Genetical and Physiological Studies on a Thermosensitive Mutant of *Escherichia coli* Defective in Cell Division

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SUMMARY

A new temperature-sensitive mutant of *E. coli*, defective in cell division, was isolated after selection for tolerance to colicin E2. The mutant strain, ASH124, growing in either minimal or complex medium, commences filament formation immediately upon shift to high temperature. High densities of bacteria or the presence of 0.44 M-sucrose prevents filament formation at 42 °C and division continues. Filament formation in the mutant is reversible and upon return to 29 °C the multinucleate filaments divide up into normal-sized bacteria by a series of rapid but sequential divisions. In the presence of chloramphenicol at 29 °C, 25% of these division sites are still expressed. A genetic locus designated *ftsH*, apparently controlling both temperature sensitivity and filament formation, was provisionally mapped at minute 80 on the *E. coli* K12 map.

INTRODUCTION

The triggering of division in *Escherichia coli* is apparently determined by a complex series of events including the completion of chromosomal replication and the sequential synthesis of specific proteins during the cell cycle (see, for example, Donachie, Jones & Teather, 1973; Slater & Schaechter, 1974). In an attempt to analyse the regulatory basis of this process genetically, Ricard & Hirota (1973) have identified seven genes in thermosensitive mutants of *E. coli*, which affect cell septation in some way. Several other reports of similar mutants have appeared in recent years (Reeve, Groves & Clark, 1970; Nagai & Tamura, 1972; Castellazzi, George & Buttin, 1972; Zusman, Inouye & Pardee, 1972; Stone, 1973), attesting to the complexity of the division process. Unfortunately, little useful information concerning the regulation of cell division has so far emerged from these studies. In embarking on the examination of yet another mutant, defective in cell division, we therefore set ourselves certain specific objectives: (i) the establishment of the uniqueness of this particular mutation by a detailed genetic analysis of its properties, including its location on the *E. coli* map; (ii) the determination, as far as possible, of the stage in the cell cycle which is primarily affected; (iii) the specific examination of the synthesis of many individual membrane proteins at both permissive and non-permissive temperatures with the improved techniques now available, in anticipation that this might lead to some understanding of the molecular basis of the mutational block. Finally, we introduced an element of selection into the isolation of the mutant by selecting initially for tolerance to an agent, colicin E2, acting on the cell surface, which blocks cell division in sensitive bacteria (see Holland, 1975). We present here the general properties of the mutant, including the mapping of the mutant locus *ftsH* at minute 80 on the *E. coli* K12 map.

METHODS

Strains. Strain ASH124, *thyA metB str-r fisH* was isolated from strain ASH1 (Buxton & Holland, 1973) after 2-amino purine mutagenesis, as tolerant to colicin E2 at 30 °C and unable to grow on nutrient broth plates at 42 °C. Subsequent tests confirmed that ASH124 formed multinucleate filaments in liquid medium after shift to 42 °C. Strain ASH124I was obtained as a spontaneous, faster-growing derivative of ASH124 which still formed filaments at 42 °C but was no longer tolerant to colicin E2 or E3. Stocks of both strains were preserved as frozen (−20 °C) cultures in nutrient broth (Oxoid No. 2) containing 20% (w/v) glycerol.

Strain ASH1 is an Hfr strain which displays both a clockwise and an anti-clockwise mode of conjugational transfer, originating near minute 11 on the chromosome (Buxton & Holland, 1973).

Strain ED1002 (F_{H} , *lac*⁺, transposition Hfr, *pro str-s*) was obtained from P. Meacock (Broda, Meacock & Achtman, 1972). A derivative of this strain mutant at the *bfe* locus (Buxton, 1971), and therefore resistant to E-colicins and bacteriophage BF23, was isolated for use in this study as described previously (Hill & Holland, 1967); BW114 (Hfr P4x, *uvrA thi str-s*) and Hfr KL16 (*recA*) were from Dr B. M. Wilkins.

Mutants ASH5 (an F^- strain, *colI^B thr leu thi try his argHBCE lacY gal malT xyl mtl str-r tsx-r λ⁻ non⁺ supE⁺*) and Hfr J4 (*bfe thr leu thiA φ80^B*) are Leicester laboratory strains (see also Threlfall & Holland, 1970; Taylor & Trotter, 1967).

Strain ASH503 (F^- *colI^B thr leu try lacY gal malT str-r λ⁻ uvrA cycA fisH*) was obtained from a cross between Hfr BW114 and ASH501 (F^- *fisH cycA argH str-r*) and selecting for *argH*⁺ [*str-r*].

Media and cultural conditions. Minimal medium (M9), supplemented with amino acids where necessary, nutrient broth (NB), and conditions for growth of aerated cultures were all as described (Buxton & Holland, 1973).

Preparation of colicins. Colicins E3 and E2 were prepared from *E. coli* CA38 (colicin E3⁺) and *Salmonella typhimurium* LT2, 906 (colicin E2-P9) respectively and assayed as described (Hill & Holland, 1967). Colicin I was prepared from *E. coli* strain BW131 (colicin I*bdrd*) kindly provided by Dr B. M. Wilkins.

Coulter counting. Total cell numbers in bacterial cultures were determined with a Coulter counter, model B, as described (Holland & Holland, 1970).

Microscopy. Examination of bacteria was carried out using a Wild phase-contrast microscope (Wild Heerbrugg Ltd, Heerbrugg, Switzerland). Bacteria were stained with polyvinylpyrrolidone as described by Stone (1973), to detect nuclear bodies. For counting bacteria and filaments, bacterial samples were mixed with 0.5% formalin, concentrated 10-fold to about 2×10^8 bacteria/ml, mixed with NB containing 0.4% (w/v) agar, and this mixture was allowed to solidify on a Thoma haemocytometer cell.

Measurement of macromolecular synthesis. Incorporation of [³H]uracil [¹⁴C]thymine, and [¹⁴C]histidine into cold trichloroacetic acid-precipitable material in both mutant and wild-type strains at 42 °C was carried out as described (Holland, 1968; Senior, Kwasniak & Holland, 1970). Cultures were pre-labelled at 29 °C with the appropriate isotope for one to two generations as indicated in the text, before temperature shift. For the measurement of phospholipid synthesis, [³H]glycerol incorporation into trichloroacetic acid-precipitable material was determined in wild-type and mutant strains at either 29 or 42 °C. Final specific activity of [³H]glycerol was 0.2 μCi/μg. Previous studies (Daniels, 1969; Dr P. Beard, personal communication) have indicated that 80 to 100% of [2(*n*)-³H]glycerol is incorporated into the phospholipid fractions of growing bacteria.

Isotopes. L-[U-¹⁴C]histidine (500 mCi/mmol), [5,6-³H]uracil (40000 mCi/mmol), [2-¹⁴C]-thymine (60 mCi/mmol) and [2(*n*)-³H]glycerol (100 to 500 mCi/mmol) were all obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

Conjugational analysis. Crosses were usually carried out at 30 °C and recombinants purified by two cycles of re-streaking on selective plates before testing. All other procedures, including methods of analysis of 3 and 4 factor crosses, have been described (Buxton & Holland, 1973).

Phenotype tests. Suspensions of recombinants in buffer were cross-streaked against bacteriophage BF23, or λ on nutrient-agar, and incubated overnight at 30 °C; colicin sensitivity tests were carried out similarly. Ultraviolet sensitivity was determined as before (Buxton & Holland, 1974). In determining sensitivity to cycloserine, samples were spotted on to M9 minimal agar containing 2.5×10^{-5} M D-cycloserine and incubated for 36 h at 30 °C. Temperature sensitivity was determined at 42 °C after spotting samples on nutrient agar and incubation for 16 h.

*Isolation of *cycA* mutants.* An exponential-phase culture of strain ASH501 grown in nutrient broth was concentrated and about 2×10^8 bacteria plated on M9 minimal agar containing 2.5×10^{-5} M D-cycloserine as described by Russell (1972). This selects for mutations at the *cycA* locus at minute 84 on the *E. coli* K12 chromosome (Taylor & Trotter, 1972). Several spontaneous mutants were picked, purified and shown to grow in the presence of 2.5×10^{-5} M-cycloserine but not at higher concentrations (10^{-4} M). One such mutant was kept and the presence of the *cycA* locus confirmed in subsequent genetic analysis.

RESULTS

Inhibition of division in strain ASH124

When the *ftsH* mutant ASH124, growing exponentially at 29 °C in either M9-glucose medium or NB, was shifted to 42 °C, multinucleate filaments which lacked detectable septa under the phase-contrast microscope were produced. The kinetics of this effect were determined by measurement of bacterial number in the Coulter counter and by the determination of E_{450} in the same culture. Virtually complete inhibition of division occurred, apparently with little or no delay, upon temperature shift (Figs. 1 and 4). In minimal glucose medium, with ASH124 and some of its derivatives, incubation at 42 °C eventually resulted in clumping and consequently in an apparent loss in number of bacteria (Fig. 1*b,c*). However, this effect occurred some time after the onset of filament formation and was not observed in cultures grown in nutrient broth.

Filament formation could be suppressed by raising the osmolarity of the growth medium (Fig. 1*c*), as found with many *Fts* mutants (Reeve, Groves & Clark, 1970; Ricard & Hirota, 1973). Filament formation at 42 °C was also absent in cultures of high bacterial density ($> 3 \times 10^7$ to 4×10^7 bacteria/ml). This effect, which was not investigated further, was previously observed with a thermosensitive mutant by Stone (1973), who suggested that growth under conditions of limiting oxygen tension might be the factor causing suppression. The *Fts* mutant BUG6, studied by Reeve *et al.* (1970), is also reported to divide normally when shifted to 42 °C at high bacterial densities (J. N. Reeve, personal communication).

Effect of temperature shift upon macromolecular synthesis

Cultures of ASH1 and ASH124 in nutrient broth were grown for several generations at 29 °C in the presence of [¹⁴C]thymine, before shifting to 42 °C in the presence of the isotope. Incorporation at 42 °C continued for at least 1.5 h, precisely in parallel with the increase

Fig. 1

Fig. 2

Fig. 1. Inhibition of division in ASH124 (*ftsH*⁻) after temperature shift. Strain ASH124 growing in M9-glucose with shaking at 29 °C was diluted in pre-warmed medium and incubation continued at 42 °C. Samples were removed at intervals and (a) extinction measured at 450 nm or (b) bacterial number determined in a Coulter counter. In (c), a similar culture of ASH124 was shifted to 42 °C in the presence or absence of 0.44 M-sucrose.

Fig. 2. Effect of temperature on DNA synthesis. Strain ASH124 (*fts*⁻) (a) and parental strain ASH1 (b) were grown with shaking in nutrient broth at 29 °C with [¹⁴C]thymine (final specific activity 0.06 μ Ci/ μ g). After 90 min, the cultures were diluted to approximately 2×10^7 bacteria/ml in medium containing [¹⁴C]thymine of identical specific activity. Incubation at 29 °C was continued and samples were removed at intervals to determine extinction or incorporation of [¹⁴C]thymine into acid-precipitable material. When the cultures reached a density of 0.5 (E_{450}), cultures were diluted 5-fold into pre-warmed medium containing [¹⁴C]thymine and labelling was continued at 42 °C. ●, acid-precipitable [¹⁴C]thymine; ○, E_{450} .

in E_{450} (Fig. 2). DNA synthesis in the mutant therefore appears to continue normally at the restrictive temperature.

The effect of temperature shift upon RNA and protein synthesis was also examined (Fig. 3a,b). In both strains the rates of incorporation initially exceeded the increase of extinction, but after about 60 min the synthesis of RNA and protein in both mutant and wild-type strains was observed to continue to increase in parallel with total cell mass as measured by E_{450} . Phospholipid synthesis in the mutant was determined by following incorporation of [³H]glycerol into acid-precipitable material. Phospholipid synthesis in the mutant also continued after temperature shift, at a rate which precisely paralleled the rate of increase in extinction (Fig. 3c). From all these experiments it may be concluded that inhibition of cell division in the mutant cannot therefore be ascribed to any permanent imbalance in the synthesis of these major cell constituents at the restrictive temperature.

Fig. 3. Effect of temperature shift on protein, RNA and phospholipid synthesis. Strains (●) ASH1 and (○) ASH124 (*fts⁻*) were grown at 29 °C in M9-glucose medium containing radioactive isotope for 60 min, or for 120 min for [³H]glycerol labelling. At time zero, cultures were diluted 3 to 4-fold into pre-warmed, fresh medium containing isotope and incubation was continued at 42 °C; samples were removed at intervals and incorporation of radioactivity into acid precipitable material determined. ---, Mass increase (E_{450}) for ASH1; —, mass increase in strain ASH124. Initial specific activities in the medium: (a) [³H]uracil, 0.4 μ Ci/ μ g; (b) [¹⁴C]histidine, 0.0013 μ Ci/ μ g; (c) [³H]glycerol, 0.2 μ Ci/ μ g.

Fig. 4. Division of ASH124 filaments upon return to 29 °C. Strain ASH124 was first grown in nutrient broth at 29 °C, before dilution and shifting to 42 °C after 2 h. Incubation was continued at 42 °C for 75 min and filament formation confirmed by phase-contrast microscopy. Samples were removed at intervals throughout for determination of extinction and number of bacteria. Finally, the culture was again diluted and shifted back to 29 °C. Half the culture received 75 μ g chloramphenicol/ml at this stage (○); half did not (●).

Fig. 5

Fig. 6

Fig. 5. Effect of temperature shift on bacterial size in strain ASH124. With the data obtained from the experiment described in Fig. 4, extinction/ 10^7 bacteria was calculated and plotted against time at various temperatures. The arrow indicates the time when the extinction had doubled after return to 29 °C.

Fig. 6. Sensitivity of Fts mutants to colicin E2. Exponential cultures of (■) ASH1 (fts^+), (○) ASH124 ($ftsH$), and (●) the faster-growing derivative ASH1241 ($ftsH$), grown in nutrient broth at 30 °C, were diluted in broth to about 2.5×10^7 bacteria/ml and treated with colicin E2 (5 units/ 10^7 bacteria) at 29 °C. Samples were removed at various times, diluted in buffer, plated in duplicate on nutrient broth agar and incubated overnight at 30 °C to determine the fraction of surviving bacteria.

Expression of division potential in ASH124 upon return to low temperature

Strain ASH124 growing in NB medium was shifted to 42 °C for 70 min (about 1.5 doublings). By the end of this period the resultant filaments had attained a length two to three times that of normal rods. The culture was then diluted into fresh medium at 29 °C and the kinetics of subsequent division determined (Fig. 4). The filaments began to divide almost immediately after the shift to low temperature. The rate of division, however, was now almost twice the rate of increase of E_{450} , and after 2 h virtually all bacteria were of normal size (Fig. 5). Thus, division potential acquired during incubation at 42 °C is rapidly expressed at low temperature. However, the rate of division observed on return to 29 °C indicated that dividing up of the filaments was occurring sequentially rather than by abrupt fragmentation into bacteria of normal size. This was confirmed by phase-contrast microscopy, which revealed that filaments at 29 °C which showed visible 'septa' (i.e. which were in the process of cell separation) almost invariably contained only a single septum. In a sample of 1311 bacteria examined under phase-contrast (Table 1A) only two filaments were observed containing two septa. These results are consistent with a random distribution of the single septum between division sites, situated at polar, subpolar or central positions within the filaments. Thus although the formation of normal-sized bacteria is initiated in a successive fashion, the position of each cleavage plane appears to be distributed randomly amongst potential division sites.

The ability of filaments to divide was also examined in the presence of chloramphenicol.

Table 1. *Analysis of septum position in ASH124 filaments upon return to 29 °C*

Time at 29 °C (min)	No. of cells counted	Central (%)	Terminal* (%)	Subterminal† (%)	Normal rods (%)	Total septa (%)
(A) Untreated culture						
0	335	1.2	1.5	0.35	6.0	3.0
5	370	4.3	1.7	2.95	8.5	8.9
10	89	3.4	2.2	6.7	14.6	12.3
15	100	8.0	8.0	8.0	25.0	24.0
30	417	5.9	5.9	2.2	30.0	15.0
(B) Culture treated with chloramphenicol (75 µg/ml) at 29 °C						
0	91	2.2	1.1	0	7.6	
5	75	2.7	2.7	2.7	12.0	
10	124	8.1	4.0	0	13.7	
30	142	2.1	7.0	1.4	21.0	

* One cell length from a pole.

† Septum between central and terminal positions.

Division of the filaments did continue in the presence of the drug but abruptly ceased when about one-quarter of all potential division sites had been utilized (Fig. 4). Analysis of the positioning of the septum under these conditions revealed no preference for central rather than polar cleavage sites (Table 1 B). However, there was some indication that subpolar localization of the septum may be rather less frequent in filaments exposed to chloramphenicol (Table 1 B).

Other properties of strain ASH124 and one of its derivatives

Mutant ASH124 is a slow-growing strain with a mean generation time of 88 min at 29 °C (in supplemented minimal glucose medium) compared with 55 min for the parent strain. In addition, growth yields from glucose grown cultures at 29 °C were found to be only 80% of those obtained with the parent strain (data not shown) which may indicate some slight defect in energy metabolism in the mutant. In the course of this study a faster-growing derivative (mean generation time for supplemented minimal glucose of 73 min) appeared spontaneously in stocks of the mutant. This strain, ASH124I, nevertheless still retained the Fts phenotype. Very slow growth rates and inability to divide at high temperatures are therefore separable properties in strain ASH124. Tolerance to colicin E2, the property originally used to select Fts mutants, was also separable from thermosensitivity since ASH124I was completely sensitive to colicin E2 (Fig. 6). Both ASH124 and ASH124I, however, gave very small indistinct plaques and reduced plating efficiencies (<10%) with bacteriophage λ compared with the parental strain at 30 °C.

Genetic mapping of fts by conjugation analysis

To facilitate the genetic analysis of the *fts* locus, strain ASH124I was crossed with ASH5, a multi-auxotrophic F⁻ strain; a *xyl*⁺ [ColI^R] recombinant (strain ASH501), which was also temperature sensitive, was obtained. The data resulting from this cross indicated that *fts* was located in the 70 to 90 minute region. Consequently a derivative of ASH501, strain ASH503, was constructed (see Methods), carrying the markers *cycA* (84 minutes) and *uvrA* (81 minutes). This strain was then crossed with Hfr ED1002 (*bfe*), whose origin of transfer is close to minute 63, and selection made for the late marker, *thr leu* (Table 2). In addition, ASH501 was crossed with Hfr J4, whose origin of transfer is at minute 81, and selection made

Table 3. *Conjugational transfer of ftsH by Hfr J4*

		Selected marker	No. tested	Unselected markers			
				<i>bfe</i> ⁻	<i>str-s</i>	<i>thy</i> ⁺	<i>fts</i> ⁺
Donor Hfr 14 (<i>bfe leu str-s</i>)							
Recipient ASH501 (<i>ftsH argH thy str-r</i>)		argH ⁺ [leu ⁺]	116	92 %	81 %	65 %	52 %
Analysis 1				Indicated order			
	<i>str-r</i> <i>str-s</i>			<i>str-s</i>	<i>arg</i> ⁺	<i>fts</i> ⁺	→
	<i>fts</i> ⁺ 10 41						
	<i>fts</i> ⁻ 21 38						
Analysis 2				<i>str-r</i>	—	—	
	<i>thy</i> ⁺ <i>thy</i> ⁻			<i>thy</i> ⁺	<i>arg</i> ⁺	<i>fts</i> ⁺	→
	<i>fts</i> ⁺ 56 7						
	<i>fts</i> ⁻ 22 30						
				—	—	—

Fig. 7. *Escherichia coli* K12 map showing point of origin of Hfr strains (Taylor & Trotter, 1967, 1972) used in this study and the approximate position of the *ftsH* locus.

for the early marker, *argH* (Table 3). In contrast to Hfr ED1002, Hfr J4 transferred in an anti-clockwise direction (see Fig. 7). The analysis of the unselected markers from these crosses demonstrated that *fts* was transferred at high frequency by both Hfr strains. This places *fts* between minutes 63 and 81, and therefore to the left of *uvrA* which is transferred as a terminal marker by Hfr J4 (Taylor & Trotter, 1967). Moreover, since Hfr J4 is λ^+ , due to the integration of the F⁻ factor at the *malB* locus (B. Low, personal communication), *fts* is also to the left of *malB*. In addition, several lines of evidence indicated that *fts* is to the right of *argH* and *bfe* at minute 79. Thus from the cross with Hfr ED1002, *fts* was mapped to the right of *bfe* (Table 2, analysis 4) and the failure unambiguously to order *fts* and *uvrA* in this cross (Table 2, analyses 5, 6, 7 and 8) indicated the closeness of these latter two markers. From the cross with Hfr J4, *fts* was mapped to the right of *argH* (Table 3, analyses 1 and 2). Inspection of the data in Table 3 reveals that *fts*, despite its presumed

proximal position on the Hfr J4 chromosome, is found less frequently amongst recombinants than the distal markers *str* and *thyA*. This, however, is to be expected if *fts* is very close to the origin of transfer of Hfr J4 and consequently subject to early marker depression of recombinant formation. On the basis of all the conjugation data we therefore conclude that *fts* is close to minute 80 on the *E. coli* chromosome and that the probable map order is *argH-bfe-fts-malB* as shown in Fig. 7.

DISCUSSION

The properties of the strains discussed here appear to fulfil the minimum requirements of putative division mutants (see review by Slater & Schaechter, 1974). Moreover, the genetic locus apparently determining filament formation in these strains is distinct from the seven loci, *ftsA* to *G* previously mapped by Ricard & Hirota (1973). In particular, *ftsD* was reported by these workers to map at minute 78 but the *fts* locus investigated in this study mapped to the right of this region at minute 80. In accordance with the nomenclature of Ricard & Hirota we therefore designate this locus as *ftsH*.

Division of ASH124 was blocked immediately upon shift to high temperature and resumed, with little or no lag, on return to low temperature, indicating a defect which is expressed very late in the cell cycle. This suggests that even cells late in the D-period (Pierucci & Helmstetter, 1969) fail to carry out some essential step in septum formation, upon temperature shift. DNA replication and nuclear segregation did, however, continue but unlike *env* mutants (Normark, Boman & Bloom, 1971) chain formation was never observed. As seen with other division mutants (e.g. Reeve & Clark, 1972) *ftsH* strains appear to accumulate division potential, i.e. to construct incomplete or inactive division sites during growth at 42 °C. Completion of these sites is accomplished rapidly at a rate faster than further mass increase upon return to the permissive temperature, and the filaments divide up to produce bacteria of normal size. Nevertheless, such divisions are clearly sequential, indicating either some physical restraint upon simultaneous, multiple ingrowths of the cell envelope or the presence of limiting amounts of a cellular constituent, which serves a catalytic role in septum formation. Unfortunately, reports in the literature on similar studies are sufficiently unclear on this point for it to be impossible to assess whether this pattern of division is the norm after division delay, or a feature unique to *ftsH* mutants. Whatever is the basis of sequential division, under these conditions, septum formation is successively triggered, apparently at random, from any one of several possible sites, within each filament.

On the basis of the limited physiological studies so far carried out with strain ASH124, any speculation upon the nature of the temperature-sensitive component, or its possible role in the normal division process is unwarranted at this stage. Since some studies have indicated (unpublished data) that *ftsH* mutants have altered envelopes, future studies will examine the synthesis of membrane proteins in the mutants under a variety of conditions. In particular, these studies will seek to establish whether any membrane changes are primary or secondary effects of mutational changes at the *ftsH* locus, and whether this necessarily indicates the specific involvement of the *ftsH* gene product in division.

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