par genes and the pathology of chromosome loss in *Vibrio cholerae*

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The causes and consequences of chromosome loss in bacteria with multiple chromosomes are unknown. *Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera, has two circular chromosomes. Like many other bacterial chromosomes, both *V. cholerae* chromosomes contain homologues of plasmid partitioning (*par*) genes. In plasmids, *par* genes act to segregate plasmid molecules to daughter cells and thereby ensure plasmid maintenance; however, the contribution of *par* genes to chromosome segregation is not clear. Here, we show that the chromosome II *parAB2* genes are essential for the segregation of chromosome II but not chromosome I. In a *parAB2* deletion mutant, chromosome II is mislocalized and frequently fails to segregate, yielding cells with only chromosome I. These cells divide once; their progeny are not viable. Instead, chromosome II-deficient cells undergo dramatic cell enlargement, nucleoid condensation and degradation, and loss of membrane integrity. The highly consistent nature of these cytologic changes suggests that prokaryotes, like eukaryotes, may possess characteristic death pathways.

chromosome segregation | *parA* | *parB* | apoptosis

The molecular mechanisms that ensure the accurate segregation of bacterial genomes are incompletely understood. Partitioning (*par*) genes have been known for many years to play an essential role in the stable inheritance of certain plasmids. Plasmid partitioning systems consist of two proteins encoded by genes, often known as *parA* and *parB*, and a cis-acting centromere-like site, *parS*. Plasmids deleted for their *par* loci no longer localize to particular regions of the cell and are readily lost from host cells (1–4). *ParB* proteins bind to their cognate *parS* site(s) and form a nucleoprotein complex. *ParA* proteins possess Walker-type ATPase motifs and show weak ATPase activity. *ParA* proteins interact with the *ParB*–*parS* complex, and their ATPase activity is essential for plasmid partitioning (5, 6). Several *ParA* homologues have been shown to form ATP-dependent polymers *in vitro* and to oscillate *in vivo* (7–10). Still, the molecular mechanisms by which the interactions of *ParA* with the *ParB*–*parS* complex mediate plasmid localization and partitioning are not well understood, although several models have been proposed to account for *Par*-mediated plasmid segregation (9–13). Homologues of plasmid *Par* proteins are also encoded in most bacterial chromosomes (11, 14); however, less is known about the function of chromosomal *parABS* loci.

It has become clear in recent years that some bacteria, including several pathogens, have complex genomes that consist of more than one circular chromosome (15). The genome of *Vibrio cholerae*, the curved Gram-negative rod that causes the severe diarrheal disease cholera, is divided between two circular chromosomes (16). Chromosome I [chrI; 2.96 megabases (Mb)] is larger than chromosome II (chrII; 1.07 Mb) and contains the majority of the genes considered essential for cell growth. ChrII contains some essential genes, including *rpmI*, *rplT*, *infC*, and *thrS*, which encode ribosome proteins L22 and L35, a translation initiation factor, and an aminoacyl tRNA synthetase, respectively (16). Interestingly, chrII also encodes 13 toxin-antitoxin (TA) loci; similar TA loci are often found in plasmids, where they ensure maintenance by preventing the survival of plasmid-free

cells (17–19). The presence of many TA loci, as well as other features of chrII, led to the suggestion that this chromosome originated as a megaplasmid acquired by an ancestral *Vibrio* species (16). However, the presence of essential genes on chrII qualifies this replicon as a bona fide chromosome (15, 16).

Although the two *V. cholerae* chromosomes initiate replication in coordinated fashion and have some shared replication requirements (20, 21), different mechanisms appear to control their segregation, and their origin regions have distinct subcellular distributions and dynamics. In newborn cells, the origin of replication of chrI (*oriCI_{vc}*) is found near one of the cell poles, whereas the origin of replication of chrII (*oriCII_{vc}*) is found near the cell center (22, 23). The origin region of chrI undergoes an asymmetric segregation process: one copy of the duplicated *oriCI_{vc}* remains at the pole while the other copy traverses the cell to the opposite pole (22–24). In contrast, the duplicated copies of *oriCII_{vc}* segregate in a symmetric fashion from the cell center to the quarter positions of the cell, the sites of the new cell centers (22, 23). The pattern of *oriCI_{vc}* segregation is very similar to that reported for the *Caulobacter crescentus* chromosome (25), whereas the pattern of *oriCII_{vc}* segregation is similar to that reported for P1 and the F plasmid (3, 26).

Each of the *V. cholerae* chromosomes has *parAB* genes near its replication origin (16). Phylogenetic analyses suggest that the *Par* proteins encoded on chrI (*ParA1* and *ParB1*) cluster with other chromosome-encoded *Par* proteins, whereas the *Par* proteins encoded on chrII (*ParA2* and *ParB2*) tend to cluster with plasmid and phage *Par* proteins (11, 14, 16). Given the importance of *par* loci in plasmid segregation, we have initiated studies to evaluate the role of *parAB1* and *parAB2* in *V. cholerae* chromosome segregation. Recently, we found that *parA1* is required for the polar localization and asymmetric segregation pattern of chrI but does not influence the segregation of chrII. Furthermore, *parA1* mutants did not have an appreciable cell growth defect and did not lose chrI (24, 27). Here, we investigated the role of *parAB2* in the maintenance and segregation of the *V. cholerae* chromosomes. We found that *ParAB2* functions in a chromosome-specific manner to promote the accurate subcellular localization and maintenance of chrII but not chrI. A *parAB2* deletion mutant yields a high frequency of cells that lack chrII. Cells containing only chrI divide once and then become “CHUB” cells (an acronym we created based on some of the cells’ properties: condensed nucleoid, hypertrophic, undividing

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Abbreviations: chrI, chromosome I; chrII, chromosome II; CHUB, condensed nucleoid, hypertrophic, undividing bacteria; TA, toxin-antitoxin.

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Fig. 1. *V. cholerae* *parABS2* stabilizes a mini-F plasmid in *E. coli*. Stability is measured as the percentage of cells harboring mini-F plasmid derivatives over time. The mini-F derivatives are pYB145 (*parABS2*⁺; filled squares, solid line), pYB095 (*parAB2*⁺; filled circles, gray line), pYB141 (*parS2*⁺; open squares, broken line), and control vector pXX705 (open circles, dotted line).

bacteria). Also, some of the characteristic cytologic changes that occur in cells lacking *chrII* appear to be attributable to the activity of some of the toxins encoded in the TA loci on *chrII*.

Results and Discussion

***parABS2* Is Functional in a Heterologous Host.** Several previous studies have introduced chromosomal *parABS* loci into an unstable derivative of the F plasmid to test their functionality in a heterologous host, *Escherichia coli* (11, 28–31). To perform parallel experiments, we defined ParB2-binding sites (*parS2*). We found that six of nine *parS2* sites on *chrII* were fairly close to *oriCII_{vc}*, but none were found within the *parAB2* genes (our unpublished work). Then we explored whether *parAB2*, along with *parS2*, can mediate the partitioning of an unstable plasmid

in *E. coli*. Although the unstable mini-F plasmid was readily lost from *E. coli* in the absence of selection, insertion of *parAB2* and *parS2* dramatically stabilized the plasmid (Fig. 1). The ability of *V. cholerae* *parABS2* to stabilize this plasmid in *E. coli* indicates that the *chrII* *par* loci encode a functional partitioning system and suggests that they may mediate DNA partitioning by the same general mechanism used by related Par proteins of some plasmids. Furthermore, this observation suggests that *parABS2* does not require any additional *V. cholerae*-specific factors to mediate partitioning. Insertion of either *parS2* or *parAB2* alone did not stabilize the mini-F plasmid (Fig. 1), suggesting that all three components of the *chrII* *par* system are required for the activity of this partitioning system.

***parAB2* Is Required for Wild-Type (WT) Cell Growth.** Using a standard allele exchange method (32), we found that we were unable to delete *parAB2* unless a WT copy of these genes was provided on a plasmid (pYB085). However, pYB085 could be lost from the host deletion mutant. Mutant cells that lost pYB085 were easily identified because they generated tiny colonies [Fig. 2A and supporting information (SI) Fig. 6]. These cells also had a severe growth defect in liquid culture (Fig. 2B) that could be complemented by providing a plasmid-borne copy of *parAB2* (SI Fig. 6 and data not shown). The severe growth defect of the *parAB2* mutant is in marked contrast to the lack of an appreciable growth defect in *parA1* mutants (24, 27).

The Δ *parAB2* strain consists of two phenotypically different types of cells. About one-third of the Δ *parAB2* cells were normal in size, shape, and nucleoid morphology, and about two-thirds of the cells were significantly larger, irregularly shaped, and had condensed nucleoids (CHUB cells; Fig. 2C and Table 1). The CHUB phenotype is directly associated with a ParAB2 deficiency, inasmuch as CHUB cells were not seen in the WT population or in Δ *parAB2* cells that contain a *parAB2* complementing plasmid. CHUB cells also have a lower density than WT cells, allowing them to be isolated by density gradient centrifugation.

Fig. 2. Growth defect and heterogeneity of cell size, shape, and nucleoid morphology in a Δ *parAB2* *V. cholerae* strain. (A) Heterogeneous colony size of a Δ *parAB2* strain harboring a *parAB2* complementing plasmid grown on nonselective LB plates. All of the normal-sized colonies were found to carry the *parAB2*-bearing plasmid, whereas all of the tiny colonies (arrowhead) lost the *parAB2*-bearing plasmid (data not shown). (B) Growth in LB at 37°C of WT (diamonds), unfractionated Δ *parAB2* cells (black circles), light fraction (blue circles), and heavy fraction (red circles). (C) Merged phase-contrast and fluorescence images of DAPI-stained fixed cells. The arrowheads point to cells with fragmented nucleoids. (Scale bar, 2 μ m.) (D) BacLight live/dead assay of fractionated cells. Cells were assayed immediately after fractionation (time 0) or after growth in LB medium at 37°C for 1 h. Normal and CHUB cells were distinguished by morphology. Depicted are normal-live cells (white), normal-dead cells (black), CHUB-live cells (green), and CHUB-dead cells (magenta).

Table 1. Characteristics of $\Delta parAB2$ *V. cholerae* before and after fractionation on a density gradient

Fraction	Morphology*		cfu (ml/OD ₆₀₀)*	qPCR†	
	Normal	CHUB		<i>oriCl_{vc}/oriCl_{vc}</i>	<i>TerII_{vc}/TerI_{vc}</i>
Unfractionated	33.6%	66.4%	5.6×10^7	0.30 ± 0.02	0.34 ± 0.09
Light	0.7%	99.3%	5.7×10^5	0.020 ± 0.006	0.020 ± 0.006
Heavy	83.0%	17.0%	1.7×10^8	0.62 ± 0.12	0.63 ± 0.12

*Average of three independent experiments.

†Mean $\pm$ SD of three independent experiments.

gation (33). The “heavy” fraction consisted of $\approx 80\%$ normal-appearing cells; DAPI staining revealed diffuse nucleoids similar to those of WT cells (Fig. 2C and Table 1). In contrast, the vast majority of the cells in the “light” fraction were large and had the condensed nucleoid(s) characteristic of CHUB cells. In addition, CHUB cells appear to be inviable; there was virtually no increase in the optical density (OD) of cultures of cells isolated from the light fraction for up to 6 h after inoculation in LB (Fig. 2B). The plating efficiency (per OD unit) of cells from the light fraction was nearly 1/300 that of cells from the heavy fraction (Table 1). Furthermore, although isolated CHUB cells were initially scored as “live” according to BacLight (Molecular Probes, Eugene, OR) live/dead analysis, after 1 h of incubation at 37°C in LB, these cells apparently lost membrane integrity, allowing propidium iodide staining and their classification as “dead” (Fig. 2D). Overall, the growth defect of the $\Delta parAB2$ mutant appears to be largely attributable to the emergence of inviable CHUB cells in this background.

$\Delta parAB2$ Is Required for Maintenance of ChrII. The ability of $\Delta parAB2$ to stabilize an unstable plasmid, and the severe growth defect of

the $\Delta parAB2$ mutant, raised the possibility that these genes were important for *V. cholerae* chromosome segregation. To examine the chromosome content of the $\Delta parAB2$ strain, we used quantitative PCR (qPCR) to measure chromosome-specific DNA at multiple sites on both chromosomes. In contrast to WT cells (for which we set the ratio chrII/chrI equal to 1), in $\Delta parAB2$ cells the ratio chrII/chrI was only ≈ 0.3 (Table 1). Thus, in the absence of $\Delta parAB2$, chrII appears to be frequently lost. Furthermore, CHUB cells appear to completely lack chrII (Table 1). The small percentage of purified CHUB cells containing chrII DNA ($\approx 2\%$) is probably contaminating non-CHUB cells. Taken together, these observations suggest that the loss of chrII due to the absence of $\Delta parAB2$ results in the development of CHUB cells.

$\Delta parAB2$ Is Required for Proper Subcellular Localization and Segregation of ChrII. A fluorescent protein fused to ParB2 (e.g., CFP-ParB2) can be used to determine the subcellular location of the *parS2* sites, which on chrII are mostly clustered near the origin of replication (our unpublished work). Similarly, the ParB1 protein binds to *parS1* sites near the origin of chrI, and a fluorescent fusion protein (e.g., YFP-ParB1) can be used to

Fig. 3. Number and subcellular location of YFP-ParB1 and CFP-ParB2 foci in WT and $\Delta parAB2$ *V. cholerae*. (A and B) Static images of representative WT (A) and $\Delta parAB2$ (B) cells expressing YFP-ParB1 and CFP-ParB2. (C) Histograms showing the localization of CFP-ParB2 foci in WT (Upper) and $\Delta parAB2$ (Lower) cells. The distance from each focus to the nearest pole was measured and divided by cell length. A total of 261 foci from 118 WT cells and 274 foci from 103 $\Delta parAB2$ cells were analyzed. (D) Time-lapse images of $\Delta parAB2$ cells taken at 12-min intervals at 35°C. The number in the lower left corner of each image indicates the time (min). YFP is shown in green, and CFP is shown in magenta. The dagger shows a cell division yielding a cell lacking chrII. The arrowheads show cells that will become CHUBs. Asterisks show enlarging CHUB cells. (Scale bar, 2 μ m.)

Fig. 5. Schematic representation of chromosome segregation and the generation of CHUB cells in $\Delta parAB2$ *V. cholerae*. (Left) Schematic representation showing the pattern of segregation of the origin regions of chrI (green) and chrII (magenta) in WT cells. (Right) The chrII origin region is randomly localized in the $\Delta parAB2$ mutant. Cell division can therefore result in daughter cells that either contain or lack this chromosome. Cells lacking chrII divide only once, and then their progeny develop into CHUB cells.

DNA gyrase (nalidixic acid, Nal) to begin to assess whether the action of one or more of the chrII toxins could account for the CHUB phenotype. Cm-treated cells were enlarged and had condensed nucleoids similar to CHUB cells, but TUNEL fluorescence in these cells was not as frequent or intense as in CHUB cells (Fig. 4B and SI Fig. 7). Nal-treated cells had similar TUNEL fluorescence as CHUB cells but were smaller and had more punctate nucleoids than CHUB cells (Fig. 4B and SI Fig. 7). Cells treated with both antibiotics also had similar TUNEL fluorescence as CHUB cells but did not entirely recapitulate the CHUB phenotype; compared with CHUB cells, these cells were usually smaller in length and width and had more punctate nucleoids (Fig. 4B). Thus, the combined action of chrII-encoded toxins likely contributes to some aspects of the CHUB phenotype.

Conclusions. There appears to be no redundancy in the mechanisms that account for chrII segregation in *V. cholerae*. In the *parAB2* mutant, chrII appears to have a random distribution (Fig. 3C) that results in its frequent missegregation and the generation of cells lacking this chromosome (Fig. 5). The high frequency of chrII loss in the *parAB2* mutant appears to explain the growth defect of this strain. In contrast, redundant mechanisms likely mediate chrI segregation; deletion of *parA1* abrogates the polar localization of *oriCI_{vc}*, but chrI is faithful partitioned in this background and cell growth is not altered (24, 27). Redundant mechanisms also seem to operate in the segregation of the *Bacillus subtilis* and *E. coli* chromosomes (37, 38). In contrast, low-copy-number plasmids usually depend on a single *par*-based partitioning mechanism. Thus, the lack of redundant mechanisms governing chrII partitioning supports the hypothesis that this chromosome arose from a plasmid or a phage. Perhaps consistent with this hypothesis is our observation that the Par systems of the two chromosomes appear to function independently of one another; i.e., there was no detectable mislocalization of *oriCI_{vc}* in the *parAB2* background and no *oriCII_{vc}* mislocalization in the *parA1* background (24, 27).

The missegregation of chrII in $\Delta parAB2$ *V. cholerae* routinely yields cells that contain only chrI, and these aneuploid cells undergo a highly consistent set of cytologic changes terminating in cell death. The cells divide once; the resulting daughter cells, which are no longer capable of division, then enlarge, undergo

nucleoid condensation and degradation, and lose membrane integrity. At least some of these cytologic changes may be attributable to the activity of the toxins encoded on the chrII superintegron. It has been proposed that TA loci can promote the genetic stability of superintegrons (39); our findings raise the possibility that TA loci may also contribute to the overall integrity of the *V. cholerae* genome. Future studies should address whether the cytologic changes observed in cells that contain only chrI can be entirely explained by the action of chrII toxins, or whether the absence of $\approx 25\%$ of the *V. cholerae* genetic information also contributes. The cytologic changes in cells containing only chrI bear some similarity to programmed cell death in eukaryotes, but it is not clear whether they reflect a particular *V. cholerae* genetic program. To date, the pathology of prokaryotic cell death has largely been ignored. Our findings suggest that characterizing such death pathways will be a fruitful area for study. Finally, it was proposed that *V. cholerae* cells containing a single chromosome could act as “drones”: metabolically active, nondividing cells that could contribute to the population (16). Our findings demonstrate that generation of *V. cholerae* cells containing only chrI is possible, but the contribution of CHUB cells to the cell population remains to be explored.

Materials and Methods

Strains and Plasmids. The strains and plasmids used in this study are listed in SI Table 3 and SI Table 4, respectively. All *V. cholerae* strains used in this study were derived from the sequenced El Tor clinical isolate N16961 (16). *E. coli* strains DH5 α , DH5a λ pir, and TOP10 were used for DNA cloning. SM10 λ pir was used to mobilize DNA into *V. cholerae*, and MC1061 was the host strain for the plasmid stability assay. The growth curves shown in Fig. 2B were obtained by using a SynergyHT microplate reader and KC4 software (BioTek, Winooski, VT).

Isolation of CHUB Cells. Growing cells (11 ml; OD₆₀₀ $\approx$ 0.3) and 3 ml of 40% (wt/vol) Ludox (Sigma, St. Louis, MO), filtered and pH-adjusted to ≈ 7.5 before use, were mixed in 15-ml cortex tubes and then centrifuged at $9,000 \times g$ for 30 min. The top band was then carefully transferred to a new tube by pipette and washed three times with PBS.

Analyses of DNA Content. Real-time qPCR to compare the content of *chrI* and *chrII* was performed as described previously (40), except that primers for *oriCII_{vc}* were 5'-CCAGT-GATGAATGGTGTGT-3' and 5'-ATGATCGAGACTTAC-GGTATG-3'. Standard curves were obtained with genomic DNA prepared from growing WT cells. Pulsed-field gel electrophoresis was carried out as described previously (41). DNA degradation was detected by TUNEL assay, using the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Indianapolis, IN) in accordance with the manufacture's instructions. Samples were analyzed with either microscopy or a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Microscopy. For fluorescence microscopy experiments, strains were grown in LB medium at 37°C to the midexponential

phase. Fluorescent-tagged proteins under the control of the *P_{BAD}* promoter were induced with 0.08% L-arabinose for 1 h before cells were transferred to an agarose pad on a microscope slide. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) was used to observe the nucleoid of cells fixed with 3% paraformaldehyde. The Bac-Light live/dead bacterial viability kit for microscopy (Molecular Probes) was used for the membrane integrity assay. All images were acquired and processed as described previously (22, 37).

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