Timing of Cold-sensitive Stages in the Cell Division Cycle of \textit{Escherichia coli} K12

\textbf{By HELOISA B. M. WOLOSKER AND D. F. DE ALMEIDA}

Laboratório de Fisiologia Celular, Instituto de Biofísica, UFRJ, Centro de Ciências da Saúde, Cidade Universitária, Rio de Janeiro, Brazil

\textit{(Received 7 June 1976)}

\textbf{SUMMARY}

Synchronous cultures obtained by selection at division were used to investigate the occurrence of cold-sensitive stages during the division cycle of \textit{Escherichia coli} (\textit{λ}\textsuperscript{-}). There are two such stages within the 50 min cycle: one (early) at 10 to 20 min and the other (late) at 40 to 45 min. Similar results were obtained from calculations based both on the age frequency distribution of cells in exponential growth and on the size of the populations which accumulate as a result of a single change of temperature. Times of about 17 and 44 min were found for the early and the late stages, respectively. It is concluded that the two-step doubling of \textit{E. coli} K12 cultures synchronized by a single cold shock is due to two cold-sensitive stages in the division cycle.

\section*{INTRODUCTION}

Synchronous growth of bacterial cultures can be induced by a single (Hotchkiss, 1954; Scott \& Chu, 1958; Perry, 1959; Doudney, 1960) or multiple change(s) of temperature (Lark \& Maaløe, 1954). Such treatments result in single-step increases in cell number approaching, to varying degrees, the theoretically ideal synchronous doubling. In a previous report we described the unusual occurrence of a two-step doubling in cultures of \textit{Escherichia coli} K12 synthesized by a single change of temperature (Wolosker \& Almeida, 1975). After a cold shock, bacterial concentration doubled by two consecutive steps, and the generation time of the synchronous culture was similar to that of an asynchronous exponential-phase culture (about 50 min). The two-step doubling was strain-specific but was not observed when synchronization methods other than a single cold shock were applied to \textit{E. coli} K12. Lysogeny did not interfere with the response. We suggested that the double step might be due to two cold-sensitive stages, or blocks, in the division cycle of \textit{E. coli} K12 (Wolosker \& Almeida, 1975). If this hypothesis is correct, a cold shock applied to \textit{E. coli} K12 cultures would result in two populations of different ages due to the accumulation of organisms at each of the two blocks in the division cycle.

In this paper, we report the results of experiments which support such a hypothesis and which establish the timing of the two cold-sensitive stages in the division cycle of \textit{E. coli} K12(\textit{λ}\textsuperscript{-}).

\section*{METHODS}

\textit{Strains.} \textit{Escherichia coli} K12 wild type was provided by L. R. Caldas and strain K12(\textit{λ}) by N. Zinder. The K12(\textit{λ}\textsuperscript{-}) strain was grown in our laboratory from the non-lysogenic wild type using phage obtained from strain \textit{AB259}(\textit{λ}\textsuperscript{-}) provided by K. B. Low.
Culture medium. Glucose minimal medium (Smith & Pardee, 1970) was used throughout. Overnight cultures were diluted 1:100 with fresh medium and aerated at 37 °C. For plating, the medium was solidified with 1.5% (w/v) agar.

Cell number determinations. Growth was followed to the exponential phase (about \(10^8\) organisms ml\(^{-1}\)) by extinction measurements with a Klett-Summerson photoelectric colorimeter. The numbers of viable organisms were counted in samples diluted with 0.05 M phosphate buffer pH 7.2 and spread on agar plates. To determine the total number of organisms, samples were diluted in 2% (w/v) formaldehyde and counted under a phase-contrast microscope in a Petroff-Hauser bacterial counting chamber.

Synchronous growth. Synchronous cultures were obtained as follows. (i) By a single change of temperature using the method of Scott & Chu (1958), as modified by Wolosker & Almeida (1975). (ii) By membrane elution as described by Helmstetter & Cummings (1964) and Cummings (1970). The entire procedure was carried out at 37 °C. A culture (50 ml) in exponential growth was filtered through a polyvinyl chloride membrane filter (0.8 μm pore size, 47 mm diam.; Sartorius). The filter was then inverted and eluted with 80 ml glucose minimal medium. Elution was controlled by adjusting the fluid column. When the flow rate reached 1 ml min\(^{-1}\), 40 ml medium was added to the eluant column. Samples (1 ml), collected after the elution rate returned to 1 ml min\(^{-1}\), were diluted in 9 ml fresh medium and grown at 37 °C. Cell numbers were determined in samples removed from these cultures at 5 to 10 min intervals.

Synchronization index (S.I.). The S.I. proposed by Scherbaum (1962) was used for quantitative descriptions of the degree of synchronization and for comparison of different strains and techniques.

RESULTS AND DISCUSSION

Synchronous growth of E. coli K12 by selection at cell division

Synchronous cultures which did not deviate from normal balanced growth were needed to determine when certain stages occurred in the cell division cycle. The best way of obtaining these cultures (Scherbaum, 1964; Mitchison, 1971) seemed to be by selection of cells at division (Helmstetter & Cummings, 1964). However, as found by Cummings (1970), E. coli K12 was not as readily synchronized by membrane elution as were strains B and B/r. Better results were obtained with a polyvinyl chloride (PVC) membrane (Cummings, 1970) than with cellulose ester filters; nevertheless, the S.I. (Table 1) indicated that the degree of synchronization was still much less than previously reported for other strains. Among the E. coli K12 derivatives tested, the λind−-lysogenic strain showed the highest S.I. (Table 1). The strain-dependence is shown in the synchronization curves which, for the wild-type and the λ-lysogenic strain, were hardly distinguishable from an asynchronous exponential growth curve (Fig. 1a, b). The shapes of these curves indicate that organisms are eluted from the membrane at several different ages in the cycle, unlike the synchronous growth curve (Fig. 1c) for E. coli K12(λind−) which corresponds to what might be expected from a culture made up entirely of new daughter cells with a generation time of about 50 min. These results confirm the dependence of E. coli K12 synchrony on a lambdoid prophage (Cox & Strack, 1972). On the other hand, the presence of the λind− prophage did not alter the two-step doubling resulting from a cold shock (Wolosker & Almeida, 1975; Wolosker, 1976). Therefore, strain K12(λind−) was used for the experiments reported below.
Cold-sensitive stages in cell division cycle

Table 1. Synchronization indices of E. coli cultures selected by membrane elution

<table>
<thead>
<tr>
<th>Strain</th>
<th>S.I.</th>
<th>Membrane type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K12 wild type</td>
<td>0.26</td>
<td>PVC</td>
<td>This paper</td>
</tr>
<tr>
<td>K12(λ)</td>
<td>0.30</td>
<td>PVC</td>
<td>Cummings, 1970</td>
</tr>
<tr>
<td>K12(λind−)</td>
<td>0.68</td>
<td>PVC</td>
<td>Helmstetter &amp; Cummings, 1964</td>
</tr>
<tr>
<td>AB253 F−</td>
<td>0.3−0.4*</td>
<td>PVC</td>
<td>Cummings, 1970</td>
</tr>
<tr>
<td>AB259 Hfr</td>
<td>0.6*</td>
<td>Cellulose ester</td>
<td>Helmstetter &amp; Cummings, 1964</td>
</tr>
</tbody>
</table>

* S.I. values calculated according to Scherbaum (1964) using data from the references given.

Fig. 1. Growth of E. coli K12 derivatives synchronized by selection of cells at division:
(a) wild-type K12; (b) K12(λ); (c) K12(λind−).

Timing of cold-sensitive stages in the cell division cycle of E. coli K12

The theoretical frequency distribution of cell ages during exponential growth (Helmstetter, 1967) is shown in Fig. 2. \( t_1 \) and \( t_2 \) indicates the ages in the cycle where the cold-sensitive blocks occur, thereby determining an early \( (t_1) \) and a late \( (t_2) \) block where cells will accumulate when a culture is subjected to the change of temperature. Three populations of cells can be distinguished by their position in the cycle relative to the blocks. Cells of ages \( 0 \) to \( t_1 \) (population \( f_1 \)) accumulate at \( t_1 \); cells of ages \( t_1 \) to \( t_2 \) (population \( f_2 \)) accumulate at \( t_2 \); cells older than \( t_2 \) (population \( f_3 \)) divide once during the shock resulting in an increase over the initial number of organisms.

**Determination of \( t_2 \).** If a synchronous culture of E. coli K12(λind−) obtained by selection at division is submitted to a cold shock, the characteristics of the ensuing growth curve will vary according to the time the shock takes place. As long as the shock is applied before the culture reaches the age of \( t_2 \), few cells will be able to divide during the shock, and little if any change will occur in the total number of organisms measured after the shock compared with the number before. When the temperature shift is applied to synchronous cultures older than \( t_2 \), most of the organisms will divide during the shock, producing a significant increase in the total number of cells measured after the shock relative to the number before the shock. The results of such experiments are shown in Fig. 3. Samples to be submitted to the temperature shift were taken from the synchronous culture (Fig. 3a) as it reached the ages indicated by the sampling times in Fig. 3b. In samples of ages \( 0 \) (new-born cells) to \( 40 \) min, only a small fraction of the cells completed a division cycle in the new environment. A sudden rise (from 15 to 75%) in the percentage of cells dividing during the shock was observed for samples taken near the end of the cell cycle (Fig. 3b). It might be argued that
Fig. 2. Age distribution of cells in an exponential-phase culture: GT, generation (or doubling) time; \( t_1 \) and \( t_2 \), ages at which the cold-sensitive stages are located; \( f_1, f_2 \) and \( f_3 \), populations distinguished by their cell ages relative to \( t_1 \) and \( t_2 \). Adapted from Helmstetter (1967).

Fig. 3. Effect of a cold shock on \( K12(\lambda\text{ind}^-) \) synchronous cells of different ages. Synchronous cultures growing in glucose minimal medium at 37 °C were subjected to 90 min cold shocks at 11 to 12 °C at various ages, and the total number of cells was measured both before and after the shock. (a) Control curve; (b) cold shock applied at the indicated times.

the transfer to 11 °C slowed the cell division processes, so that only the oldest cells were able to divide during the 90 min provided. To eliminate this possibility, the experiments were repeated with the cold shock extended from 90 min to 120 and 180 min. Again, there was no significant increase in the number of cells in samples submitted to cold shock at times earlier than the calculated \( t_3 \). The results indicate that there is a cold-sensitive stage between 40 and 45 min of the cell division cycle with a generation time of 50 min. We assign this approximate location to the late block \( (t_3) \).

Determination of \( t_1 \). A similar experiment was designed to determine the time \( t_1 \), where the early block occurs. Samples taken at regular intervals (0, 10, 20 and 30 min) from the synchronous culture were submitted to the temperature shift and their growth at 37 °C was followed. The relative numbers of cells in the steps of the resulting two-stepped doubling curve were estimated. For shocks applied before \( t_1 \), we predicted that most of the organisms would accumulate at the early block and comparatively few at the late block (see following discussion on the dispersion of generation times). This should cause an increase in the number of cells making up the second step of the two-step doubling and a corresponding decrease in the first step. For shocks applied at times after \( t_1 \) but before \( t_2 \), the trend would be reversed and the accumulation should occur preferentially at \( t_2 \), thus causing an increase.
Cold-sensitive stages in cell division cycle

Table 2. Characteristics of the two-step doubling resulting from a cold-shock applied to synchronous cultures of E. coli K12(λind−)

<table>
<thead>
<tr>
<th>Age of synchronous culture (min)</th>
<th>Percentage of cells in synchrony</th>
<th>Percentage of cells in two-step doubling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Step 1</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>35</td>
</tr>
<tr>
<td>10</td>
<td>94</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>64</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>67</td>
</tr>
</tbody>
</table>

in the number of cells in the first step of the two-step doubling curve. The expected reversal of the ratio occurred as the synchronous culture age was increased from 10 to 20 min. The cell concentration after the second step was always about twice the initial value (Table 2). Therefore, the early cold-sensitive stage (t1) must be between 10 and 20 min in the 50 min cell division cycle.

It was not possible to determine t1 and t2 more accurately in view of the statistical distribution of individual cell doubling times (Powell, 1955). Although the environmental factors causing such a dispersion can be controlled, there is a minimum intrinsic variability in the doubling times of individual organisms (Engelberg, 1964) which cannot be reduced. Hence, approximate values were determined in the experiments described above.

Timing of the cold-sensitive stages derived from characteristics of the cold-induced synchronous growth

The age distribution shown in Fig. 2 is defined by the equation:

\[ N(i) = \frac{2 \ln 2}{GT} \times 2^{-i/\sigma_T} \]

where \( N \) is the relative number of organisms at the cycle stage \( i \) (age in min) and \( GT \) is the generation time (Helmstetter, 1967). The increase in the number of cells during the cold shock will be equal to \( f_3 \) (Fig. 2). The number of cells blocked at \( t_1 \) and \( t_2 \) min will then be related to \( f_1 + 2f_3 \) and to \( f_3 \), respectively. The values of fractions \( f_1, f_2 \) and \( f_3 \) can therefore be obtained from experimental data provided one determines both \( f_3 \) and the percentage of the total number of cells that constitutes the first step of the synchronous growth curve (Table 3).

From the values presented in Table 3 for E. coli K12(λind−) it is found that:

\[ f_3 = \phi_3 = 0.09 \]
\[ f_2 = \phi_3 (1 + \phi_3) = 0.51 \]
\[ f_1 = 1 - (f_3 + f_3) = 0.40 \]

Since \( f_1 \) is the population of organisms whose ages range from 0 to \( t_1 \) min (Fig. 2), it follows that

\[ f_1 = \int_0^{t_1} N(i) \, di = \int_0^{t_1} \frac{2 \ln 2}{GT} \times 2^{-\frac{i}{\sigma_T}} \, di \]

which gives a value for \( t_1 \) of about 17 min.

\( f_3 \) corresponds to the population of organisms of ages greater than \( t_2 \) min (Fig. 2). Thus

\[ f_3 = \int_{t_2}^{GT} N(i) \, di = \int_{t_2}^{GT} \frac{2 \ln 2}{GT} \times 2^{-\frac{i}{\sigma_T}} \, di \]

giving a value for \( t_2 \) of about 44 min.
Table 3. Characteristics of the two-step doubling of synchronous cultures of E. coli K12 derivatives induced by a single change of temperature

Results are expressed as the percentage increase in the total number of cells: \( \phi_1 \), in the first step; \( \phi_2 \), in the second step; \( \phi_s \), during cold shock.

<table>
<thead>
<tr>
<th>Strain</th>
<th>( \phi_1 )</th>
<th>( \phi_2 )</th>
<th>( \phi_s )</th>
<th>( \phi_s )</th>
</tr>
</thead>
<tbody>
<tr>
<td>K12 wild type</td>
<td>42</td>
<td>38</td>
<td>95</td>
<td>8</td>
</tr>
<tr>
<td>K12(( \lambda ))</td>
<td>38</td>
<td>52</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>K12(( \lambda^{-} ))</td>
<td>47</td>
<td>36</td>
<td>100</td>
<td>9</td>
</tr>
</tbody>
</table>

Analogous calculations have been performed to determine \( t_1 \) and \( t_2 \) values for the wild-type and \( \lambda \)-lysogenic strains, using the data in Table 3: \( t_1 \) was about 19 min for wild-type K12 and 24 min for K12(\( \lambda \)); \( t_2 \) was about 45 min for wild-type K12 and 47 min for K12(\( \lambda \)). Therefore, \( t_1 \) and \( t_2 \) are about 25 min apart in all K12 derivatives tested, regardless of the individual genetic characteristics.

**Conclusions**

In spite of the intrinsic variability of synchronous growth (Engelberg, 1964), there is reasonably good agreement between values found experimentally for the timing of cold-sensitive stages (10 to 20 min and 40 to 45 min for the early and late blocks, respectively) and those determined from the theoretical frequency distribution of organisms at different stages of the K12(\( \lambda^{-} \)) division cycle (about 17 and 44 min for the early and late blocks, respectively).

Such a relationship could not be established for the other K12 derivatives studied here because the membrane elution technique of synchronization does not apply to them (Fig. 1; Cox & Strack, 1972). However, evidence from the quantitative analysis of induced synchronous cultures of wild-type K12 and K12(\( \lambda \)) (Table 3) seems to support the notion that the strain-specific two-step doubling described earlier (Wolosker & Almeida, 1975) is due to two cold-sensitive stages in the cell division cycle. It is suggested that the sensitive stages are due to functionally distinct cell products needed for processes taking place at different times. As yet we are unable to characterize such products.

We thank Hernani Rosa and Celso Pereira for technical assistance. This work was supported by grants from the CNEN, the FINEP/CNPq (project SIP/04/051), the BNDE (FUNTEC-241) and the CEPG (UFRJ). H.B.M.W. holds a scholarship from CAPES. This paper is part of a thesis submitted by H.B.M.W. to the Federal University of Rio de Janeiro, Brazil.

**References**


Cold-sensitive stages in cell division cycle


