A Bidimensional Segregation Mode Maintains Symbiont Chromosome Orientation toward Its Host

Graphical Abstract

Highlights
- Symbiont sister ori segregate along the short and the long cell axis (diagonally)
- ParB recapitulates ori localization and binds an ori-proximal parS site in vitro
- Septal sister ter migrate to midcell concomitantly with septation progression
- Bidimensional segregation endows maintenance of chromosome configuration

Authors
Philipp M. Weber, Friedrich Moessel, Gabriela F. Paredes, Tobias Viehboeck, Norbert O.E. Vischer, Silvia Bulgheresi

Correspondence
silvia.bulgheresi@univie.ac.at

In Brief
Weber et al. report on the chromosome segregation of a longitudinally dividing gammaproteobacterium that lives attached to marine nematodes. They show that sister ori segregate along both the short and the length axis. Their data are consistent with the centromeric protein ParB mediating transgenerational maintenance of chromosome configuration.
A Bidimensional Segregation Mode Maintains Symbiont Chromosome Orientation toward Its Host

Philipp M. Weber,1 Friedrich Moessel,1,3 Gabriela F. Paredes,1,3 Tobias Viehboeck,1 Norbert O.E. Vischer,2 and Silvia Bulgheresi1,4,*
1Environmental Cell Biology Group, Department of Ecogenomics and Systems Biology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria
2Bacterial Cell Biology & Physiology, Swammerdam Institute of Life Sciences, Faculty of Science, University of Amsterdam, Science Park 904, 1098 Amsterdam, the Netherlands
3These authors contributed equally
4Lead Contact
*Correspondence: silvia.bulgheresi@univie.ac.at
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SUMMARY

All living organisms require accurate segregation of their genetic material. However, in microbes, chromosome segregation is less understood than replication and cell division, which makes its decipherment a compelling research frontier. Furthermore, it has only been studied in free-living microbes so far. Here, we investigated this fundamental process in a rod-shaped symbiont, Candidatus Thiosymbion oneisti. This gammaproteobacterium divides longitudinally as to form a columnar epithelium ensheathing its nematode host. We hypothesized that unimpeded host attachment would affect bacterial chromosome dynamics and set out to localize specific chromosomal loci and putative DNA-segregating proteins by fluorescence in situ hybridization and immunostaining, respectively. First, DNA replication origins (ori) number per cell demonstrated symbiont monoploidy. Second, we showed that sister ori segregate diagonally prior to septation onset. Moreover, the localization pattern of the centromere-binding protein ParB recapitulates that of ori, and consistently, we showed recombinant ParB to specifically bind an ori-proximal site (parS) in vitro. Third, chromosome replication ends prior to cell fission, and as the poles start to invaginate, termination of replication (ter) sites localize medially, at the leading edges of the growing septum. They then migrate to midcell, concomitantly with septation progression and until this is completed. In conclusion, we propose that symbiont ParB might drive chromosome segregation along the short axis and that tethering of sister ter regions to the growing septum mediates their migration along the long axis. Crucially, active bidimensional segregation of the chromosome allows transgenerational maintenance of its configuration, and therefore, it may represent an adaptation to symbiosis.

INTRODUCTION

The investigation of microorganisms with non-canonical growth modes is necessary to identify the conserved mechanisms underlying bacterial proliferation. Candidatus Thiosymbion oneisti forms a single-species community on the surface of its nematode host Laxus oneistus (Stilbonematinae) [1]. More precisely, each rod is attached by one pole (the proximal pole) to the host so that its long axis is perpendicular to the animal surface. Although Stilbonematinae regularly occur in tropical and temperate shallow water sediment, no Ca. Thiosymbion has been isolated in pure culture so far. Despite the impossibility of genetic manipulation, the reproductive mode of these Gammaproteobacteria is unique as Ca. Thiosymbion rods are the only prokaryotes reported to widen and undergo FtsZ-based longitudinal fission [2–4]. Because longitudinal fission allows for continuous attachment of the bacterial cell to the animal host, we hypothesized that (1) the orientation of the chromosome is invariable and transmitted from mother to daughter cells and that (2) this would require a DNA segregation mode different from that observed in bacteria that may adopt a free-living lifestyle, such as all model bacteria. Studies carried out on the latter revealed that, even though chromosome segregation occurs concomitantly with DNA replication, it follows a defined choreography [5–8]. Namely, it can be divided into three major steps: (1) separation and translocation of the region where replication starts (ori), (2) segregation of the bulk of the chromosome, and (3) separation of the region where replication terminates (ter) [9–11].

Segregation of the ori regions imposes the directionality of chromosome segregation and establishes the final configuration of the chromosome. A chromosomal parABS system drives or assists ori translocation in some bacterial species. This is closely related to the parABS plasmid system and consists of three components: a centromere site on the DNA (parS), a centromere-binding protein (ParB), and a Walker type ATPase (ParA) [10]. For instance, in the case of the Vibrio cholerae’s large chromosome (chr1) and of Caulobacter crescentus, one of the replicated ParB-bound ori regions stays at one pole, and the other moves to the opposite one following a retracting wave of nucleoid-bound ParB over the nucleoid [12–15]. Subsequently, a direct interaction between ParB and a pole-organizing
element, PopZ in C. crescentus [16, 17] and HupB in V. cholerae [18], anchors the translocated ParB-bound ori to the pole. In Actinobacteria, such as Corynebacterium glutamicum and in Bacillus subtilis, DivIVA appears to be the polar tether for the ParB-bound ori, albeit in the latter RacA is the functional homolog of ParB [19–24]. In order to propel itself along the ParA gradient, the ParB-bound ori region likely exploits the chromosomal elastic and/or the proteophoretic force through iterative interactions with ParA [25–27]. Notably, not only the ParB anchors but also the ParA and ParB localization patterns differ among bacteria. For example, instead of being tethered at the cell poles, ParB-bound ori is found near midcell in Mycobacterium smegmatis and displays a subpolar position in non-replicating Myxococcus xanthus [28–31]. Moreover, in M. xanthus, ParA is not nucleoid bound but instead is mainly confined to the DNA-free regions between the cell poles and the nucleoid [29, 30]. In bacteria lacking parA and parB genes, such as Escherichia coli [32], physical mechanisms underlie the translocation of the ori regions. Polymer dynamics in confined space anticipate that entropic forces will mediate the separation of freshly duplicated ori regions [33, 34]. Based on the finding that freshly replicated ori regions remain in close proximity for a protracted amount of time before they suddenly separate [35, 36], a “snap-release” mechanism has been put forward for E. coli ori segregation, by which entropic repulsion of DNA segments would drive the sister ori regions apart [37]. In B. subtilis, structural maintenance of chromosome (SMC) complexes that load in the vicinity of ori via Spo0J (the ParB homolog) may drive self-condensation and disentanglement of ori-proximal DNA loops [38–42]. Physical rather than biochemical phenomena were also invoked to explain bulk chromosome segregation [33, 34, 37, 43–48].

To guarantee complete segregation of sister chromosomes, DNA pumps, such as the FtsK protein (SpoIVFe, during B. subtilis sporulation), actively translocate DNA from one perspective daughter cell to the other [49–51]. FtsK binds the divisome at the growing septum, as well as specific FtsK-orienting polarized sequences (KOPS) on the chromosome [52, 53]. Numerous KOPS are distributed along both arms, and their ori-to-ter orientation confers directionality to the DNA translocation process [54–58]. KOPS guide the translocation of FtsK toward the dif site, another genetic element contained in the ter region that controls chromosome dynamics. The dif site serves indeed as the recognition sequence for the site-specific recombinase XerCD, which ensures resolution of chromosome dimers prior to cell division. The assembly of the recombination complex is facilitated by the aforementioned septum-tethered DNA translocase FtsK, which ensures the capture of the two recombining dif sites in the vicinity of the septum and activates the complex for recombination [59, 60]. About half of all presently sequenced bacterial genomes contain a canonical dif site, and several alternative ones were found in other bacteria [61]. Notably, special sites (matS) in E. coli ter bind MatP, which causes compaction of the region and tethers the domain to the closing division septum [62]. matS-containing chr1 and chr2 ter regions (ter1 and ter2, respectively) are also compacted by MatP in V. cholerae, another gammaproteobacterium [63].

As for the overall arrangement of the genetic material, bacterial chromosomes can assume two major configurations, ori-ter (longitudinal) and left-ori-right (transverse). In ori-ter, the origin is located at one cell pole, the terminus at the other pole, and both left and right chromosomal arms are parallel to the long axis of the cell [7]. This pattern has been observed in C. crescentus and V. cholerae chr I, for example. The left-ori-right pattern is rotated 90° with respect to ori-ter; namely, both ori and ter are centrally positioned so that the left and right arms reside in opposite cell halves. E. coli and other bacteria can switch between longitudinal and transverse arrangements, depending on the cell cycle stage and the growth conditions [35, 64–66].

Here, we present data indicating that, in longitudinally dividing Gammaproteobacteria, ParB/parS binding may mediate ori segregation along the cell short axis. Furthermore, we propose that tethering of sister ter regions to the newly assembled divisome makes them migrate toward one another along the cell long axis as septation proceeds and until, immediately prior to daughter cells’ separation, they reach the center of the cell. Crucially, this bidimensional DNA segregation mode allows transgenerational maintenance of the transverse configuration in an animal symbiont.

RESULTS

Longitudinally Dividing Ca. T. oneisti Is Monoploid, and Its ori Is Segregated along the Cell Short Axis

Both the phylogenetic placement of Ca. T. oneisti and the characterization of its genome suggested that the symbiont genetic material is contained in a single, circular chromosome and that no extrachromosomal genetic elements (plasmids) are present [67] (Table S1). Moreover, the DNA appeared to be homogeneously distributed throughout the cell, except for an 8% drop in fluorescence signal in the polar cell fourths (Figure S1A). To determine the ploidy of Ca. T. oneisti and the localization pattern of its ori, we fixed it and subjected it to DNA fluorescence in situ hybridization (FISH) with a set of nine fluorescent probes targeting a 2,874-nt-long chromosomal region containing the dnaA and dnaN genes both predicted to be ori-proximal [68, 69] (ori probe; Figure S1B; Tables S3 and S5). This region also contained a duplex-unwinding region (also referred to as DNA unwinding element [DUE]) [70, 71] and binding sites for the initiator protein DnaA (DnaA boxes) [72] (Figure S1B), 69% (226) of the 327 cells analyzed by epifluorescence microscopy displayed one fluorescence focus, 25% (81) two foci, and 6% (20) three foci (Figure 1A), with this latter fraction accounting mostly for cells that started a second round of chromosome replication. Therefore, if we assume that one fluorescent focus equals one ori copy, we may conclude that the nematode symbiont is monoploid. This conclusion was also supported by counting the number of fluorescence foci obtained with an anti-ParB antibody and with probes targeting tfsQAZ- and ter-containing chromosomal regions (see below and Figures 1C, S1C, and 3A, respectively).

After determining Ca. T. oneisti ploidy, we analyzed the ori localization pattern throughout the symbiont life cycle. DNA-FISH-based visualization of ori revealed that, in non-dividing cells displaying only one focus, it occupies a near-midcell position (Figure 1B, left panel, and plot in Figure 1E). Moreover, the segregation of sister ori regions starts before the onset of septation, a process concomitant with symbiont cell growth [2]
 Upon replication, sister ori regions are segregated along the short but also along the long axis (from here on, we will refer to this segregation mode as diagonal; Figure 1F; Table S2). Localization pattern of a 3,273-nt-long chromosomal region (Tables S3 and S5) containing the single-copy genes ftsQ, ftsA, and ftsZ, which are ori-proximal in E. coli [73, 74], revealed a number of fluorescence foci (Figure 1C) and a diagonal migration pattern similar to that observed with the ori probe (Figures S1D and S1E). However, at least one of the two foci observed with the ftsQAZ probe appeared more polar, irrespectively of the cell cycle stage (Figure S1E).
Symbiont ParB Recapitulates ori Localization Pattern

One of the generalities of bacterial chromosome segregation is that, when present, the parABS system contributes to it [32, 75–77]. The alignment of the predicted Ca. T. oneisti ParB protein sequence with other bacterial ParB proteins showed conservation of the box I and II and of the HTH motif required for the binding to parS [5, 78] (Figure 2A). Furthermore, probing western blots of symbiont protein extracts with a specific anti-ParB antibody showed that ParB is expressed (Figure 2B). Therefore, we tested whether ParB localization pattern would be consistent with a role in DNA segregation by immunostaining (Figure 1). Epifluorescence-microscopy-based analysis of fixed cells with a specific anti-ParB antibody showed that 82% (414), 15% (78), and 3% (16) of the 508 analyzed cells displayed one, two, or three fluorescence foci, respectively (Figure 1C). The fact that the percentages of cells bearing one, two, or three ori or ParB foci were similar (Figures 1A and 1C, respectively) and that ParB foci localization pattern resembled that of the ori region (see their apparent diagonal segregation in Figure 1D and plots in Figures 1G and 1H) indicated that symbiont ParB might bind the predicted centromeric parS and drive chromosome segregation. Consistently, phylogenetic analysis revealed that, among all ParB proteins reported to mediate DNA segregation, symbiont ParB is mostly related to those of Pseudomonas and Vibrio, that is to chromosome- and not to plasmid-encoded ParB proteins [79, 80–83] (Figure 2A).

In conclusion, our data indicate that native symbiont ParB may bind the chromosome in the vicinity of the ori.

Symbiont ParB Binds an ori-Proximal parS Site In Vitro

To further support a possible role of Ca. T. oneisti ParB in chromosome segregation, we tested its capacity to specifically bind symbiont parS in vitro. Therefore, we expressed and purified a His-tagged version of ParB (Figure S2B) and identified one ori-proximal parS site located between the dnaN and gyrB genes by searching the genome draft for the consensus sequence [32] (Figure 2B). Figure 2C shows that >0.125 μM symbiont recombinant ParB decreased the electrophoretic mobility of an 827-nt-long parS site-containing DNA fragment. However, recombinant ParB could not appreciably retard a DNA fragment bearing a parS site in which the 11 most conserved nucleotides [32] were mutated (Figure 2B). Thus, recombinant ParB appeared to specifically bind parS in vitro.

In conclusion, both ParB localization pattern and the electrophoretic mobility shift assay (EMSA) are consistent with native ParB binding an ori-proximal parS site in live symbiont cells.

Chromosome Transverse Configuration and Segregation of Sister ter

To determine the position of the ter region and, thereby, the overall arrangement of the symbiont chromosome, we took advantage of a 28-nt-long consensus sequence obtained by comparing 161 dif-related sequences from 137 proteobacterial species [84] (see STAR Methods for the symbiont dif site nucleotide sequence). We then subjected fixed Ca. T. oneisti to a set of 12 fluorescent probes targeting a 3,916-nt-long chromosomal region containing the predicted dif site (ter probe; Tables S3 and S5). First of all, the majority of the cells (56.7%) displayed one fluorescence focus only, confirming the monoploidy of the...
symbiont (Figure 3A). Secondly, in these non-dividing cells, the ter position on the long axis resembled that of ori, whereas, on the short axis, ter appeared closer to the cell envelope than ori (leftmost panel in Figure 3B and Figures 3C and 3F). Given that both ori and ter occupied a near-midcell position at the beginning and at the end of the cell cycle, we concluded that the symbiont chromosome is not longitudinally but likely transversally organized (see schematic representation in Figure 4).

To analyze the localization pattern of the ter throughout the cell cycle, we grouped the cells into three width classes and plotted the total fluorescence emitted by each class against the normalized short axis or long axis (Figures 3D and 3E, respectively; in Figure 3E, we further subdivided the 263 widest cells of 3D into three width classes). Based on our analysis, sister ter regions appeared to localize medially in dividing cells (two rightmost representative cells in Figure 3B; demograph in Figure 3D; demograph in Figure S3A) and to move to the center of the cell concomitantly with the invagination of the cell envelope and the growing septum (two leftmost representative cells in Figure 3B; plot in Figure 3E; demograph in Figure S3B). To better resolve the ter localization pattern in dividing cells, we also grouped them into three classes, depending on the position of the ter foci along the long axis, and plotted their total fluorescence against the short or the long axis, which confirmed rapprochement of the sister ter at midcell (left and right plot in Figure S3C, respectively). Notably, the presence of non-dividing cells bearing two fluorescence foci indicated that sister ter may segregate diagonally prior to septation onset (second leftmost panel in Figure 3B and leftmost panel in Figure 3G).

We conclude that sister ter may migrate away from each other diagonally before Ca. T. oneisti starts dividing and that, as septation begins, they localize medially at the divisome leading edges. Finally, as septation progresses, each ter migrates toward midcell.

**DISCUSSION**

We investigated chromosome dynamics in a longitudinally dividing bacterium and made a number of observations challenging simple predictions about DNA replication and segregation: (1) rod-shaped *Gammaproteobacteria* segregate their DNA along their short axis prior to cell division and likely via a parABS system; of note, this system has so far been known to mediate DNA segregation along the long axis only; (2) sister ter regions segregate away from one another in non-dividing cells, whereas in dividing cells sister ter are medial and migrate toward one another; and (3) diagonal (bidimensional) segregation of ori-, ftsQAZ-, and ter-containing regions in non-dividing cells and rapprochement of medial sister ter in dividing cells allow transgenerational maintenance of the chromosome configuration; this implies that the intracellular localization of specific genetic loci may be transmitted from mother to daughter cells (Figure 4).

According to the DNA-relay model [85], the ParB-translocating force is derived from the elastic dynamics of the DNA, which are harnessed each time ParA interacts with ParB. Therefore, as long as there is a molecule that interacts with and anchors the duplicated ParB at its final destination—the opposite lateral membrane—ParB motion could occur across the cell width. As we could not identify any of the genes encoding for the known ParB anchors [16–18, 23, 24], a yet to be discovered molecule might mediate the attachment of ParB to the lateral sides of the cell. Moreover, the fact that (1) the segregation of both the symbiont ori and the ori-proximal ftsQAZ operon are diagonal and (2) the fact that ori and ParB foci are spread along the long axis suggest that, if existing, the putative ori anchor is not confined to the center of the lateral membrane. Namely, in non-dividing cells showing a single ori focus, we observed that 47% of the foci (n = 124) localized at 40%–50% of the long axis, 26% at 30%–40%, and 27% at 0%–30%.

An additional reason for the spreading of ori foci along the long axis could be the technique itself, as immunostaining-detected symbiont ParB foci were less spread than DNA-FISH-detected ori foci throughout the cell cycle (Figure 1). Along the same line, [86] observed a noisier localization pattern of chromosomal loci when applying DNA FISH versus fluorescent repressor-operator system (FROS) on *C. crescentus*, despite ori/ParB being tethered to the poles by PopZ. As a final remark on the spreading of ori signal along the long axis, it should be highlighted that we analyzed the localization patterns of chromosomal loci in cells that were not grown in culture. Instead, we fixed the symbiont cells upon collection from the environment while they were still attached to the worms. Therefore, it is possible that, at the moment of fixation, different symbiont cells were experiencing different environmental conditions and/or nutrients’ availability. As reported for culturable bacteria, such as *E. coli*, growth conditions may significantly affect the position of their chromosomal loci [5].

From an evolutionary perspective, the existence of anchors on the symbiont lateral sides would imply that these physiologically correspond to the poles of parABS-utilizing Gammaproteobacteria, such as *Pseudomonas* and *Vibrio* species [81, 83]. It is tempting to speculate that the Ca. T. oneisti ancestor, likely a basal free-living flagellated gammaproteobacterium [87] possessing a long axis-oriented parABS system, attached to the nematode surface by one of its two lateral sides. Subsequently, as the bacterium-animal association became more and more stable, the Ca. T. oneisti predecessor would lose its flagellum [88], and the evolutionary pressure to maximize the number of cells per unit of nematode surface (or other yet unknown physiological constraints) would “squeeze” it laterally [89, 90]. In such an evolutionary scenario, the ancestrally polar ori and ter would have ended up occupying the cell center of the symbiont, or in other words, the typical longitudinal chromosome arrangement characteristic of flagellated, polar rods [12, 83, 91] would become transverse. Furthermore, the ancestral polar ParB anchor would become a lateral one and mediate chromosome segregation along the short cell axis. However, it should also be noted that membrane anchors, either polar or lateral, could be dispensable, as it is known, for instance, that plasmids are positioned to specific subcellular locations in a self-organizing manner [92, 93].

As for ParA, according to the reaction-diffusion model [94], it could form a lateral instead of a polar gradient. However, the establishment of a stable ParA gradient before DNA replication and segregation is not needed for directional segregation of ParB-parS. Indeed, the motion of ParB could be sufficient to establish asymmetry in ParA localization (i.e., between the back and the front of ParB-parS) and to drive directional motion. Although transcriptomics and proteomics revealed that the parA
Figure 3. DNA FISH-Based Localization Pattern of Ca. T. oneisti ter

(A) Histogram showing number of ter fluorescence foci per cell. Total number of analyzed cells (n) is 1,454.

(B) Five representative cells probed with a ter probe arranged from the youngest to the oldest from left to right. Upper panels show phase-contrast images of five cells arranged from the thinnest (youngest) to the thickest (oldest) cell, from left to right. Lower panels show the corresponding epifluorescence images of the ter signal (white). Cell outlines were deduced from the corresponding phase-contrast images (white dotted lines). Scale bar, 1 μm.

(C) Histogram shows the distribution and abundances (%) of ori (n = 346) and ter (n = 824) fluorescence foci along the normalized cell width (%). 0% corresponds to the cell center, and 100% corresponds to the lateral membrane.

(D) We plotted the total fluorescence emitted by cells belonging to three width classes (0.51–0.90 μm, n = 1,293, dotted line; 0.90–1.29 μm, n = 871, dashed line; 1.29–1.69 μm, n = 263, full line) along the short axis.

(E) The 263 widest cells analyzed in (D) (1.29–1.69 μm) were subdivided into three additional width classes (1.29–1.42 μm, n = 136, dotted line; 1.42–1.55 μm, n = 91, dashed line; 1.55–1.69 μm, n = 36, full line) and their fluorescence plotted along the long axis.

(F and G) Subcellular localization of ter fluorescence foci in cells displaying only one (F) or two fluorescence foci (G). In (G), ter foci found in non-dividing cells are shown in the left plot, foci found in dividing cells in the middle plot, and foci found in both non-dividing and dividing cells in the right plot. The position of each focus is plotted against the normalized cell width and length (%) of the cell that contained them. Dashed lines represent the long and the short cell axis, and midcell is defined as the point in which they intersect.

See also Figure S3 and Tables S1–S3 and S5.
gene is expressed (L. König, S. Markert, and S.B., unpublished data), the symbiont ParA localization pattern is currently unknown.

Given that several species (with the exception of C. crescentus, M. xanthus, and V. cholerae chromosome II) still segregate their chromosome normally in most cells, even when parABS is non-functional [12, 95–99], additional mechanisms might be in place to guarantee symbiont chromosome segregation in the absence of this system. For example, it has been proposed that the bacterial actin homolog contributes to DNA segregation in some bacteria [100–103] and that the Min system mediates active segregation of ori in E. coli [104]. As both mreB [2] and the min system are present and expressed in Ca. T. oneisti (L. König, S. Markert, and S.B., unpublished data), it will be compelling to assess their role in symbiont chromosome organization and segregation.

In E. coli, sister ter segregation immediately precedes daughter cells’ separation so that DNA replication and segregation are concomitant [91, 105]. In contrast, in Ca. T. oneisti, sister ter appear already segregated in non-dividing cells, suggesting that a longer fraction of the chromosome segregation process occurs in the absence of DNA replication. Concerning what captures and tethers the dif site-containing ter region to the symbiont septum, Ca. T. oneisti FtsK is the best candidate, although no antibody is currently available to analyze the intracellular localization of this DNA translocase. In most bacteria, the assembly of the recombination complex is indeed facilitated by FtsK, which ensures the capture of the two dif sites in the vicinity of the septum and activates the complex for recombination [106]. This is likely mediated by DNA topoisomerases and the XerCD tyrosine recombinases, of which we have genomic-based evidence (GenBank: SMSC00000000.1), and would result in decatenation of duplicated intertwined circular chromosome. Despite both E. coli and V. cholerae possessing a MatP/matS system that causes compaction and tethering of the ter region to the closing division septum [62, 63], Ca. T. oneisti sister ter do not appear to be compacted. They indeed segregate from one another in non-dividing and toward one another in dividing cells. Consistently, we could identify neither a matP gene nor a matS site in the available genome draft (GenBank: SMSC00000000.1).

It was previously reported that different fluorochromes yielded different gene detection efficiencies, with Alexa 488 and Alexa Fluor 594 giving the strongest and most stable signal and the highest gene detection efficiency [107]. In our experimental system, only FISH probes labeled with Alexa Fluor 594 allowed us to detect an ori-, ter-, or ftsQAZ-containing chromosomal region. Therefore, given the impossibility of utilizing fluorochromes other than Alexa Fluor 594, the ori-, ter-, and ftsQAZ-specific probes could not be applied together but were each applied singularly.

In line with the hypothesis that Ca. T. oneisti evolved from a “squeezed” flagellated rod bearing a longitudinally (ori-ter) arranged chromosome, we expect each chromosome arm to be contained in either the proximal or the distal cell half of the symbiont. The fact that the ftsQAZ operon is in the polar cell third throughout the cell cycle indicates that not only ori and ter but also other chromosomal loci are non-randomly positioned and segregated. In B. subtilis and E. coli, midcell positioning of ori-proximal regions is maintained by the condensins SMC and MukB (a structural homolog of SMC), respectively [108–110]. As we do have genomics-based evidence (GenBank: SMSC00000000.1) that SMC is present in Ca. T. oneisti, this could compact large regions of the symbiont chromosome and, by interacting with ParB, organize the ori-proximal regions as observed in B. subtilis [38].

If we compare the symbiont nucleoid structure to that of other model bacteria, reports of both E. coli and of B. subtilis have suggested a variety of organizational patterns, including ori-ter or left-ori-right arrangement, as well as alternation between the two (see Introduction). Reports in C. crescentus and V. cholerae have been more consistent, with both bacteria invariably possessing ori-ter chromosome arrangement [12, 83, 91]. Despite assuming a transverse arrangement, the configuration of the symbiont genetic material is maintained throughout the cell cycle and therefore, in this regard, more similar to what observed in polarized bacteria.

As for why the symbiont chromosome configuration is maintained, we hypothesize that it might be advantageous to permanently position genetic loci mediating host interaction in the vicinity of the host (proximal cell half) and to confine loci involved in environmental response to the distal cell half. Determination of the 3D structure of the chromosome by chromosome conformation capture and development of live imaging techniques are necessary to unambiguously correlate the existence of a specific chromosome configuration with symbiont physiology. If we will indeed prove the existence of such a correlation, the range of cell biological adaptations to the symbiotic lifestyle will have to include positioning of the genetic material, a fundamental feature of cellular life. Moreover, from a chromosome-centric evolutionary perspective, even the peculiar reproductive mode of the symbiont (longitudinal division) might have evolved from the need to maintain a specific chromosome configuration.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- LEAD CONTACT AND MATERIALS AVAILABILITY
AUTHOR CONTRIBUTIONS

P.M.W. did most of the experiments, methodology, formal analysis, and visualization; provided resources; and reviewed and edited the manuscript. F.M. expressed and purified ParB recombinantly, performed the EMSA, and reviewed and edited the manuscript. G.F.P. contributed to the establishment of the DNA FISH protocol and reviewed and edited the manuscript. T.V. did the hybrid assembly of the symbiont genome and its annotation, ParB phylogenetic analysis, and data curation, and reviewed and edited the manuscript. G.F.P. contributed to the establishment of the DNA FISH protocol and reviewed and edited the manuscript. G.F.P. contributed to the establishment of the DNA FISH protocol and reviewed and edited the manuscript.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-Ca. T. oneist ParB | This paper; Eurogentec | N/A |
| Monoclonal anti-His antibody | Sigma | Product number: SAB1305538; RRID: AB_2687993 |
| **Bacterial and Virus Strains** |        |            |
| *Escherichia coli* Strain number: B21 (DE3) | Invitrogen | Catalog number: C601003 |
| *Escherichia coli* Strain number: Top10 | Invitrogen | Catalog number: C404010 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Ampicillin | Duchefa | Catalog number: A0104 |
| IPTG | This study | N/A |
| H- Thr-ParB | This study | N/A |
| **Critical Commercial Assays** |        |            |
| HisTrap HP | GE Healthcare | Catalog number: 17524701 |
| Ulysis Nucleic Acid Labeling Kit | ThermoFisher | Catalog number: U21654 |
| **Deposited Data** |        |            |
| Ca. T. oneisti genome draft | This paper | GenBank: SMSC00000000; https://www.ncbi.nlm.nih.gov/nuccore/SMSC00000000 |
| Ca. T. oneisti parS containing fragment | This paper | GenBank: MK650415; https://www.ncbi.nlm.nih.gov/nuccore/MK650415 |
| Ca. T. hypermnestrae ParB amino acid sequence | This paper | GenBank: MK650416; https://www.ncbi.nlm.nih.gov/nuccore/MK650416 |
| **Experimental Models: Organisms/Strains** |        |            |
| *Candidatus Thiosymbion oneisti* Environmental sample | N/A |
| **Oligonucleotides** |        |            |
| T.oneisti_parB_F = (5'-ATGTCTACGAAGAAAAAGGGC-3') | This paper | N/A |
| T.oneisti_parB_R = (5'-CTACTTTATATGAGCAAG-3') | This paper | N/A |
| FWD_primer_parB = (5'-GAGGCCCCAAGGGGTATACTAGTACCTTATAGAAGAAGAAGG-3') | This paper | N/A |
| REV_primer_parB = (5'-GTGCGGCGCGCGCCGCGACGAGCAGAGAAGAAGG-3') | This paper | N/A |
| FWD_To_pET-15b = (5'-GCTGCCGCGCGCGCGACCAGG-3') | This paper | N/A |
| REV_To_pET-15b = (5'-CTAGCATAACCCCTTGGGG-3') | This paper | N/A |
| To_parS_EMSA_dnaN_F1 = (5'-GTGGTAATGCCGATGAGACTTTG-3') | This paper | N/A |
| To_parS_EMSA_R1.1 = (5'-AACCATATGATGAAGGGAAGTCGGC-3') | This paper | N/A |
| To_parSmut11l_F1 = (5'-GCAGGTTAATGTTCCGGGATGTCCG-3') | This paper | N/A |
| To_parSmut11l_R = (5'-[Phosphorylation]ACA GGG TAC ATA GAA CAG CAC TGT CCA GCG TT-3') | This paper | N/A |
| Oligonucleotides for FISH probe synthesis (see Table S3) | This manuscript | N/A |
| **Recombinant DNA** |        |            |
| Plasmid pET-15b-His-ParB, see Method Details | This paper | N/A |
| Plasmid pJET1.2-parS fragment | This paper | N/A |
| Plasmid pJET1.2-parS-mut11 fragment | This paper | N/A |
| **Software and Algorithms** |        |            |
| ImageJ | NIH | https://imagej.nih.gov/ij/ |
| ObjectJ | University of Amsterdam | https://sils.fnwi.uva.nl/bcb/objectj/ |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Silvia Bulgheresi (silvia.bulgheresi@univie.ac.at).

This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Sediment samples were collected on multiple field trips (2015-2019) in ~1 m depth from a sand bar off Carrie Bow Cay, Belize (16°48′11.01″N, 88°4’54.42″W). Specimens of *L. oneistus* were extracted from the sediment by stirring the sand in seawater and pouring the supernatant through a mesh sieve (125 µm opening size). The retained material was transferred into a Petri dish and single nematodes were handpicked under a dissecting microscope. For DNA FISH, nematodes were fixed in 3 or 4% PFA for 12-14 hr at 4°C, washed with 70% ethanol and stored at −20°C. For Western Blotting and immunostaining, nematodes were transferred to methanol and stored at −20°C. Symbiotic nematodes were transported from Carrie Bow Cay to the University of Vienna deep-frozen.

METHOD DETAILS

**Oxford Nanopore Technologies sequencing**

Total genomic DNA was extracted using Genomic-tip 20/G (QIAGEN) following the manufacturer’s instruction, with elution into 20 µL of PCR molecular grade water. After clean-up using 1X AMPure XP beads (Beckman Coulter), the library was prepared using the ONT 1D ligation sequencing kit (SQK-LSK109) and subsequently 70 ng were loaded onto a R9.4 flow cell (FLO-MIN106) and sequenced on a MiniON for 26 hr. Base calling was performed locally with ONT’s Albacore v2.3.3, and resulting fastq-files were trimmed using Porechop v0.2.1 (https://github.com/rrwick/Porechop). For the hybrid assembly, metagenomic Illumina HiSeq 2000 paired-end reads were mapped to the symbiont *Ca. T. oneisti* genome [88] using the bwa v0.7.16a aln algorithm [111] and the mapped reads together with the reads acquired by ONT sequencing were assembled using Unicycler v0.4.6 [112]. Completeness, contamination and strain heterogeneity was calculated in CheckM v1.0.7 with the lineage_wf workflow. Fasta statistics were calculated using genome-tools v1.5.9 seqstat module [113]. The assembly was annotated automatically on the MicroScope platform [114]. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession GenBank: SMSC00000000.1.

**DNA Fluorescence In Situ Hybridization**

We used the genome draft of *Ca. T. oneisti* (SMSC00000000.1) to design specific primers against the *Ca. T. oneisti* ori and ter regions, as well as against a region containing the *ftsQAZ* operon (Table S3; symbiont *dif* site sequence: ATTCGCGATAATGTATATTATG TAAAAGT). Worms were rehydrated in phosphate-buffered saline (PBS) and bacteria were detached from the worms by sonication. Subsequently, 1 µL of bacterial suspension was used as template in each 25 µL PCR reaction (primers’ sequences and PCR conditions are listed in Tables S3 and S5). A 2,874 nt-long fragment containing the *dnaA* and *dnaB* genes (Figure S1B), a 3,273 nt-long fragment containing the *ftsQAZ* operon and a 3,916 nt-long fragment containing the predicted *dif* site were amplified from *Ca. T. oneisti* genomic DNA. Each purified fragment was then used as template to PCR-amplify dsDNA polynucleotide probes (referred to as *ori*, *ftsQAZ* and *ter*, respectively) that were subsequently chemically labeled with the Alexa Fluor 594 using the Ulysis Nucleic Acid Labeling Kit (ThermoFisher) following the same modifications to the manufacturers’ protocol as in [107]. Single *L. oneistus* nematodes were rehydrated in PBS and bacteria were detached by sonication. For the hybridization procedure, we followed a slightly modified version of the direct-gene FISH protocol [107]. After letting the cell suspension dry onto a well of a Poly-L-lysine coated Epoxy-slide, cells were dehydrated in a series of increasing ethanol concentrations and permeabilized with freshly prepared lysozyme solution for 1 hr on ice. *Ca. T. oneisti* *ori*, *ftsQAZ* and *ter* probes were diluted in hybridization buffer containing 35% (*ori* and *ftsQAZ* probes) or 45% (*ter* probe) formamide to a final concentration of 62 pg/µl and each probe was applied to the cells individually. Slides were transferred into a hybridization chamber and incubated for 40 min at 85°C and subsequently at 46°C for 2 hr. Washing buffer was applied...
to the cells once briefly and once for 15 min at 48 °C and, finally, cells were incubated in PBS for 20 min at room temperature. Upon a quick wash in ddH2O and, subsequently, in absolute ethanol, cells were air-dried and mounted in 4.5 µl Vectashield mounting medium (Vector Labs) per microscopic slide well.

**ParB alignment and phylogenetic tree**

Selected ParB sequences (Table S4) were retrieved from GenBank and aligned using mafft v7.397 [115]. The alignment was visually inspected and poorly aligned or misaligned regions were removed. The maximum-likelihood tree with SH-like aLRT support values (10,000 replicates) was inferred using IQ-TREE v1.6.2 [116].

**Expression of recombinant Ca. T. oneisti ParB**

The 912 nt-long parB gene was amplified from Ca. T. oneisti and used as a template to obtain the fragments for the Gibson assembly (see Table S5 for primers’ sequences and PCR conditions). The Ca. T. oneisti parB gene was cloned into the pET-15b vector (Novagen) by Gibson assembly and a His-tagged ParB recombinant protein (His-ToParB) was expressed in E. coli strain BL21 (DE3) (Invitrogen). The parB recombinant strain was grown at 37 °C in Lysogeny Broth (LB) medium with 100 µg/ml ampicillin. The recombinant protein expression was induced at OD600 = 0.6 in 1 mM IPTG and 0.2% L-arabinose. After 3 hr induction, the cell culture (125 ml) was pelleted by centrifuging 10 min at 4,500 rpm and resuspended in 4 mL lysis buffer (20 mM Na2HPO4-NaH2PO4, 15 mM imidazole, 0.5 M NaCl, pH 7.4) supplemented with 4 µl of proteinase inhibitor cocktail (Sigma). Cell lysates were obtained by sonication and after a 45 min-long centrifugation at 30,000 rpm at 4 °C soluble fractions were applied to a HisTrap (GE Healthcare) column packed with nickel chelate resin and connected to an EP-1 Econo pump (BioRAD) at 4 °C at a flow rate of 0.2 ml/min. Washing was performed with 4 ml lysis buffer at a flow rate of 0.5 ml/min. Bound recombinant ParB was eluted by applying 5 mL elution buffer (20 mM Na2HPO4-NaH2PO4, 0.5 M imidazole, 0.5 M NaCl, pH 7.4) at a flow rate of 0.2 ml/min. Eluted 1 mL fractions were subjected to SDS-PAGE to select those that appeared to contain recombinant ParB only. Finally, 1 mL of eluate was dialyzed against 250 mL PBS overnight at 4 °C and subsequently used for functional assays.

**Antibodies and western blots**

Methanol fixed deep-frozen worms were rehydrated in PBS and, subsequently, proteins from Ca. T. oneisti dissociated from its host or from E. coli cells expressing recombinant ParB were separated by reduced sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on NuPAGE 4%-12% Bis-Tris pre-cast MOPS gel (Invitrogen), respectively, and each blotted onto Hybond ECL nitrocellulose membranes (Amersham Biosciences). Membranes were blocked for 45 min in PBS containing 5% (wt/vol) nonfat milk (Bovine) at room temperature and incubated overnight at 4 °C. Membranes were washed with PBS containing 0.1% Tween20, the blots were incubated for 1 hr at room temperature with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:10,000; Amersham Biosciences) in PBSM. Protein-antibody complexes were visualized using ECL Plus detection reagents (Amersham Biosciences).

**Identification of Ca. T. oneisti parS and electrophoretic mobility shift assay (EMSA)**

To identify Ca. T. oneisti parS we used the consensus sequence from 1,030 parS sequences identified from 276 prokaryotic genomes [32] against the genome draft (Figure 2B). Sequence logos of parS sequences were generated via the WebLogo3 webpage (http://weblogo.threeplusone.com/).

An 827 nt-long parS-containing DNA fragment was amplified from Ca. T. oneisti (GenBank: MK650415; see Table S5 for PCR cycling conditions), cloned into pJET1.2/blunt vector (ThermoFisher, K1231) and used as a template to synthesize the DNA fragment used in the EMSA. The 827 nt-long parS-containing DNA fragment was subsequently PCR amplified out of the vector and residual vector was digested with DpnI (New England Biolabs, R0176S) for 5 min at RT and 1 hr at 37 °C. For the negative control, the eleven most conserved nucleotides of the parS consensus sequence GTTCAGTGAAC (enlarged nucleotides in Figure 2B) were mutated into, respectively, ACCTGACAGGT via PCR mutagenesis using the ToparSmut11_F1 and ToparSmut11_R primers. After a digest with DpnI for 5 min at RT and 1 hr at 37 °C and a clean-up, the pJET1.2-parS-mut11 fragment was circularized for 20 min at RT with T4 DNA ligase (ThermoFisher, EL0011) and transformed into competent E. coli Top10 cells. The cleaned-up plasmid was then used to PCR amplify the 827 nt-long parS-mut11-containing DNA fragment, which was subsequently digested with DpnI to remove the residual vector.

500 nM, 250 nM or 125 nM affinity purified recombinant ParB was incubated with 200 ng DNA containing either the wild-type or the mutated parS site in 20 µl at 30 °C for 30 min. As an additional negative control, 500 nM ParB was heat-inactivated by incubating 30 min at 94 °C in the presence of 1 mM DTT prior incubation with the DNA fragment containing the wild-type or the mutated parS site. Samples were mixed with 2x Native Dye (100 mM NaCl, 100 mM imidazole, 4 mM 6-aminocaproic acid, 2 mM EDTA, 20% glycerol) and 20 µl of each were loaded onto a 4%-16% Native PAGE Bis-Tris Gels (Invitrogen). The gel was run in 1x Anode (50 mM Bis-Tris, pH 7.0) and 1x Cathode (50 mM Tricine, 15 mM Bis-Tris) buffer at 4 °C. Sample separation was done with a stepwise increase of voltage (i.e., 50 V for 20 min, 100 V for 30 min, 150 V for 30 min, 200 V for 30 min). The SDS-PAGE was stained in an ethidium bromide gel bath for 15 min and visualized under UV light.
**Immunostaining and DNA staining**

Deep-frozen methanol fixed nematodes were rehydrated and washed in PBS containing 0.1% Tween 20 (PBT), followed by permeabilization of the bacterial peptidoglycan by incubation for 15 min with 0.1% (wt/vol) lysozyme at room temperature. Blocking was carried out for 1 hr in PBT containing 2% (wt/vol) bovine serum albumin (blocking solution) at room temperature. Ca. T. oneisti was incubated with a 1:500 dilution of peptide rabbit polyclonal anti-Ca. T. oneisti ParB antibody (Eurogentec). All primary antibodies were incubated in blocking solution overnight at 4°C. Upon incubation with primary antibody (or without, in the case of the negative control) samples were washed three times in PBT and incubated with a 1:500 dilution of secondary Alexa 488-conjugated anti-rabbit antibody (Jackson ImmunoResearch, USA) in blocking solution for 1 hr at room temperature. Unbound secondary antibody was removed by three washing steps in PBT and thereupon incubated in 5 µg/ml Hoechst 33342 PBT for 15 min. After two washing steps to remove unbound DNA stain, worms were sonicated for 45 s in order to dissociate Ca. T. oneisti from its host prior mounting. 1.5 µl of the bacterial suspension was mixed with 0.75 µl of Vectashield mounting medium (Vector Labs) and applied to a 1% agarose covered microscopy slide.

**Fluorescence microscopy**

Slides containing symbiont cells subjected to DNA FISH or immunostaining were imaged using a Nikon Eclipse Ni-U microscope equipped with a MFCool camera (Jenoptik). Images were acquired using the ProgRes Capture Pro 2.8.8 software (Jenoptik).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Microscopic images were processed using the public domain program ImageJ in combination with plugin ObjectJ and XY-shape inspector. Cell outlines were traced and morphometric measurements recorded. Fluorescent intensities were measured within the cell boundaries and the positions of the fluorescence foci (i.e., points of maximal fluorescent emissions) were plotted as fraction of the normalized cell width and length of the cell that contained them. Automatic cell recognition was double-checked manually. For the average fluorescence plots, cells were automatically grouped into morphological classes based on phase-contrast images, each cell was resampled to the same length and the fluorescence intensities added up and averaged. For representative images, the background subtraction function of ImageJ was used and brightness and contrast were adjusted for better visibility. Data analysis was performed using Excel 2017 (Microsoft Corporation, USA), plots were created with Excel 2017 and figures were compiled using Photoshop CC and Illustrator CC (Adobe Systems, USA). Gene features were plotted using the DNAFeaturesViewer Python library (https://github.com/Edinburgh-Genome-Foundry/DnaFeaturesViewer).

**DATA AND CODE AVAILABILITY**

The symbiont genome has been deposited at DDBJ/ENA/GenBank under the accession number GenBank: SMSC00000000.1. Ca. T. oneisti parS-containing DNA fragment and the ParB amino acid sequence have been deposited in DDBJ/ENA/GenBank under the accession numbers GenBank: MK650415 and MK650416, respectively. The documentation for the ImageJ plugin XY-shape inspector can be accessed here: https://sils.fnwi.uva.nl/bcb/objectj/examples/XY-Shape-Inspector/MD/xy-shape-inspector.html.