Molec. gen. Genet. 177, 103–112 (1979)

MGG

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# Isolation and Characterisation of a Strain Carrying a Conditional Lethal Mutation in the *cou* Gene of *Escherichia coli* K12

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**Summary.** A strain which carries a mutation conferring clorobiocin resistance and temperature sensitivity for growth was isolated from *Escherichia coli* K12. Genetic mapping and the molecular weight of the gene product suggest that the mutation is in the *cou* gene, specifying a sub-unit of DNA gyrase. Nuclear organisation and segregation and placement of septa are grossly abnormal in the mutant at 42° C. RNA synthesis and initiation of DNA replication are also affected at the restrictive temperature but the rate of DNA chain elongation continues almost undisturbed.

## Introduction

Coumermycin $A_1$ and novobiocin are related coumarin and carbohydrate-containing antibiotics which inhibit the growth of *Escherichia coli.* Both antibiotics have also been found to block the activity in vitro of DNA gyrase – an enzyme which introduces supercoils into relaxed covalently closed circular DNA molecules (Gellert et al., 1976a). The growth of *Escherichia coli* cells becomes insensitive to these drugs as the result of a mutation which also renders DNA gyrase activity drug resistant (Ryan, 1976; Gellert et al., 1976b). Consequently the genetic locus *cou,* which determines resistance to novobiocin and coumermycin $A_1$ and maps near the *dnaA* gene, has been identified as the structural gene of a DNA gyrase sub-unit (Gellert et al., 1976b). Upon further purification of DNA gyrase it became evident that the enzyme is a complex of at least two polypeptides, the *cou* gene product and the *nalA* gene product which have molecular weights of about 95,000 and 110,000 respectively (Higgins et al., 1978; Mizuuchi et al., 1978a).

Attempts to determine the physiological role of DNA gyrase by studying the mode of action of novobiocin and coumermycin $A_1$ have been complicated by the pleiotropic effects of these drugs on bacterial growth, as will be described elsewhere (Fairweather, Orr and Holland, in preparation). However, involvement in the elongation step in DNA synthesis has been considered to be a major role of this enzyme (Gellert et al., 1976b; Itoh and Tomizawa, 1977; Gellert et al., 1977; Sugino et al., 1977).

In the hope of obtaining clearer evidence about the physiological role of DNA gyrase we have sought to isolate temperature sensitive gyrase mutants, making the assumption that simultaneous acquisition of temperature sensitivity for growth and drug resistance would identify such a mutation. Using a closely related compound to novobiocin – clorobiocin Fairweather et al., in preparation), several mutants with this phenotype were isolated. The physiological properties of one such strain in which the mutation was mapped near *dnaA* are described here.

## Materials and Methods

*Strains, Media and Cell Culture Conditions. Escherichia coli* K12 strains which were used in this work (Table 1) were cultivated in M9 minimal salts medium supplemented with glucose (0.2%), the required L-amino acids (50 µg/ml) and vitamin-free casamino acids (0.5%) when necessary. For the selection of D-galactonate positive transductants (Cooper, 1978), glucose was replaced by D-galactonate (10 mM) as carbon source. Colony counts were made on minimal agar or nutrient broth plates. Incubation of liquid cultures was in a New Brunswick gyrotary shaker.

*Isolation of Mutants.* Clorobiocin resistant mutants were selected spontaneously on minimal agar plates containing various concentrations of clorobiocin (isolated by Dr. L. Ninet, Rhone-Poulenc S.A., Vitry France) freshly dissolved in ethyl alcohol. Resistance

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**Table 1.** Strains used

| Strain | Genotype | Source |
|---|---|---|
| LE234 | $F^-$ *metB argE ilv tna supE*(?) | E. Orr |
| LE316 | LE234 *ts* (clorobiocin resistant) | |
| A3 | $F^-$ *thr leu thi lacY supE dnaA* T46 | R.H. Pritchard |
| CO509 | $F^-$ *metB dgoD* | R.A. Cooper |
| 159 | $F^-$ *uvrA* $\lambda ind^-$ | W.J. Brammar |
| 4258 (F′111) | F′ *(pyrE→malB)/argG metB his leu recA mtl xyl malA gal lacY str tonA* $\lambda^R$ $\lambda^-$ *supE* | B.J. Bachmann |
| CO101 | HfrC *thi dgoD relA* | R.A. Cooper |
| LE311 | A3 *dgoD* | R.A. Cooper |
| LE315 | LE234 *dnaA* T46 $ilv^+$ | E. Orr |
| 833 | $F^-$ *Δ(gal λatt bio trpED) ΔlacZ tonA his str tsx* | W.J. Brammar |
| LE707 | LE316 *recA* | N.F. Fairweather |

to clorobiocin and coumermycin $A_1$ (gifts from May & Baker Ltd. Dagenham, Essex, U.K.) was always tested on minimal agar plates.

*Measurements.* Absorbance was measured at 450 nm with a Gilford microsample spectrophotometer and particle number was determined with a Coulter Counter Model B. The relative volume distribution was estimated and plotted with a Coulter Counter Model ZB1 and Coulter Channelyzer C-1000. Viable cells were estimated from samples diluted in 10 mM phosphate buffer pH 7.2 and spread on the appropriate plates at 30°C. DNA synthesis was determined by adding [$^3$H]-thymidine (1 µCi/ml; 1 µg/ml; Amersham Radiochemicals) and uridine (1.5 mM) to the medium at least seven generations before assaying samples by standard procedures. The increment of DNA in "run out" experiments was measured using the same labelling regime with cultures to which rifampicin (Sigma) at 150 µg/ml was added. Rates of DNA and RNA synthesis were determined by withdrawing 0.5 ml of culture at intervals and labelling with radioactive thymidine and uridine respectively for 2 min.

*Staining of Nuclei.* Cell suspensions were thermally fixed and hydrolized with N HCl at 60° C before being stained with Giemsa R66 (Gurr).

*Isolation of λ Transducing Phages.* Transducing lysates of phage λ, kindly donated by Dr. B.G. Spratt, were prepared as previously described (Schrenck and Weissberg, 1975) by infecting the *Δatt λ* strain 833 (Table 1) with phage λCI857 *S7* followed by infection with λch82 and induced by thermal (42° C, 15 min.) and UV treatments. $dgoD^+$ transductants of strain CO509 ($\lambda^+$) were selected for growth on D-galactonate as sole carbon source at 37°C, after infection with the transducing lysate. The transductants were purified under selective conditions and grown in Luria Broth medium for phage induction. Screening for $cou^+$ and $dnaA^+$ genes was done by spotting lysates on nutrient agar plates at 42° C containing a lawn of LE316 or LE315 (Table 1) respectively lysogenized with $\lambda^+$.

*Proteins Specified by λ Transducing Phages.* Labelling of proteins synthesised after infection with transducing phages was performed as described by Ptashne (1967), with some modifications. Strain 159 ($\lambda ind^-$) was grown in M9 minimal salts medium supplemented with 0.4% (w/v) maltose and 0.4% (v/v) glycerol to an $A_{450}=0.6$. Cells were irradiated with UV (12,000 erg/mm$^2$) and concentrated five-fold in the same medium containing 10 mM $MgSO_4$. Infection (m.o.i. of 5 to 10) was allowed to proceed 15 min at 37° C before a five-fold dilution into prewarmed medium. After aeration for 10 min at 37° C [$^{35}$S]-methionine (100–200 µCi/ml; $2 \times 10^{-7}$ µg/ml) was added and chased (in ice) after approximately 30 min, with non-radioactive methionine (1.5 mg/ml) and chloramphenicol (200 µg/ml). Cells were harvested, washed in phosphate buffer, and lysed in SDS-buffer (0.06 M tris HCL, pH 6.8, 20% w/v glycerol, 2% w/v SDS, 5% v/v mercaptoethanol) and boiled for 5 min. SDS polyacrylamide gel electrophoresis was carried out essentially as described by Laemmli (1970), and fluorographs prepared according to Laskey and Mills (1975).

## Results

### *Isolation of Mutants*

Approximately $10^{10}$ cells from an overnight culture of LE234 were plated on glucose minimal medium which contained increasing concentrations of clorobiocin from 0 to 150 µg/ml. The plates were incubated at 30° C for 3–5 days and the colonies purified on the same medium and tested for their ability to grow at 42° C. Several temperature sensitive mutants were isolated and one – LE316 – was studied further.

### *Drug Resistance and Viability of LE316*

LE316 can form colonies on glucose minimal medium plates at the permissive temperature in the presence of 50 µg/ml clorobiocin, a concentration which totally abolishes the growth of LE234. LE316 and other clorobiocin resistant mutants also grew in the presence of coumermycin $A_1$ at concentrations which inhibited LE234. This strongly suggested that clorobiocin resistance was due to a mutation at the *cou* locus and therefore close to *dnaA*. LE316 plated with high efficiency on both nutrient and minimal agar when tested at temperatures up to 37° C. In contrast, at 42° C the plating efficiency was $< 10^{-7}$ that of LE234.

**Table 2.** Mapping of the mutation in LE316 by P1 transduction

| Donor | Recipient | Selected marker | Unselected markers | | | | | | Number of transductants |
|---|---|---|---|---|---|---|---|---|---|
| | | | *ilv* | *tna* | *cry* | *ts* | *clb* | *dgoD* | |
| CO101 | LE316 | ilv$^+$ | | + | | + | | + | 6 |
| | | | | + | | − | | + | 6 |
| | | | | + | | + | | − | 3 |
| | | | | − | | − | | + | 84 |
| | | | | − | | − | | − | 1 |
| CO101 | LE316 | ts$^+$ | + | + | | | + | − | 7 |
| | | | − | + | | | + | − | 97 |
| | | | + | + | | | + | + | 13 |
| | | | − | + | | | + | + | 6 |
| | | | − | − | | | + | − | 5 |
| | | | − | − | | | + | + | 4 |
| A3 | LE316 | ilv$^+$ | | + | − | | − | | 6 |
| | | | | + | + | | + | | 31 |
| | | | | − | − | | − | | 4 |
| | | | | − | + | | + | | 359 |

*clb*$^+$: designates resistance to clorobiocin. *cry*$^-$: indicates cold sensitivity.

## *Genetic Analysis of LE316*

The following experiments suggest that a single mutation leads to both thermosensitivity and drug resistance. LE316 was transduced with P1*vir* grown on the wild type strain and temperature resistant transductants selected. All transductants were drug sensitive. When spontaneous temperature resistant revertants were selected, a proportion of them were also drug sensitive.

The F-prime plasmid F111 carries chromosomal DNA between genes *pyrE* and *malB* and is known to carry the *dnaA* gene (Orr, unpublished data). When F111 was introduced into a *recA* derivative of LE316 (LE707) at a permissive temperature by selecting for *ilv*$^+$ colonies, the resulting clones were able to grow at 42° C and became sensitive to clorobiocin. Elimination of F111, which was readily achieved by growing the culture under non-selective conditions, restored temperature sensitivity and drug resistance. These results support the earlier data that both phenotypes are the result of a single mutation. Both phenotypes were recessive in plate tests.

The mutation carried by LE316 was mapped by means of P1 transduction. Unfortunately, phage P1 did not grow on LE316 even at permissive temperatures and it was only possible to perform transductions in which this strain was used as a recipient. Table 2 shows that the mutation in LE316 is cotransducible with *ilv, tna, dnaA* and *dgoD*. When strain A3 (*dnaA*) was used as the donor and *ilv*$^+$ as the selected marker in the recipient (Table 2) no recombinants between drug resistance and cold sensitivity (a phenotype resulting from the *dnaA* lesion in strain A3; Orr et al., 1978) were found among 400 transductants.

**Table 3.** Mapping of thermosensitivity of LE316 in relation to *dnaA*46

| Donor | Recipient | Selected marker | Unselected markers | | | Number of transductants |
|---|---|---|---|---|---|---|
| | | | *ilv* | *tna* | *dgoD* | |
| LE311 | LE316 | ts$^+$ | + | + | + | 2 |
| | | | − | + | + | 7 |
| | | | + | + | − | 1 |
| | | | − | + | − | 13 |
| | | | + | − | − | 11 |
| | | | − | − | − | 66 |
| | | | + | − | + | 12 |
| | | | − | − | + | 35 |

$^+$ indicates growth.

A further attempt was made to order the *ts* locus in LE316 and the *dnaA* locus by transducing *dnaA*T46 into LE316 and selecting for cross-overs between the two loci to produce temperature resistant colonies. From the analysis of the least frequent recombinant classes amongst the unselected markers (Table 3) together with the data in Table 2, we deduced the complete map order to be *dgoD, ts, dnaA, tna, ilv*. This agrees with the reported position of the *cou* locus to the left of *dnaA* (Ryan, 1976) and we therefore

conclude that LE316 is mutant at the *cou* locus. The positioning of *ts* (*cou*) to the left of *dnaA* was further supported by the observation that a small proportion of λ*dgoD* transducing phages abolished temperature sensitivity and drug resistance in LE316 but not the temperature sensitivity in the *dnaA* mutant.

### *Use of Transducing Phage to Identify the Gene Product of the ts Mutation*

λ transducing phages (λ*dgoD* and λ*dgoD, cou*) were constructed which as prophages complemented either *dgoD* or both *dgoD* and the *ts* lesion of LE316. An ultraviolet light-irradiated λ*ind*$^-$ lysogen was infected with both types of transducing phage and the proteins synthesised were labelled with [$^{35}$S]-methionine. Only phages which abolished the temperature sensitivity of LE316 coded for a polypeptide with a molecular weight of 92,000 (Fig. 1). The *dgo* proteins were not induced in this system. The polypeptide unique to λ*cou* has the same molecular weight as that reported for the *cou* gene product (Mizuuchi et al., 1978a; Higgins et al., 1978).

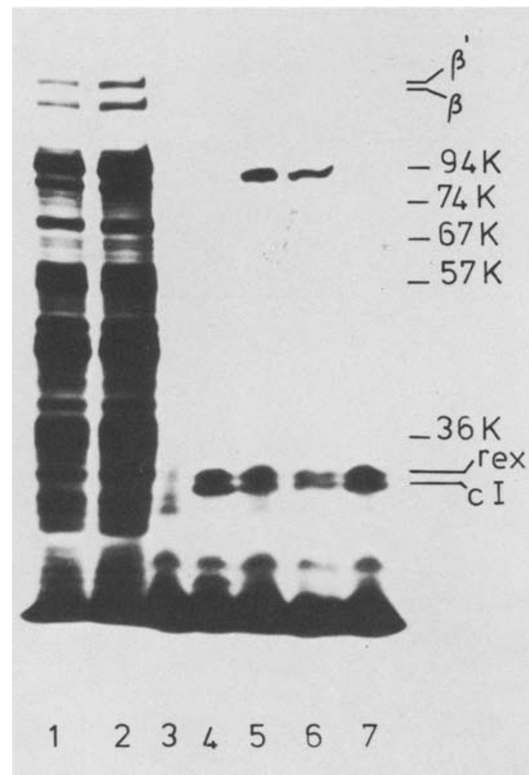



**Fig. 1.** Polyacrylamide (11% w/v) gel electrophoresis of proteins specified by λ transducing phages. The gel columns are fluorographs of proteins from (1) and (2) whole cell lysates with no irradiation; (3) irradiated cells with no phage; (4) host infected with wild type λ; (5) and (6) host infected with independent isolates of λ*dgo*D, *cou*; (7) host infected with λ*dgoD*. The arrows indicate the position of the following proteins which were visualized by Coomassie Brilliant Blue staining: lactic dehydrogenase (36K); bovine serum albumin (62K); ovalbumin (43K); pyruvate kinase (57K) transferrin (74K); phosphorylase A (94K); β, β′ subunits of RNA polymerase (155K; 160K respectively)

### *DNA Synthesis at Non-permissive Temperature*

Since DNA gyrase has been reported to play an essential role in the elongation step of DNA replication, it might be expected that the metabolism of DNA in LE316 would be disturbed. A preliminary observation which favoured this assumption was the increased length of the mutant cells at both permissive and non-permissive temperatures since inhibition of either initiation of chromosome replication or of DNA chain elongation is known to cause filamentation. A diagnostic feature of specific inhibition of DNA synthesis in *E. coli* is normally that, following such inhibition, mass increase continues relatively unperturbed for a considerable time. When the increment in both DNA and mass (absorbance) was followed after a temperature shift from 30° C to 42° C it was found that the increase in both parameters in LE316 slowed down in parallel (Fig. 2). DNA and mass in LE234 increased at a new exponential rate after the temperature shift (see Fig. 2 for mass, DNA not shown). Thus no clear-cut evidence for a specific inhibition of DNA synthesis was provided by this experiment.

In an attempt to look more specifically for an inhibition of DNA chain elongation the time taken to terminate rounds of chromosome replication in the presence of rifampicin (to block initiation) in LE316 compared with LE234 was measured at 42° C

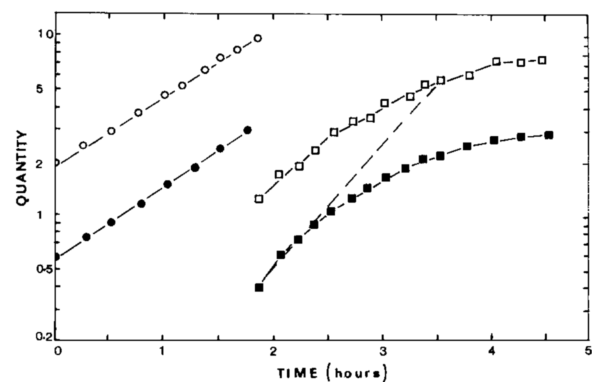



**Fig. 2.** DNA synthesis and mass increase of LE316 and mass of LE234 at 30° C and 42° C in M9 glucose minimal medium. The cultures were diluted at the time of the temperature shift, into prewarmed medium. (○) $10^3 \times$ cpm/ml at 30° C, LE316; (□) $10^3 \times$ cpm at 42° C, LE316; (●) absorbance of LE316 at 450 nm × $10^{-1}$ at 30° C similar results obtained with LE234; (■) absorbance at 450 nm × $10^{-1}$ at 42° C, LE316. The increase in absorbance of LE234 at 42° C is indicated by the broken line

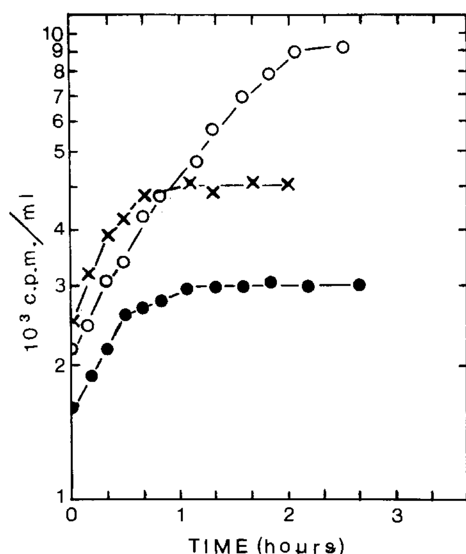



**Fig. 3.** DNA synthesis of LE234 and LE316 at 42° C in M9 glucose minimal medium in the presence of rifampicin added at time zero: (●) LE316+rifampicin; (○) LE316; (×) LE234+rifampicin

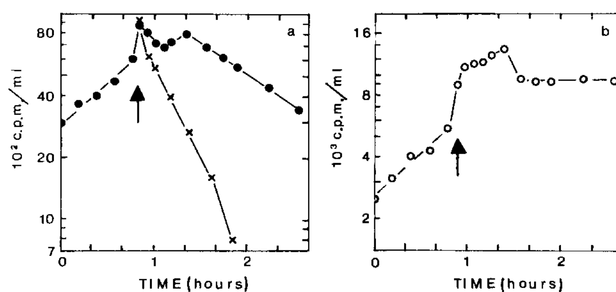



**Fig. 4a and b.** Rate of DNA and RNA synthesis of LE316 in M9 glucose minimal medium. The arrows indicate the time of the temperature shift from 30° C to 42° C. DNA was labelled with [$^3$H]-thymidine (2 µCi/ml; $10^{-5}$ µg/ml) and RNA with [$^{14}$C]-uridine (2 µCi/ml; 4 µg/ml). **a** DNA synthesis; (●) without rifampicin; (×) with rifampicin; **b** RNA synthesis

(Fig. 3). The results suggested that the thermosensitive mutation had no major effect on DNA chain elongation although some reduction in rate is apparent at later times.

*Kinetics of Macromolecular Synthesis after a Temperature Shift*

The experiments described in the previous section did not indicate any major effect of the thermosensitive mutation on DNA chain elongation. On the other hand the gradual reduction in accumulation of DNA at 42° C might indicate a reduction in the frequency of initiation. If so, this could be an indirect effect of a reduced rate of RNA or protein synthesis or a specific effect upon initiation.

We therefore sought to apply a more sensitive method to determine whether the onset of the reduction in the rate of DNA synthesis precedes that of any effect on RNA synthesis. Cultures were pulse-labelled with [$^{14}$C]-uridine and [$^3$H]-thymidine (or with reversed isotopes) to label RNA and DNA respectively. Figure 4 shows that after the temperature shift there was an immediate enhancement of RNA and DNA synthesis presumably as a direct consequence of the raised temperature. The rate of RNA synthesis then became essentially linear after about 30 min. However, the rate of DNA synthesis was reduced very shortly after the shift and continued to fall during incubation at 42° C although at a rate less than that following addition of rifampicin. This result, together with the finding that elongation of DNA chains is not substantially affected at early times after the shift, indicates that the frequency of initiation of replication is reduced at 42° C. Since the synthesis of RNA was less affected than that of DNA we conclude that any effect upon initiation is not simply due to a reduction in RNA synthesis.

*DNA Concentration and Replication Times at Permissive Temperatures*

Growth of thermosensitive mutants at various permissive temperatures which allows partial expression of the mutation, without loss of colony forming ability, has been employed before to study the physiological properties of a *dnaA* mutant (Orr et al., 1978). As mentioned above, cells of LE316 are longer than the wild type cells at permissive temperature but do not lose viability when incubated at temperatures up to 37° C. We therefore felt that a study of DNA synthesis at various permissive temperatures could lead to a better understanding of the physiological role of the *cou* gene product.

DNA concentration (DNA/mass) has been shown to be determined by these parameters: the average mass per chromosome origin at which initiation of rounds of chromosome replication takes place; the replication time (C) of the chromosome; and the growth rate (Pritchard and Zaritsky, 1970). An increase in the initiation mass, an increase in C and an increase in growth rate all reduce the DNA concentration (Pritchard and Zaritsky, 1970).

Cultures of LE316 and LE234 were grown exponentially in glucose minimal medium supplemented with a casamino acids mixture, at 30° C, 34° C and 36° C. The DNA concentration of LE316 at all temperatures was lower than that of LE234 (Table 4) but did not change substantially when the tempera-

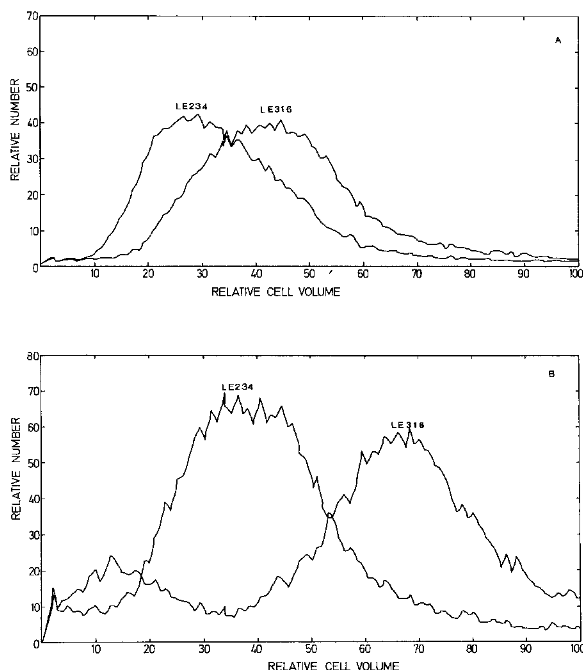



**Fig. 5A and B.** Relative distribution of cell volumes of LE234 and LE316 grown in M9 glucose minimal medium: **A** at 30° C; **B** at 36° C

ture was raised. The average cell colume of LE316 at 30° C was larger than that of LE234 (Fig. 5a) and the difference increased as the temperature was raised (Fig. 5b). The above experiments were performed by labelling with radioactive thymidine in the presence of uridine. As the strains which we have used are *thy*$^+$, incorporation of the nucleotides from the medium could be affected by changes in the internal pools of nucleotides and therefore could give misleading results. The DNA concentration was therefore measured chemically at 30° C using the diphenylamine method. The results (data not shown) were similar.

C times of exponentially growing cultures of LE234 and LE316 at 30° C, 34° C and 36° C were estimated by incubating them in the presence of rifampicin and determining the increment of DNA. The time taken for DNA synthesis to cease (the run-out time) was also estimated.

Replication times were calculated from the increment or estimated directly from the run-out time. To eliminate any possibility of non specific effects of rifampicin, the same experiment at 34° C was done in the presence of chloramphenicol. The results were identical to those obtained with rifampicin.

Table 4 shows that the C times of LE316 and LE234 were not substantially different at any temperature tested. To put these data in perspective, it would require about a 3-fold change in C to reduce the DNA concentration of LE316 at 36° C to that found in LE234. The observed changes in DNA concentration were hence much greater than could be accounted for by the calculated changes in the C times. Since the growth rate of LE316 was similar to LE234 at all temperatures (Table 4), the major changes in DNA concentration were probably the result of an altered initiation mass.

### *Cell Division and Cell Morphology in LE316*

Cell division continued after a temperature shift at a linear rate after a delay of 20–40 min (Fig. 6). However, no increase in the viable population could be detected. The number of viable cells stayed constant for a few hours before declining.

Further analysis revealed that most of the newborn cells after 2 h at the restrictive temperature were smaller in average cell volume than either LE316 or LE234 (Fig. 7; see below). Initially after the shift to 42° C the cells became longer because of a reduced rate of cell division (confirmed by phase contrast microscopy), but an hour later a new population with a smaller average cell volume was formed.

As the *cou* gene product is assumed to be involved in the maintenance of DNA in a supercoiled state, we investigated the state of the mutant nuclei at both permissive and restrictive temperatures. For this pur-

**Table 4.** Growth parameters

| Temp. °C | $\tau$ (min) | | DNA/MASS[a] | | $\Delta G$ (%) | | $C_1$ (min) | | $C_2$ (min) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LE234 | LE316 | LE234 | LE316 | LE234 | LE316 | LE234 | LE316 | LE234 | LE316 |
| 30 | 51 | 51 | 1.0 | 0.72 | 99 | 90 | 112 | 106 | 110 | 105 |
| 34 | 40 | 40 | 1.0 | 0.66 | 51 | 61 | 51 | 59 | 55 | 55 |
| 36 | 35 | 41 | 1.0 | 0.58 | 48 | 61 | 42 | 60 | 45 | 50 |

$C_1$ – calculated from $\Delta G$. $C_2$ – calculated from run out times.

[a] The values of DNA/mass for LE316 were calculated relatively to those of LE234. The DNA/mass of LE234 was chosen independently as 1.0 at each temperature.

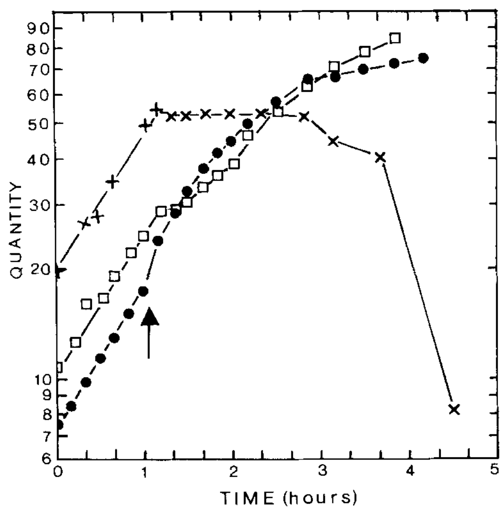



Fig. 6. Growth and viability of LE316 in M9 glucose minimal medium. The arrow indicates the time of the temperature shift from 30° C to 42° C. (●) absorbance at 450 nm × $10^{-1}$; (×) $10^7$ × viable particles per ml; (□) $10^7$ × particles per ml, determined by Coulter counter

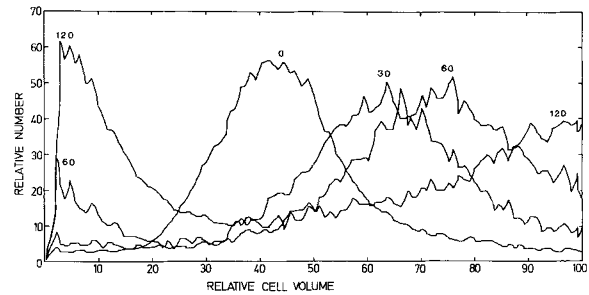
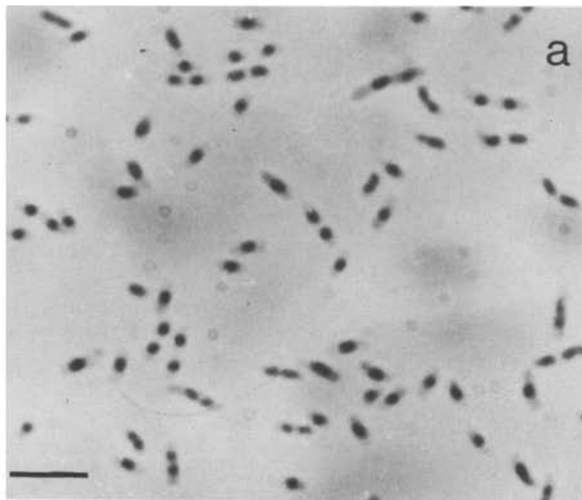
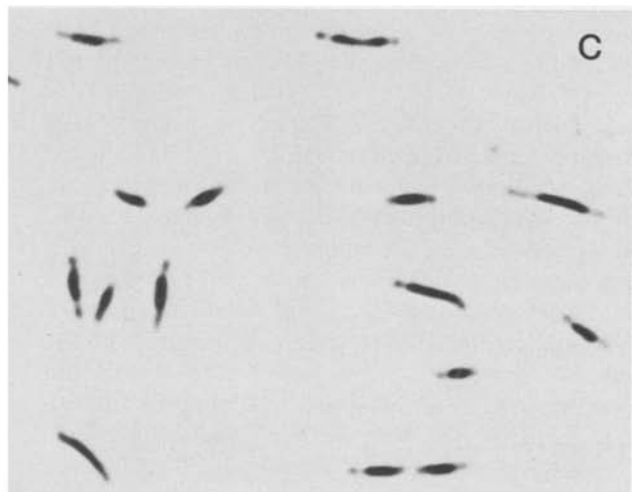
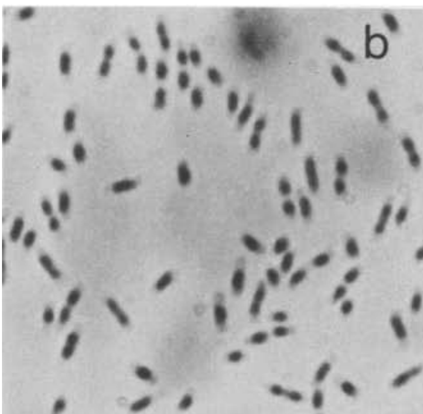



Fig. 7. Relative distribution of cell volumes of LE316 after a temperature shift to 42° C in M9 glucose minimal medium. The times (in min.) after the shift are indicated



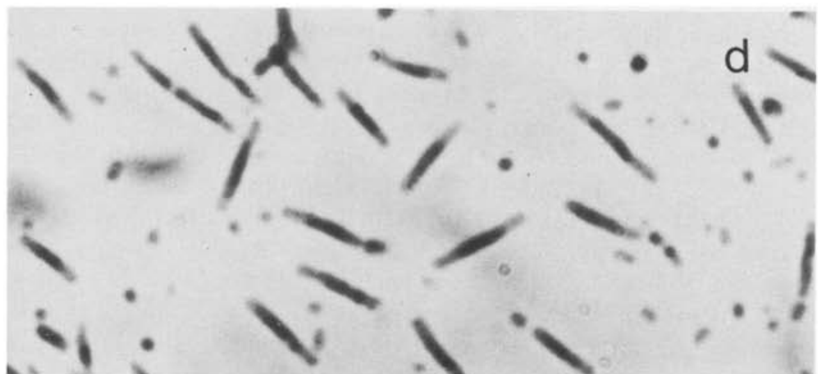




Fig. 8a–d. Nuclei of LE234 and LE316 grown in M9 glucose minimal medium stained with Giemsa after HCl hydrolysis. **a** LE234 at 30° C; **b** LE316 at 30° C; **c** LE316 after 60 min. at 42° C; **d** LE316 after 150 min. at 42° C. Bar represents 3.5 μm

pose we used the staining technique by Giemsa after HCl hydrolysis. Figure 8 shows that nuclei of LE234 at 30° C are small and compact, similar to the picture found 42° C (not shown). The mutant, however, had quite elongated nuclei even at permissive temperature. After a shift to 42° C, cells of LE316 elongated and the nuclei appeared to spread out along the length of the cells (Fig. 8). The staining technique further demonstrated that the small cells which appeared at 42° C were invariably anucleate.

These nuclear changes were not an artefact of the staining procedure as similar results were observed when staining with the fluorescent dye, 4′,6-diamidino-2-phenyl indole-dihydrochloride, without hydrolysing the RNA (Williamson and Fennell, 1975). These results suggest therefore that the *cou* gene product is involved in the maintenance or the organisation of the DNA as a specific structure.

## Discussion

A single genetic locus – *cou* – which determined resistance to coumermycin $A_1$ and novobiocin has been identified as a structural gene for one of the subunit polypeptides of DNA gyrase (Gellert et al., 1976b). The evidence presented in this paper suggests that the conditional lethal mutation in LE316 is located in the same gene. The evidence so far, suggests that although the *dnaA* and *cou* loci are very closely linked they are nevertheless distinct genes. At the same time, it is interesting that both the *dnaA* and *cou* products (see discussion below) are involved in initiation of DNA replication. It is perhaps significant that the *dnaA* T46 mutant is more sensitive to clorobiocin than the isogenic strain $dnaA^+$ (unpublished data). These results suggest that the *dnaA* and *cou* products interact during initation of chromosomal replication in *Escherichia coli.*

Although extensively studied in vitro, the physiological role of DNA gyrase is not clear. It has been suggested that the inhibitory effect of novobiocin and coumermycin $A_1$ on DNA replication (Ryan, 1976; Staudenbauer, 1975; Smith and Davis, 1967), transcription (Ryan, 1976; Smith et al., 1978; Sanzey, 1979; DeWyngaert and Hinkle, 1979), and maintenance of DNA in supercoiled form (Gellert et al., 1976b; Drlica and Snyder, 1978) reflect the importance of DNA gyrase for these processes and its presumed role in illegitimate recombination (Mitzuuchi et al., 1978b).

The data presented in this paper are not consistent with a major role for the *cou* gene product in DNA chain elongation. However, the reduced initiation mass of the mutant as well as of other clorobiocin resistant mutants (Fairweather et al., in preparation), suggests that initiation of replication is delayed in the mutant even at permissive temperature, whilst aberrant division and disorganisation of nuclei at 42° C suggest that DNA gyrase is essential for the structural organisation of the chromosome and its normal association with the cell membrane.

The early reduction in the rate of DNA synthesis in LE316 at 42° C appears to be primarily due to a further reduction in the rate of initiation of rounds of replication. This did not appear to be caused by the reduction in bulk RNA synthesis (and hence mass) since the effects on DNA preceded those on RNA synthesis. Delayed initiation in the mutant could simply indicate a specific requirement for supercoiled DNA at the origin; to facilitate attack by initiation enzymes analogous to the endonuclease (A protein), coded by phage $\Phi$X174, which is inactive with relaxed DNA (Marians et al., 1977); or to promote specific transcription at the origin which is considered essential for initiation in both *E. coli* (see Messer et al., 1975; Orr et al., 1978) and phage $\lambda$ replication (Dove et al., 1971).

Consistent with the latter are several recent reports which indicate that supercoiled DNA enhances transcription in vitro (Botchan et al., 1973; Richardson, 1975; Wang, 1974), and more significantly that novobiocin-like drugs have varying effects on different promoters (Smith et al., 1978; Sanzey, 1979).

The discrepancy between the effect of the mutation in LE316 on DNA replication at 42° C and the immediate inhibition of DNA synthesis by novobiocin, coumermycin and by clorobiocin (Fairweather et al., in preparation) is not understood. It is possible that the mutation is leaky although several physiological changes are detectable immediately after the shift to the restrictive temperature. One possible explanation arises from the suggestion that DNA gyrase is not released from the DNA in the presence of novobiocin and coumermycin (Sugino et al., 1978); these drugs (and clorobiocin) might therefore trap the enzyme in a complex on the DNA which would prevent strand separation and therefore on-going replication. The mutation in LE316 might lead to the release of DNA gyrase from the DNA at non permissive temperature with consequent loss of overall supercoiling of the chromosome, but with little effect on replication fork movement as discussed below.

The changes in nuclear morphology of strain LE316 at 42° C were quite dramatic and indicate that the DNA is unfolded or, more likely, has greatly reduced superhelicity due to an inactive DNA gyrase. The alternative, that the DNA is nicked and therefore

relaxed, appears unlikely since fragmentation of newly synthesised DNA was not detected (unpublished results). It is perhaps significant that novobiocin and coumermycin $A_1$ reduce the superhelical density of *E. coli* (Drlica and Snyder, 1978) and phage λ DNA (Gellert et al., 1976b), consistent with a role for DNA gyrase in maintaining the many supercoiled domains of the chromosome (Worcel and Burgi, 1972). Furthermore, this suggests that DNA gyrase is distributed throughout the chromosome rather than simply confined to the replication fork. Indeed it is the paradox of our results that whilst the structural disorganisation of the chromosome can readily explain all the pleiotropic effects of the mutation in LE316 DNA chain elongation continues almost undisturbed. We suggest therefore that the possible role of DNA gyrase or at least the *cou* subunit, in strand separation during replication (see Champoux, 1978) should be re-evaluated, with the probability that the enzyme is primarily involved in maintaining the required superhelical density of the chromosome for purposes other than DNA fork movement.

As in many mutants which exhibit defective metabolism of nucleic acids, cell division is also abnormal in LE316. Interestingly, however, it is not the frequency of divisions which is primarily affected but the positioning of the septa, generating anucleate cells about one third of the normal length. The reason for this effect is not clear, although the simplest explanation is that it follows directly from the physical changes in the structure of the chromosome preventing both nuclear segregation and the formation of septa at the correct sites.

*Acknowledgements.* We wish to thank Dr. B.G. Spratt and Mr. G.S. Plastow for λ strains, and their help with λ methodology, and Dr. Michael Sargent for helping with nuclei staining and for the fluorescent dye 4′6-Diamidino-2-phenyl-indole dihydrochloride. We are also grateful to Dr. R.A. Cooper for the *dgo* strains. This work was supported by a grant from the Medical Research Council to R.H.P. N.F.F. was supported by a Science Research Council CASE studentship in collaboration with May & Baker Ltd.

## References

Botchan, T., Wang, J.C., Echols, H.: Effect of circularity and superhelicity on transcription from bacteriophage λ DNA. Proc. Natl. Acad. Sci., U.S.A. **70**, 3077–3801 (1973)

Champoux, J.J.: Proteins that affect DNA conformation. Ann. Rev. Biochem. **47**, 449–479 (1978)

Cooper, R.A.: The utilization of D-galactonate and D-2-Oxo-3-Deoxygalactonate by *Escherichia coli* K-12. Arch. Microbiol. **118**, 199–206 (1978)

De Wyngaert, M.A., Hinkle, D.C.: Involvement of DNA gyrase in replication and transcription of bacteriophage T7 DNA. J. Virol. **29**, 529–535 (1979)

Dove, M., Inokuchi, H., Stevens, W.: Replication control in phage λ. In: The Bacteriophage lambda (ed. A.D. Hershey) pp. 747–771. Cold Spring Harbor, N.Y.: Cold Spring Harbor Press 1971

Drlica, K., Snyder, M.: Superhelical *Escherichia coli* DNA: Relaxation by coumermycin. J. Mol. Biol. **120**, 145–154 (1978)

Gellert, M., Mizuuchi, K., O'Dea, M.H., Nash, H.A.: DNA gyrase: An enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. U.S.A. **73**, 3872–3876 (1976a)

Gellert, M., O'Dea, M.H., Itoh, T., Tomizawa, J.: Novobiocin and coumermycin inhibit DNA supercoiling catalysed by DNA gyrase. Proc. Natl. Acad. Sci. U.S.A. **73**, 4474–4478 (1976b)

Gellert, M., Mizuuchi, K., O'Dea, M.H., Itoh, T., Tomizawa, J.: Nalidixic acid resistance: A second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci., U.S.A. **74**, 4772–4776 (1977)

Higgins, N.P., Peebles, C.L., Sugino, A., Cozzarelli, N.R.: Purification of subunits of *Escherichia coli* DNA gyrase and reconstitution of enzymatic activity. Proc. Natl. Acad. Sci., U.S.A. **75**, 1773–1777 (1978)

Itoh, T., Tomizawa, J.: Involvement of DNA gyrase in bacteriophage T7 DNA replication. Nature (London) **270**, 78–79 (1977)

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685 (1970)

Laskey, R.A., Mills, A.D.: Quantitative film detection of $^3H$ and $^{14}C$ in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**, 335–341 (1975)

Marians, K.J., Ikeda, J.E., Schlagman, S., Hurwitz, J.: Role of DNA gyrase in ΦX replicative-form replication. Proc. Natl. Acad. Sci. U.S.A. **74**, 1965–1968 (1977)

Messer, W., Dankworth, L., Tippe-Schnidler, R., Womack, J.E., Zahn, G.: In: DNA synthesis and its regulation (eds. M. Goulian and P. Hanawalt) pp. 602–617, Menlow Park, Calif.: W.A. Benjamin 1975

Mizuuchi, K., O'Dea, M.H., Gellert, M.: DNA gyrase: subunit structure and ATPase activity of the purified enzyme. Proc. Natl. Acad. Sci., U.S.A. **75**, 3960–3963 (1978a)

Mizuuchi, K., Gellert, M., Nash, H.A.: Involvement of supertwisted DNA in integrative recombination of bacteriophage lambda. J. Mol. Biol. **121**, 375–392 (1978b)

Orr, E., Meacock, P.A., Pritchard, R.H.: Genetic and physiological behaviour of an *Escherichia coli* strain carrying the *dnaA* mutation T46. In: DNA synthesis – present and future. (eds. I.J. Molineux and M. Kohiyama) pp. 85–100, New York: Plenum Press. 1978

Pritchard, R.H., Zaritsky, A.: Effect of thymine concentration on the replication velocity of DNA in a thymineless mutant of *E. coli.* Nature (London) **226**, 127–131 (1970)

Ptashne, M.: Isolation of the λ phage repressor. Proc. Natl. Acad. Sci. U.S.A. **57**, 306–313 (1967)

Richardson, J.P.: Initiation of transcription by *Escherichia coli* RNA polymerase from supercoiled and non-supercoiled bacteriophage PM2 DNA. J. Mol. Biol. **91**, 477–487 (1975)

Ryan, M.J.: Coumermycin $A_1$; a preferential inhibitor of replicative DNA synthesis in *Escherichia coli*: *In vivo* characterisation. Biochemistry **15**, 3769–3777 (1976)

Sanzey, B.: Modulation of gene expression by drugs affecting deoxyribonucleic acid gyrase. J. Bacteriol. **138**, 40–47 (1979)

Schrenck, W.J., Weissberg, R.A.: A simple method for making new transducing lines of coliphage λ. Molec. Gen. Genet. **137**, 161–167 (1975)

Smith, C.L., Kubo, M., Imamoto, F.: Promoter-specific inhibition

of transcription by antibiotics which act on DNA gyrase. Nature (London) **275**, 420–423 (1978)

Smith, D.H., Davis, B.D.: Mode of action of novobiocin in *Escherichia coli*. J. Bacteriol. **93**, 71–79 (1967)

Staudenbauer, W.L.: Novobiocin-specific inhibitor of semiconservative DNA replication in permeabilized *Escherichia coli* cells. J. Mol. Biol. **96**, 201–205 (1975)

Sugino, A., Peebles, C.L., Kreuzer, K.N., Cozzarelli, N.R.: Mechanism of action of nalidixic acid. Purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. U.S.A. **74**, 4767–4771 (1977)

Sugino, A., Higgins, N.P., Brown, P.O., Peebles, C.L., Cozzarelli, N.R.: Energy coupling in DNA gyrase and the mechanism of action of novobiocin. Proc. Natl. Acad. Sci. U.S.A. **75**, 4838–4842 (1978)

Wang, J.C.: Interaction between twisted DNA's and enzymes: the effect of superhelical turns. J. Mol. Biol. **87**, 797–816 (1974)

Williamson, D.H., Fenell, D.J.: The use of a fluorescent DNA binding agent for detecting and separating yeast mitochondrial DNA. In: Methods in cell biology (ed. D. Prescott) pp. 335–351. New York: Academic Press 1975

Worcel, A., Burgi, E.: On the structure of the folded chromosome of *Escherichia coli*. J. Mol. Biol. **71**, 127–147 (1972)

Communicated by B.A. Bridges

Received September 6, 1979