The two chromosomes of *Vibrio cholerae* are initiated at different time points in the cell cycle

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The bacterium *Vibrio cholerae*, the cause of the diarrhoeal disease cholera, has its genome divided between two chromosomes, a feature uncommon for bacteria. The two chromosomes are of different sizes and different initiator molecules control their replication independently. Using novel methods for analysing flow cytometry data and marker frequency analysis, we show that the small chromosome II is replicated late in the C period of the cell cycle, where most of chromosome I has been replicated. Owing to the delay in initiation of chromosome II, the two chromosomes terminate replication at approximately the same time and the average number of replication origins per cell is higher for chromosome I than for chromosome II. Analysis of cell-cycle parameters shows that chromosome replication and segregation is exceptionally fast in *V. cholerae*. The divided genome and delayed replication of chromosome II may reduce the metabolic burden and complexity of chromosome replication by postponing DNA synthesis to the last part of the cell cycle and reducing the need for overlapping replication cycles during rapid proliferation.

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Introduction

Most bacteria contain only one circular chromosome and bidirectional replication is initiated at a single origin. However, a few bacteria contain a genome distributed on multiple chromosomes and some have unusual genome structures like linear chromosomes. What are the evolutionary advantages of dividing the genome into several chromosomes, when most bacteria have not evolved so? Multiple chromosomes are found in diverse prokaryotic phyla includ-

ing the Deinococcus–Thermus, Spirochaetes and the α -, γ - and β -subgroups of Proteobacteria. Interestingly, most of the bacteria with multiple chromosomes have several different life cycles and interact with host organisms, like *Vibrio cholerae* that lives in brackish water and within human hosts, making it likely that dividing the genome into several different replicons may be favourable for organisms living under variable environmental conditions (Egan *et al*, 2005). The genome of *V. cholerae* is distributed onto two circular chromosomes (Trucksis *et al*, 1998; Heidelberg *et al*, 2000), with a large 2.96 Mb chromosome I and a small 1.07 Mb chromosome II. This division is seen in other *Vibrio* bacteria as well, but the relative sizes of the secondary chromosomes vary significantly between different *Vibrio* species (Okada *et al*, 2005). The splitting of the *V. cholerae* genome onto two chromosomes could facilitate an up- or downregulation of genes present on one of the chromosomes by an amplification or elimination of one of the chromosomes under certain environmental conditions (Heidelberg *et al*, 2000). A study of gene expression in *V. cholerae* shows a preferential increase in the transcription of genes from the small chromosome II during colonisation compared to cells growing *in vitro* under aerobic conditions (Xu *et al*, 2003). Thus, possessing multiple chromosomes may give an additional mechanism for switching between different portfolios of genes needed in various environments.

Bacteria tightly regulate genome replication and this process is primarily controlled at the level of initiation of chromosomal DNA replication. The presence of several chromosomes makes the coordination of genome replication more difficult compared to when only a single chromosome is present. Some bacteria grow very fast under optimal conditions, with a doubling time much shorter than the time required for DNA replication and segregation, and therefore have overlapping DNA replication cycles. *V. cholerae* cells are able to divide every 18 min under optimal conditions. How do bacteria with multiple chromosomes and fast cell cycles ensure the faithful replication and distribution of several chromosomes to daughter cells?

Most of the experimental data on chromosome replication and segregation in bacteria with divided genomes have been obtained using *V. cholerae* as model organism. A Meselson–Stahl density shift experiment showed that both *V. cholerae* chromosomes are replicated only once per cell cycle and with an inter-replication time equal to the doubling time of the cells (Egan *et al*, 2004). Thus, the replication of both chromosomes in *V. cholerae* are linked to the cell cycle (Egan *et al*, 2005). In contrast, plasmid replication is normally not tied to the cell cycle; plasmids tend to replicate at random time points during the cell cycle. Flow cytometry analysis of samples of slow growing cells treated with rifampicin and cephalixin indicated that the two chromosomes are initiated at the same time in the cell cycle (Egan *et al*, 2004). Examination of the replication control systems of the two

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V. cholerae chromosomes showed that each chromosome is controlled by its own initiator molecule: replication of chromosome I is controlled by the DnaA protein, similarly to the control of chromosome replication in *Escherichia coli* (Egan and Waldor, 2003). Increased expression of the DnaA protein stimulates initiation of chromosome I, but not of chromosome II (Duigou *et al*, 2006). Chromosome II has a plasmid-like origin, its replication is controlled by the RctB protein and requires the DnaA protein (Egan and Waldor, 2003). P1 and F plasmids shows a similar requirement for DnaA protein (Hansen and Yarmolinsky, 1986) despite that replication is controlled by the respective replication proteins RepA and RepE. The role of DnaA protein in these and other plasmids is strand opening, helicase loading or an architectural role in forming the replication complex (reviewed by Messer, 2002) and a similar role for the DnaA protein in chromosome II replication is probable. The RctB protein is autoregulated and the activity of RctB is further controlled by the *rctA* titration sites. The titration activity of *rctA* is controlled by the RctB protein (Venkova-Canova *et al*, 2006). Induction of the RctB protein stimulates initiation of chromosome II, but not chromosome I (Duigou *et al*, 2006). The separate control of replication initiation of the two *V. cholerae* chromosomes makes it difficult to understand how initiation synchrony can be obtained in this organism. This led us to re-evaluate the finding of initiation synchrony by Egan *et al* (2004). Using novel flow cytometry methods and marker frequency analysis, we show that initiation of the smaller chromosome II is delayed compared to initiation of chromosome I, resulting in completion of replication of the two chromosomes at approximately the same time in the cell cycle.

Results and discussion

Models for coordinating replication of the two chromosomes in *V. cholerae*

The cell cycle of slowly growing bacteria is divided into three time periods with respect to chromosomal DNA replication: B is the period from cell birth to initiation of chromosome replication, C is the period from initiation until termination

of chromosome replication and D is the period between termination and cell division (Figure 1). Thus, cells in the B period are small and contain one genome equivalent of DNA, cells in the C period are intermediate in size and contain between one and two genome equivalents of DNA and cells in the D period are large and contain two genome equivalents of DNA.

Both of the chromosomes in *V. cholerae* are replicated once and only once during the cell cycle and replication run-off experiments support an 'initiation synchrony' model (see Figure 1), where the two origins are initiated at the same time and DNA replication proceeds bidirectionally on both chromosomes until the terminus of each chromosome is reached (Egan *et al*, 2004). Owing to the smaller size, replication of chromosome II will terminate earlier than replication of chromosome I. According to the initiation synchrony model, the number of *oriCI* will equal the number of *oriCII*, whereas the number of *terI* will be lower than the number of *terII*. The total rate of DNA accumulation will be highest at the onset of the C period when both chromosomes are replicating and decrease when replication of chromosome II has terminated.

Alternatively, initiation of the smaller chromosome II could take place at a later point in the cell cycle. In the 'termination synchrony' model (see Figure 1), chromosome II is initiated when the size of the non-replicated part of chromosome I is roughly equivalent to the size of chromosome II. Replication of both chromosomes then proceeds until the replication forks reach the termini at approximately the same time in both chromosomes. According to the termination synchrony model, the number of *terI* will equal the number of *terII*, whereas the number of *oriCI* will be higher than the number of *oriCII*. The rate of DNA accumulation will be highest late in the C period when both chromosomes are replicating.

The DNA replication rate increases abruptly during the C period

To determine which of the two models in Figure 1 that best describes the timing of replication of the two chromosomes in *V. cholerae*, we measured the DNA accumulation rate as a

Figure 1 Models of the DNA replication patterns during the *V. cholerae* cell cycle assuming either initiation or termination synchrony. Shown are chromosome I (large circles) and chromosome II (small circles). Origins are marked with filled circles and termini are marked with triangles. The replication speed is assumed to be equal for both chromosomes and to be constant during the entire C period.

function of cell age. The DNA content and cell size of exponentially growing cells were measured using flow cytometry and the data are visualised by the 3-dimensional (3D) presentations in Figure 2A. The ridges of the 3D distributions were computationally determined as described in Materials and methods. The graph for exponentially growing *Caulobacter crescentus* cells, a one-chromosome bacteria with distinct B, C and D periods, is shown in Figure 2A. The first horizontal part of the ridge graph represents the B period, where only cell size increases. This is followed by the C period where both cell size and DNA content gradually increase, and the final horizontal line is the D period. Similar results were obtained with an *E. coli* strain (Supplementary Figure S1). When the same analysis is performed on an exponentially growing *V. cholerae* culture, the graph still displays B, C and D periods, but a bend is evident in the C period part of the graph (Figure 2A and Supplementary Figure S1). Since the cell size most likely increases gradually during the cell cycle, this bend must correspond to a change in the rate of DNA synthesis (Figure 2B). The slope of the ridge plot increases in the last part of the C period, indicating that *V. cholerae* cells abruptly increase their rate of DNA synthesis late in the C period. We repeated this experiment with varying carbon sources to change the growth rate (Table I) and in all cases we observed a very similar bend of the ridge representing the cells in the C period (Supplementary Figure S1).

Simultaneous initiation of the two chromosomes would have led to a high rate of DNA replication at the onset of the C period and a decreased rate of DNA replication after termination of chromosome II (Figures 1 and 2B, Initiation synchrony) in contradiction with our results. In contrast, the increased rate of DNA replication late in the C period corresponds well with later initiation of chromosome II than chromosome I, leading to a doubling of the number of active DNA replication forks late in the C period (Figures 1 and 2B, Termination synchrony).

Computer simulations of DNA contents distributions

To support our conclusion that chromosome II replicate during the late part of the C phase and to determine basic cell-cycle parameters of *V. cholerae*, we compared the experimental DNA histograms obtained by flow cytometry of exponentially growing cells to computer simulations of DNA contents in ideal cultures using the approach described by Michelsen *et al* (2003) that was modified to accommodate two-chromosome bacteria. In these simulations, the DNA histograms are resolved into the contributions from cells in the B, C and D periods by varying the basic cell-cycle parameters and the time of initiation of chromosome II. We simulated the DNA contents assuming that replication of the two chromosomes either initiate or terminate simultaneously. We only obtained good fits between experimental and simulated DNA histograms when the two chromosomes terminate at approximately the same time in the computer simulations (Figure 2C). Similar results were obtained when *V. cholerae* cells were grown in other media, resulting in different doubling times (Supplementary Figure S2).

The fitting of simulations of the DNA histograms to experimental data gave estimates of the basic cell-cycle parameters of *V. cholerae* cells growing at medium and slow growth rates (Table I). In cells growing at a moderate rate

(42–55 min doubling time), the replication time for chromosome I was nearly 30 min (27–32 min) and the replication time for chromosome II was nearly 10 min (9–11 min), giving a replication speed of approximately 800 bp per second per replication fork, very similar to the replication speed in *E. coli* when grown at the same temperature (37°C). The D period was 12–14 min, which is similar to that observed in *E. coli* B/r cells and shorter than that observed in *E. coli* K-12 cells at similar growth rates (Michelsen *et al*, 2003).

The *oriCI* to *oriCII* ratio is increased in fast-growing *V. cholerae* cells and is growth rate dependent

The different sizes of the two chromosomes will lead to different copy numbers of either the origins or the termini of the two chromosomes (Figure 1). The initiation synchrony model predicts an *oriCI* to *oriCII* ratio of one and a *terI* to *terII* ratio less than one (Figure 1). The termination synchrony model predicts an *oriCI* to *oriCII* ratio higher than one and a *terI* to *terII* ratio of one (Figure 1). The differences in *ter* and *oriC* ratios, respectively, are most pronounced during rapid growth, where a larger fraction of the cells will be in the part of the cell cycle where chromosome I is under replication and replication of chromosome II is either finished or not initiated yet. This difference is modelled as described in Materials and methods and shown in Figure 3B and C.

We measured the ratios of *oriCI* to *oriCII* and the ratios of *terI* to *terII* in *V. cholerae* cells where the doubling time was modulated between 18 and 164 min by varying the carbon source and the supplements of amino acids in the culture medium (Figure 3A). The *oriCI* to *oriCII* ratio dropped from ~2 at fast growth rates to just above one at doubling times longer than 100 min, whereas the *terI* to *terII* ratio was almost invariant and near 1 for all doubling times. The results from the marker frequency analysis fit a termination synchrony model significantly better than it fit an initiation synchrony model (Figure 3).

Initiation of replication at *oriCI* and *oriCII* responds differently to the transcription inhibitor rifampicin

Our finding of later initiation at *oriCII* than at *oriCI* contradicts with the initiation synchrony observed by Egan *et al* (2004). Their observation was based on flow cytometry analysis of slowly growing *V. cholerae* cells followed by treatment with rifampicin and cephalixin. Rifampicin inhibits transcription, which then inhibits initiation of DNA replication, and cephalixin inhibits cell division. If chromosome II is initiated later than chromosome I, it was expected to observe cells that had started replication of chromosome I but not replication of chromosome II in the rifampicin run-off experiments. These cells should contain approximately 7.2 Mb DNA after rifampicin incubation, corresponding to two copies of chromosome I and one copy of chromosome II. However, Egan *et al* (2004) observed that all cells contained either 4.1 or 8.3 Mb DNA after rifampicin treatment, corresponding to either one or two copies of each chromosome and they therefore concluded that the two chromosomes are initiated synchronously.

To resolve this contradiction, we measured the ratio of *oriCI* to *oriCII* in very fast-growing *V. cholerae* cells (doubling time of 18 min) at different time points after addition of rifampicin (Figure 4). We found that the *oriCI* to *oriCII* ratio was ~2.6 before addition of rifampicin as expected from the

Figure 2 Flow cytometry analysis of chromosome replication. (A) 3D flow cytometry analysis of DNA replication patterns in *C. crescentus* and *V. cholerae*. The left panels show the raw 3D flow cytometry data with cell size on the x-axis, the DNA contents on the y-axis and the number of cells colour-coded as shown in the bar. The right panels show the ridges of the 3D graphs. The ridge graph for *C. crescentus*, a bacterium with one chromosome, shows three distinct parts, corresponding to the birth (B), chromosome replication (C) and division (D) periods of the cell cycle. The graph for *V. cholerae* shows a bend in the C period part of the ridge graph. (B) Models of the expected ridge graphs for *V. cholerae* with either initiation or termination synchrony. A higher slope of the ridge graph is expected in the part of the C period where both chromosomes replicate, since more replication forks are present. (C) Fitting of an experimental *V. cholerae* DNA histogram of exponentially growing cells to computer simulations of DNA contents in cells of an ideal culture assuming an equal speed of the replication forks throughout the entire C phase and different initiation times of chromosome II. Purple dots are actual DNA contents data, green lines are simulated B period cells, blue lines are simulated C period cells, red lines are simulated D period cells and black lines are the combined simulated cells. The left graph shows a simulation based on both chromosomes initiating at the same time. In the right graph, the simulation is based on a delay of initiation of chromosome II until two-thirds of chromosome I is replicated, resulting in simultaneous termination of the two chromosomes.

experiment in Figure 3. After addition of rifampicin, this ratio dropped to near 1 after 10 min of incubation with rifampicin. This indicates that initiation at *oriCII* continued for a longer

period after addition of rifampicin than initiation of chromosome I, until the two chromosomes had the same copy number. A double-sided *t*-test shows that the means of the

1 and 30 min points are different with 99% confidence. Similarly, the ratio of *oriCI* to *oriCII* in *V. cholerae* cells growing with doubling time of 55 min dropped from 1.4 to 1.0 after addition of rifampicin (data not shown). These results can only be explained if rifampicin has a delayed effect on initiation of chromosome II or if the chromosome I DNA is degraded during the rifampicin incubation. This second option is highly unlikely as the raw data show a constant amount of *oriCI* and an increasing amount *oriCII*. We therefore conclude that the initiations at the two origins are not equally sensitive to rifampicin, which explains the discrepancy between our results and the finding of initiation synchrony by Egan *et al* (2004).

Bremer and Churchward (1977b) measured a delay of 6–11 min before the inhibition of initiation of *E. coli* chromosomal DNA replication by either rifampicin (inhibiting transcription) or chloramphenicol (inhibiting translation) was complete, indicating that cells near initiation can complete this process in the presence of the drugs. Further, there are several demonstrations of rifampicin-resistant initiation in *E. coli* strains where DnaA is in surplus (Pierucci *et al*, 1987; Atlung and Hansen, 1993; von Freiesleben *et al*, 2000;

Morigen *et al*, 2005), indicating that the primary action of rifampicin in chromosome replication is the inhibition of *de novo* DnaA protein synthesis. As initiation at *oriCI* is controlled by the activity of the DnaA protein and initiation at *oriCII* is controlled by the activity of the RctB protein (Duigou *et al*, 2006), initiation at both origins is expected to require ongoing protein synthesis and therefore they should both be sensitive to rifampicin. Our demonstration that rifampicin inhibition is delayed for *oriCII* may indicate that the RctB protein bound to the *rctA* titration sites can be released during transcription arrest and initiate replication.

Mechanisms ensuring approximately simultaneous termination of the two chromosomes

A mechanistically simple mechanism for ensuring approximately simultaneous termination of the two chromosomes would be to link initiation of both chromosomes to cell mass, but with a different initiation mass for the two chromosomes.

Table 1 Estimates of the B, C and D periods of *V. cholerae* cells growing at moderate growth rates

Growth medium	Doubling time (min)	B period (min)	C period (min)	D period (min)
AB + sucrose	42	2	27	13
AB + maltose	55	10	31	14
AB + fructose	55	11	32	12
AB + glycerol + serine	80	22	39	19
AB + glycerol	114	40	49	25

The cell-cycle parameters were estimated by fitting simulations of DNA contents distributions (Figure 2C and Supplementary Figure S2) assuming termination synchrony to experimental flow cytometry DNA histograms.

Figure 4 Differential effect of rifampicin addition on the two *V. cholerae* chromosomes. The *oriCI/oriCII* ratios at various time points after addition of rifampicin to a culture growing in LB glucose media ($\tau = 18$ min). The measurements were repeated at least four times and the s.e.m. are represented on the graphs.

Figure 3 Relative marker frequencies (*oriCI/oriCII* and *terI/terII* ratios) at different growth rates. (A) Wild-type *V. cholerae* cells were grown in media containing different carbon sources, resulting in a range of doubling times. Cells from exponentially growing cultures were fixed and the relative marker frequencies were determined using quantitative PCR. Shown are the relative *oriCI/oriCII* (■) and *terI/terII* (▲) marker frequencies. The line shows the predicted *oriCI/oriCII* ratio and the broken line shows the predicted *terI/terII* ratio assuming termination synchrony. (B) Predicted *oriCI/oriCII* ratio (line) and predicted *terI/terII* ratio (broken line) assuming initiation synchrony. (C) Predicted *oriCI/oriCII* ratio (line) and predicted *terI/terII* ratio (broken line) assuming termination synchrony. Predicted relative marker frequencies were calculated as described in Materials and methods assuming a constant C period at all growth rates.

The two chromosomes are independently controlled by distinct replication initiators, DnaA for chromosome I and RctB for chromosome II (Duigou *et al*, 2006). The activity of RctB is controlled in much of the same fashion as the activity of DnaA is controlled in *E. coli* with both initiator autorepression and titration (Pal *et al*, 2005; Egan *et al*, 2006; Venkova-Canova *et al*, 2006). Thus, it is likely that chromosome II initiation is linked to cell mass in a similar fashion as the chromosome of *E. coli* (Donachie, 1968) or to a lesser degree plasmid P1 (Keasling *et al*, 1992; Bogan *et al*, 2001).

Another possible molecular mechanism ensuring termination of the two chromosomes at approximately the same time during the cell cycle could be the presence of a timer on chromosome I that when replicated sends a signal to chromosome II. In other bacteria, a change in methylation status is known to effect transcription of a number of genes. In *C. crescentus*, the passage of the replication fork leads to the presence of hemimethylated DNA, which activates the promoter of the cell-cycle control gene *ctrA* (Reisenauer and Shapiro, 2002; reviewed by Wion and Casadesus, 2006). Dam methylation could have a similar function at an unknown activator gene in *V. cholerae*.

Concluding remarks

In the current work, we have determined the replication timing for both of the chromosomes in *V. cholerae* by flow cytometry analysis of samples harvested from exponentially growing cultures. Two different data analysis methods led to the conclusion that the rate of DNA replication increases late in the C period in congruence with a model where approximately two-thirds of the large chromosome I is replicated before the smaller chromosome II is initiated, leading to approximately simultaneous termination of replication of the two chromosomes. We have further tested this conclusion independently by measuring the ratio of the copy numbers of the two chromosomal origins, *oriCI* to *oriCII*, and the ratio of the two termini, *terI* to *terII*, as a function of the doubling time. These data show that the origin ratio varies with growth rate, whereas the termini ratio is invariant. This finding further supports that replication of the two chromosomes is initiated at different points of the cell cycle, whereas termination of the two chromosomes happens at approximately the same time in the cell cycle. Our finding of approximate termination synchrony conflicts with the finding of initiation synchrony by Egan *et al* (2004) based on flow cytometry analysis of a rifampicin-treated sample. We show here that it takes longer time before rifampicin inhibits initiation at *oriCII* than at *oriCI* and that the copy number of the two chromosomes will be the same after a long rifampicin treatment, explaining the discrepancy between the results.

Fogel and Waldor (2005) studied the intracellular locations of the two origins in fast-growing cells and found that 78% of the cells had two *oriCI* foci and only 30% of the cells had two *oriCII* foci. Consistent with this, Fiebig *et al* (2006) found that mainly mid-sized cells have two *oriCI* foci and one *oriCII* focus, long cells had two foci of each origin and short cells had one focus of each origin. The finding of fewer *oriCII* foci than expected from the initiation synchrony model was explained by much earlier segregation of *oriCI* than of *oriCII* during the cell cycle. Our finding of later initiation at *oriCII* than at *oriCI* and the subsequently higher copy number of *oriCI* compared to *oriCII* is consistent with the origin

localisation data. Thus, both *oriCI* and *oriCII* foci are likely to segregate away from each other shortly after initiation.

Srivastava *et al* (2006) studied the intracellular locations of the two termini in cells growing in either L broth or minimal glucose medium. They observed that most cells had only one focus of each terminus and that these foci colocalised in these cells. Only 2–3% of the cells had two *terI* foci and 17–18% of the cells had two *terII* foci, indicating that *terII* foci separates earlier than *terI* foci. With the age distribution (Powell, 1958), the cell age at *terI* and *terII* foci separation can be calculated to be later in the cell cycle than the termination of chromosome replication (calculations not shown). The earlier segregation of *terII* is therefore not in conflict with an approximately termination synchrony of the two chromosomes.

Evolutionary advantages of having a divided genome

Over-initiation in *E. coli* leads to a decreased replication speed (Atlung *et al*, 1987) and under-initiation results in an increased DNA replication rate (Atlung and Hansen, 2002; Morigen *et al*, 2003; Skovgaard and Løbner-Olesen, 2005), demonstrating that the normal replication rate is limited by the supply of energy, nucleotides, replication factors or other components. By delaying initiation at *oriCII* until as late as possible in the cell cycle, the cell postpones DNA synthesis until it has increased its metabolic capacity for providing the required energy, dNTPs and/or replication factors, potentially making replication more efficient.

The simulations of DNA content distributions provide estimates of the cell-cycle parameters for *V. cholerae* (Figure 2C and Table I and Supplementary Figure S2). For the fastest growing cells that can be analysed using this approach (42 min doubling time), a C period of approximately 27 min and a D period of approximately 13 min was observed (Table I). Since there is a short B period at a doubling time of 42 min, the minimal C+D period is 40 min or a little shorter. This is very fast compared to *E. coli*, in which the minimum C+D period is 50–60 min (Cooper and Helmstetter, 1968; Michelsen *et al*, 2003). Bacteria growing with a doubling time shorter than the minimal C+D period initiate replication in the previous cell cycle, so cells are born with a partially duplicated genome. Division of the genome into several replicons shortens the C+D period, and thereby reduces the number of overlapping replication cycles needed during fast growth. When grown in rich media, *V. cholerae* only need two overlapping replication cycles of chromosome I and no overlapping replication cycles of chromosome II, whereas *E. coli* need three overlapping rounds of replication. This reduces the number of *oriCI* copies in newborn fast-growing *V. cholerae* cells to two, compared to four *oriC* copies in newborn fast-growing *E. coli* cells. This also reduces the number of replication forks from fourteen to six (eight when both chromosome I and II replicate) after initiation of a new round of DNA replication. The lower number of overlapping replication cycles eases the regulatory problem of ensuring initiation at multiple identical origins within a very short time period (the initiation cascade; Løbner-Olesen *et al*, 1994) and makes chromosome segregation less complicated by reducing the complexity of properly localising and detangling branched, partially replicated chromosomes during rapid growth.

Materials and methods

Bacterial strains and growth conditions

The *V. cholerae* strain used was AC-V304 (Hava and Camilli, 2001). The cells were grown in AB minimal medium (Clark and Maaløe, 1967) supplemented with different carbon sources: 0.2% glutamate; 0.2% glycerol; 0.2% glycerol, 100 µg/ml serine; 0.2% fructose; 0.2% maltose; 0.2% sucrose; 0.2% glucose; or 0.2% glucose, 100 µg/ml serine, 0.5% casamino acids. Alternatively, cells were grown in LB media with 0.2% glucose added. The *E. coli* strain FH1218 (Løbner-Olesen *et al*, 1989) was grown in AB minimal medium containing 0.2% glycerol, 25 µg/ml histidine, 25 µg/ml tryptophan, 25 µg/ml uracil and 10 µg/ml thiamine. All growth experiments were conducted at 37°C in shaking water baths. The *C. crescentus* strain used was CB15N (Evinger and Agabian, 1977) and the cells were grown in PYE (Peptone-Yeast Extract) medium (Ely, 1991) at 30°C. Cell growth was monitored by measuring optical density at 450 nm (OD₄₅₀).

Flow cytometry

Samples were taken from exponentially growing cells (OD₄₅₀ ~ 0.2), incubated on ice to arrest growth and fixed in 75% ethanol. Samples for rifampicin arrest were harvested from exponentially growing cultures at OD₄₅₀ ~ 0.2 and incubated with rifampicin (300 µg/ml; Novartis Pharma Inc.) and cephalixin (36 µg/ml; Sigma Chemical Co.) to inhibit initiation of DNA replication and cell division, respectively (Skarstad *et al*, 1986; Boye and Løbner-Olesen, 1991). Samples were taken at 1, 5, 10, 20 and 30 min after arrest and fixed in 75% ethanol. Flow cytometry was performed as described by Løbner-Olesen *et al* (1989) using an Apogee A10 instrument (Apogee Flow Systems Inc.). The ridge in the 3D flow cytometry graphs was extracted using a custom Perl script. For each light scatter channel, the fluorescence in a sliding window covering 11 fluorescence channels was calculated and the midpoint channel of the window with the highest fluorescence was plotted as the ridge of the graph.

Cell-cycle simulations

The DNA distributions in ideal cultures were simulated using a custom Excel worksheet based on the method described by Skarstad *et al* (1985) and Michelsen *et al* (2003) that was modified to accommodate for the presence of two chromosomes. The age distributions of ideal bacterial cultures growing without overlapping DNA replication cycles were calculated as described (Powell, 1958). B- and D-phase cells were assumed to contain one or two genome equivalents, respectively. To calculate the total DNA amount originating from the two chromosomes during the DNA replication phase of the cell cycle, the C period cells were divided into 100 sub-populations, where the amount of chromosome I DNA increased linearly from one to two during the C period. The time of replication of chromosome II was controlled through an input parameter. Before this time was reached, the amount of chromosome II DNA was set at one. Then it increased linearly until it reached two chromosome II equivalents and stayed at two until the end of the C period. For the simulations, the progression rates of all replication forks were equal and constant during the entire C period. Instrumental variability was included into the simulations as described by Michelsen *et al* (2003) by approximating peaks with normal distributions. The input parameters to the simulations are the lengths of the C and D periods, the relative timing of chromosome II initiation during the C period and the standard deviations of the one and two chromosome peaks. These parameters were varied to obtain the best possible fits between experimental DNA histograms and simulated DNA distributions.

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Marker frequency determination

DNA templates were prepared from ethanol-fixed cells. The cells were harvested, lysed by resuspension in TE-buffer and vigorously vortexing, and the DNA template was diluted 10-fold with H₂O before use. Quantitative PCR was conducted on a Light-Cycler 2.0 instrument (Roche Inc.) using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I PCR kit (Roche Inc.) according to the instructions.

The following primer pairs amplifying ~130 bp fragments located near the origin (*oriC*) or terminus (*ter*) on chromosome I (I) or chromosome II (II) were used for marker frequency determinations: *oriCI* (VC2775, *gidA*) CGCCAACCGAGTTTGGATTTC and AAAAAG CGCGTGAGCTTGG; *terI* (VC1410, *vceA*) CTGAGGCGGATTTGGCACTC and GCTTGCGCGCTTTTAACGT; *oriCII* (VCA1114, *parB*) GCTCCACC TTCCGTGTTTCG and TGGTTTCGTGTGGCAGCAAT; *terII* (VCA0563) TATCCGCACAGCCTCAGCAA and CACGCAAACAGACCGACACC.

Each measurement was made in triplicate, Cp (crossing point) values were extracted and used for calculating the *oriCI/oriCII* and *terI/terII* ratios. Marker ratios were normalised to a culture grown to stationary phase in AB + 0.2% glucose medium, where flow cytometry showed that DNA replication was complete and the cells had the same number of chromosome I and chromosome II.

Marker frequency model

Marker frequencies generated by quantitative PCR are compared with a model based on the equation of Bremer and Churchward (1977a):

$$oriC/ter = 2^{C/\tau} \quad (1)$$

where τ is the doubling time and C is the replication period. It is assumed that the C period is constant at different doubling times.

For bacteria with two chromosomes, I and II, we get:

$$\frac{oriCI/terI}{oriCII/terII} = \frac{2^{C_I/\tau}}{2^{C_{II}/\tau}} = 2^{(C_I - C_{II})/\tau} \quad (2)$$

Assuming initiation synchrony, *oriCI/oriCII* will equal 1 (see Figure 1) and can be removed:

$$terI/terII = 2^{-(C_I - C_{II})/\tau} \quad (3)$$

Assuming termination synchrony, *terI/terII* will equal 1 (see Figure 1) and can be removed.

$$oriCI/oriCII = 2^{(C_I - C_{II})/\tau} \quad (4)$$

Equations (3) and (4) are used to predict the changes in *oriI/oriII* and *terI/terII* ratios at different doubling times. The model is based on the assumption of a constant difference in replication time ($C_I - C_{II}$), which is likely to be constant at 18–20 min in the doubling time range 18–55 min as observed in *E. coli* (Donachie, 1968). At doubling times >55 min, the C period increase. This probably also results in an increase in $C_I - C_{II}$, but the differences is negligible due to the comparatively larger increase in doubling time.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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