Cell Division in a Mutant of *Salmonella typhimurium* which is Temperature-sensitive for DNA Synthesis

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SUMMARY

In *Salmonella typhimurium* PG154 the time taken for a round of DNA replication (*C*) at 37° is approximately 48 min. The time between the end of a round of DNA replication and the following cell division (*D*) is approximately 25 min. at 37°. In a temperature-sensitive mutant of *S. typhimurium* defective in the initiation of DNA synthesis, cell division at the restrictive temperature occurs at an approximately normal rate for 75 min., suggesting that $C + D = 75$ min.; the cells then elongate and form filaments. After about a further 1 h. in minimal medium at 38° cell division recommences at the ends of the filaments to form small cells. Filaments can divide at each end to produce more than one small cell. Once formed, small cells do not grow further. They do not synthesize DNA, RNA or protein and contain little DNA. Complete inhibition of DNA synthesis at the time of the shift stops cell division within about *D* min. and almost completely stops the production of small cells. A period of growth at 38° before the inhibition of DNA synthesis allows more division and the production of an almost normal number of small cells. Both the early divisions and the production of small cells are considered to be the result of the termination of rounds of DNA replication in the absence of new initiations.

INTRODUCTION

Although bacterial cells usually contain only one or two nuclear bodies, cells lacking DNA are extremely rare under normal conditions (Hirota *et al.* 1968*a*). This has suggested to many workers that DNA synthesis and cell division are tightly coupled (Kuempel & Pardee, 1963; Clark, 1968; Walker & Pardee, 1968). The nature of the relation between DNA replication and cell division has been studied by Helmstetter and his co-workers, who have shown that at growth rates greater than 1 doubling/h. at 37° a constant time elapses between the end of a round of DNA replication and the following cell division (Cooper & Helmstetter, 1968). At 37° in *Escherichia coli* B/r this time is about 22 min. and is called the *D* period. Once a cell has finished a round of replication, division can occur even if further DNA synthesis is inhibited (Clark, 1968). Thus inhibition of DNA synthesis results in residual cell division at the normal rate for a further 20 min. (Clark, 1968; Helmstetter & Pierucci, 1968). This residual division is considered to be in cells which were in the *D* period at the time of inhibition of DNA synthesis.

A second parameter of cell growth studied by Helmstetter and his co-workers (Cooper & Helmstetter, 1968) is the time required for a round of DNA replication.

They have shown that at growth rates greater than 1 doubling/h. a round of DNA replication takes a constant time, C . At 37° in *Escherichia coli* B/r C is about 44 min. As C and D are constants at these growth rates (at a particular temperature), it follows that the rate of cell division is determined by the frequency of initiations of rounds of DNA replication (Maaløe & Kjeldgaard, 1966).

We have reported a temperature-sensitive mutant of *Salmonella typhimurium* that appears to be defective in the initiation of DNA synthesis at the restrictive temperature (Spratt & Rowbury, 1970). In this paper we report the cell division pattern of the mutant at the restrictive temperature and some properties of the small cells produced under these conditions, and compare this behaviour with that predicted by the model of cell growth proposed by Cooper & Helmstetter (1968) and with the behaviour of other DNA synthesis mutants.

METHODS

Organisms. *Salmonella typhimurium* PG154 was obtained from Dr K. Sanderson and has the following genetic constitution: F⁻, *metA22*, *tryB2*, *ilv-90*, *xyl-1*, *fla-66*, *strA210*, *malA1*, *tre-12*, *h1-b*, *h2-e,n,x*. The mutant 11G was obtained from PG154 by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Spratt & Rowbury, 1970).

Media. Nutrient broth (Oxoid NB2) was used as a rich medium. The minimal medium (MM) was that of Davis & Mingioli (1950) with 0.2% glucose and 40 µg./ml. of the DL-form of the required amino acids. Difco vitamin-free Casamino acids (0.1%) were added to enrich the MM in some experiments. These media were solidified with Difco Bacto agar (2%). For phage assays the basal agar consisted of 1% trypticase agar base, 1.4% Difco Bacto agar and 0.5% NaCl with a soft agar overlay of 0.2% MgCl₂, 1% Oxoid no. 2 broth, 0.3% NaCl and 0.75% Difco Bacto agar.

Growth of organisms. Organisms were subcultured from nutrient agar slopes into the required medium and incubated overnight in a shaking water-bath. To obtain exponentially growing organisms, such cultures were diluted into fresh medium and incubated for several hours with shaking. Extinction of cultures was measured in a Hilger photoelectric colorimeter against an uninoculated medium blank (490 nm. for MM and 550 nm. for nutrient broth). Total cell number was measured using a Coulter model F electronic particle counter. Samples were diluted into Isoton diluent containing 0.1% sodium azide (Coulter Electronics Ltd), and 0.1 ml. counted with the 30 µm. orifice with a threshold of 4, attenuation 1 and aperture 8.

Cell division. Cell division was studied either by Coulter-counting or by following the growth of individual cells under the phase-contrast microscope. In the latter case a suitably diluted culture of cells growing exponentially in the required medium at the required temperature was poured on to a thin film of agar (made from the same medium solidified with 2% Difco Bacto agar). The agar was transferred to a clean microscope slide, kept moist by surrounding the film of agar with blocks of the same agar, covered with a coverslip, and examined at approximately 15 min. intervals under a phase-contrast microscope at ×1500. All the procedures were performed in a constant-temperature room maintained at 38°. The cell-division pattern of 20 to 30 well-separated cells could be studied in this way and the agar could be kept moist for over 4 h. at 38°.

Synthesis of DNA, RNA and protein. Macromolecular syntheses were measured by

following the incorporation of [^3H]thymidine, [^{14}C]uracil and [^{14}C]phenylalanine as described previously (Spratt & Rowbury, 1970).

Chemical estimation of DNA. Cell samples for DNA estimation (approximately 10^{10} cells) were cooled, centrifuged, washed in cold tris-HCl buffer (0.05 M, pH 7.5), resuspended in 3 ml. of 5% TCA and the DNA extracted at 90° for 20 min. The supernatant of the extraction was assayed for deoxyribose by the modified diphenylamine method of Giles & Myers (1965). Calf thymus DNA was extracted similarly and used for a standard curve.

Separation of small cells. Strain IIG was grown in the required medium at 38° for about 4 h., centrifuged at 3000 rev./min. for 10 min., washed with 0.05 M-tris-HCl buffer, pH 7.5, and resuspended at about 10^{10} organisms/ml. The suspension was then gently layered on to a 5 to 50% linear sucrose gradient and spun at 1500 rev./min. in 15 ml. glass tubes in the swing-out head of a bench centrifuge for 10 min. After this treatment the filaments were in the pellet and in the bottom half of the tube, whereas the small cells formed a homogeneous band in the top half of the tube. The small cells were removed carefully with a sterile syringe. This method of purification of small cells gave a homogeneous preparation with less than 1% contaminating filaments.

Chemicals. Radioisotopes were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, [^{14}C]phenylalanine was supplied at 12.6 mCi/mM, [^{14}C]uracil at 54.9 mCi/mM, and methyl [^3H]thymidine at 5.0 Ci/mM. Nalidixic acid was kindly donated by the Bayer Products Company.

RESULTS

Growth parameters of Escherichia coli and Salmonella typhimurium

The growth parameters C (time for a round of DNA replication) and D (time between the end of a round of DNA replication and the following cell division) have been shown to be useful for describing cellular growth of *Escherichia coli* (Cooper & Helmstetter, 1968; Helmstetter, 1969; Marr, Painter & Nilson, 1969). We have made estimates of C and D for *Salmonella typhimurium*. D was estimated by measuring the residual cell division after the inhibition of DNA synthesis with nalidixic acid. Fig. 1 shows the effect of 15 $\mu\text{g./ml.}$ nalidixic acid on cell division in *S. typhimurium* PG 154 at 37° in Casamino acid MM and nutrient broth. Fifteen $\mu\text{g./ml.}$ nalidixic acid stops DNA synthesis immediately in this strain without having very much effect on RNA or protein synthesis. The value for D , i.e. the time before residual cell division stops, is between 23 and 26 min. and the residual cell division is about 50% in Casamino acid MM and 100% in nutrient broth. Thus in nutrient broth every cell can proceed from birth to division in the absence of DNA synthesis, i.e. the mean generation time in this medium is approximately equal to D (Helmstetter & Pierucci, 1968).

A value for C can be obtained in several ways (see Pritchard & Zaritsky, 1970). The percentage increment (ΔDNA) in DNA synthesis after inhibition of protein synthesis by chloramphenicol is given by

$$\Delta\text{DNA} = 100 \left(\frac{2^n \ln 2}{2^n - 1} - 1 \right)$$

(Sueoka & Yoshikawa, 1965; Donachie, 1969) and n can be shown to be equal to C/τ , where C is the time for a round of replication of DNA and τ is the growth rate of the organism in the medium used (Pritchard & Zaritsky, 1970). *Salmonella typhi-*

murium growing exponentially with a mean generation time of 27 min. gives $\Delta\text{DNA} = 73\%$ when $150\ \mu\text{g./ml.}$ chloramphenicol is added, and this increment is reached in about 40 min. (Fig. 2). Substituting these values in the equation above gives a value for C of 47 min. for *S. typhimurium* PG154 at 37° .

Fig. 1

Fig. 1. Effect of DNA synthesis inhibition on cell division in strain PG154. Cell number was followed using the Coulter counter for cultures of *Salmonella typhimurium* PG154 growing exponentially at 37° in Casamino acid MM (○) and nutrient broth (□). At 40 min. $15\ \mu\text{g./ml.}$ nalidixic acid (nal) was added and cell division followed for a further 60 min.

Fig. 2

Fig. 2. The increment in DNA synthesis in strain PG154 at 37° in the absence of protein synthesis. A culture of *Salmonella typhimurium* PG154 growing exponentially in Casamino acid MM + $1.5\ \text{mM}$ uridine at 37° was split into two portions. $[^3\text{H}]\text{Thymidine}$ was added at $0.2\ \mu\text{Ci}/0.2\ \mu\text{g./ml.}$ and incorporation followed at 37° in the presence (○) and in the absence (□) of $150\ \mu\text{g./ml.}$ chloramphenicol.

A second value for C can be obtained by knowing D and the DNA content per cell at a particular growth rate and using the equation

$$\bar{G} = \frac{\tau}{C \ln 2} \left(2^{(C+D)/\tau} - 2^{D/\tau} \right)$$

(Cooper & Helmstetter, 1968), where $\bar{G}$ is the DNA content per cell in genome equivalents. To obtain $\bar{G}$ the DNA content of a non-replicating genome must be known. Cooper & Helmstetter (1968) give a value of $4.2 \times 10^{-15}\ \text{g./genome}$ for *Escherichia coli* B/r and the value for *Salmonella typhimurium* is probably almost identical, as Maaløe & Kjeldgaard (1966) give a value of 4 to $5 \times 10^{-15}\ \text{g./genome}$. We have used the value of $4.2 \times 10^{-15}\ \text{g./genome}$ for calculating $\bar{G}$. *Salmonella typhimurium* PG154 growing exponentially at 37° was harvested and the DNA/cell measured as described in Methods. At a mean generation time of 29.5 min. at 37° we obtained a DNA content of $14.2 \times 10^{-15}\ \text{g./cell}$ (DNA expressed as free acid). This gives a value for C of 49 min. based on $D = 25\ \text{min.}$

Effect of inhibition of DNA synthesis on cell division in strain 11 G

Cell division in *Salmonella typhimurium* PG 154 stops after a 25 min. residual division period at 37°, when DNA synthesis is stopped with nalidixic acid. When DNA synthesis is stopped in strain 11 G by shifting a culture from 25° to 38° cell division continues for several hours. In MM at 38° nearly all the cells are seen to divide once under the phase-contrast microscope and to produce two apparently normal daughter cells (Table 1) which both elongate to form short filaments, able to divide terminally forming a shorter filament and a small cell. In nutrient broth at 38° most cells of 11 G are able to divide twice and produce four apparently normal daughter cells (Table 1) which elongate and start to divide at the ends in the same way as the MM grown cells. Some of these daughter cells produced in the early divisions must be viable as the viable count increases during the first hour at 38° before falling off exponentially (Spratt & Rowbury, 1970). Studies with the Coulter counter show that a culture of 11 G in MM when

Table 1. *Division of individual cells of 11 G on solid media*

Organisms growing exponentially in liquid media at 25° were shifted to the same solid media at 38° and cell division of individual cells followed as described in Methods. In the experiments with nalidixic acid the solid media contained 25 µg./ml. nalidixic acid. In all cases the division of more than 50 cells was followed. Small cells were those that were produced terminally and which failed to increase in size. Median 'normal' divisions were those that produced two equal-sized daughter cells that both grew further.

Medium	Number of normal divisions (%)					Normal daughter cells producing small cells (%)
	0	1	2	3	4	
MM	15	77	8	0	0	39
Casamino MM	10	34	51	5	0	—
Nutrient broth	3	29	53	15	0	52
Nutrient broth + nalidixic acid	61	39	0	0	0	5
Nutrient broth + nalidixic acid added after 60 min. at 38°	—	—	—	—	—	48

shifted from 25° to 38° continues to increase in cell number for about 75 min.; cell number then remains static for about another 70 min. and then increases again (Fig. 3). The plateau value reached after 75 min. represents an increase of 160% in cell number over the number present at the time of the shift in this medium. During the initial 75 min. period nearly all cells can be observed under the microscope to divide once (the extent of division appears to be slightly greater in liquid medium than on agar) and the increase in cell number after 140 min. represents the recommencement of cell division after the period of elongation. As the growth rate afforded by the medium increases, so the amount of cell division in the first 75 min. after the shift increases and the production of small cells occurs earlier until in nutrient broth the plateau at 75 min. is only seen as a shoulder in the curve (Fig. 3). The value of the shoulder in nutrient broth represents an increase in cell number of about 300%, corresponding to the direct microscopic observation that nearly all cells can divide twice in this medium after a shift to 38°, before the production of small cells.

Effect of chemical inhibition of DNA synthesis on cell division in strain 11 G at 38°

Strain 11 G after a shift from 25° to 38° continues DNA synthesis for about 40 min., resulting in the termination of rounds of DNA replication in the absence of new initiations of DNA synthesis (Spratt & Rowbury, 1970). If the termination of rounds of DNA replication is necessary for the continuing cell division at 38°, then immediate inhibition of DNA synthesis with nalidixic acid at the time of the temperature shift should drastically reduce the amount of cell division. In fact cell division should stop within about *D* min. Table 1 shows the effect on cell division of shifting 11 G growing in nutrient broth at 25° to nutrient agar plates at 38° containing 25 µg./ml. nalidixic

Fig. 3. Cell division in strain 11 G at 38°. Cell number was followed using the Coulter counter for cultures of strain 11 G growing exponentially in MM (○) and nutrient broth (□) after a shift from 25° to 38°.

acid. None of the cells can divide more than once, whereas without nalidixic acid 68% of cells can divide twice or three times. These cells that do divide in the presence of nalidixic acid were presumably in the *D* period at the time of the shift. The 61% of cells that fail to divide would not be in a *D* period. The addition of nalidixic acid at the time of the temperature shift reduces the number of small cells produced from 52% to 5%. In liquid media plus 15 µg./ml. nalidixic acid at 38° residual cell division continues for about *D* min. as expected (data not shown).

Thus the termination of rounds of DNA replication in 11 G at 38° would seem to be necessary for continued cell division. If the termination of rounds is not only responsible for the normal divisions but also is involved in the subsequent production of small

cells at 38° then a period of growth at 38° before the addition of nalidixic acid should allow rounds to be finished and the production of small cells. Table 1 shows that 60 min. growth at 38° before addition of nalidixic acid allows the almost normal production of small cells.

Properties of small cells

Small cells were separated by sucrose density centrifugation from a culture obtained by growing 11G at 38° in nutrient broth for 4 h. The small cells, washed to remove sucrose and resuspended in nutrient broth, showed no increase in extinction when shaken at 38° for at least 2 h. Similarly, DNA, RNA and protein synthesis in purified small cells was negligible. Incorporations of [³H]thymidine, [¹⁴C]uracil and [¹⁴C]phenylalanine in the small cells at 38° were 0, 0.6 and 1.1 % respectively compared with that in strain PG154 for the same initial cell density during a 2 h. period.

Less than 0.5 % of the small cells formed colonies on nutrient agar at 25°. These few colonies were probably due to contaminants obtained during the purification of the small cells, to temperature-resistant revertants of 11G which would band with the small cells in the sucrose gradient and to small filaments.

DNA Content of small cells. Three methods were used to determine whether the small cells derived from strain 11G after growth at 38° contained any DNA. First, DNA was estimated chemically by the modified diphenylamine assay (Giles & Myers, 1965); secondly, we measured the retention in small cells of radioactivity from cells of 11G previously labelled at 25° with [³H]thymidine; and thirdly, thin sections of the small cells were examined in the electron microscope. All methods indicated a reduced DNA content in the small cells and electron microscopy demonstrated the absence of discrete nuclear bodies.

DISCUSSION

It would seem that the regulation of the growth of *Salmonella typhimurium* can be described by the same growth parameters as *Escherichia coli*. The data of Schaechter, Maaløe & Kjeldgaard (1958) on DNA contents of *S. typhimurium* at different growth rates fits well with the theoretical curves and the experimental results of Cooper & Helmstetter (1968). Our values of *C* and *D* for *S. typhimurium* PG154 are also very similar to those of *E. coli* B/r. Similarly, a value for *C*+*D* of 60 to 70 min. can be obtained from the data of Kjeldgaard, Maaløe & Schaechter (1958) (Pritchard, Barth & Collins, 1969). We would consider that the model derived by Cooper & Helmstetter (1968) for *E. coli* can be usefully employed for describing and predicting the growth behaviour of *S. typhimurium*.

Since the temperature-sensitive lesion in strain 11G is in the initiation of DNA replication (Spratt & Rowbury, 1970), we would predict that cell division after a shift from 25° to 38° would continue for *C*+*D* min., i.e. any cells that had initiated a round of DNA replication before the shift + any cells in the *D* period at the time of the shift would be able to divide. The last cells to divide would be those that had just started a round of DNA replication at the time of the shift. These cells would finish the round *C* min. after the shift and then divide after a further *D* min. This is the actual behaviour of strain 11G when shifted to 38° (Fig. 3) and this suggests that the DNA lesion in 11G does not affect cell division *per se*. Furthermore, the extent of cell division before the 75 min. plateau is reached after growth in different media supports the view that

rounds of replication are completed at the restrictive temperature and that normal segregation occurs. Further evidence that the 75 min. residual cell division is due to the termination of rounds of DNA replication in 11G at 38° is suggested by the cell division pattern in the presence of nalidixic acid. Immediate inhibition of DNA synthesis at the time of the shift should result in a drastic reduction in cell division. This is seen to occur (Table 1).

The recommencement of cell division and the production of small cells would also seem to be connected to the residual DNA synthesis in 11G at 38°, since small cell production is almost nil if nalidixic acid is added at the time of the temperature shift from 25° to 38° whereas if nalidixic acid is added after a period of growth at 38° (allowing rounds of replication to be completed) an almost normal number of small cells can be produced. Hirota, Ryter & Jacob (1968*b*) have shown that in two temperature-sensitive DNA synthesis mutants of *Escherichia coli* the production of DNA-less cells is the result of a second mutation (*div*). We have no evidence that strain 11G contains two lesions but a search (at present in progress) among *ts*⁻ recombinants from genetic crosses for the loss of the ability to produce DNA-less cells should clarify the matter.

In MM, according to the model of Cooper & Helmstetter (1968), *Escherichia coli* B/r has a chromosome with a single replication point. If this model is applied to the behaviour of strain 11G, inhibition of the initiation of DNA synthesis at 38° should result in the production of two completed chromosomes in MM. Within the first 75 min. at 38° in MM nearly all cells divide once and this would result in the segregation of the two completed chromosomes, one per daughter cell. Any further cell division should result in the production of cells lacking DNA. The fact that the small cells, in contrast to the filaments, fail to grow at 38° supports this interpretation. Results of three methods to detect DNA show that the small cells have little DNA. These small cells, as expected, fail to synthesize DNA, RNA or protein.

F' derivatives of strain 11G fail to form colonies at 38°, but are able to transfer poorly the *F'* into *F*⁻ recipients and to segregate the episome into small cells at 38° (R. J. Rowbury & B. G. Spratt, unpublished). Thus some transfer and segregation of the independent *F'* replicon can occur at 38° in the absence of chromosomal DNA synthesis, a result similar to that obtained with the segregation of *F*-primes into minicells in the minicell-producing strain (Adler, Fisher, Cohen & Hardigree, 1967), where chromosome segregation is abnormal (Kass & Yarmolinsky, 1970).

The fact that strain 11G behaves as predicted by the model of Cooper & Helmstetter (1968) suggests that the lesion in DNA synthesis in 11G is not affecting cell division *per se*. We also believe that the cell division pattern of strain 11G is further evidence that it is the termination of rounds of DNA replication that is required for the triggering of cell division. Some DNA synthesis mutants behave, as regards cell division, in accordance with this model (Clark, 1968; Fangman & Novick, 1968; Gross, Karamata & Hempstead, 1968). Other DNA synthesis mutants would seem to be affected in a function that is common to DNA synthesis and cell division (Inouye, 1969; Khatchatourians & Clark, 1970). The cell division pattern of the temperature-sensitive DNA synthesis initiation mutants of Hirota *et al.* (1968*a*) is not clear from the literature. Strains T46 and T83 of Hirota *et al.* (1968*b*) seem to be affected in a common function (Shapiro, Siccardi, Hirota & Jacob, 1970) but they do differ in that T46 has a *div* mutation and T83 has not. Khatchatourians & Clark (1970) briefly

report that T83 fails to divide at the restrictive temperature but can be made to divide to a certain extent at the restrictive temperature by osmotic shock. These authors suggest that the lesion in T83 is in a factor that is involved in both the initiation of DNA synthesis and in cell division. The observation (B. G. Spratt & R. J. Rowbury, unpublished) that the *ts* lesion in 11G maps some distance to the right of *metA* on the *Salmonella* genetic map suggests that the lesion in 11G is different from that in T83 and T46.

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