms. The left panel depicts the origin of replication of Chr1, *oriC1*, and the demonstrated (*dam*/SeqA) or putative (RIDA, *datA*, ATP and phospholipid synthesis, DARS) mechanisms controlling its unwinding. The role of *dam* and SeqA in Chr1 replication initiation was analysed. The controls exerted by RIDA, *datA*, ATP and phospholipid synthesis and DARS in *E. coli* were added on the basis that they should operate in *V. cholerae* since *oriC1* can functionally replace the origin of replication of the *E. coli* chromosome, *oriC*. The origin of replication of Chr2, *oriC2*, is depicted on the right panel. Two chromosome-like regulatory mechanisms control its unwinding, *dam* methylation, which directly affects RctB binding to its 11-12mers binding site and *crtS*, which places Chr2 replication initiation under the control of Chr1 replication elongation. **B.** Plasmid-like regulatory mechanisms of Chr2 replication (right panel). A scheme of the origin of replication of P1 and of the mechanisms regulating its unwinding is shown on the left panel for comparison. Black arrow-head and T-head lines: initiation activating mechanisms; red arrow-head and T-head lines: initiation inhibitory mechanisms; grey circles: DnaA; grey pentagons: DnaA boxes; black circles: methylated GATC sites; red rectangles with curved angles: SeqA; jelly-fish shapes: phospholipids; yellow circles: IHF; yellow squares: IHF binding site; pink circle: ParB2; pink square: *parS2*; orange circles: RctB (top and bottom right panels) or RepA (bottom left panel); small orange arrow box: *rctA*; large orange arrow box: *rctB* or *repA*, as indicated; cyan arrow boxes: 11-12mers; dark blue diamond boxes: 29-39mers.

way to how RIDA prevents re-initiation of Chr1 replication during the elongation phase [2,49]. The molecular mechanism of how *crtS* replication triggers initiation of Chr2 replication is still unknown. However, RctB directly binds *crtS* and, by analogy to DARS, might help convert inhibitory RctB dimers to active RctB monomers [2].

DciA probably controls the loading and release of the replicative helicase, DnaB, on either side of the origins of Chr1 and Chr2, to start bidirectional replication [8]. As Chr1 and Chr2 are circular, replication terminates with the merging of the opposite replication forks. Marker frequency analysis and GC-skew studies suggest that termination generally occurs in a region opposite to their origins, *ter1* and *ter2*, respectively.

Cellular arrangement and choreography of segregation of Chr1 and Chr2

The organization of bacterial chromosomes can be stereotypically divided into two categories: a transversal arrangement, as described in slow-growing *E. coli* cells [38,56,58] and a longitudinal arrangement, as described in *C. crescentus* and in *B. subtilis* during sporulation [58,59]. In the transversal arrangement, the origin of replication is located at mid-cell and is flanked by the left and right arms of the chromosome in newborn cells, which creates a left-*oriC*-right pattern. In the longitudinal arrangement, the origin and terminus of replication are located at opposite poles and the two chromosome arms reside beside each other along the long axis of newborn cells, which creates an *oriC-ter* pattern. During a division event, each of the two daughter cells inherits one of the pre-existing poles of the mother cell, the "old pole" and one of the two poles originating from the constriction event at the division site, the "new pole". In the *oriC-ter* configuration, the origin of replication is specifically located at the old pole and the terminus of replication at the new pole in newborn cells [42,58].

Despite the close relationship of *V. cholerae* and *E. coli*, Chr1 and Chr2 are both longitudinally arranged [35]. Systematic cytological analysis of multiple chromosomal loci showed that Chr1 covers the entirety of the cell, with *oriC1* at the old pole and *ter1* at the new pole, whereas Chr2 only resides in the younger half of the cell, with *oriC2* at mid-cell and *ter2* towards the new pole [11] (Figure 3).

Specific factors and protein complexes contribute to determine and to maintain the chromosomal organization during the entire cell cycle, from the beginning of the replication cycle to the end of the division cycle. In particular, the localization of specific chromosomal regions such as the origin and terminus of replication often rely on dedicated systems that control their segregation timing and positioning. In bacteria displaying an *oriC-ter* arrangement, the origin regions are segregated to opposite cell halves and maintained in proximity of the old poles by

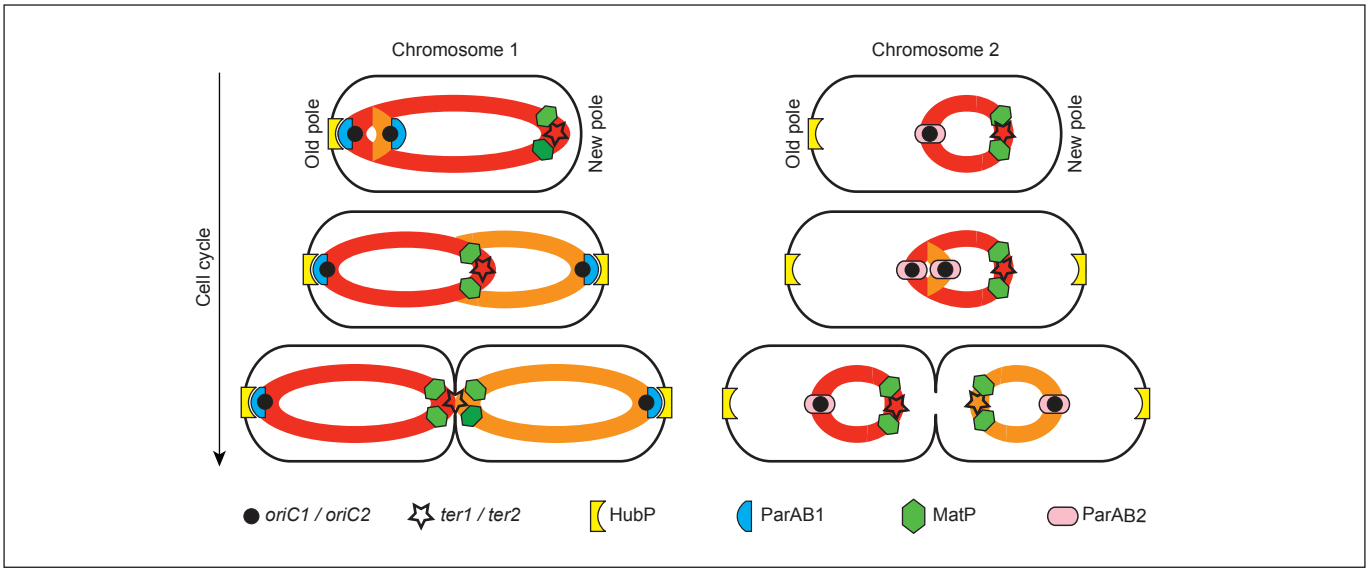


Figure 3 Schematic representations of Chr1 and Chr2 segregation and arrangement in *V. cholerae*. The segregation cycle and chromosome arrangement are depicted for *V. cholerae* Chr1 and Chr2, respectively in the left and right panel. Left panel: in newborn cells the origin of replication of Chr1, *oriC1*, is anchored by HubP and the ParAB1-*parS1* system to the old pole and the terminus of replication *ter1* is kept in proximity of the new pole by MatP. During the cell cycle it is HubP the first factor transitioning towards the opposite pole, soon followed by ParAB1 and one sister copy of the newly replicated *oriC1*. While the replicated *oriC1* copies are segregated in opposite cell halves, the *ter1* region bound by MatP relocates to mid-cell where the newly duplicated *ter1* regions remain together until the end of the cell cycle. Right panel: in newborn cells Chr2 occupies the younger half of the cell, its origin of replication, *oriC2*, is maintained at mid-cell by the ParAB2-*parS2* system and the *ter2* region close to the new cell pole by MatP. After duplication, in older pre-divisional cells, the *oriC2* sister copies are segregated at the quarter positions by the parAB2-*parS2* system and the movements of segregated *ter2* sister copies are restricted around the division site by MatP.

an origin-specific partition system [42,58]. Partition systems consist of a Walker-type ATPase, ParA, and a protein, ParB, which binds to specific *cis*-acting centromere-like sites, *parS*, located in the *oriC* region of bacterial chromosomes [42,58]. ParA interacts with ParB-*parS* complexes and drives one of the newly duplicated *oriC* copies towards the opposite cell pole, initiating the chromosome segregation cycle [58]. The extent to which Par systems contribute to the organization and segregation of *oriC* sister copies differs widely between bacteria. Inactivation of the Par system leads to small defects in origin segregation and positioning during the vegetative growth of *B. subtilis* [57] whereas it is critical for chromosome partitioning in *C. crescentus* [46]. In *V. cholerae*, two distinct ParAB-*parS* systems, ParAB1-*parS1* and ParAB2-*parS2*, drive the localization and segregation patterns of *oriC1* and *oriC2*, respectively [21,62] (Figure 3).

ParAB1-*parS1* imposes an asymmetric segregation process similar to that described for the origin of replication of the *C. crescentus* chromosome [11,21,55]. A transmembrane protein, HubP, acts as a polar organization factor in *V. cholerae*, like TipN in *C. crescentus* and DiIVA in *B. subtilis* [61]. HubP interacts directly with ParA1, which in turn recruits the ParB1-*parS1* complexes [12] (Figure 3, left panel). Correspondingly, HubP, ParB1 and *oriC1* co-localize at the old pole during the entire cell cycle [11,23,61]. As the cell cycle progresses, HubP proteins start accumulating at the new pole, shortly followed by ParB1 and in turn by one copy of the newly duplicated *oriC1* [23]. Even though disruption of the HubP-ParAB1-*parS1* partition system perturbs *oriC1* localization, Chr1 segregation is not impaired [11,21,61]. In addition, Chr1 remains longitudinally arranged within the cell, with *oriC1* near the old pole of newborn cells [11].

In contrast to *oriC1*, *oriC2* follows a symmetric segregation process similar to that of P1 and F plasmids [25,39,62]. After duplication at the centre of the cell, the two *oriC2* copies move to ¼ and ¾ cell positions, i.e. to the future cell centres of the two daughter cells [62] (Figure 3, right panel). In addition, the ParAB2-*parS2* partitioning system is essential for segregation of Chr2. In its absence, aberrant unviable Chr2-deficient cells are produced [62].

The organization, positioning and segregation dynamics of the *E. coli* chromosome terminus of replication, *ter*, depends on the MatP macrodomain protein. MatP binds to specific DNA motifs, *matS*, which are exclusively present and overrepresented in the chromosome terminus region [36]. MatP interacts directly with a specific component of the divisome machinery that co-localizes with the Z-ring [20]. As a result, MatP maintains newly replicated *ter* copies at mid-cell. Reciprocally, MatP plays a role in the selection of the division site and the licensing of divisome assembly [34]. *V. cholerae* codes for an ortholog of *E. coli* MatP. *ter1* and *ter2* both harbour *E. coli matS* motifs with a density similar to *E. coli ter* [16,36]. However, *ter1* and *ter2* behave differently. Sister *ter1* copies remain together at mid-cell until a very late stage of the division cycle, when septa are clearly visible (Figure 3, left panel), whereas sister *ter2* copies segregate in the two cell halves before initiation of septation [16] (Figure 3, right panel). Careful inspection of the segregation dynamics of *ter1* and *ter2* loci showed that even though sister copies of *ter2* loci separate earlier than sister copies of *ter1*, they remain in the vicinity of the division site and keep colliding with each other during the septation process. When MatP is inactivated the position of *ter2* sisters is no longer restricted, dramatically reducing collision events. In the absence of MatP, *ter1* sister copies separate early in the cell cycle. However, they remain in the vicinity of the cell centre [16].

When a chromosome is circular, as it is the case for most bacterial chromosomes, homologous recombination events between sister chromatids can generate chromosome dimers, which threaten chromosome segregation [35]. In *E. coli*, chromosome dimers are resolved by the addition of a crossover at a specific site within the terminus region, *dif*, by two tyrosine recombinases, XerC and XerD [37]. A cell division protein, FtsK, plays two roles in the process. First, it uses the energy from binding and/or hydrolysis of ATP to pump DNA between daughter cell compartments after the assembly of the divisome but before final scission [15]. Polar DNA motifs, the KOPS, orient the loading of FtsK on DNA, which directs the direction of translocation [4,5]. KOPS are overrepresented in the *E. coli* genome and point from the origin of replication towards *dif* [5]. As a result, FtsK brings together the two *dif* sites of a chromosome dimer at mid-cell. Second, FtsK activates Xer recombination by a direct interaction with XerD [37]. The dimer resolution machinery described in *E. coli* is conserved in almost all bacteria. Chr1 and Chr2 harbour two specific and incompatible *dif* sites in their terminus region, *dif1* and *dif2*, which are used for the resolution of chromosome dimers by a common Xer/FtsK machinery at the time of cell division [16,48]. In *E. coli*, FtsK contributes to the segregation of sister chromosomes independently of chromosome dimer formation under slow growth conditions [22,44]. However, the action of FtsK is mainly restricted to chromosome dimers in fast growth conditions in *E. coli* because sister *ter* separate before the onset of cell division [22]. In *V. cholerae*, FtsK also processes sister Chr1 copies independently of chromosome dimer formation in slow growing conditions. However, it remains implicated in the process also under fast growth because *ter1* sister copies persist at mid-cell for a prolonged length of time independently of the growth rate [22]. Future work will be necessary to elucidate the behaviour of *ter2* sister copies and how they are managed by FtsK at different growth rate.

Cell division cycle and division site placement

The cell division process has been depicted in detail in *E. coli*, *B. subtilis* and *C. crescentus*. In these species, the divisome is a dynamic protein complex comprising at least a dozen highly conserved proteins, which are recruited to the division site in

an almost linear pathway [12,17]. Divisome assembly can be schematically divided into two distinct sequential steps [12,17]. First, a tubulin homologue, FtsZ, polymerizes into a ring-like structure, the Z-ring, at mid-cell at about 25-38% of the cell cycle. The Z-ring is stabilized and anchored to the membrane by a set of proteins that are recruited at the same time. Second, periplasmic and integral membrane proteins, which are involved in cell wall remodelling or in safe keeping sister chromosome replication termini (FtsK) are recruited at about 48-52% of the cell cycle. The septation process starts soon after the arrival of this second set of proteins and lasts throughout the remaining half of the cell cycle [12,17].

V. cholerae harbours homologues of most of the *E. coli* cell division proteins. However, their cell cycle choreography is considerably different. All divisome components are specifically located at the new pole at the beginning of the cell cycle. FtsZ molecules, soon followed by the other early cell division proteins, only relocate to mid-cell at about 50% of the cell cycle, where they form a loose pre-divisional Z-ring. The remaining cell division proteins leave the new pole and join the early divisome complex at mid-cell at about 80% of the cell cycle. The pre-divisional FtsZ structures concomitantly coalescence into a compact Z-ring. Cell wall constriction initiates at about 90% of the cell cycle, leaving a very short time to complete cell scission [23,24] (Figure 4A).

In *E. coli*, the combined action of two FtsZ-polymerization inhibitory systems, Min and nucleoid occlusion (NO), specifically licenses cell division at mid-cell at the end of each round of replication/segregation cycle. Min couples the longitudinal positioning of the Z-ring to the geometrical shape of the cell. It prevents FtsZ polymerization at the cell poles, which directs it to mid-cell [33]. NO couples Z-ring formation to the replication/segregation cycle of the *E. coli* chromosome. It prevents Z-ring formation over the bulk of the nucleoid, which directs it to the low DNA-density zone that develops between newly forming nucleoids [3,60]. Min is the major regulator of division site placement. Min-deficient mutants form filamentous cells. Z-rings can assemble at the poles of the filaments, which generates anucleated mini-cells [33,12,65]. In contrast, inactivation of NO does not generate noticeable phenotypes, unless it is

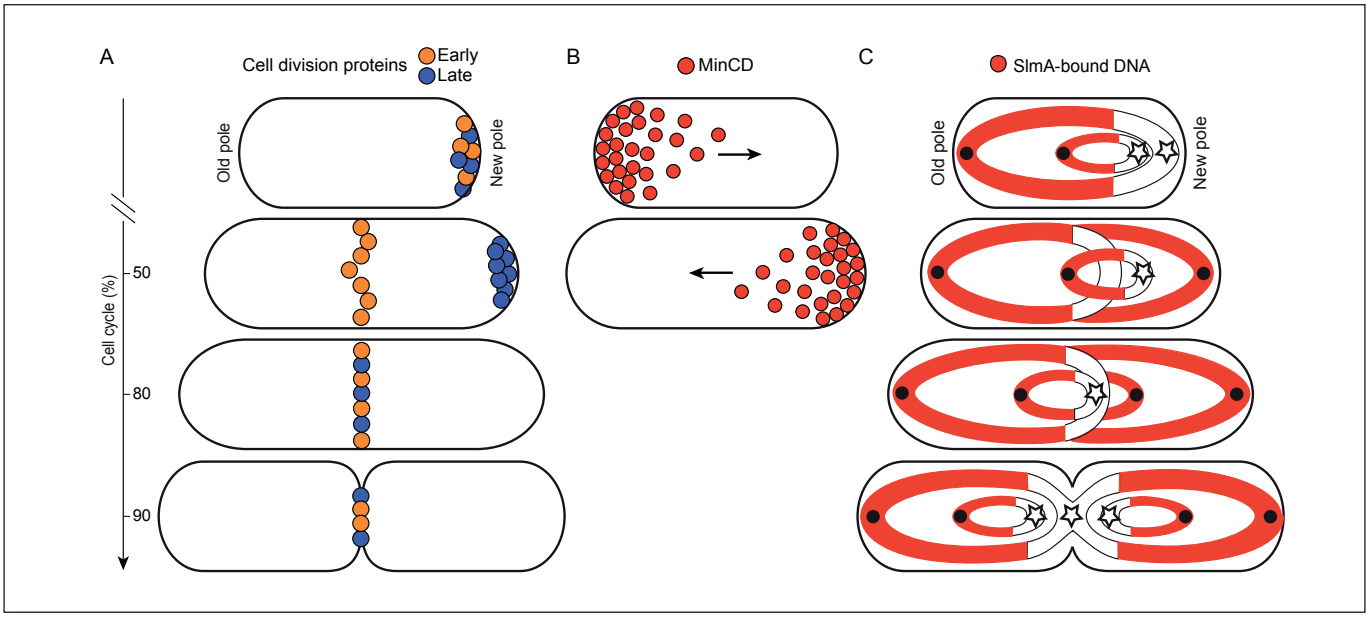


Fig. 4 Divisome assembly and regulation of division site placement in *V. cholerae*. A. Schematic representation of the divisome assembly. All division proteins are located at the new pole in newborn cells. At about 50% of the cell cycle FtsZ and the early cell division proteins leave the cell pole and relocate to mid-cell where they form a loose pre-divisional structure. The Z-ring coalescences into a compact structure at about 80% of the cell cycle, concomitantly with the arrival of the late cell division proteins at mid-cell. Cell constriction characterized by visible cell wall indentations starts at about 90% of the cell cycle. **B.** Schematic representation of the Min system, spatial regulator of division site placement. Throughout the cell cycle MinCD oscillate between the cell poles creating a gradient of MinC, inhibitor of FtsZ polymerization, which is lowest at mid-cell and highest at the poles. Z-rings can only assemble at mid-cell, the geometrical centre of the cell, characterized by the lowest MinC concentration over time. **C.** Schematic representation of the NO system, spatiotemporal regulator of division site placement. The effector of NO and inhibitor of Z-ring assembly SlmA binds to specific DNA sequences distributed all around Chr1 and Chr2 (SlmA-bound DNA) with the exception of *ter1* and *ter2* regions. During the cell cycle the spatial arrangement and segregation timing of Chr1 and Chr2 direct FtsZ molecules and assembly of divisional Z-rings to the SlmA-free zones. In newborn cells the SlmA-free *ter1* and *ter2* regions are both located at the new cell pole. Chr1 SlmA-free DNA is located at the centre of the cell starting from about 50% of the cell cycle, however Chr2 SlmA-bound DNA is still located at mid-cell at this stage, delaying the formation of compact Z-ring structures. It is only at about 80% of the cell cycle that both *ter1* and *ter2* regions co-localize at mid-cell, permitting assembly of divisional Z-rings at the future division site.

combined with defects in initiation of replication, segregation, or the disruption of Min [3]. *E. coli* Min is composed of three proteins: MinC, MinD and MinE. MinC is the factor responsible for blocking Z-ring formation, MinD is the activator of MinC, and MinE is the topological regulator of MinCD [33]. Specific inhibition of FtsZ polymerization at the cell poles is achieved through the regulated oscillation of the Min proteins between the two cell poles. MinD is an ATPase. Its ATP-form binds to the membrane where it recruits MinC. MinE stimulates ATP hydrolysis, which releases MinD-ADP and MinC from the membrane. MinD and MinC then migrate towards the opposite pole where, after nucleotide exchange in the cytosol, MinD-ATP re-associates to the membrane [33]. Continuous shuttling of MinCD between the poles creates a concentration gradient of MinC with a minimum at mid-cell (Figure 4B). NO couples the timing and assembly of the Z-ring to the replication/segregation cycle. The nucleoid serves as a scaffold for the positioning of a DNA binding protein that inhibits FtsZ polymerization, SlmA. SlmA binding sites (SBS) are asymmetrically distributed on the *E. coli* chromosome and essentially absent from *ter*. As a result, cell division can only initiate at the very end of the chromosome duplication/segregation cycle when sister *ter*, devoid of SBS, are the only chromosomal regions left at mid-cell [9,12,45] (Figure 4C).

V. cholerae carries orthologs of both the Min and NO effectors, MinCDE and SlmA. *V. cholerae* MinD was shown to shuttle between poles as reported for *E. coli* [24]. However, Min-inactivation does not generate any apparent phenotype unless additional mutations perturbs the cellular arrangement of chromosomes, suggesting that NO is the major regulator of division site placement in *V. cholerae* [24]. Indeed, SBS sites were identified on both Chr1 and Chr2 and their distribution was shown to drive the choreography of the cell division proteins and the timing of assembly and maturation of the divisome [24] (Figure 4C). As the partition machinery of Chr1 is conserved in most bacteria with the notable exception of Enterobacteriales, it seems reasonable to argue that NO was probably the primary cell division regulation mechanism in the Enterobacteriales/Vibrionaceae ancestor and that Min superseded it in the Enterobacteriales because they lost their origin partition machinery in the course of evolution.

Concluding remarks and open questions

A recurrent question about multi-chromosomal bacteria concerns the definition of a secondary domesticated chromosome and how they can be distinguished from plasmids. The presence of essential genes is not sufficient because large portions of the genome can be moved from one replicon to another. Likewise the size of the replicon is insufficient because of the existence of large mega-plasmids. Some criteria can be proposed based on the *V. cholerae* model. Preventing the over-initiation of Chr1 and Chr2 replication relies on *dam* while the final stages of Chr1 and Chr2 segregation depend on the same FtsK/XerCD machinery. Thus, a first criterion could be to exploit the replication/segregation regulatory systems of the primary chromosome. Duplication of a small sequence on Chr1, *crtS*, serves to license Chr2 replication. Thus, a second criterion could be to integrate replication cycle coordination. Third, Chr1 and Chr2 form a single nucleoid, with the territory occupied by Chr2 in the cell comprised within the territory occupied by Chr1, and Chr2 harbours SBS that are essential for the regulation of cell division by nucleoid occlusion. Thus, direct participation of secondary chromosomes to the regulation of the cell cycle could be added to the list of criteria. However, the validity of each of these criteria cannot be assessed without any insight in the domestication process. Chr2 harbours many features of Chr1: a *dif* site, KOPS directed towards *dif* on both replichores, *matS* sites in its terminus region, SBS sites outside of the terminus region and *dam* methylation sites to control RctB binding to its origin region. Were they all acquired during the domestication process or were some of them already present in the plasmid ancestor of Chr2 to permit its maintenance? It would be advantageous for large replicons to use FtsK oriented DNA translocation to align dimer resolution. It would mean acquiring properly oriented KOPS but probably also synchronizing replication termination with the formation of the divisome. The addition of *matS* sites would help maintain sister *ter* in the vicinity of the divisome and harbouring SBS sites avoid septum closure before replication termination. In contrast, it is difficult to imagine how Chr1 *crtS* could have pre-existed. We can thus question if some of the "chromosome-like" features of Chr2 were acquired during domestication or pre-existed. RctB does not belong to the classical replication initiator family of plasmids and seems quite specific to Vibrionaceae. To answer this question, it would be interesting to find a plasmid relying on an RctB-like replication initiator and study the regulation of its replication and segregation.

Another recurrent question concerns the size of secondary replicons. As bacterial chromosomes harbour a single origin of replication, splitting the genome on several chromosomes reduces the length of time necessary for the duplication of genetic information. With a replication speed of 1000 bp/sec, replication of the 4.5 Mbp *E. coli* chromosome takes ~38' while replication of Chr1 and Chr2 only takes ~25' min and ~8', respectively. *V. cholerae* can thus multiply as fast as *E. coli* in rich growth conditions while running less replication circles in parallel than *E. coli* [22]. From this point of view, it is surprising that no multi-chromosomal species was found that harboured chromosomes of similar size. It is now explained in the case of the Vibrionaceae: their secondary chromosome must be smaller than their primary chromosome for the *crtS* regulation mechanism to operate.

Finally, a complex unexpected mechanism has evolved to enforce synchronization of the replication termination of Chr1 and Chr2. As stated earlier, this is probably linked to the role FtsK plays in the management of *ter1* and *ter2*. In this regard,

it seems surprising that Vibrios lack a homologue of the *E. coli* replication fork trap machinery [27]. Did another system evolved in the Vibrios? Likewise, what differences between the *E. coli* and *V. cholerae ter* macrodomain organization system explain why MatP, which acts on both *ter1* and *ter2* and was shown to directly link sister copies of *E. coli ter* to the divisome, seems unable to maintain *ter2* at mid-cell? In addition to further our understanding of the *V. cholerae* cell cycle, answering these questions could help unmask the primordial role of regulatory mechanisms common to *E. coli* and *V. cholerae* from any additional role they might have adopted during speciation, as illustrated by the cell division studies.

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