Chromosome replication dynamics in the archaeon *Sulfolobus acidocaldarius*

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The “baby machine” provides a means of generating synchronized cultures of minimally perturbed cells. We describe the use of this technique to establish the key cell-cycle parameters of hyperthermophilic archaea of the genus *Sulfolobus*. The 3 DNA replication origins of *Sulfolobus acidocaldarius* were mapped by 2D gel analysis to near 0 (*oriC2*), 579 (*oriC1*), and 1,197 kb (*oriC3*) on the 2,226-kb circular genome, and we present a direct demonstration of their activity within the first few minutes of a synchronous cell cycle. We also detected X-shaped DNA molecules at the origins in log-phase cells, but these were not directly associated with replication initiation or ongoing chromosome replication in synchronized cells. Whole-genome marker frequency analyses of both synchronous and log-phase cultures showed that origin utilization was close to 100% for all 3 origins per round of replication. However, *oriC2* was activated slightly later on average compared with *oriC1* and *oriC3*. The DNA replication forks moved bidirectionally away from each origin at ≈ 88 bp per second in synchronous culture. Analysis of the 3 *Orc1/Cdc6* initiator proteins showed a uniformity of cellular abundance and origin binding throughout the cell cycle. In contrast, although levels of the MCM helicase were constant across the cell cycle, its origin localization was regulated, because it was strongly enriched at all 3 origins in early S phase.

Archaea | cell cycle | DNA replication

During initiation of chromosome replication, initiator proteins bind to and act on DNA replication origins to control DNA unwinding for new DNA synthesis. The genomes of the hyperthermophilic archaeal genus *Sulfolobus* each encode 3 initiator proteins: *Orc1*-1, -2, and -3 (also known as *Cdc6*-1, -2, and -3). Like most archaeal proteins involved in DNA replication, the *Orc1* proteins are homologous to their eukaryotic counterparts, *Orc1* and *Cdc6*. In *Sulfolobus solfataricus*, there are 3 specific origins of replication located around the ≈ 3 -Mb single circular genome, and the 3 *Orc1* proteins bind with distinct patterns and affinities to the different origins (1–3). The *Orc1* proteins contain AAA+ domains, suggesting they probably remodel origin DNA during their ATPase cycle (4). However, almost nothing is known about how the origins are regulated.

Sulfolobus genomes also contain a gene encoding a homolog of eukaryotic MCM2–7, another AAA+ domain protein involved in the initiation and elongation stages of DNA replication. MCM2–7 is thought to be the central component of the helicase assembly that unwinds DNA for ongoing DNA synthesis. Biochemical assays with purified recombinant protein have demonstrated that *Sulfolobus* MCM forms a homo-hexamers and has ATP-dependent helicase activity in vitro (5). However, it is still not known whether *Sulfolobus* MCM functions at replication origins and acts as the replicative helicase in vivo.

Chromosome replication in *Sulfolobus* initiates very soon after the preceding cell division, to give a very short G₁ phase of $\leq 5\%$ of the generation time (6). Once chromosome replication is complete (normally taking ≈ 35 – 45% of the cell cycle), a relatively long G₂ phase persists until chromosome segregation and cytokinesis complete cell duplication (2, 6, 7).

The above parameters were established by using asynchronous cultures, but the ability to synchronize the growth and division cycle of a population of cells is essential for further studies of chromosome replication and cell cycle. A synchronization method for *Sulfolobus acidocaldarius* was previously described that involves an initial treatment of a midlog-phase culture with 3 mM acetate (2). Cells stop growth and accumulate with a 2N genomic content, suggesting a G₂ or stationary phase-like arrested state (2, 6). Upon “release” of the cells by resuspension in fresh growth medium, a fraction of the cells resume growth, resulting in a broad wave of cell division and subsequent chromosome replication very reminiscent of the partially synchronous growth out from stationary phase (8). Although such “arrest and release” techniques are simple to do and can be of use for certain experimental approaches, they frequently cause ambiguous or artifactual results, because a cellular response may be the direct consequence of the treatment or an incomplete cell cycle arrest (at the subcellular level), rather than normal cell cycle progression (see refs. 9–11).

To synchronize cells at the start of G₁ phase and to avoid the problems associated with arrest and release, we adapted the membrane elution or “baby machine” synchronization technique for high temperature use with *Sulfolobus*. In the baby machine, an asynchronously dividing culture of cells bound to the surface of a membrane is continually perfused with medium. As each cell divides, 1 daughter cell remains bound to the membrane and the other is shed into the liquid medium (12). The liquid eluate thus contains early-G₁ synchronized cells that have a very high degree of synchrony and are physiologically minimally perturbed (see refs. 13 and 14). We have used the technique to study replication origin usage during the early stages of chromosome replication in *S. acidocaldarius*, and we have measured the relative cellular levels of the above initiator proteins and their binding to replication origins throughout the cell cycle. Our findings offer new insights into chromosome replication and the cell cycle in the archaea and open the door to unambiguous cell cycle analyses in this domain of life.

Results

Cell-Cycle Synchronization of *S. acidocaldarius*. Each baby machine experiment was initiated by filtering midlog phase cells onto a membrane. The membrane was then inverted, and medium pumped through to culture the cells on the lower surface. Fig. 1A shows cell concentrations in the effluent in 3 independent experiments initiated with different cell numbers. Overall yields of cells in the effluent did not greatly increase when $>10^{10}$

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Fig. 1. Baby machine synchronization of *S. acidocaldarius*. (A) Cell numbers eluting from the baby machine were recorded with a Coulter counter. Three independent experiments were initiated with either 2.5×10^9 (square), 5×10^9 (filled circle), or 1.5×10^{10} (triangle) midlog-phase cells. The vertical dashed line and the gray region indicate the optimal time and acceptable time period, respectively, for collection. (B) Coulter cell size distributions for asynchronous log phase and synchronized cell populations. (C) Growth of a synchronized culture was monitored by using a Coulter cell counter/sizer. The cell concentration (filled square) and mean cell diameter (square) are plotted over time. (D) DNA content analysis of cells in the culture in C. Samples were withdrawn at the indicated time-points (min) and analyzed by flow cytometry. The relative DNA content of cells in the G₁, S, and G₂ phases of the cell cycle are indicated in the log phase prebaby machine sample (Top).

starting cells were used, and the purity of synchronized cells, containing ≈ 1 chromosome equivalent, was generally lower when compared with subsaturating experiments. During the early stages of elution the cell concentration declined (Fig. 1A), and we observed a relatively poor, but steadily improving, enrichment of newborn cells (data not shown). After 60–120 min, the cell concentration then steadily increased (Fig. 1A), and there was significant improvement in the selection for newborn cells. The maximum purity of newborn cells was achieved after ≈ 180 min, as judged by analysis of cell size (Fig. 1B) and cellular DNA content (Fig. 1D Upper). The proportion of newborn cells in the population was typically $\approx 70\%$ (at 180 min), and the modal fluorescence value of the sharp G₁ peak in the DNA content distribution was 49% of that for the G₂ peak derived from the smaller population of contaminating asynchronous cells (Fig. 1D, 0 min). We conclude that the baby machine produces sharply synchronized cells of *S. acidocaldarius*.

Synchronized cells collected from the baby machine were

grown at 75 °C in a rotary water bath, and the cell concentration, cell size distribution and DNA content distribution were recorded from samples withdrawn at intervals (Fig. 1C and D). The results of these time courses were highly reproducible. The mean cell diameter began to increase immediately after the start of the incubation, and it increased linearly throughout the S-phase and the early G₂ phase of the first synchronous cell cycle (Fig. 1C). The first round of DNA replication (S phase) also started very soon after the experiment was started, with significant progression into S-phase clearly evident at the first time-point (15 min), and it took ≈ 90 min to complete (Fig. 1D). At 135 min, the first cells to divide were becoming evident, because there was both a downturn in the mean cell size of the population (Fig. 1C), and a reappearance of cells with a G₁ DNA-content (Fig. 1D). The midpoint of the first wave of cell division of the population was ≈ 180 min. A second wave of chromosome replication was also in progress in many cells by the 180-min stage, because replication initiates around the time of cytokinesis (Fig. 1D). Flow cytometry analysis of longer-term incubated cultures indicated that at least 3 successively poorer quality synchronous rounds of DNA replication were detectable (data not shown), and the culture eventually reached a stationary phase of normal cell density.

Mapping *S. acidocaldarius* Origins of Replication. Three replication origins in *S. acidocaldarius* were previously localized to 3 ≈ 50 -kb regions around the 2.2-Mb circular genome (2). To examine timing of replication origin usage during initiation of chromosome replication by using baby machine synchronization, we first sought to map the replication origins to a greater accuracy. The replication origins were located by sequence homology to the precisely known *S. solfataricus* origins (1, 3, 15). Samples of total cellular DNA extracted from a midlog phase culture embedded in agarose plugs were digested with restriction enzymes to give fragments that contained each of the putative origins within ≈ 4 –7 kb fragments. DNA replication intermediates were analyzed by 2D gel electrophoresis and Southern blot analysis by using probes for the putative origin loci; and for 1 control locus near the expected terminus region (Fig. 2A). Three different types of branched DNA structures were identified (Fig. 2A). First, the classical “bubble arc” that is representative of DNA fragments in various stages of DNA replication after initiation from an origin within the fragment (see ref. 16) was clearly present for the fragments located at 0 (*oriC2*), 579 (*oriC1*), and 1,197 kb (*oriC3*) but not for the control locus at 1,727 kb. Second, all 4 Southern blots displayed the common “fork arc,” representing Y-shaped molecules with a single replication fork at various locations in the population of fragments. Third, the origin-containing fragments displayed X-shaped molecules (the “X-spike” on the 2D pattern), consisting of 2 unit-length DNA segments linked together at various positions.

Timing of Replication Origin Firing During Synchronous Growth. We next sought to analyze replication origin activity during the cell cycle. Samples were withdrawn from a synchronized culture after 0-, 30-, 60-, and 90-min incubation, and DNA was analyzed by 2D gel electrophoresis and Southern blot analysis with a probe for *oriC1* (Fig. 3A). There was clear origin activity at 0 min, which contains newborn cells (Fig. 1). No origin firing was detected at 30, 60, or 90 min, which suggested that initiation of replication in the synchronous population had ended before 30 min, consistent with the flow cytometry in Fig. 1D. Also, the fork arc was extremely faint at the latter 3 time points, and the X-spike was not detectable in any of the Southern blots. Next, we examined the timing of each origin’s firing at a narrower time window very early in the cell cycle. Samples were collected at 0, 2, 6, and 10 min after starting growth of a baby machine synchronized culture, and all 3 origins were analyzed by 2D gel electrophoresis

Fig. 4. Whole-genome MFA of chromosome replication dynamics in *S. acidocaldarius*. The normalized ratios of replicating to stationary phase DNA hybridization signals are plotted against chromosome position (kb). (A) Cells were collected at the indicated synchronous growth times (six independent experiments) for MFA by microarray hybridization. (B) Data obtained from analysis of an asynchronous midlog phase culture ($A_{600} = 0.24$). The difference in frequency between *oriC2* and the other 2 origins was seen consistently in 3 independent experiments. (C) Simulations of log-phase MFA data with a fractional S phase length of 0.45 (determined from flow cytometry analysis of the culture used for the experiment shown in B). The dashed gray line simulates 100% usage of all 3 origins, the solid black line simulates 100% usage of *oriC1* and *oriC3* and 90% usage of *oriC2*, whereas the solid gray line simulates 80% usage of all 3 origins per round of replication.

chromosomes at later stages because of continual cell/chromosome duplication in the population). A good correspondence between the simulation and the log-phase data were achieved when probabilities of 100% usage for *oriC1* and *oriC3* and 90% usage for *oriC2* were simulated (compare Fig. 4B with C). Importantly, this differential usage effect could have been caused by a slightly later average replication of *oriC2*, rather than a reduced probability of firing per round of replication as modeled in Fig. 4C, the log phase data alone do not allow such a distinction to be drawn. However, a comparison of the relative frequency of the origin regions during synchronous growth reveals that the frequency of *oriC2* reaches that of *oriC1* and *oriC3* later in the S phase (Fig. 4A). We conclude that *oriC2* has a slightly later average replication than *oriC1* and *oriC3*, and that it too would have close to 100% efficiency when considered at the level of the whole cell cycle.

Levels of Initiator Proteins Throughout the Cell Cycle. To begin to study the regulation of origin activity, we wished to determine

Fig. 5. Orc1-1, -2, -3, and MCM protein levels do not fluctuate significantly during the cell cycle. Baby machine synchronized cells were analyzed by Western blot analysis after samples were withdrawn from a culture at the indicated time-points (min), washed, and then cell density was normalized to equivalent absorbencies (600 nm). Blots were reprobed by using anti-TATA-box-binding protein (TBP) anti-serum as a loading control.

whether there were cell-cycle regulated protein level fluctuations of the candidate replication initiator proteins Orc1-1, -2, 1–3, and MCM. We withdrew samples from a synchronized culture at regular time intervals and analyzed the relative levels of the 4 proteins by using quantitative Western blot analysis. As can be seen in Fig. 5, the levels of these 4 proteins were uniform throughout the cell cycle and were thus proportional to culture biomass (as measured by A_{600}), and not cell cycle position. Replicate experiments using cells collected from the baby machine for a 15-min period at 75 °C before incubation, rather than the normal collection on ice, gave the same results (data not shown).

Association of Initiator Proteins with Replication Origins During the Cell Cycle. To determine whether there were changes in the levels of binding of Orc1-1, -2, -3, and MCM to each of the origins during different cell-cycle phases in vivo, we carried out ChIP assays, in which affinity-purified antibodies were used to isolate specific protein–DNA complexes from extracts of formaldehyde-treated cells. The relative amount of each origin bound to each protein was then measured by quantitative PCR (qPCR). We observed clear binding patterns for each of the 4 proteins to the different origins in asynchronous log-phase cells, as described below (Fig. 6A). MCM was clearly detected at all 3 replication origins but was detected only at very low levels at the control locus, *lrs14* (≈ 70 kb from *oriC2*). The amount of DNA recovered from the MCM ChIP varied for the 3 origins, although influencing this would be both the stoichiometry and affinity of the various MCM–origin complexes and possible technical factors such as potential differences in antibody accessibility to epitopes and protein–DNA cross-linking efficiency in the various protein–origin complexes.

The Orc1-1 ChIP reaction in Fig. 6A showed clear binding of *oriC1*, but, unexpectedly, the levels of *oriC2* and *oriC3* recovered were very low. In the Orc1-2 ChIP reaction, the amount of origin DNA recovered was only marginally above background for all 3 origins. Orc1-3 clearly bound *oriC2* (Fig. 6A), but only very low levels of the other 2 origins were detected. Western blot analysis experiments confirmed that all 4 proteins were efficiently recovered in these ChIP reactions, and, in a further control for the Orc1-2 ChIP, we detected significant binding around the *orc1-2* gene promoter region, consistent with previous findings in *S. solfataricus* (S.D.B., unpublished results) and demonstrating that Orc1-2 copurified with at least 1 expected locus. Finally, we found that the *mcm* gene was not detected significantly above background in the MCM ChIP reaction, ruling out coupled transcription/translation as a general factor contributing to DNA recovery (data not shown). We suspect that the failure to detect several expected Orc1 interactions that have been observed at the homologous *S. solfataricus* origins by ChIP, footprinting, and in a corresponding X-ray crystal structure of an *oriC2*/Orc1-1/

Orc1–3 subcomplex (1, 3, 4) may reflect a detection limitation of the ChIP reported here. It is clear that the highest affinity interactions seen in *S. solfataricus* (1) were those clearly detected in the present ChIP study of the *S. acidocaldarius* homologs. However, we cannot formally exclude the possibility that the origin binding patterns are significantly different in these 2 species. Nevertheless, our aim in the present study was to determine whether the detected binding patterns varied throughout the cell cycle, as described below.

The results of ChIP experiments using cells collected at 0 (G_1), 30 (S), and 110 min (G_2) during synchronous growth are shown in Fig. 6B. When the binding patterns were compared between the various time points, we observed that both Orc1–1 binding at *oriC1* and Orc1–3 binding at *oriC2* remained bound at a constant level throughout the cell cycle, and the levels of binding were very similar to those seen in the log-phase sample. Significantly, however, MCM levels at all 3 origins were significantly elevated at the G_1 (0 min) time point compared with the asynchronous log-phase situation (see Fig. 6B Bottom), strongly implicating MCM function at the origins during initiation of replication. The levels of MCM dropped significantly after 0 min, with levels detected at the 30 (S) and 110 min (G_2) time points similar to the levels seen for the log-phase sample.

Discussion

In the current work, we describe the use of the baby machine as a means of obtaining a nonchemically perturbed synchronized culture of an archaeon. Previous attempts to synchronize archaea have relied on either DNA replication inhibitors or treatment of cultures with acetate (2, 18, 19). The latter treatment leads to arrest of *Sulfolobus* cells in the G_2 phase of the cell cycle. The mechanism of the G_2 arrest is not yet known (although it may be due to respiration uncoupling; see ref. 20), and thus it is not possible to deconvolute genuine cell cycle effects from acetate-induced perturbations of normal cell physiology. Such ambiguity is well illustrated by the difference between results obtained when measuring levels of Orc1 proteins in baby machine vs. acetate treated cells. We observed in baby machine synchronized cells that Orc1 levels remained constant across the cell cycle (Fig. 5), whereas cells arrested by acetate showed elevated levels of Orc1–2 and low levels of Orc1–1 and Orc1–3 (1); resumption of growth after arrest resulted in elevated Orc1–1 and -3 and greatly reduced Orc1–2. Several laboratories have reported microarray analyses of the response of the *Sulfolobus* transcriptome to a range of physiological stresses such as UV treatment, viral infection, and heat shock (21–24). Interestingly, these studies have revealed modulation of Orc1 gene transcript and/or protein levels; similar to that seen with microarray analysis of acetate-treated cells (25). It seems likely, therefore, that the modulation of Orc1/Cdc6 levels induced by acetate treatment and subsequent resumption of growth reflects a stress response in *Sulfolobus* cells rather than a true cell cycle effect.

The origin occupancy of the Orc1/Cdc6 initiator proteins also did not vary across the cycle, as detected by ChIP (Fig. 6). This is reminiscent of the situation in budding yeast where ORC remains chromatin-associated through the cell cycle and differs from higher eukaryotes where ORC1 levels and localization are controlled during the cell cycle (26). In contrast to the invariance of Orc1 at origins, we observe significant enrichment of MCM at all 3 origins in G_1 and early S-phase cells, in strong agreement with the proposed role of archaeal MCM as the replicative helicase. We note that the ChIP analysis of MCM at *oriC2* yields the greatest recovery of DNA. Although this may be simply due to technical reasons, it is also compatible with our observation that *oriC2* shows delayed kinetics of firing in a significant population of cells. Perhaps this reflects prolonged occupancy of this origin by the MCM protein in prereplicative complexes.

Fig. 6. Interactions of the Orc1–1, Orc1–2, Orc1–3 and MCM proteins with replication origins in vivo. (A) DNA recovered from the indicated chromatin immunoprecipitates from a midlog phase cell extract was analyzed by quantitative PCR by using primer sets for *oriC1*, *oriC2*, *oriC3*, and *lrs14*. The quantified results are displayed in the right-hand panel. (B) Results of reactions using synchronized cell extracts. Quantified data for the MCM ChIP reactions are shown in Bottom.

2D gel analysis of DNA replication intermediates from synchronized cells demonstrated that activation of all 3 replication origins occurs very early in the cell cycle, as predicted based on flow cytometry analysis of asynchronous cultures (6). Our analyses also identified important differences between the DNA replication intermediates detected at origins in log-phase vs. synchronized cells. In addition to replication initiation “bubbles” at origins, we clearly detected a similar abundance of Y- and X-shaped molecules in log-phase *S. acidocaldarius* (Fig. 2). The Y-shaped molecules were strongly associated with initiation of replication and were almost undetectable at *oriC1* in mid and late S phase synchronized cells (Fig. 3A). The presence of Y-shaped molecules during initiation of replication at *S. acidocaldarius* replication origins could be explained by a degree of heterogeneity in the timing of each replication fork’s departure from the origins; the greater relative abundance of the large Y-shaped molecules is consistent with this interpretation (Fig. 3). In contrast, the X-shaped molecules were not detected during initiation of replication in synchronized cultures and were seen only in log-phase samples in which much larger quantities of total DNA were analyzed (Figs. 2A and 3). Thus, the X-shaped molecules seen at replication origins, which appear to be hemicanenanes rather than Holliday junctions in *S. solfataricus* (3) and budding yeast (27) are not directly associated with replication initiation or fork movement in synchronized *S. acidocaldarius*.

Quantitative analyses of chromosome replication dynamics by MFA revealed that, in both log-phase cells and baby machine

synchronized cells, a great majority of cells use all 3 origins in chromosome replication (Fig. 4). The kinetics of firing suggested a degree of coordination, with the majority of firing occurring within a few minutes of cytokinesis. This very tight temporal association between cytokinesis and initiation of chromosome replication suggests there might be a direct link between cytokinesis and the regulation of origin firing. The MFA of asynchronous cells revealed an underrepresentation of *oriC2* sequences compared with *oriC1* and *oriC3*. Interestingly, this phenomenon was apparent in a previous study but was not commented on (2). The underrepresentation of *oriC2* sequences could be due to either a reduced frequency of usage of *oriC2* per round of replication or a later average firing of *oriC2*. Our MFA data from synchronized cells strongly support the latter possibility, because by 60 min, the relative frequency of the 3 origin peaks had equalized. Thus, *oriC2* of *S. acidocaldarius* can be considered a somewhat later-firing origin compared with *oriC1* and *oriC3*.

Materials and Methods

Baby Machine Synchronization of *Sulfolobus*. The baby machine and synchronization technique were similar to those described (13, 14), with modifications as described in [supporting information \(SI\) Materials and Methods](#).

Coulter Counting and Flow Cytometry. Cell counting and sizing were done by using a Multisizer 3 Coulter counter with a 20- μ m aperture tube, calibrated using 2- μ m diameter latex beads (Beckman-Coulter). Cells were diluted 1/100 by using Isoton II diluent (Beckman-Coulter) that had been filtered by using a 0.2- μ m filter unit. Volumetric cell count analysis was carried out within 10 min of dilution to avoid any cell volume changes that occurred after extended periods after dilution. For flow cytometry, cells were fixed with ice-cold

ethanol (70% final concentration) and then prepared as described (21). Approximately 10^5 cells were analyzed to generate DNA content distributions by using a MoFlo cell sorter (Dako), as described (3).

Neutral-Neutral 2D Gel Electrophoresis. 2D gel analysis was performed essentially as described (1), with modifications described in [SI Materials and Methods](#).

Marker Frequency Analysis by Microarray Hybridization. Genomic DNA preparation and labeling, and microarray hybridizations were performed by using standard techniques, with modifications described in [SI Materials and Methods](#). The marker frequency equation was used to generate all marker frequency simulations (equation 4 in ref. 17), as detailed in [SI Materials and Methods](#).

Analysis of Protein Levels by Western Blot Analysis. Cells from 5 mL of culture were washed with 1 mL of PBS then resuspended in 0.1 mL of PBS, and then the A_{600} was measured and the sample stored at -20°C . Thawed samples were diluted with PBS as necessary to give equivalent A_{600} values for all samples and were then analyzed by SDS/PAGE and Western blot analysis by using standard techniques and enhanced chemiluminescence (Amersham Biosciences) detection with preflashed autoradiography film.

Chromatin Immunoprecipitation and qPCR. Formaldehyde cross-linked cell extracts were prepared, and immunoprecipitation reactions were carried out essentially as described (1), with modifications described in [SI Materials and Methods](#).

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