Replication patterns and organization of replication forks in *Vibrio cholerae*

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We have investigated the replication patterns of the two chromosomes of the bacterium *Vibrio cholerae* grown in four different media. By combining flow cytometry and quantitative real-time PCR with computer simulations, we show that in rich media, *V. cholerae* cells grow with overlapping replication cycles of both the large chromosome (ChrI) and the small chromosome (ChrII). In Luria–Bertani (LB) medium, initiation occurs at four copies of the ChrI origin and two copies of the ChrII origin. Replication of ChrII was found to occur at the end of the ChrI replication period in all four growth conditions. Novel cell-sorting experiments with marker frequency analysis support these conclusions. Incubation with protein synthesis inhibitors indicated that the potential for initiation of replication of ChrII was present at the same time as that of ChrI, but was actively delayed until much of ChrI was replicated. Investigations of the localization of SeqA bound to new DNA at replication forks indicated that the forks were co-localized in pairs when cells grew without overlapping replication cycles and in higher-order structures during more rapid growth. The increased degree of fork organization during rapid growth may be a means by which correct segregation of daughter molecules is facilitated.

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INTRODUCTION

All cells must replicate their genome before division so that each daughter cell receives a copy of the genetic material. In eukaryotic cells, many origins on many chromosomes initiate throughout the replication period (Gilbert, 2001; Woodfine *et al.*, 2004). Prokaryotic cells seem simpler and often have only one chromosome with one origin. There are, however, also many examples of bacteria with multiple chromosomes, such as *Rhodobacter sphaeroides* (Suwanto & Kaplan, 1989), *Agrobacterium tumefaciens* (Allardet-Servent *et al.*, 1993), members of the genus *Burkholderia* (Wigley & Burton, 2000) and all known *Vibrio* species (Okada *et al.*, 2005).

Here, we set out to investigate in detail the replication of one chromosome relative to the other in *Vibrio cholerae*. This bacterium has two chromosomes, ChrI (2.96 Mb) and ChrII (1.07 Mb), each with its unique origin of replication, *oriI* and *oriII* (Heidelberg *et al.*, 2000; Trucksis *et al.*, 1998). In slowly growing cells, replication of ChrII has been reported to initiate either simultaneously with that of ChrI (Egan *et al.*, 2004) or much later (Rasmussen *et al.*, 2007). In an earlier study of rapidly growing cells, ChrI replication was found to span two generations, while ChrII was

reported to limit its replication to the current generation (Srivastava & Chattoraj, 2007). ChrI is initiated by the DnaA protein, possibly in a way similar to initiation in *Escherichia coli*, and ChrII is initiated by the RctB protein in a fashion which seems to be similar to the initiation of some plasmids (Egan & Waldor, 2003). Overproduction of DnaA and RctB leads to specific overreplication of ChrI and ChrII, respectively (Duigou *et al.*, 2006).

One of the mechanisms that prevents reinitiation of already initiated origins in *E. coli* is sequestration (Campbell & Kleckner, 1990; Lu *et al.*, 1994; von Freiesleben *et al.*, 1994). This process functions by specific inactivation of newly replicated origins by the protein SeqA (Slater *et al.*, 1995). Newly synthesized DNA in *E. coli* is hemimethylated, methylated (by Dam methylase) on the old strand and unmethylated on the new strand, and SeqA binds preferentially to hemimethylated DNA (Brendler *et al.*, 1995; Slater *et al.*, 1995). In *V. cholerae*, similar control mechanisms may operate, since this organism also harbours *seqA* and *dam* genes (Hiraga *et al.*, 2000; Løbner-Olesen *et al.*, 2005). Dam has recently been shown to be required for once-per-cell-cycle initiation of both chromosomes (Demarre & Chattoraj, 2010; Koch *et al.*, 2010). Both origins of *V. cholerae* have been found to remain hemimethylated longer than other DNA and may thus be subject to sequestration (Saint-Dic *et al.*, 2008). Studies of the intracellular localization of SeqA in *E. coli* indicate that most of the protein binds to newly synthesized DNA at the replication forks (Molina &

Abbreviations: τ , generation time; FACS, fluorescence-activated cell sorting; Q-PCR, quantitative real-time PCR.

Five supplementary figures and a supplementary table are available with the online version of this paper.

Skarstad, 2004). The SeqA protein bound at replication forks forms structures that appear as distinct foci when visualized by fluorescence microscopy (Hiraga *et al.*, 1998; Onogi *et al.*, 1999).

In this work we have investigated the timing of initiation of replication in wild-type *V. cholerae* grown in four different growth media, as well as the localization of replication forks in these cells, visualized as SeqA foci. We find that regulation of replication of ChrII seems to be dependent on the replication of ChrI and that replication forks are co-localized.

METHODS

Bacterial strains and growth conditions. The bacterial strain used was *V. cholerae* 2740-80 (CTX ϕ ⁻) (Pearson *et al.*, 1993). Cells were grown in four different media at 37 °C. These were AB minimal medium (Clark & Maaloe, 1967) with 1 µg thiamine ml⁻¹ supplemented with either 0.2 % (v/v) glycerol [glycerol medium, generation time (τ)=107 min], 0.2 % fructose (fructose medium, τ =46 min) or 0.2 % glucose and 0.5 % casamino acids (glucose/CAA medium, τ =27 min), and LB medium (τ =19 min). At least three independent experiments were performed for each medium.

At OD₄₅₀ [or OD₆₀₀ for Luria–Bertani (LB) medium] 0.15, exponentially growing cells were harvested or treated with 150 µg rifampicin ml⁻¹ (Fluka) and 10 µg cephalixin ml⁻¹ (Eli Lilly) for four to five generations to allow completion of ongoing rounds of replication. Samples were harvested after the drug treatment. Both exponentially growing cells and cells treated with rifampicin and cephalixin were resuspended in TBSE buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 130 mM NaCl) and fixed in 70 % ethanol.

Flow cytometry. Cells fixed in ethanol were washed in 0.1 M phosphate buffer (PB), pH 9.0, and stained overnight (4 °C) in the same buffer with 3 µg FITC ml⁻¹. The cells were washed in Tris-buffered saline (TBS) (20 mM Tris/HCl, pH 7.5, 130 mM NaCl) and stained in the same buffer containing 1.5 µg Hoechst 33258 ml⁻¹ (Sigma) for at least 30 min.

An ethanol-fixed sample of slowly growing cells from the glycerol medium (the majority of which contained either 1+1 or 2+2 chromosomes) was used as an internal standard for calibration of the DNA measurement. The standard was included with every sample during incubation with the Hoechst 33258 stain and was not stained with FITC.

Flow cytometry analysis was then performed using an LSR II flow cytometer (Becton Dickinson) equipped with a 488 nm argon ion laser and a 355 nm krypton laser (both Spectra Physics). The FITC fluorescence was collected through a 530/30 nm bandpass filter. A 450/50 nm bandpass filter was used to collect Hoechst 33258 fluorescence. The peaks of the FITC-negative standard were positioned at fixed channels using the photomultiplier tube (PMT) voltage, allowing the DNA content of the FITC-positive sample cells to be accurately calculated (Torheim *et al.*, 2000).

The data obtained from the flow cytometry measurement were analysed by WinMDI or FlowJo software.

Fluorescence-activated cell sorting (FACS). Cells fixed in ethanol were washed in 0.02 M TBS, pH 7.5, and stained in the same buffer containing 1.5 µg Hoechst 33258 ml⁻¹. The sorting was performed using a FACS DiVa cell sorter (Becton Dickinson) equipped with a

351 nm krypton laser. A 440/40 nm bandpass filter was used for collecting Hoechst 33258 fluorescence.

For the visualization of SeqA foci, cells from the different intervals (Fig. 6a) were sorted directly onto glass slides for subsequent immunostaining. The glass slides were washed with ethanol and treated with poly-L-lysine (Sigma) in advance. A total of 25 000 cells were sorted from each interval.

For the quantitative real-time PCR (Q-PCR), at least 5 × 10⁶ cells from each interval were sorted into tubes for subsequent isolation of DNA.

Calculation of cell cycle parameters and replication fork distributions. Samples were collected at a point where cells were in balanced exponential growth, and it was assumed that all cells grew in the same way (same replication pattern, cell division pattern, etc.). The sequence of the non-pathogenic strain 2740-80 is highly similar to that of the pathogenic strain N16961 (Vesth *et al.*, 2010). We therefore assumed that the 2740-80 ChrI and ChrII are 2.97 and 1.06 Mb, respectively.

For cells grown slowly in fructose or glycerol medium (with replication only in the 'current' k generation), an Excel-based simulation routine, adapted from the one used by Rasmussen *et al.* (2007), was used to simulate the theoretical DNA distributions (Supplementary Fig. S1a, b). In our version of the program, the rate of ChrII replication relative to the rate of ChrI replication could be varied. The percentage of cells in B, C and D phase could be iterated until the simulations matched the experimental histograms measured by flow cytometry. These percentages of cells can be used to calculate the duration of the phases:

$$p(t) = \frac{2 \ln 2}{\tau} 2^{-\frac{t}{\tau}} \quad (1)$$

The age distribution (equation 1) gives the probability, $p(t)$, of any cell being age t , t ranging from 0 (newborn) to τ (dividing). τ is known from the optical density measurements. The fraction of cells in B is thus given by:

$$P_B = \int_{t_{\text{Bstart}}}^{t_{\text{Bstop}}} p(t) dt$$

where t_{Bstart} is 0, and we want to find t_{Bstop} . So:

$$P_B = -2 \times 2^{-\frac{t}{\tau}} \Big|_0^{t_{\text{Bstop}}}$$

and accordingly:

$$t_{\text{Bstop}} = -\tau \frac{\ln(1 - \frac{P_B}{2})}{\ln 2}$$

Likewise, the same calculations can be performed for the C phases ($t_{\text{C1 start}} = t_{\text{B stop}}$) and D. The program WinMDI was used to find the point at which the shift of cells per DNA interval is observed and the initiation of ChrII replication occurs (DNA content of cells at the start of C_{II}).

A new Excel-based simulation routine for *V. cholerae* was built, and works in the same way as a previous simulation routine for *E. coli* (Skarstad *et al.*, 1985). It was used for simulation of rapidly growing cells (cells grown in LB and glucose/CAA medium), which have started replication in previous generations (k-1 or k-2). The new computer routine was used for simulation of the theoretical DNA distributions when (C+D)> τ . C periods obtained by Q-PCR were used as the starting point for the simulations. For cells grown in glucose/CAA, we also calculated a preliminary time point for

initiation of ChrI from the histogram of cultures treated with rifampicin/cephalexin (Fig. 3b). The number of cells in the first major peak represents the fraction of cells that has not yet initiated replication of ChrI. This percentage ($P_{8.0\text{Mb}}$) and the age distribution (equation 1) can thus be used to find the time point in the cell cycle at which ChrI initiates, $t_{C_i \text{ start}}$, by the equation:

$$P_{8.0\text{Mb}} = \int_0^{C_i \text{ start}} p(t) dt$$

The parameters of the C- and D-phase durations and the initiation point for ChrII were then iterated in the computer routines until the simulated histograms matched the experimental histograms. Different sets of parameters gave different time points in the cell cycle at which one or both chromosomes initiated or terminated replication. The DNA content of the cells was calculated with formulae deduced from the chromosome content of the cells at different time points. For example, newborn cells grown in glucose/CAA medium contain one copy of ChrI and one copy of ChrII, and in addition newly replicated arms of each chromosome. Replication of ChrI has been ongoing for 22 min of the total ChrI replication period of 30 min, and replication of a new ChrII has been ongoing for 4 min of the total ChrII replication period of 9 min (Fig. 1d). The DNA content of the cells at $t=0$ is thus:

$$\left(1 + \frac{22}{30}\right) \text{size of ChrI} + \left(1 + \frac{4}{9}\right) \text{size of ChrII} = \left(1 + \frac{22}{30}\right) 2.96 \text{ Mb} + \left(1 + \frac{4}{9}\right) 1.07 \text{ Mb} = 6.7 \text{ Mb}$$

All the formulae used to calculate the amount of DNA in the simulations shown in Fig. 1 are indicated in Supplementary Fig. S2.

Between time points at which one or both of the chromosomes initiates or terminates replication, the number of replication forks, and thus the total replication rate, is constant. Integration of the age distribution (equation 1) over these different periods in the cell cycle gives the fraction of cells within each interval. According to the age distribution, these fractions are plotted in subintervals. A variable standard deviation was applied to the theoretical histograms to simulate biological and methodological variation in the cell cultures. A theoretical histogram was fitted to each experimental histogram, and a distribution of replication forks calculated. Best fit was found using a standard deviation of about 7%.

As explained above, cells will have different numbers of forks in different periods in the cell cycle. For example, integration of the age distribution (equation 1) between $t=0$ and $t=5$ min for the cells in glucose/CAA medium resulted in a fraction of cells with four replication forks (Fig. 1d). However, these cells also had four forks at a later stage, from $t=8$ min to $t=23$ min, and we must therefore add up the fractions to find the total amount of cells with four forks. The mean values and standard deviations were calculated on the basis of three different experiments (Fig. 5). Cells grown in LB medium produced the same DNA histogram for each experiment, so that the standard deviations (error bars) cannot be distinguished in the figure.

Purification of SeqA antiserum. The SeqA antiserum was raised against purified SeqA protein from *E. coli* and was found by Western blotting to bind SeqA from *V. cholerae* with little cross-reaction (data not shown).

Cell extract for non-specific absorption of antibody was prepared from a ΔseqA strain, EBO193 (Torheim *et al.*, 2000). Cells from 1000 ml of exponentially growing culture were harvested, washed once in 10 ml 50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.4, and resuspended in 1.5 ml of the same buffer. Cells were lysed by addition of 250 μg egg white lysozyme ml^{-1} (30 min on ice) followed by freezing and

thawing (on ice). The supernatant was collected by centrifugation (18 500 g, 30 min, 4 °C), and cell extract was frozen in liquid nitrogen and stored at -80 °C.

Precipitation of non-specific antibody was performed by dilution of SeqA antibody about 100-fold with ΔseqA cell extract to a final volume of 470 μl . A 30 μl volume of PBST (140 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.05 % Tween 20) containing 10 mM EDTA was added followed by incubation on ice for 1 h. The supernatant was collected by centrifugation (21 000 g, 15 min, 4 °C) and used as SeqA antibody solution.

Immunostaining and fluorescence microscopy. A glass slide (cleansed with ethanol) was incubated with 20 μl poly-L-lysine for 5 min before the slide was washed with distilled water and air-dried. A 10 μl volume of the ethanol-fixed cell suspension was dropped onto the slide and air-dried. After washing and drying, the dried cell area was covered with 100 μl lysozyme solution [2 mg lysozyme ml^{-1} in 25 mM Tris/HCl (pH 7.5), 50 mM glucose, 10 mM EDTA] and incubated at room temperature for 8 min. Then, the slide was covered with 1 ml PBST for 30 s and inclined to remove the solution. This step was repeated five times. The slide was covered with 1 ml 99 % methanol for 1 min, inclined and air-dried. A 100 μl volume of PBST containing 2 % BSA (PBST-BSA) was placed on the slide to block the sample and was removed after 15 min by inclining the slide. A 25 μl volume of the SeqA antibody solution (first step antibody) was placed on the slide, covered with a cover glass and left for 1 h at room temperature in a moisture chamber. The cover glass was then removed and the slide washed 15 times with 1 ml PBST. The sample on the slide was blocked again with PBST-BSA for 15 min and air-dried. A 20 μl volume of 500-fold-diluted Cy3 anti-rabbit IgG red fluorescence (second step antibody) was placed on the slide, covered with a cover glass and incubated for 1 h in a dark moisture chamber. The slide was then washed as before, 15 times with PBST. The sample was covered with 10 μl mounting medium containing Hoechst 33258 [40 % (v/v) glycerol, 0.02 M PBS (pH 7.5), 5 μg Hoechst 33258 ml^{-1}] and covered with a cover glass.

Visualization of immunostained cells was performed using a Zeiss AxioPlan 2 phase-contrast/fluorescence microscope equipped with a numerical aperture 1.40 objective and a BP546/12 excitation filter. DNA was visualized with a BP365/12 excitation filter. Pictures were taken using a MicroMax charge-coupled device (CCD) camera (Princeton Instruments) that was connected to a computerized image analysis system (Zeiss AxioVision 4.6). The optical images, which showed cell outlines, were merged with the fluorescent images, which showed SeqA proteins.

Determination of GATC-site distributions. SeqA binds to pairs of hemimethylated GATC sites. There are at least 17 different spacings between the sites that are bound by *E. coli* SeqA in *in vitro* gel-shift experiments (Brendler *et al.*, 2000). These are 6, 7, 8, 10, 11, 12, 13, 14, 18, 19, 20, 21, 22, 23, 29, 30 and 31 bp (for example, the sequence GATCTGATC has a spacing of 5 bp).

A program was written that identified all the pairs of GATC sites with favourable spacings along each of the *V. cholerae* chromosomes. The maps in Supplementary Fig. S3 show potential SeqA binding sites as a black bar for each GATC pair, on the two chromosomes.

Isolation of chromosomal DNA and Q-PCR. A 7.5 ml volume of *V. cholerae* culture was mixed with 7.5 ml ice-cold killing buffer (20 mM Tris, 5 mM MgCl_2 , 20 mM NaN_3) and centrifuged for 3 min at 6000 g and 4 °C. The supernatant was discarded and the pellet resuspended in 300 μl Tris-EDTA (TE) buffer containing 40 μl 10 % SDS and 3 μl 0.5 M EDTA. After incubation for 5 min at 65 °C, 750 μl 2-propanol was added before centrifugation at 15 600 g for 5 min. The pellet was resuspended in 500 μl TE buffer, and 2 μl

Fig. 1. Overlapping replication rounds in *V. cholerae* cells during rapid growth. (a, c, e) DNA histograms of cells grown in different media obtained by flow cytometry. (a) In poor medium (fructose), cells contained from 4.0 to 8.0 Mb DNA, which corresponds to one copy of *ChrI* and one of *ChrII* or two copies of each. (c, e) When grown in richer media, the cells contained more DNA, and thus had overlapping rounds of replication. (b, d, f) Calculated replication patterns at the three different growth rates. The replication patterns were determined from the best-fit simulated DNA distributions. The corresponding simulated distributions are shown in Fig. 2(a) and Supplementary Fig. S1(b, c). The black horizontal arrows indicate time axes. Each pair of lines above these axes represents one generation, where the 'current' generation is denoted *k*, the 'mother' *k*-1 and the 'grandmother' *k*-2. The dark-blue lines represent periods of *ChrI* replication (C_I), and the light-blue lines periods of *ChrII* replication (C_{II}). D_I , D_{II} and B phases are shown in grey. Time points at which initiation or termination occurs are marked on the time axes, and the numbers of replication forks between these points are indicated below the axes (green boxes). Cells are drawn schematically for each life cycle with *ChrI* in dark blue, *ChrII* in light blue, and the origins as black dots [in (f), the chromosome configuration in one interval, age 8–10 min, is not shown]. The purple arrow indicates the point of initiation of *ChrII* in cells grown in fructose medium.

RNase A (25 mg ml⁻¹) was added and incubated for 30 min at 65 °C. Subsequently, 2 µl proteinase K (25 mg ml⁻¹) was added and samples were incubated at 37 °C for 15 min followed by phenol extraction and precipitation with ethanol and sodium acetate. Precipitated DNA was resuspended in 50 µl double-distilled H₂O (ddH₂O). Q-PCRs were carried out in triplicates of 25 µl each. Chromosomal DNA was digested with *EcoRI* to allow optimal access of primers to the template. About 10 ng digested DNA was used as template in 10 µl ddH₂O and added to a mixture of 12.5 µl TaqMan Gene Expression mix (Applied Biosystems) and 2.5 µl three-primer mix (9 µM each forward and reverse primer and 2.5 µM probe) in 96-well PCR plates. Primer pairs for *oriI* (*oriI*fw, *oriI*rv and *oriI*probe), *terI* (*terI*Cfw, *terI*Crv, *terI*Cprobe), *oriII* (*oriII*fw, *oriII*rv, *oriII*probe) and *terII* (*terII*Rfw, *terII*Rrv, *terII*Rprobe) were used in separate reactions. For a list of primers, see Supplementary Table S1. Reactions were carried out with a 7500 Real-Time PCR system (Applied Biosystems). The system software was used to calculate the cycle number at which the fluorescence crossed the threshold (*C_t* value), which was transformed to a relative value of template DNA. The standard deviation for three replicates was about 3 %. The relative values for three different experiments for each growth condition were used to calculate *oriI/terI*, *oriI/oriII* and *terI/terII* ratios (Table 1) relative to the respective ratios of DNA from standard samples (cells grown in glycerol medium to stationary phase). Primer pairs for *oriII*, *terI* and *terII* gave comparable signals in the measurement of standard DNA, whereas the primer pair for *oriI* gave a somewhat lower signal. The ratio of origin copies to terminus copies in an exponentially growing population is given by the formula $ori/ter = 2^{\frac{C}{\tau}}$, where *C* is the time it takes to replicate the chromosome from the origin to the terminus and τ is the doubling time of the population (Bremer & Churchward, 1977). *C* periods were calculated using this formula with the indicated variation according to the standard deviation. The Q-PCR analysis of sorted cells was carried out twice for each of two different cell sortings.

RESULTS

V. cholerae 2740-80 was grown in media that gave four different growth rates (see Methods). Cells in exponential steady-state growth were harvested at an optical density of 0.15 and subsequently used for at least three types of measurement: (i) DNA contents of cells were measured by flow cytometry, (ii) the duration of replication periods was estimated by Q-PCR, and (iii) the localization of the SeqA

protein was determined by immunofluorescence microscopy. The DNA histograms and replication period durations were used in computer simulations to calculate the different patterns of replication.

ChrII replication occurs at the end of the ChrI replication period

The DNA histogram of *V. cholerae* grown in fructose medium (with $\tau = 46$ min) shows that through the cell cycle cells contain either 4.0 or 8.0 Mb DNA, or amounts of DNA in between these values (Fig. 1a). This indicates that cells in the first peak contain one copy of ChrI and one copy of ChrII, and are thus in the pre-replication phase (B period). Cells in the second peak contain two copies, one of both ChrI and II, and are in the post-replication, D, phase. From the shape of the DNA distribution in the C phase, between the peaks, it can be seen that the number of cells per DNA channel is lower in the second part of the replication period than in the first. The reason for lower numbers of cells per channel is a higher total rate of DNA synthesis in the cells in late C phase compared with the cells in early C phase (i.e. replication at four forks compared with replication at two forks). This observation indicates that replication of ChrI is initiated first and goes on for a while before the replication of ChrII is initiated. The exact point at which the shift to a lower number of cells per channel occurs can be seen in the DNA histograms (marked by an arrow, Fig. 1a), and the time point of initiation of ChrII in the cell cycle can be calculated from this DNA amount (see arrow, Fig. 1b). Computer simulations were performed to obtain the fractions of cells in the four intervals B, early C, late C and D that gave the best fit to the experimental histogram (see Methods). The durations of cell cycle periods can be calculated from these fractions, taking the exponential age distribution into consideration. The result, that ChrII replication (with duration $C_{II} = 12$ min) is performed in the late part of the C phase ($C_I = 26$ min) (Fig. 1b), is in accordance with the results of Rasmussen *et al.* (2007).

Table 1. *oriI/terI*, *oriI/oriII* and *terI/terII* ratios and *C* period durations derived from Q-PCR

Ratio or period	Fructose medium ($\tau = 46$ min)	Glucose/CAA medium ($\tau = 27$ min)	LB medium ($\tau = 19$ min)
<i>oriII/terI</i> ratio	1.6 ± 0.1	2.5 ± 0.1	3.6 ± 0.5
<i>oriII/terII</i> ratio	1.1 ± 0.2	1.2 ± 0.2	1.3 ± 0.0
<i>oriI/oriII</i> ratio	1.4 ± 0.2	1.9 ± 0.3	2.5 ± 0.3
<i>terI/terII</i> ratio	1.0 ± 0.1	0.9 ± 0.0	0.9 ± 0.1
<i>C_I</i> period (min)	30 (26–33)*	35 (34–37)*	35 (31–38)*
<i>C_{II}</i> period (min)	9 (0–19)*†	7 (0–13)*	8 (7–8)*

**C* period values are calculated from the original *oriI/terI* ratios to two decimal places by the formula $ori/ter = 2^{\frac{C}{\tau}}$. The values in parentheses are the estimated range of *C* values given by the *oriI/terI* ratio ± SD.

†Subtraction of the SD from the *oriI/terI* ratio gave a ratio below one and thus a negative value for the *C_{II}* period. The minimal value was therefore set at 0 min. The difference between the copy numbers of *oriII* and *terII* was considered too small for measurements of *C* with Q-PCR in this case, and the value obtained was not used further.

The DNA histogram of cells growing more slowly (in glycerol medium, $\tau=107$ min) yielded similar values for C and D periods and considerably extended B periods ($B_I=60$ min and $B_{II}=76$ min) (Supplementary Fig. S4). Replication of ChrII ($C_{II}=13$ min) also seemed to occur during the late part of C ($C_I=29$ min) under these conditions.

Both ChrI and ChrII have increased copy numbers during rapid growth

Bacteria that are capable of rapid growth, such as *E. coli* and *Bacillus subtilis*, allow replication to span more than one generation, and can in this way achieve generation times that are much shorter than the time required to complete duplication of the chromosome (Cooper & Helmstetter, 1968; Skarstad *et al.*, 1985). *V. cholerae* ChrI has also been reported to initiate replication in the mother cell, whereas ChrII has been reported to limit replication to the 'current' generation (Srivastava & Chattoraj, 2007). Here, we found that newborn *V. cholerae* cells grown in the rich media glucose/CAA and LB contained about 6.7 and 9.9 Mb DNA, respectively (Fig. 1c, e). These DNA contents correspond to much more than one copy of the genome, and the cells must therefore initiate replication in previous generations. We simulated theoretical DNA distributions, and estimated the best fit to the experimental histograms, to determine the details of the replication patterns.

Cells grown in LB ($\tau=19$ min) gave DNA distributions ranging from 9.9 to 19.8 Mb DNA (Fig. 1e). To obtain an independent measure of the duration of the C phase, we performed Q-PCR of the origin and terminus regions (Table 1). The ratio of origin copies to terminus copies in an exponentially growing population is given by the formula $ori/ter=2^{\frac{C}{\tau}}$, where C is the time it takes to replicate the chromosome from the origin to the terminus and τ is the doubling time of the population (Bremer & Churchward, 1977). In the following simulations, the C and D values were iterated to find the best theoretical fits to the experimental DNA histograms. At different time points of the cell cycle, the corresponding DNA content was calculated by our Excel-based simulation program. The number of cells in the intervals between these points was found and then plotted in subintervals according to the age distribution. Calculation points were whenever origins fire (which will start new replication forks, cause an increase in the total replication rate and thus fewer cells per DNA interval), replication rounds finish (causing a decrease in the number of replication forks) or cells divide. Our estimated C_I duration was 32 min and that of C_{II} was 8 min; this is in accordance with both our Q-PCR values and earlier findings (Dryselius *et al.*, 2008; Srivastava & Chattoraj, 2007). With this replication pattern ($C_I=32$, $C_{II}=8$, $D_I=17$ and $D_{II}=20$ min; simulation in Fig. 2a), cells initiate replication of ChrI at four origins in the middle of generation k-2 (i.e. in the 'grandmother'), whereas initiation of ChrII occurs in

generation k-1 (the 'mother') at two origins (Fig. 1f). Simulations in which the two copies of ChrII did not initiate replication at the same time, but some minutes after each other, also fit the experimental histogram relatively well (data not shown). These results thus indicate that the cells grow with between two and four *oriII* in LB medium. It is, however, not possible from these measurements to determine whether the two ChrII origins initiate at exactly the same time or not.

A simulation with D-phase parameters so that ChrI initiated replication in the k-1 generation (i.e. in the 'mother') and ChrII in the current, k, generation ($D_I=6$ min and $D_{II}=11$ min; see Supplementary Fig. S5a for corresponding replication pattern) was made to test the earlier-suggested replication model (Srivastava & Chattoraj, 2007). This simulated DNA histogram did not fit well, both because the shape was clearly wrong and because the DNA contents became too low (5.8–11.6 Mb; Fig. 2b). A simulation with the same D_I -phase duration as that of the cells grown in fructose medium ($D_I=12$) and a D_{II} phase ($D_{II}=15$ min) which allows termination of ChrII slightly before termination of ChrI (and is in accordance with *oriI/oriII* results from the Q-PCR), also did not fit well and gave DNA values that were too low (8.0–16.0 Mb; Fig. 2c and Supplementary Fig. S5b). These badly fitting simulations are included here to demonstrate that the distribution of DNA contents in the cell culture yields precise information about the degree of replication cycle overlap.

Cells grown in glucose/CAA medium ($\tau=27$ min) contained DNA amounts from about 6.7 to 13.4 Mb DNA (Fig. 1c). A similar procedure of simulation was undertaken as for the cells grown in LB, and the resulting histogram is shown in Supplementary Fig. S1(c). The parameters that gave the best fit to the shape and DNA values of this distribution were $C_I=30$, $D_I=19$, $C_{II}=9$ and $D_{II}=22$ min (Fig. 1d), which are quite similar to those found for cells grown in LB. Replication of both chromosomes was found to span two generations.

The origin of ChrII fires much later than the origin of ChrI, but is ready at the same time

In the analysis of chromosome replication in *E. coli*, drug treatment which inhibits initiation of replication but allows ongoing replication forks to finish is often used to determine the numbers of origins per cell (Skarstad *et al.*, 1986). Rifampicin inhibits the transcription necessary for initiation at *oriC*, while ongoing forks are allowed to finish. Cephalixin is also added to inhibit cell division (Boye & Løbner-Olesen, 1991). Thus, after a few hours of drug treatment, cells will contain 2^n ($n=0, 1, 2, 3, \dots$) fully replicated chromosomes if initiation is synchronous. The number of chromosomes in a cell then corresponds to the number of origins present in the cell at the time of drug action.

We subjected *V. cholerae* 2740-80 cells to this treatment and found that cells grown in fructose responded in a

Fig. 2. Flow cytometry analyses indicate high copy numbers of both *V. cholerae* chromosomes. (a–c) The experimental flow cytometry histograms of cells growing exponentially in LB medium (grey connected dots) were compared with histograms from different simulations of replication patterns (black lines). (a) Simulation with iterations of C-phase durations and D-phase values until a good fit was obtained. The corresponding replication pattern is shown in Fig. 1(f). (b) Simulation with the same C-phase durations, assuming initiation of ChrI in the ‘mother’ generation and initiation of ChrII in the ‘current’ generation (see Supplementary Fig. S5a for replication pattern). (c) Simulation with the same C-phase durations, and D-phase length as for cells in fructose medium (see Supplementary Fig. S5b for replication pattern). Details of simulations can be found in Methods.

initiation of replication from *oriI*. If rifampicin also inhibited initiation from *oriII*, about 30 % of the cells would have two ChrI but only one ChrII (7.0 Mb). Cells with this amount of DNA were not found. Since this population was missing, *oriII* can be initiated even in the absence of transcription and protein synthesis.

In accordance with earlier reports we found that cells grown in LB did not finish ongoing rounds of ChrI replication in the presence of rifampicin and cephalixin (Srivastava & Chattoraj, 2007). A subpopulation of the cells was capable of finishing with 4 + 4 chromosomes, while a large proportion of the rest of the cells contained a higher amount of DNA (data not shown).

Cells grown in glucose/CAA medium finished ongoing rounds of replication in the majority of the cells in the presence of rifampicin and cephalixin (Fig. 3b). Thirty per cent of the cells contained an amount of DNA equivalent to two ChrI and two ChrII, and the rest were judged to contain four ChrI and four ChrII, although this was not seen clearly because some cells had not completed ongoing rounds. The reason for this is not known, but it may be that the reservoir of nucleotides is not large enough to support completion of all replication forks in the absence of protein synthesis. Adding nucleosides to the culture for improvement of the run-out histogram did not, however, yield any effect (data not shown). The result supports the above finding that the *oriII* initiations, which allow ChrII to reach the same number as ChrI, are not inhibited by rifampicin. The result also shows that when a matching number of chromosomes was reached, further initiation of *oriII* was inhibited.

***ori/ter* ratios of sorted subpopulations of *V. cholerae* cells confirm that ChrII can initiate replication in previous generations**

To independently verify the replication pattern with the high copy numbers of ChrII found for *V. cholerae* cells grown in LB medium, we sorted cells by FACS and measured the marker frequencies of origins and termini

similar way to *E. coli* cells and in accordance with results reported for *V. cholerae* (Egan *et al.*, 2004; Rasmussen *et al.*, 2007). Cell counting by Coulter Counter showed that cephalixin inhibits cell division in *V. cholerae* also (data not shown). About 20 % of the cells had a DNA content corresponding to one ChrI plus one ChrII (4.0 Mb) and about 80 % had a DNA content corresponding to two ChrI and two ChrII (8.0 Mb) (Fig. 3a). The number of cells with one and two ChrI was as expected from the replication pattern (Fig. 1b), indicating that rifampicin inhibits

Fig. 3. *V. cholerae* cells accumulate matching numbers of chromosomes after replication run-out. DNA histograms of cells grown in fructose (Fig. 1a) and glucose/CAA medium (Fig. 1c) and incubated with rifampicin and cephalixin.

in the subpopulations by Q-PCR. Cells from two intervals, P1 and P2, were collected (Fig. 4b), and the *oriI/terI*, *oriIII/terII*, *oriI/oriII* and *terI/terII* ratios determined by Q-PCR (Fig. 4c). According to our simulated replication pattern (Fig. 1f), cells in P1 contain four *oriI* and either one or two *terI* (which gives a theoretical *oriI/terI* ratio of about 3), and two *oriII* and two *terII*. The cells in P2 have initiated another round of replication of both ChrI and ChrII. We calculated the theoretical *ori* and *ter* frequencies for the two intervals (grey bars in Fig. 4c) and found the agreement with the experimental data (purple and yellow bars in Fig. 4c) to be good. The result supports our proposed replication pattern (Fig. 1f). Notice especially that the high copy number of ChrI, combined with the *oriI/oriII* ratio of 2, suggests that ChrII initiates replication of two copies simultaneously. If one copy of ChrII were replicated first, during the first half of a cell's life, and then the other copy during the second half, the *oriI/oriII* value would have been 4/3, i.e. about

1.3, in the young cells and 2 in the old. The Q-PCR experiments indicate that the value is around 2 in both young and old cells.

Co-localization of the replication forks in *V. cholerae* cells grown in poor media

In *E. coli* the new DNA at replication forks can be identified by immunostaining with anti-SeqA antibody (Molina & Skarstad, 2004). The *V. cholerae* SeqA protein is 69% similar to SeqA in *E. coli*. It can therefore be assumed that most of the SeqA protein in the *V. cholerae* cell is also bound to newly replicated DNA if the distribution of GATC sites is similar to that of *E. coli*. A computer routine which identifies pairs of GATC sites with distances favourable for SeqA binding was constructed, using the same criteria as those used earlier for such analyses in *E. coli* (Brendler *et al.*, 2000). It was found that both ChrI and ChrII of *V. cholerae* have a slightly higher frequency of such pairs of GATC sites than the genome of *E. coli* (Supplementary Fig. S3). We therefore assume that SeqA binding also occurs at the replication forks in *V. cholerae*.

V. cholerae cells analysed by flow cytometry were also subjected to immunofluorescence microscopy to determine the localization of SeqA, and thus the localization of newly replicated, hemimethylated DNA. The SeqA foci distributions (Fig. 5a, d, g, j) were compared with the replication fork distributions (Fig. 5b, e, h, k) to estimate the degree of co-localization for each medium. The replication fork distributions were determined from the calculated replication patterns (see Methods).

V. cholerae grown with glycerol as carbon source had a doubling time of 107 min and no overlapping replication rounds (Supplementary Fig. S4b). Calculation of cell cycle parameters yielded 22% C-phase cells and about 6% in late C (with both ChrI and ChrII replicating). Immunofluorescence microscopy showed that about 74% of the cells had no foci, 21% contained one and 5% two SeqA foci (Fig. 5a). The results indicate that most cells in early C phase (replicating only ChrI) contain one SeqA focus and most cells in late C (replicating both ChrI and ChrII) contain two SeqA foci during slow growth. This indicates that pairs of replication forks stay colocalized during most of the replication period.

Similar results were found for cells grown in fructose medium. Here, cells with no, one, two or three foci were observed (Fig. 5d, f). The cells with two or three foci were larger than the cells with no or one focus. This further indicates that cells with one SeqA focus were in early C phase and contained two forks, whereas the cells with two or three foci were in late C with four forks. During the above experiments it was noticed that whereas most cells with one focus were relatively small, about 13% of them were large, had a septum, and had the focus positioned near the septum (cell with arrow in Fig. 5f). It is possible either that these cells are still in late C phase or that a SeqA structure persists after replication is finished. It is likely that in the individual cell,

Fig. 4. Marker frequency analyses of sorted cells confirm the replication pattern of LB-grown *V. cholerae*. (a, b) Cells from the DNA intervals P1 (purple) and P2 (yellow) were collected by FACS. Schematic of theoretical chromosome configurations at the indicated intervals in the DNA distribution (based on the cell cycle parameters shown in Fig. 1f). Note that P1 will contain cells with both the first and the second chromosome configuration. (c) Q-PCR measurements of origin and terminus frequencies in cells from the two intervals (purple and yellow bars) compared with the calculated theoretical values (grey bars).

one chromosome terminates replication slightly before the other, with methylation of the GATC sites of this chromosome in the terminus region following 1–2 min later (Waldminghaus & Skarstad, 2010). The terminus region of the other chromosome will then contain the only hemimethylated DNA in the cell. SeqA bound here might therefore be more difficult to displace, because no other hemimethylated binding sites exist to compete for binding, thus possibly giving rise to persisting SeqA structures.

Replication fork organization during the cell cycle in *V. cholerae*

Cells grown in fructose medium were sorted by FACS according to DNA content. Narrow intervals from the left

side of the B-phase peak, the right side of the D-phase peak and from early and late C phase (see narrow coloured stripes in Fig. 6a) were chosen in order to collect cells as representative as possible. The sorting procedure typically yielded about 96 % purity. The sorted cells were subjected to immunofluorescence microscopy and the numbers of SeqA foci per cell scored (Fig. 6c). Most of the B-phase cells (79 %) had no SeqA focus, whereas most cells (50–65 %) from the other categories had one focus. Many of the late C-phase cells had two foci (31 %) and some had three (10 %). Most of the D-phase cells contained one intense focus at midcell, and resembled the cells with a focus at the septum referred to above. From the SeqA focus distributions in the four categories of cells a total focus distribution was calculated and found to be essentially the same as that

Fig. 5. Replication fork co-localization. Fixed cell samples used for flow cytometry in Fig. 1 and Supplementary Fig. S4 were also immunostained using anti-SeqA antibody and subjected to fluorescence microscopy. (a, d, g, j) Distributions of SeqA foci per cell. At least 100 cells were counted in each experiment. (b, e, h, k) Replication fork distributions were determined from the replication patterns of the simulations. (c, f, i, l) Representative cells from each of the four growth media. Some of the cells grown in poor media had one focus positioned at the septum [see arrow in (f)]. Bars, 1 μ m (applies to all images).

of the unsorted cells. The results support the above indication that pairs of *V. cholerae* replication forks are co-localized during most of the replication period when the cells grow in poor medium.

A more extensive organization of replication forks during rapid growth

The rapidly growing cells were also subjected to immunofluorescence microscopy and the numbers of SeqA foci per cell were scored. A comparison of the numbers of forks per cell with the numbers of SeqA foci indicated that the increase in fork numbers was much larger than the increase

in numbers of SeqA foci (Fig. 5g–l). Cells grown in glucose/CAA medium had four, six or eight replication forks and most often one, two or three foci, and most cells grown in LB medium had four or 16 forks and two, three or four SeqA foci. The results indicate that during rapid growth more than two replication forks are often co-localized.

DISCUSSION

A method for direct detection of late-firing origins

We report here a method for detecting the exact point of initiation of ChrII replication in *V. cholerae*. In principle

Fig. 6. Cell cycle distribution of SeqA foci in *V. cholerae*. (a) Cells grown in fructose medium were sorted from four narrow DNA intervals, as indicated by the four coloured lines on the DNA distribution. (b) Illustration of chromosome configurations in each sorted interval (Egan *et al.*, 2005; Srivastava *et al.*, 2006). In the B phase (red FACS interval), cells contain one ChrI (blue) and one ChrII (purple) but no replication forks. In early C (blue FACS interval), cells contain two ChrI replication forks, and in late C (green FACS interval), also two ChrII replication forks, i.e. four forks in total. In the D phase (yellow FACS interval), cells contain two of each chromosome and no forks. (c) Cells from these intervals were immunostained with anti-SeqA antibody and subjected to fluorescence microscopy, and the numbers of SeqA foci per cell were determined.

cells per channel. Likewise the number of cells per channel will increase when replication forks finish. Thus, the initiation and termination points can be read directly from the shape of the DNA histogram.

Simultaneous replication of two copies of ChrII

In rich media the duration of the ChrII phases ($C_{II} + D_{II}$) was found to be longer than one generation (one doubling time). The initiation of ChrII replication then occurred in the ‘mother’ generation, at two origins. It has earlier been reported that only ChrI (and not ChrII) can replicate with overlapping cycles; this is based on a microscopy study yielding mainly one and two *oriII* foci (Srivastava & Chattoraj, 2007). A reason for this apparent discrepancy could be that the numbers of origin foci were taken by the authors to be equivalent to numbers of origins. However, if *V. cholerae* origins stay co-localized for some time after initiation, as shown for origins in rapidly growing *E. coli* (Fossum *et al.*, 2007), the number of origins will be underestimated by this approach. Three and four *oriII* foci have been observed in another similar microscopy study of LB-grown cells (Saint-Dic *et al.*, 2008), supporting our result. Also, in *E. coli*, both separate and co-localized origins have been observed in the same cell (giving the unexpected number of three foci), depending on the growth conditions (Adachi *et al.*, 2008; Fossum *et al.*, 2007; Nielsen *et al.*, 2007).

The control of ChrII copy number may be coupled to ChrI replication

Our results suggest that ChrII replicates at the end of the ChrI replication period at all four growth rates tested, but that inhibition of transcription and protein synthesis leads to equal numbers of ChrI and ChrII in all cells. For instance, cells grown in glucose/CAA medium end with two ChrI and two ChrII or four ChrI and four ChrII after treatment with rifampicin and cephalexin. Most cells in the exponentially growing culture contained four *oriI* and two *oriII*. The absence of a population of cells with four ChrI and two ChrII indicates that initiation of ChrII is

this method can be used for any organism, provided replication occurs in a regulated manner from specific origins. The method is based on the fact that in a theoretical DNA histogram, the number of cells with a given DNA content will drop to around half (from one channel to the next) when the number of replication forks (and the rate of DNA synthesis) is doubled. This means that the higher the total DNA synthesis rate, the fewer the

regulated in such a way that one ChrII is initiated for each copy of ChrI. The same tendency is seen for LB-grown cultures, in which cells harbour four *oriI* and two *oriII* or eight *oriI* and four *oriII*. After treatment with rifampicin and cephalaxin, a subpopulation ends up with 4+4 chromosomes, while the rest seem to continue towards a higher DNA content. We suggest that initiation of ChrII replication is directly or indirectly coupled to initiation of ChrI, but then inhibited until most of ChrI is replicated. The mechanisms for this regulation remain to be investigated and may involve components that to date are unknown. However, such regulation could in principle be achieved with the already known initiator proteins of ChrI and ChrII, DnaA and RctB. These proteins seem to be switched on and off by opposite signals. Whereas the active form of DnaA is the ATP-bound form, the active RctB has ADP bound (Duigou *et al.*, 2008). It is possible that a mechanism similar to the *E. coli* RIDA mechanism (Katayama *et al.*, 2001) exists in *V. cholerae* and causes hydrolysis of active ATP–DnaA to inactive ADP–DnaA as soon as ChrI forks are under way, preventing further initiation of ChrI. If the launching of ChrI forks also causes a gradual hydrolysis of ATP–RctB to the active ADP form, the mechanism could explain how replication of the two chromosomes can be coordinated without initiating at the same time. Such a mechanism would also explain why matching numbers of the two chromosomes accumulate after rifampicin treatment. In the presence of rifampicin, protein synthesis will cease, and also the synthesis of RctB. However, since all necessary RctB would already be present (but inactive), initiation can occur when ChrI forks have replicated for long enough and enough RctB–ATP is hydrolysed.

Alternatively, it could be that ChrII initiation is regulated independently of ChrI, but governed by common regulatory restrictions. For instance, a prolonged sequestration of *oriII* might explain the delay in ChrII replication. For such a mechanism to function, *oriII* would have to be sequestered for an entire generation (and then released after exactly one generation). If *oriI* were also sequestered for exactly one generation, the release from these sequestration events would be staggered and generate the delay. Measurements of the amounts of hemimethylated origin DNA in exponentially growing cultures indicate that either 40 and 20 % (Saint-Dic *et al.*, 2008) or 10 and 50–70 % (Demarre & Chattoraj, 2010) of *oriI* and *oriII* sequences, respectively, are hemimethylated. The measurements depend on which diagnostic GATC site is used in the measurement, and especially the latter result may support the idea that *oriII* initiation is prevented for an entire generation. It is, however, not clear how separate, staggered sequestration windows (or only the *oriII* sequestration window) would operate in a precise fashion. The release from sequestration of the last GATC site in *oriII* (or at least the last site that prevents initiation) would in this case have to be quite precisely timed. Such a prolonged sequestration mechanism would also imply that during slow growth all

B-period cells would contain origins in sequestration. In the present experiments we found only a few B-period cells with SeqA foci. Thus, if *oriII*–SeqA structures are present during the B period, they are for some reason not detected by the immunofluorescence microscopy.

Flexible co-localization of pairs of replication forks during slow growth

Segregation of ChrI in *V. cholerae* is dependent on the ParAI and ParBI proteins, which act on a centromere-like site near *oriI* and pull the origin region of one of the replicated chromosomes from the old pole to the new pole (Fiebig *et al.*, 2006; Fogel & Waldor, 2005, 2006). The origin of ChrII is localized at the cell centre and is reported to move independently of ChrI to the quarter positions of the cell after replication (Fiebig *et al.*, 2006; Fogel & Waldor, 2005). Thus, the segregation of the two chromosomes seems to be by separate mechanisms.

For *V. cholerae* cells grown in glycerol medium ($\tau=107$ min), our results indicate that pairs of replication forks co-localize. Since short cells in early C phase (with replication of ChrI alone) contain one SeqA focus, and longer cells in late C (with replication of both chromosomes) two foci, replication forks originating from the same origin are probably co-localized. The indication that the two ChrI forks are localized separately from the two ChrII forks supports the notion that the two chromosomes segregate by separate mechanisms. For cells grown in fructose medium ($\tau=46$ min), we observed in addition a few cells with three foci, which may suggest that the co-localization is somewhat flexible. The sorting confirmed that the cells with three foci contained a DNA amount corresponding to late C phase, which supports the finding that replication of ChrII occurs during the latter part of C. Many cells sorted from late C contained only one SeqA focus. This may have been caused by co-localization of all of the replication forks. Due to biological variation some cells sorted from the late C-phase DNA interval may also actually be early C- or D-phase cells. Note that the resolution of the microscope allows a determination of co-localization within a distance of not less than 200 nm. Thus, it is not clear whether the single focus represents one or several separate SeqA structures at this resolution.

Further co-localization of replication forks during segregation of multi-fork chromosomes

In *E. coli* it has been found that the SeqA protein may be involved in the organization of new DNA at the replication forks (Fossum *et al.*, 2007; Hiraga *et al.*, 1998; Molina & Skarstad, 2004). The structures formed by SeqA are largely independent of the exact numbers of replication forks, and seem to be present in numbers roughly proportional to cell size (Morigen *et al.*, 2009; Odsbu *et al.*, 2009). In cells with multi-forked chromosomes, it has been suggested that new replication forks latch onto existing SeqA structures, and are

thus confined to the correct cell half (Morigen *et al.*, 2009). Our findings of limited numbers of SeqA foci during rapid growth may support the idea of both separate localization and segregation of the two *V. cholerae* chromosomes, and an extensive co-localization of the forks on the same multi-fork chromosome. The fact that we only found cells with 2+2 or 4+4 chromosomes after drug treatment may be a consequence of regulatory mechanisms that ensure that matching numbers of the two chromosomes are replicated. This result is compatible with separate mechanisms for segregation of ChrI and ChrII.

In *E. coli*, deletion of the *seqA* gene leads to reinitiation at already initiated origins, changes in the superhelicity and organization of the chromosome, and a reduced growth rate in rich medium (Lu *et al.*, 1994; Waldminghaus & Skarstad, 2009; Weitao *et al.*, 2000). In *V. cholerae*, it has been reported that a *seqA* null mutant cannot be constructed, indicating that the SeqA protein is essential and thus more important for this organism than it is for *E. coli* (Egan & Waldor, 2003; Saint-Dic *et al.*, 2008). Even though a *seqA* null mutant has since been studied, showing that under certain conditions loss of SeqA can be tolerated (Demarre & Chatteraj, 2010), the SeqA protein may still be more important in *V. cholerae* than in *E. coli*. It is possible that because of the two-chromosome organization of the *V. cholerae* genome, this organism has a requirement for a more extensive origin sequestration or a more advanced degree of fork organization.

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