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Quorum-sensing acts at initiation of chromosomal replication in *Escherichia coli*

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ABSTRACT **Chromosomal replication in *Escherichia coli* was studied by flow cytometry and was found to be inhibited by an extracellular factor present in conditioned media collected during late exponential and early stationary phase, i.e., via a quorum-sensing mechanism. Our results suggest that the inhibitory activity of the extracellular factor is exerted during initiation of DNA replication rather than during elongation. Furthermore, we present evidence that this interaction may occur directly at each of the replication forks. Unlike other quorum-sensing systems described so far for Gram-negative bacteria, this inhibitory activity does not require transcription or translation to be effective. Implications of quorum-sensing regulation of DNA replication are discussed.**

Bacteria have been shown to utilize diffusible extracellular factors to regulate transcription of specific genes, thereby altering the phenotypic characteristics of the population when it has attained a cell-density threshold (1, 2). Quorum-sensing is known to regulate a diverse range of physiological processes including bioluminescence (3), antibiotic biosynthesis (4), virulence determinants (5), and plasmid conjugal transfer (6). In Gram-negative bacteria, many of these factors or autoinducers belong to the *N*-acylhomoserine lactone (N-AHL) family of compounds, which vary in the composition and length of the acyl group (1, 2). This type of cell-to-cell communication or quorum-sensing allows an individual bacterium to monitor its own population density and possibly the total bacterial population density in its environment and regulate specific gene expression accordingly. In *Vibrio harveyi*, for example, two independent, quorum-sensing systems regulate bioluminescence. The first of these systems (signaling system 1), regulated by a homoserine lactone derivative (AI-1), is species-specific, whereas the second (signaling system 2) is non-species-specific and regulated by an as-yet-unidentified signal molecule (AI-2; ref. 7). Both *Escherichia coli* and *Salmonella typhimurium* recently have been shown to produce a small soluble organic molecule when grown in the presence of glucose, which is involved in intercellular communication during pre-stationary-phase growth (8). In this case, the signaling molecule is degraded at the onset of stationary phase or upon depletion of glucose and can interact with signaling system 2 from *V. harveyi*. Quorum-sensing in *E. coli* also has been implicated in the regulation of one of the promoters of the *ftsQAZ* cluster of cell-division genes (9, 10) and in the expression of the transcriptional regulator SdiA (11). Here we show by flow cytometry that initiation of chromosomal replication is regulated, during late exponential and early stationary phase, by an extracellular factor.

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MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions. All bacteria used in this study were derivatives of *E. coli* K-12, specifically, MG1655 (12). The bacteria were grown in M9 minimal medium (13). Unless otherwise stated, supplements were added at the following concentrations: 0.2% (wt/vol) glucose; 0.5% (wt/vol) Casamino acids; 10 $\mu g \cdot ml^{-1}$ threonine. All strains were grown at 30°C. Any variation from these growth conditions is indicated in the text.

Temperature-Shift Experiments. To obtain cultures of MG1655*dnaC2* that were synchronized for initiation of chromosome replication, overnight cultures were diluted into fresh medium and allowed to grow at permissive temperature (30°C) for at least three generations. Cell concentration was monitored as OD at 600 nm. At an OD_{600} of 0.05 the cultures were shifted to nonpermissive temperature (38°C) for 120 min. The cultures then were transferred to the permissive temperature, either (*i*) for the duration of the experiment or (*ii*) transiently for 10 min and then shifted back to the nonpermissive temperature (38°C).

Preparation of Conditioned Medium (CM). Cells were grown to an OD_{600} of 1.0 in supplemented M9 medium at 30°C. The culture was centrifuged to remove the cells, and the supernatant then was filtered through a 0.2-μm sterile filter. This formed standard CM, which was stored at −20°C until required. Before use, standard CM was concentrated 20× by using a vacuum evaporator (cCM). This then was added to cultures to give either a 1× or 2× concentration.

Flow Cytometry. For flow cytometry (14), 100-μl samples were collected from bacterial cultures, and the cells were fixed by the addition of 1 ml of 77% ice-cold ethanol and stored at 4°C. Before measurements, the cells were centrifuged, washed in 1 ml of 10 mM Tris buffer (pH 7.5) containing 10 mM $MgCl_2$, centrifuged again, and resuspended in 75 μl of the same solution. For DNA staining, an equal volume of stain containing 40 μg of ethidium bromide ml^{-1} and 200 μg of mithramycin A ml^{-1} in Tris/$MgCl_2$ buffer was added to the sample. Sample analysis was carried out on a Bryte-HS flow cytometer (Bio-Rad). The instrument was calibrated with plastic beads (Bio-Rad) with a diameter of 1.5 μm and labeled with the fluorescent coumarin 6 dye. The beads were uniform, with coefficients of variation of <1.2 and $<1.5\%$ for size and fluorescence, respectively.

RESULTS

***E. coli* Produces an Extracellular Factor in Early Stationary Phase That Affects DNA Replication.** During a systematic study of the thermosensitive DNA replication mutant *dnaC2* of *E. coli*, it became apparent that the ability of cells to reinitiate replication after run-out of replication at the non-

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Abbreviations: CM, conditioned medium; cCM, concentrated CM.
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permissive temperature depended on the cell density of the culture. At optical densities greater than 0.4, reinitiation of replication did not occur (15). We now used CM (supernatant prepared from an early stationary phase culture) to determine whether this inability to reinitiate replication was due to the presence of an extracellular factor. Conditioned media traditionally are prepared by growing a culture into stationary phase. The cells were removed by centrifugation, followed by filtration through a 0.2-μm filter. Test cultures were grown into exponential phase and the cells were harvested by centrifugation. These cells were resuspended in CM to which glucose and other supplements had been added. Alternatively, conditioned medium was concentrated by evaporation and then added to the exponentially growing cells at the desired concentration. Although we obtained identical results in both cases, we found that addition of a concentrated extract resulted in less disturbance to the growing culture. The salt could not be removed from the extract by dialysis against deionized water, since the extracellular factor moved with the low molecular weight fraction. Therefore, to compensate for the increase in salts, the cells were grown in supplemented 0.25× M9 minimal medium. All experiments reported here used this procedure.

Flow cytometry can be used to study DNA replication. In this analyses, the DNA is stained, resulting in a fluorescence that is proportional to the content of DNA in each cell; the analysis shows the DNA–content distribution in the population. We used this method to follow the progression of chromosomal replication in a culture synchronized with respect to DNA replication. The bacteria were grown exponentially at 30°C, shifted to nonpermissive temperature (38°C) for 2 hr to allow run-out of replication (as shown by the single 1-chromosome-equivalent peak, Fig. 1 *Left Top*), and then shifted back to permissive temperature. In the control culture, DNA replication began immediately after the downshift to the permissive temperature as seen by the rightward progression of the peak (Fig. 1, solid line). The DNA distribution continued to shift to the right and also to broaden as a second and possibly a third round of replication was initiated. Addition of cCM, to give a final concentration of 2×, to a MG1655*dnaC2* culture that was synchronized for replication, just before the shift to the permissive temperature (30°C) resulted in a transient inhibition of replication as seen in Fig. 1 (dashed line). After 15 min in the presence of cCM, most of the cells remained at the 1-chromosome-equivalent position (Fig. 1; compare 0 and 15 min) with no discernible increase in the DNA content until approximately 30 min. At 30 min, some of the cells had initiated DNA replication as seen by the rightwards broadening in the peak; however, replication in other cells remained inhibited since the left side of the peak remained static as the right expanded. This transient inhibition of replication lasted for approximately 30 min under these conditions. Addition of concentrated fresh medium to a synchronized culture did not affect the restart of DNA replication (data not shown). The inhibitory effect was also concentration-dependent, since the addition of cCM to give a 1× final concentration resulted in a shorter transient inhibition (data not shown).

To determine whether this inhibition in DNA replication was specific to cCM isolated from stationary-phase culture, CM was also prepared from an exponentially growing culture at an OD_{600} of 0.05 and 0.1. In both cases, when added to give a 1× concentration to synchronized MG1655*dnaC2*, no effect on DNA replication was detected (data not shown). When added at a 2× concentration, a lower level of inhibition of replication was observed with conditioned medium isolated at OD_{600} 0.1, suggesting that the extracellular factor also may be present at low levels during exponential growth (data not shown).

To determine whether this effect on replication was related to the mutation in the *dnaC* gene, which caused the temperature-sensitive phenotype, we added cCM to give a 2× concentration to an exponentially growing MG1655 culture (Fig. 2). At the time of addition the OD_{600} of the culture was 0.1. Samples were taken every 10 min from the time of addition for 1 hr. Addition of cCM to the culture caused a dramatic change in the DNA content of the population. After only 10 min, a major peak began to resolve at the left-hand side of the main exponential population, at approximately the 2-chromosome-equivalent position (Fig. 2*B*). This resolution continued until the majority of the population contained 2 chromosome-equivalents at around 40 min. At this stage a small subpopulation of cells were able to restart replication as seen by the broadening at the base of the left-hand peak. Sixty minutes after the addition of cCM, the culture had returned to a DNA content characteristic of an exponentially growing population. The presence of a quorum-sensing autoinducer produced by the test culture itself was observed in the latter stages of the experiment (Fig. 2*A*). At approximately 50 min, a change in the DNA content profile of the control culture was observed, suggesting a slowing down in the rate of initiation of replication. Although there were observable changes in the amount of DNA, the growth rates of cultures with and without cCM were the same over the initial 20-min period of the experiment when the changes in initiation of DNA replication were observed (Fig. 3). After this period a drop in growth rate was observed for the cultures containing cCM. cCM prepared from cultures that do not produce the extracellular factor also had the same effect on growth (data not shown). In conclusion, our data suggest that the inhibition in DNA replication was

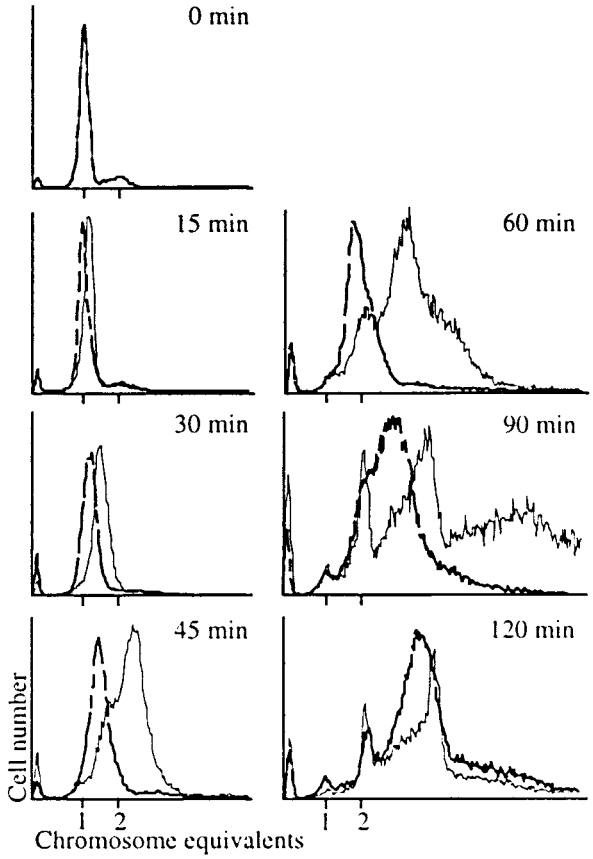

FIG. 1. MG1655*dnaC2* was synchronized for replication by incubation at nonpermissive temperature. At 0 min the culture was split into two and either no addition (solid line) was made or 1 ml of cCM per 10 ml of culture (dashed line) was added before the cultures were shifted to the permissive temperature. Samples were taken for flow cytometry at the times indicated.

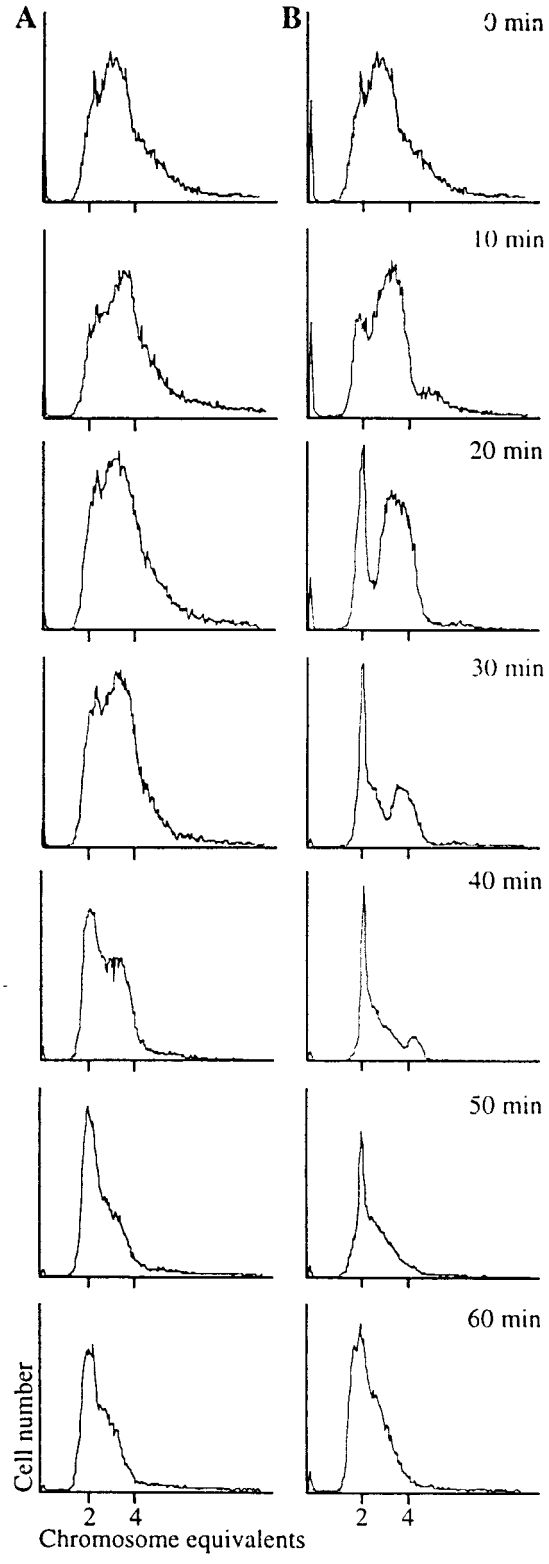

FIG. 2. MG1655 was grown at 37°C to an OD_{600} of 0.1. The culture was split into two and either no addition *(A)* was made or 1 ml of cCM per 10 ml of culture *(B)* was added. Samples were taken for flow cytometry at the times indicated.

occurring during the initiation process rather than later, during ongoing replication, since distinct peaks were resolved.

Restart of DNA Replication in the Presence of cCM After a Transient Shift to Permissive Temperature. To determine whether inhibition was occurring during initiation of replication rather than during elongation, a transient shift experiment was

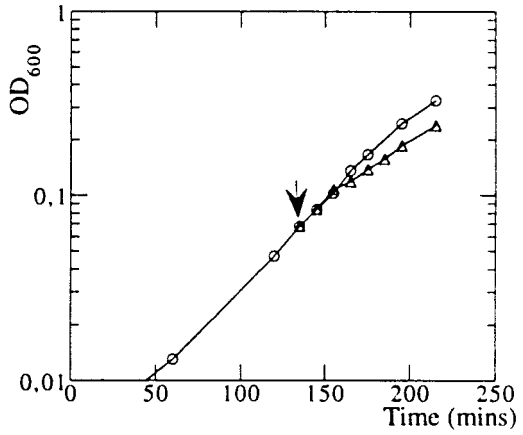

FIG. 3. MG1655 was grown in M9 medium supplemented with glucose and Casamino acids at 37°C to the indicated OD (↓), whereupon the culture was split into two. Either 1 ml of sterile distilled water (○) or 1 ml of cCM (△) per 10 ml of culture was added. Samples were taken at regular intervals throughout the experiment, and the OD was measured at 600 nm.

carried out using replication-synchronized MG1655*dnaC2*. In this experiment, the culture was synchronized for replication by a 2-hr incubation at the nonpermissive temperature (38°C). The culture was then shifted to the permissive temperature (30°C) for 10 min before being returned to the nonpermissive temperature. This allowed a single round of DNA replication to be initiated (15). The left-hand column of Fig. 4*A* shows the normal DNA profile of a transient shift, with the single peak moving from the 1-chromosome-equivalent position to the 2-chromosome-equivalent position over a period of approximately 60 min. The subsequent build-up of the cells in the leftmost peak was due to cell division, which began to be detectable at 60 min and continued to increase until 120 min, where a maximum level was reached. Addition of cCM just before the transient shift caused complete inhibition of replication in approximately 50% of the population as seen by the number of cells that remained at the 1-chromosome-equivalent position during the entire experiment (Fig. 4*A Right*). The remaining 50% were able to initiate replication, with approximately 50% of those moving to the 2-chromosome-equivalent position over a period of 60 min. The remaining 25% of cells were located at a position corresponding to a DNA content of 1.5 chromosome equivalents. This profile remained the same from 45–120 min, with a small decrease in the 2-chromosome-equivalent peak at 120 min, probably due to cell division. This result would suggest that there were three possible scenarios that could occur at the shift to permissive temperature in the presence of cCM: no initiation occurred (cells in Peak 1), initiation occurred but only one fork proceeded to the terminus region where it was blocked (cells in Peak 2), and, finally, initiation occurred with both replication forks and proceeded to completion (cells in Peak 3). If this is indeed the case, a strain deleted for the *tus* gene should have either 1 or 2 chromosome equivalents per cell since those cells containing a single replication fork (those that formed Peak 2 of the Tus^+ strain above) should continue through the terminus region to complete replication (16). Fig. 4*B* shows that Peak 2 was present at 60 min but had disappeared by 90 min, consistent with a unidirectional replication fork moving through the terminus region unimpeded, and continuing around the chromosome to finally generate two complete chromosomes (120 min). The fact that a 1.5-chromosome-equivalent peak was detectable in a Tus^+ strain is consistent with there being a window of opportunity for initiating replication (in this case limited to the transient shift to permissive temperature), with first a single active replication fork followed after a discrete time interval by the second. It is also worth noting that the ratio between cells containing 1 and 2 chromosome

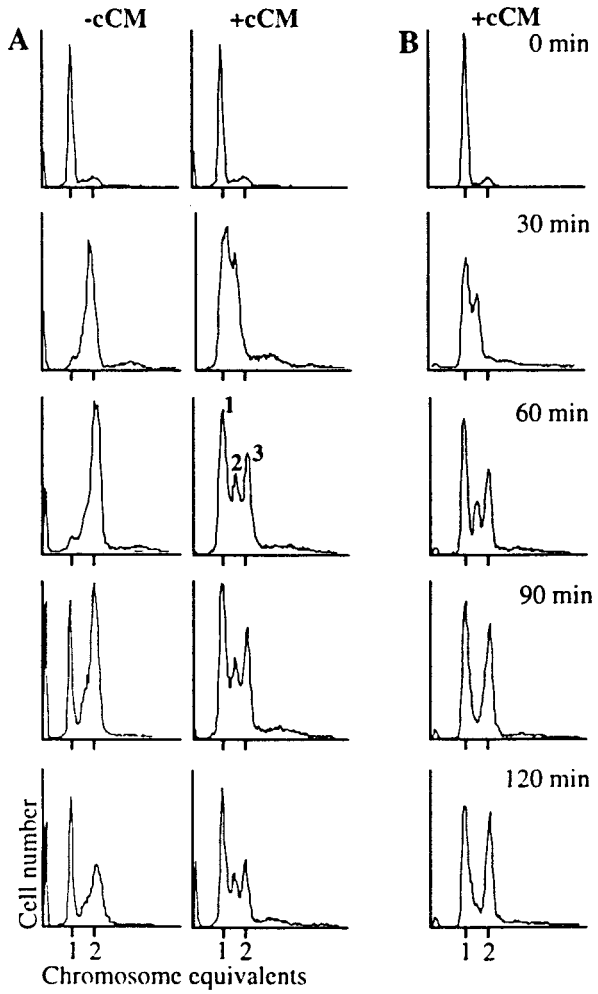

FIG. 4. MG1655*dnaC2 (A)* and MG1655*dnaC2tus (B)* were synchronized for replication by incubation at nonpermissive temperature. Both cultures were handled as follows. At 0 min the cultures were split into equal portions and either no addition was made or cCM was added to a final concentration of 2×. All cultures then were shifted to the permissive temperature for 10 min before being returned to the nonpermissive temperature for the duration of the experiment. Peaks 1, 2, and 3 referred to in the text are indicated on graphs where appropriate. Samples were taken for flow cytometry at the times indicated.

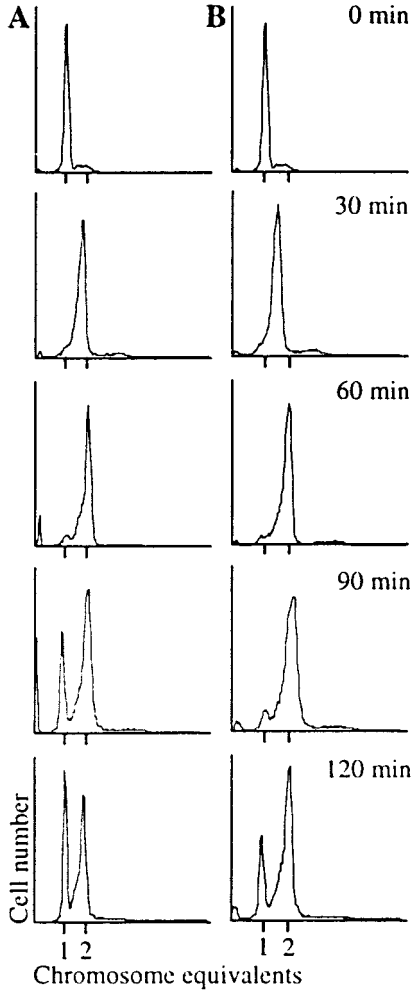

FIG. 5. MG1655*dnaC2* was synchronized by incubation at the nonpermissive temperature. At 0 min, the culture was divided into two equal parts. Either no addition (*A*) or cCM was added to a final concentration of 2× after the cultures were shifted to the permissive temperature (30°C) for 10 min and returned to the nonpermissive temperature (38°C) for a further 5-min incubation (*B*). Samples were taken for flow cytometry at the times indicated.

equivalents observed at 90 min remained the same at 120 min, suggesting that there was little or no cell division occurring in cultures that cCM had been added to.

Extracellular Factor Does Not Affect Replication Elongation. To confirm that the observed block in replication is at the point of initiation and does not impede elongation, a transient shift experiment was performed as described previously, but cCM was added 15 min after the initial shift to the permissive temperature. Withers and Bernander (15) showed that 84–88% of the cells will initiate replication during the 10 min spent at the permissive temperature. If the block is at initiation rather than in the early stages of elongation, all the cells would initiate replication and proceed to completion when the cCM is added after the transient shift. If, on the other hand, replication is blocked during elongation, replication would cease upon addition of cCM. Fig. 5*B* shows that the first scenario was true since the majority of the cells (84–88%) were able to replicate their DNA to completion when cCM was added 15 min after the downshift to the permissive temperature (0 min), as compared with 25% when cCM was added at the downshift to the permissive temperature (0 min, Fig. 4*B*). Although there was no detectable difference in DNA replication, the amount of cell division was lower in those cells that had been exposed to cCM. The level of cell division observed after 90 and 120 min in the presence of cCM (Fig. 5*B*) was equivalent to that observed at 60 and 90 min in the control, respectively (Fig. 5*A*).

Control of DNA Replication Via Quorum Sensing Does Not Require Transcription or Translation. It has been shown previously that reinitiation of DNA replication in MG1655*dnaC2* after synchronization does not initially require transcription or translation (15). Chloramphenicol and rifampin can be added to a culture to inhibit the initiation of chromosomal replication by inhibiting protein synthesis and transcription, respectively. Additionally, cephalexin is added to the cultures to block cell division during the run-out period. After the run-out period, the DNA content is measured by flow cytometry, where the number of cells that have been able to initiate replication can be calculated from change in the relative size of the peaks in the flow cytogram. To determine whether RNA synthesis was required for inhibition of reinitiation of replication after incubation at the nonpermissive temperature, rifampin and cephalexin (300 and 10 $\mu g \cdot ml^{-1}$, respectively) were added to the culture either with or without cCM at 2× concentration. Aliquots were then shifted to the

permissive temperature at different times after the addition of rifampin. All shifted aliquots were incubated a further 120 min at 30°C before fixation for flow cytometry. In a culture shifted immediately to 30°C in the presence of rifampin, 80–85% of cells had moved to the 2-chromosome-equivalent position, confirming that under these conditions the majority of cells competent to initiate replication did so (Fig. 6*A Upper Center*; ref. 15). However, in the presence of cCM (plus rifampin and cephalexin), only 35–38% moved to the 2-chromosome position, indicating that most cells were unable to initiate replication upon shift to the permissive temperature. In cultures that had been incubated for a further 15 min at 38°C in the presence of rifampin, before the shift to 30°C, most of the cells were unable to initiate replication probably because of an increased requirement for transcription (Fig. 6*A Upper*). When cCM was added, there was a further decrease in the number of cells that were able to initiate replication.

To determine whether protein synthesis was required for inhibition of the initiation of replication in MG1655*dnaC2*, chloramphenicol and cephalexin (200 μg and 10 μg·ml^{-1}, respectively) were added before the shift to the permissive temperature. Under these conditions, the majority of cells were able to initiate at least one round of replication, with many initiating a second round as seen by cells that contained either 3 or 4 chromosome equivalents after run-out (Fig. 6*B Upper Center*). In the presence of cCM (chloramphenicol and cephalexin), approximately 38% of the cells were inhibited for initiation of replication as compared with 4% in absence of cCM. The remaining cells initiated replication, most reaching the 2-chromosome-equivalent position. Some cells that had initiated replication did not complete elongation, probably because of the lack of protein synthesis as seen by the slope on the left-hand side of the second peak. As had been observed previously (15), protein synthesis is required during elongation in a *dnaC2* mutant, particularly in those cells that contain more than 2 chromosome equivalents, but not for reinitiation of replication.

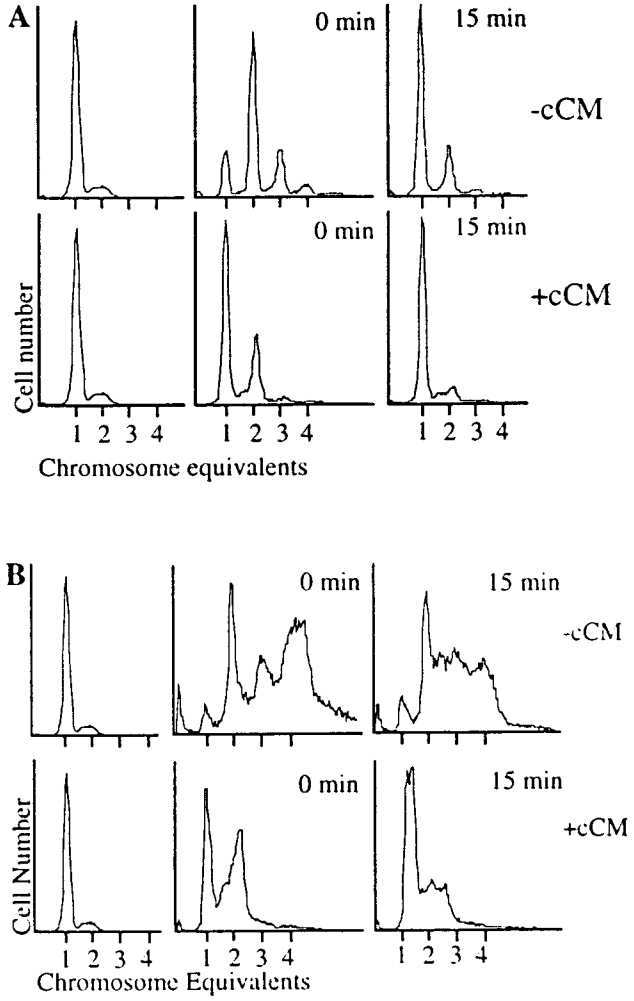

FIG. 6. Analysis of transcriptional and translational requirements for inhibition of DNA replication by an extracellular factor. MG1655*dnaC2* was synchronized at 38°C for 120 min. *(A)* After synchronization the culture was subdivided, and rifampin and cephalexin were added to both. At the same time cCM was added to one aliquot (*Lower*) to a final concentration of 2×; the other formed the control (*Upper*). In both cases, aliquots were incubated at 38°C for 0 or 15 min before being shifted to 30°C and incubated for a further 120 min before sampling for flow cytometry. *(B)* Same as for *A* except that chloramphenicol was added instead of rifampin.

In cells that had been incubated for a further 15 min at 38°C before the shift to 30°C (Fig. 6*B Right*), many of the cells in the presence of cCM appeared to be inhibited for initiation of replication, shown by the cells remaining in the 1-chromosome-equivalent position. However, this peak was notably broader than that observed after 0 min at 38°C, suggesting that initiation may have occurred in some of these cells but these cells had been rapidly blocked for elongation probably because of the presence of chloramphenicol. It therefore was not possible to determine whether there was a definite increase in the level of inhibition of reinitiation of replication.

In conclusion, these data suggested that neither transcription nor translation was required for the inhibitory activity of the extracellular factor.

DISCUSSION

E. coli is the first prokaryote in which fundamental cell processes have been shown to be regulated by a quorum-sensing mechanism. In this report, we have shown that chromosomal DNA replication is negatively influenced by the presence of an extracellular factor. Analysis of CM made at different stages through the growth cycle indicated that it was at a sufficient concentration to inhibit DNA replication at late exponential into early stationary with a decrease in concentration during late stationary phase (data not shown). In this respect, this quorum-sensing system may differ from that described by Surette and Bassler (8), which was maximal during mid-exponential phase. Data gathered from experiments that utilized cCM from different stages of the growth cycle suggest that there was a low level of extracellular factor present at all stages that was below the critical threshold for inhibition of DNA replication (data not shown).

The effect on initiation of DNA replication was transient, since replication could resume in most cells after approximately 30 min of exposure to cCM (Fig. 1). This transient effect is probably because there are insufficient cells to maintain the extracellular factor concentration above the required threshold, thus releasing the block. This combined with its degradation or inactivation by interaction with its target would provide a means for rapid reinitiation under changing environmental conditions. The transient nature of the replication block would be physiologically important, because if it was too prolonged the cells would not be able to respond quickly to changes in their environment. In a complex, competitive environment, it would be detrimental for the cells to be delayed in the restart of DNA replication by first having to degrade an inhibitor. This would make the bacteria less fit and they would be quickly out-competed. There is evidence in most experiments presented here that not all cells were blocked for initiation of DNA replication, suggesting that the threshold concentration of extracellular factor is not maintained under the experimental conditions used presumably because of insufficient cell density (Figs. 1, 4, and 6). The intracellular

concentration could vary from cell to cell; initially, it would be high in all cells, but as it is degraded or inactivated the concentration would decrease, allowing replication to initiate. The time it would take for this to occur probably would vary from cell to cell, and, therefore, initiation would be staggered throughout the population (Fig. 1). Those above the threshold would be inhibited while those below may have the opportunity to initiate replication.

We have demonstrated that quorum-sensing regulation of DNA replication occurs during the initiation process, but how this is achieved remains a mystery. Data presented here would suggest that it may be through direct interaction with the replication machinery rather than via a transcription/translation mechanism. The speed at which the inhibition occurs and the lack of sensitivity to transcription/translation inhibitors would further support a direct interaction. Finally, the appearance of cells containing 1.5 chromosome equivalents during transient shift experiments would suggest that each replication fork can be inhibited independently at the point of initiation (Fig. 4). This further supports the idea of direct interaction with a component of the replisome. Data from the transient shift experiments in the presence of cCM suggest that there is a discrete period of time between the establishment of the first and second replication forks that we can interrupt by the addition of cCM (Fig. 4). With this type of experiment, it will be possible to examine the directionality of the first fork as well as the factors that effect the switch from uni- to bidirectionality. Since this inhibitory effect on replication first was detected in a *dnaC2* mutant, the block must be either at the point of DnaC involvement or downstream of this point in the initiation process.

Why then would *E. coli* want to use a quorum-sensing mechanism to regulate initiation of DNA replication? It might be of value for a culture to slow down replication well in advance of entering into stationary phase since it takes a long time for ongoing replication to proceed to completion. Cell survival after entering stationary phase and the ability to respond quickly to environmental changes may be impaired by the presence of stalled forks arising because of insufficient available nutrients to complete ongoing replication upon entry into stationary phase. During rapid growth, bacteria may have to make the decision of whether to initiate a round of replication in a previous generation; therefore, the ability to sense early how many bacteria are occupying a specific niche may provide valuable information. It may not be sufficient for a bacterium to sense only how much nutrient(s) is available, but it may also be important to be able to detect the number of bacteria, of any species occupying the same environment, that will utilize those resources and regulate its replication accordingly. If a bacterium fails to initiate a round of replication it may prematurely enter stationary phase and thus will not utilize all available resources to the maximum, or, if it initiates too late, it may enter stationary phase with incomplete replication—both scenarios could cause a decrease in competitiveness of an individual bacterium. It also remains to be shown whether the quorum-sensing regulation of cell division observed here and elsewhere is in some way coupled to the quorum-sensing control of DNA replication. Coordination of both of these events into a single "cell cycle" would be advantageous.

We currently are investigating the mode of action, as well as attempting the purification, of this extracellular factor. Initial analysis has revealed that it does not extract into organic solvents but remains in the aqueous phase. Preliminary biochemical analyses suggest that it is a very stable molecule of low molecular weight. Identification of the extracellular factor will allow detailed analysis of the mechanism of inhibition of DNA replication and its significance in the maintenance of a viable culture.

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