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Process of Cellular Division in *Escherichia coli*: Physiological Study on Thermosensitive Mutants Defective in Cell Division

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Thermosensitive *fts* mutants of *Escherichia coli* belonging to seven previously identified genetic classes (*ftsA* to *ftsG*) were studied from a physiological standpoint. These mutants immediately stopped dividing and formed multinucleated filaments when the temperature was shifted to 41 C. Macromolecular syntheses (deoxyribonucleic acid), ribonucleic acid, cell mass, and murein) continued exponentially for at least 40 to 120 min. The number of surviving bacteria remained constant during the time of incubation, and this number began to decrease exponentially, as the rate of cell mass increase leveled off from the initial rate. The recovery of cell division at 30 C in these filamentous cells was studied after 60 min of incubation at 41 C. The existence of three types of mutants was shown. The *ftsA* and *ftsE* mutants resumed cell division without new protein synthesis; *ftsD* mutants resumed cell division only if new protein synthesis occured, while *ftsB*, *C*, *F* and *G* mutants did not resume cell division at all. No alteration in the cell envelope was detected by the method used here, although the *ftsA*, *B*, *D*, *F* and *G* mutations, in contrast with *ftsC* and *E*, caused an increased resistance to penicillin G. It was also shown that the *recA* mutation did not suppress the effect of the *fts* mutations and that none of the lysogenic *fts* mutants induced prophage multiplication while forming filaments. The effects of osmotic pressure and salts which rescue the mutant phenotype is described.

While the genetic study of microorganisms has led to the description of a large number of important cell functions, the mechanisms of cell division and their regulation remain almost unknown.

To analyze the regulatory mechanisms involved in cell division, a genetic approach was first taken. By the isolation and genetic characterization of conditional thermosensitive mutants of *Escherichia coli* defective in cell septation, seven genes (*ftsA* to *ftsG*) were identified (16, 17, 18, 40; Ricard and Hirta, in press). Mutations in these genes cause the formation of multinucleated filaments.

To gain insight into the mechanism of the septation process, a physiological study of new *fts* mutants was undertaken.

MATERIALS AND METHODS

The bacterial strains and media used in this study will be described in the next paper (Ricard and Hirota, in press).

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Thymidine-*methyl*-^3H, ^{14}C-uracil, and DL-meso-2,6-diaminopimelic acid (DAP) ^3H were purchased from Service des Molécules Marquées, C.E.A., France. Penicillin G (specilline G) was purchased from Specia, Paris, and sodium deoxycholate was from Serlabo, Paris.

To 1 ml of an *E. coli* culture, a 4-min pulse of ^3H-thymidine (3 μCi/ml final) was given in a broth medium. Samples were shaken in a water bath at 30 or 41 C, and the reaction was stopped by adding 1 ml of ice-cold 10% trichloroacetic acid. Samples were kept in ice for 1 h and then filtered through membrane filters (Millipore Corp., type HA) and washed with 30 ml of 5% ice-cold trichloroacetic acid. Filters were dried and counted in vials containing 10 ml of a scintillation counting fluid (3 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2(5-phenyloxazolyl)-benzene in 1 liter of toluene) in an Intertechnique SL-30 scintillation counter (Intertechnique Instruments, France).

The procedure for the measurement of ribonucleic acid (RNA) synthesis was the same as that used for the measurement of deoxyribonucleic acid (DNA) synthesis, except that ^{14}C-uracil (1 μCi/ml final) was used as the precursor.

A series of *fts* mutants also carrying Dap$^-$ Lys$^-$

double mutations were constructed by mating. These strains were grown overnight in a broth medium containing 4 μCi of ^{3}H-DAP per ml and 40 μg of cold DAP per ml. The next morning, the cultures were diluted 1/20 in the same medium. The experiments were carried out with cultures in exponential growth phase. A 1-ml sample was taken at each time, added to 1 ml of ice-cold 10% trichloroacetic acid, and kept on ice for 1 h. Further manipulations were carried out as previously described.

Coulter counter model F (Coultronics, France) was used. Samples having a cell concentration between 6 $\times$ 10^6 and 6 $\times$ 10^8 cells/ml were diluted 1:200 in M63 (15). Formaldehyde was added to the M63 at a final concentration of 0.1%. This solution of M63 was filtered through a type HA membrane filter. A 50-μm diameter orifice was used; the setting used was A = 0.5; O = 4; S = 7.

Variation of the intensity of fluorescence after addition of 1-anilino-8-naphtalene sulfonic acid (ANS) was measured by the procedure described previously (15).

RESULTS

In all the *fts* mutants studied, septation was arrested immediately when the temperature of incubation was raised from 30 to 41 C. During the first 2 h of incubation at high temperature, the residual increase in cell number of the mutants used in this study did not exceed 5 to 20% of the initial number. During the same time, both the cell mass (optical density measured at 550 nm) at 41 and 30 C and the cell number at 30 C increased approximately 10-fold.

Mutants MFT108 (*ftsC*) and MFT84 (*ftsB*) segregated some DNA-less bacteria from the ends of the filaments due to residual septation at 41 C. A residual increase in cell number, about 15% after 2 h at 41 C, was observed in these mutants. The morphology of these filamentous cells will be given in the next paper, and the results will not be described here.

The survival of the mutant strains at nonpermissive temperature was measured. Samples were taken from a culture after various times of incubation at 41 C and plated on the nutrient agar medium at 30 C. The number of colonies formed after about 30 h of incubation at 30 C was counted. The number of surviving cells remained constant during the first 60 min of incubation at 41 C in all the *fts* mutants examined. Then, after variable times, this number began to decrease exponentially as the rate of cell-mass increase leveled off from the initial rate. This time varies depending upon the mutation as well as the rate of growth at 41 C (Fig. 1).

The increase of cell mass and the rates of RNA and DNA synthesis were compared at

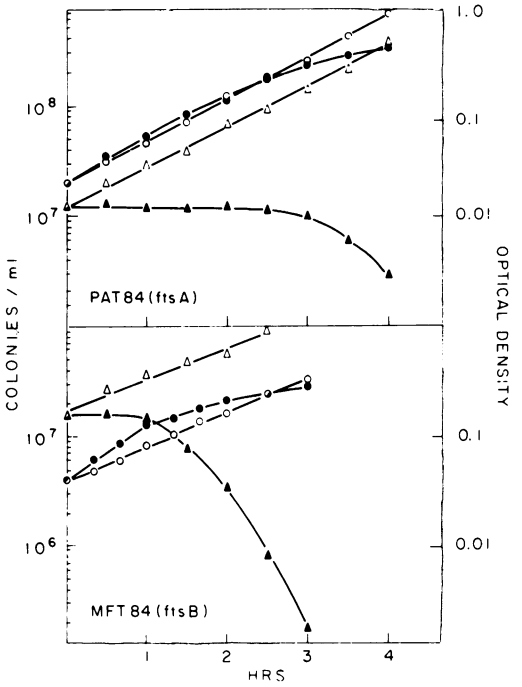

FIG. 1. *Survival of two representative strains, PAT84 (ftsA) and MFT84 (ftsB). Mutant bacteria were cultured for various times in liquid media at 30 or 41 C and then plated on agar solid medium and incubated at 30 C. The number of surviving cells per milliliter was estimated by counting the number of colonies formed after 24 to 36 h. Number of cells per milliliter in the 30 C culture* (△) *and in the 41 C culture* (▲)*; optial density (absorption at 550 nm in cuvette of 0.5-cm light path) of the 30 C culture* (○) *and of the 41 C culture* (●).

30 and 41 C by following optical density of the culture and the incorporation of ^{3}H-thymidine and ^{14}C-uracil into cold trichloroacetic acid-insoluble fractions. The synthesis of murein was also measured by following the incorporation of ^{3}H-DAP into trichloroacetic acid-insoluble fractions of cultures of Dap$^-$-Lys$^-$ derivatives of *ftsA* to *G*. The results have shown that for all the mutant classes, except *ftsC* (MFT108), the macromolecular syntheses tested continued to increase exponentially for at least 40 min at 41 C (Fig. 2). The rate of ^{14}C-uracil incorporation of *ftsD* was decreased only after 40 min at 41 C, although the septation process stopped immediately after the temperature shift. In case of the mutant MFT108, *ftsC*, DNA synthesis proceeded at a reduced rate at the nonpermissive temperature.

These experiments demonstrate that the defect in the *fts* mutants is specific for the septation process and that the filament formation is not a secondary effect due to the arrest of

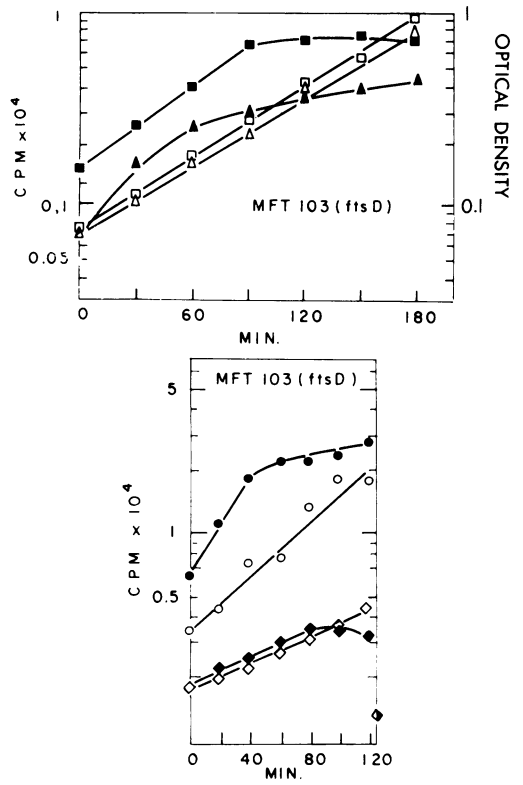

FIG. 2. *Macromolecular synthesis (DNA, RNA, optical density [OD], murein) of a representative strain, MFT103 (ftsD). Optical density (absorption at 550 nm in cuvette of 0.5-cm light path) at 30 C (△) and at 41 C (▲); incorporation of ^{3}H-thymidine during 4-min pulse labeling at 30 C (□) and at 41 C (■); incorporation of ^{14}C-uracil during 4-min pulse (○) and at 41 C (●); total incorporation of ^{14}C-α-ε-diaminopimelic acid at 30 C (◇) and at 41 C (◆).*

any of the macromolecular syntheses examined above.

The recovery of cell division at 30 C, in the presence or in the absence of chloramphenicol, was examined as follows. Filamentous cells were formed by growing the mutants in broth at 41 C for 60 to 80 min; the culture was then divided into four parts. The first was kept at 41 C; the second was kept at 41 C, and chloramphenicol (100 μg/ml) was added; the third was brought back to 30 C; and the fourth was brought back to 30 C, and chloramphenicol was added at the same time. A culture was grown at 30 C, without preincubation at 41 C, as a control. Cell number and size were followed with the use of a Coulter counter, and the cell mass increase was measured by a spectrophotometer.

The results show a wide variety of responses ranging from those mutants which did not recover at all at 30 C after 60 min of preincubation at 41 C, to those which divided very actively after 120 min of preincubation at 41 C. The type of response depended upon the various parameters, i.e., the mutant class, the time of preincubation at 41 C, and the growth rate of the strain both at non-permissive and permissive temperatures. However, when the time of preincubation was fixed within a range of 60 to 80 min, the following three types of mutants could be recognized. (i) Mutants of the first type resumed division at 30 C, even in the presence of chloramphenicol (100 μg/ml). (ii) Mutants of the second type recovered only in the absence of chloramphenicol. (iii) Mutants of the third type recovered very poorly whether chloramphenicol was added or not (Fig. 3, 4, and 5).

Cell division resumed when a culture of *ftsA* (MFT96, MFT1182, and PAT84 in Fig. 3) and *ftsE* (MFT1181) mutants was shifted to 30 C after a rather short lag (10–20 min) and pro-

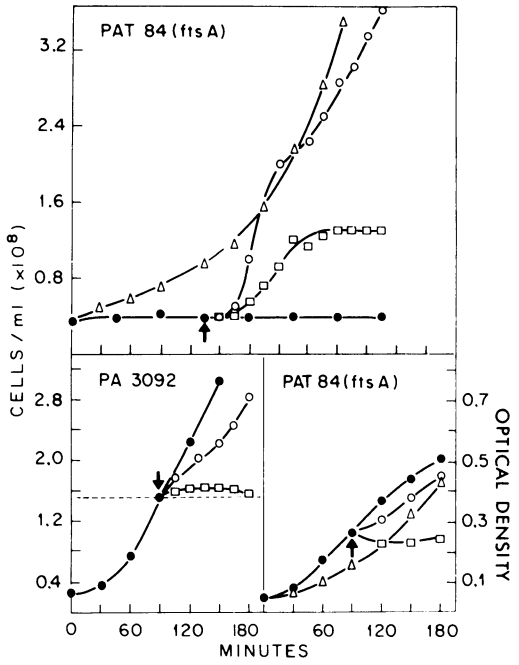

FIG. 3

FIG. 3, 4, 5. *Kinetics of cell division of PAT84 (ftsA), MFT103 (ftsD) and MFT84 (ftsB). Cell numbers are estimated with a Coulter counter. Optical densities are measured at 550 nm in 0.5-cm light path cuvettes. Cells incubated at 30 C (△); cells incubated at 41 C (●); cells incubated at 41 C and then shifted to 30 C (○); cells incubated at 41 C and then shifted to 30 C with addition of chloramphenicol (75 γ/ml) (□). Arrow indicates the time of temperature shift from 41 to 30 C.*

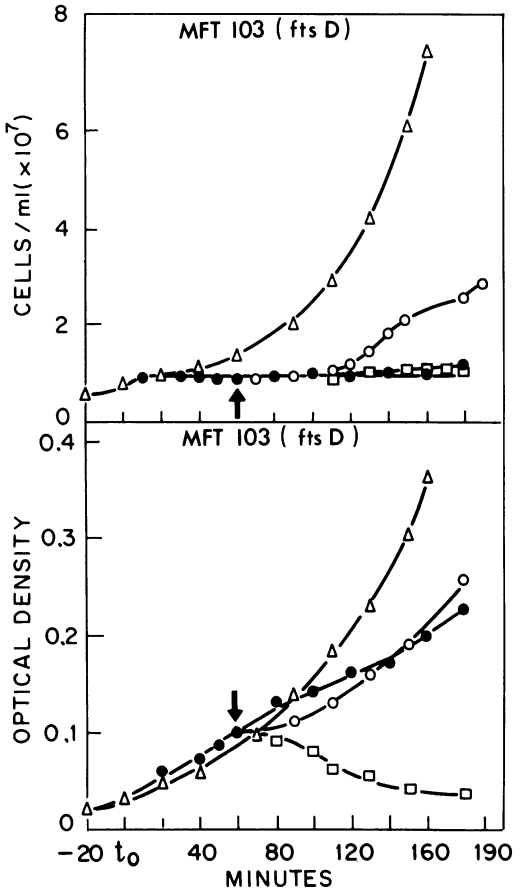

FIG. 4

ceeded for a while at a rate higher than that of the control culture kept at 30 C (Fig. 3). The addition of chloramphenicol (100 μg/ml) to the culture at the time of the temperature shift still allowed a significant increase of about 100 to 400% of the cell number while the cell mass increase stopped. The addition of the same concentration of chloramphenicol to the thermoresistant parental strain (PA 3092) does not allow more than 10 to 20% residual division.

After a longer lag (≃ 60 min), the capacity of cell division by *ftsD* (MFT103 and MFT1111) resumed and proceeded at a rate similar to that of the 30 C control culture (Fig. 4). However, the addition of chloramphenicol (100 μg/ml final) inhibited the recovery completely.

The recovery of cell division by *ftsB* (MFT84), *ftsC* (MFT108), *ftsF* (MFT114), and *ftsG* (CRT115) at 30 C was very poor, and if the time of preincubation at 41 C was within a range of 60 to 80 min no recovery occured (Fig. 5). The recovery observed for shorter time of preincubation is very slow and poor.

When cultures of the mutants of these three types were kept at 41 C, with chloramphenicol added after 60 min of preincubation, cell mass increase stopped immediately and the cell number remained constant.

In conclusion, filaments of the *ftsA* and *ftsE* formed after 60 min of preincubation at 41 C do not require new protein synthesis to recover the capacity for cell division to resume at 30 C. In contrast, *ftsD* mutants do need new protein synthesis to resume cell division. In the third case (*ftsB*, *C*, *F*, and *G* mutants), the incubation at high temperature causes rapid and irreversible damage to the division mechanism of the mutant cells. Similar mutants of these types have been described previously (1, 17, 18, 26, 29, 30, 40).

ANS produces a slight fluorescence when

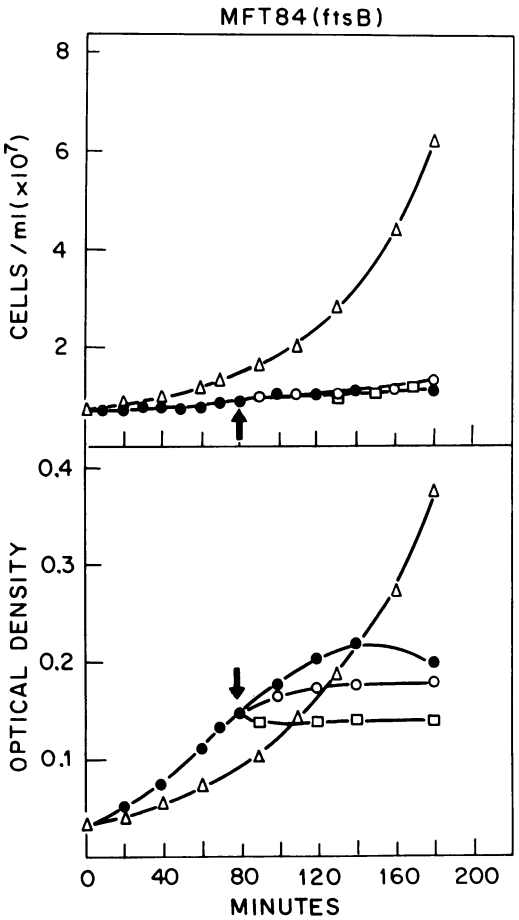

FIG. 5

added to pure water and an intense fluorescence when added to a solution of proteins, due to its fixation to hydrophobic groups (41). When added to a washed cell suspension of a mutant, CRT257, which accumulates abnormal membrane in the cell (19), ANS causes an increase in fluorescence significantly higher (×40) than that which is obtained with the parental strain. A significant difference was also observed with a DNA mutant (15). It has been postulated that a modification of the membrane of these mutants allows a greater accessibility of ANS to the hydrophobic sites of the membrane.

When a similar procedure was applied to our *fts* mutants (MFT1 and MFT1182, *ftsA*; MFT84, *ftsB*; MFT10338, *ftsD*; MFT29, *ftsG*), as well as their parental strain, no significant difference was found.

Some other mutants of *E. coli* altered in their cell envelope have been found hypersensitive to sodium deoxycholate (15, 17, 19, 27, 33). None of the *fts* mutants examined (from *ftsA* to *E*) showed an increased sensitivity to sodium deoxycholate (0.5 to 1%) compared with their parental strain PA3092, both at 30 and 41 C (data not shown).

It has been reported that penicillin G has two distinct morphological effects on *E. coli* (34, 37, 39). At low concentrations (20–100 U/ml), cell division is blocked and filament formation occurs; at high concentrations, however, cell wall synthesis is inhibited and the cells lyse. The effect of penicillin G on various thermosensitive *fts* mutants and on their parental strain was therefore analyzed. Penicillin (0, 50, 100, or 250 U/ml) was added to samples grown initially at 30 C and subsequently cultured either at 30 or 41 C. Cell mass increase and cell lysis were followed by measuring the optical density of the cultures and examining the cell morphology with the light microscope.

These experiments have shown differences between the various *fts* classes examined. Mutants of *fts* classes *A*, *B*, *D*, *F*, and *G*, but not *C* and *E*, were significantly more resistant to the penicillin lysis than that of their parental strain PA 3092 (Fig. 6).

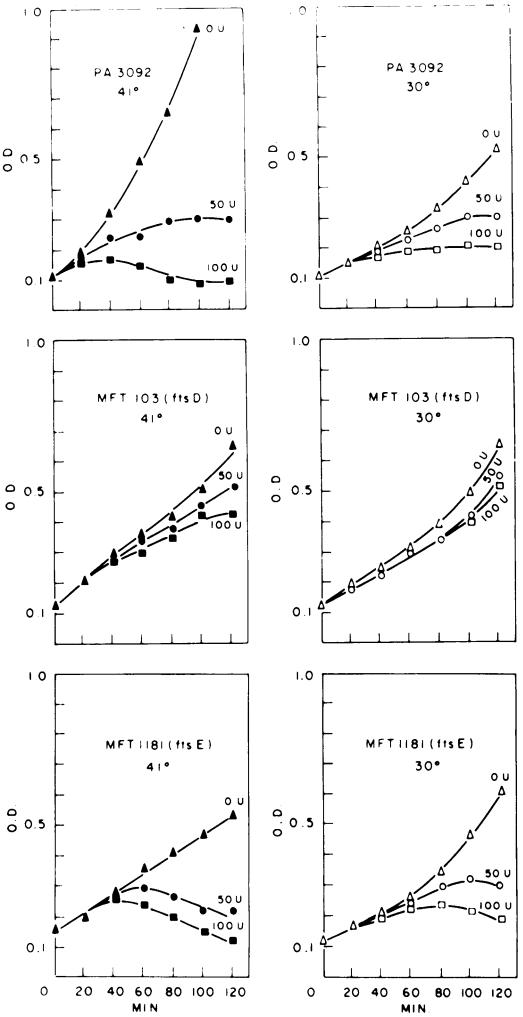

FIG. 6. *Growth of the cells at 30 and 41 C with or without penicillin G added. Lysis is followed by measuring the optical densities of the cultures at 550 nm in cuvettes of 0.5-cm light path. Symbols:* (▲) *41 C and* (△) *30 C without penicillin;* (●) *41 C and* (○) *30 C with penicillin G, 50 IU/ml;* (■) *41 C and* (□) *30 C with penicillin G, 100 IU/ml.*

A series of isogenic strains having different *fts*-mutations was constructed by transduction with phage P1. *fts* mutations (i.e., strains PAT84 and MFT1182) were introduced by P1 transduction into a recipient strain (C-600). Sensitivity of these constructed mutant cells to the penicillin lysis was completely parallel to the *fts* mutants but not to that of the recipient genotype. This phenomenon seems, therefore, to be specific for the *fts* mutations involved. It should be noted that the resistance of the mutants to penicillin lysis was found at both temperatures (30 and 41 C). In other words, the mutant phenotype was partially expressed even at 30 C. The *fts* alteration could be sufficient to cause the mutant cells to be resistant to the penicillin lysis at 30 C, but not to arrest cell division. Furthermore, the fact that *ftsC* and *E* were sensitive to penicillin lysis at 41 C demonstrated that the filament formation per se did not cause the resistance to penicillin.

Green et al. (7) showed that the *recA* mutation can suppress filament formation of *lon*

strains irradiated with small doses of ultraviolet light. In another connection, Inouye (22, 43) reported that the presence of a *recA* mutation in a *thy*$^-$ strain allows the filaments formed during thymine starvation to divide. The author concluded that *recA* gene function exerts a negative control on cell division.

We constructed a series of double mutants carrying one of the *fts* thermoconditional mutations (from *ftsA* to *ftsG*) as well as the *recA* mutation (*ftsA:* MFT1-*recA*, MFT96-*recA*, MFT1182-*recA*, PAT84-*recA; ftsB:* MFT84-*recA; ftsC:* MFT108-*recA; ftsD:* MFT103-*recA*, MFT1111-*recA; ftsE:* MFT1181-*recA*, MFT99-*recA*, MFT123-*recA; ftsF:* MFT1141-*recA; ftsG:* MFT29-*recA*). In addition, a double mutant PAT84 (*ftsA-recB*) was constructed. All the *fts-recA* double mutants and the *ftsA-recB* double mutants remained thermosensitive for colony formation at 41 C.

Cell number and size of all of these double mutants after being shifted to 41 C were measured with the Coulter counter, cell shape was examined under the microscope, and cell mass was measured by a spectrophotometer.

All of these double mutants remained thermosensitive, and cell division was arrested at 41 C immediately after the temperature shift. Filaments were formed at high temperature in the same manner as in the *fts-rec*$^+$ strains.

If the *recA*$^+$ function exerts a negative control on cell division, it is not the *recA*$^+$ function which inhibits cell division in the strains carrying an *fts* mutation (from *ftsA* to *ftsG*).

A mutant has been described (25) which produces multinucleate filaments at high temperature when nonlysogenic, but induces the development of a variety of inducible prophages when lysogenic. It is shown that this phenotype is caused by a single mutation located on the *E. coli* chromosome near the *recA* locus (2, 3).

We have constructed a series of *fts* mutants lysogenic for a phage λh434 (24). In all the *fts* lysogenes examined, the thermoinduction of prophage was not observed.

Therefore, as has already been shown by Kirby et al. (25), filament formation and λ induction can be a related process (42), but the arrest of cell division of *fts* mutants per se is not the cause of prophage induction.

The correction of various phenotypes of *E. coli* mutants by the addition of salts or sucrose to the growth medium has already been reported (29–32, 36). A similar effect has been described in the case of auxotrophic mutants of yeast (9). The majority of *fts* mutants described here divided normally at 41 C when the growth medium had an NaCl concentration higher than a critical concentration (Table 1). These mutants were able to form colonies at 41 C, as well as the wild-type parental strain (PA3092). The effect seems to be specific for the mutations involved (32). A plausible explanation is that an osmotic effect corrects the altered protein which may contain a missense mutation, probably by changing the conformation of the altered protein (9, 32). It is also likely that, if the altered gene products reside on the cell surface, such a correction can be efficient.

DISCUSSION

For a bacterial cell to cleave and separate, a considerable number of events must occur, i.e., the cleavage of covalent bonds of the cell wall, the growth of cell wall and membrane at the septum site, the invagination of the cytoplasmic membrane, the completion of cell termini, and the separation of daughter cells. The timing of such reactions, the triggering, and coordination of these events also require precise regulation. It is evident that many biochemical, structural or regulatory defects can therefore cause the production of multinucleated filaments.

Many gene mutations altered in these different processes are, therefore, expected to be found. Seven *fts* genes have already been identified which are involved in septum formation (17, 40, Ricard and Hirota, in press). These defects can be classified a priori into three operational categories in which either enzymes, building blocks, or regulatory circuits are affected. The results obtained and the experimental approaches used to analyze the character of the *fts* mutants will therefore be discussed within the framework of these categories.

From the present state of our knowledge, it would be difficult to list a priori all the enzymes and compounds which play a specific role in the septation process. One may, however, consider some of these enzymes, the intervention of which would seem to be necessary for cell division.

The *E. coli* cell is enclosed by a single macromolecule, called murein, which forms a rigid, covalently bonded structure. To divide, the cell has to modify this structure, specifically at the location of the septum and to insert new murein subunits (12, 34, 35). These reactions could be catalyzed by a set of murein hydrolases and synthetase(s). Among the division mutants, one can expect to find mutants altered specifically in one or several of these enzymatic activities. The measurement of the thermosensitivity of several murein hydrolase activities in crude extract in the *fts* mutants and their parental strain did not show significant differ-

TABLE 1. *Characters of fts mutants of E. coli*[a]

Gene	Mutation	Location on *E. coli* chromosomic map (min)	Dominance	Reversibility[b] at 30 C after 60 min at 41 C		Resistance to penicillin G	Nuclear segregation	Salt repair[c] (36)	
				− CAM	+ CAM			Critical concn	NaCl (%)
ftsA	MT1	1–1.5	R	+	+	R	+	+	1
	MT33							−	
	MT96							+	0, 55
	MT109							−	
	MT1182							+	1, 8
	T84							+	0, 65
ftsB	MT84	29–31	—	±	−	R	±	+	0, 6
ftsC	MT108	3–8	R	±	−	S	±	+	0, 5
ftsD	MT103	77.5	D	+	−	R	+	−	
	MT1111							+	0, 7
ftsE	MT99							+	0, 3
	MT1181	66–69	R	+	+	S	+	−	
	MT123							+	0, 7
ftsF	MT1141	73.5	—	±	−	R	+	+	0, 65
ftsG	MT29	26–28	R	±	−	R	+	−	
	T115							−	

[a] Abbreviations: R, recessive; D, dominant; — not known; + CAM, with chloramphenicol; − CAM, no chloramphenicol.

[b] Recovery of cell division upon temperature shift down, preincubation was made at 40 C then transferred to 30 C (see text); +, high recovery; −, no detectable recovery; ±, poor recovery.

[c] Colony formation on nutrient agar containing NaCl after 24 h at 40 C; +, rescued; −, not rescued (3).

ences (U. Schwarz and Van Heijenoort, personal communication). Assays with partially purified enzyme fractions will be carried out.

The use of specific inhibitors of cell septation could provide information as to the functions involved in that process. Penicillin G, for instance, is known to affect cell division at low concentration and cause cell lysis at higher concentration (34, 37, 39, 40). It is known that penicillin inhibits activities of several enzymes acting on murein (8, 39). Certain classes of *fts* mutants, *ftsA*, *B*, *D*, *F*, and *G*, were significantly more resistant to penicillin than their parental strain and the *ftsC* and *E* mutants. It is therefore possible that the modification of the murein is an essential step of cell division and that the *ftsA*, *B*, *D*, *F*, and *G* mutations cause alteration of the murein growth in such a way as to cause resistance to the action of penicillin G. That is, the penicillin-sensitive enzymes (8, 34, 39) might be resistant in these mutants.

A direct approach to the identification of factors involved in cell division could be to repair a mutant phenotypically by the addition of an extract of wild-type cells, as has been reported by Fisher and his associates (6).

Modifications of the membrane affecting, for example, topology of membrane growth could result in defective cell division; mutants with normal chemical composition of the cell envelope but an incorrect structure could exist.

A physicochemical technique to measure alteration of cell surface can be used, i.e., the increase of fluorescence which follows the addition of ANS to a suspension of bacterial cells. No differences were found among the *fts* mutants analyzed, and the more sensitive techniques involving different parameters must therefore be required to examine the alteration of cell envelope of the mutants. A fluorescent dye, primulin, which has been shown to specifically stain the bud scar of yeast (38), for example, might distinguish localized alteration of the cell surface of the *E. coli* mutants. The use of a high-resolution autoradiographic technique, involving pulse labeling of the murein with ^{3}H-DAP, has shown recently that murein growth is localized at the equatorial plane of the

wild-type cell (Ryter, Hirota, and Schwarz, in press). When DNA synthesis was arrested, the localized growth zone was abolished (to be published). It has now become possible to detect the alteration of the growth pattern of the *fts* mutants.

The presence of some regulatory mechanisms determining precise time span of the cell cycle have already been postulated, i.e., the temporal relationship between the DNA replication and cell division (4, 5, 10, 11); the existence of *div* genes which loosens the coupling of cell division with DNA synthesis and nuclear partition (14, 16, 17, 18, 21), the effects of defective *recA* function, ultraviolet light, and mitomycin C; and the induction of prophage with respect to the cell division, demonstrate some complex and intricate inter-relationships (2, 3, 7, 13, 14, 20, 22, 25, 36). The fact that none of the seven classes of *fts* mutants is either corrected by the introduction of the *recA* mutation or induces the multiplication of λ prophage, emphasizes the large variety of gene functions involved in the septation process.

To identify some regulatory gene functions for the cell division cycle, one can also analyze the mutational defect as to whether it is dominant with respect to the wild-type allele. Mutations on the *ftsD* locus, which is different from other *fts* loci, are dominant (Ricard and Hirota, in press), and in addition the filaments formed at 41 C can resume cell division at 30 C only if new protein synthesis occurs. One may cite two alternatives which can explain these characteristics. The *ftsD* gene may code for a regulatory protein, and the altered protein may act as a "super-repressor", is, irreversibly blocking the expression of one or several gene functions. Alternatively, the product of such a dominant gene could also be part of a compound structure of the division machinery in which the mixture of normal and defective elements causes irreversible damage to the whole machinery. Protein synthesis may be required to correct the damage by the substitution of normal elements.

In conclusion, it is evident that a large number of functions can be specifically involved in the processes of septum formation and its regulation. In particular, the variety of these functions was shown by the physiological study of the *fts* mutants. In fact, each of the seven genetic classes analyzed in this paper presents a unique set of characteristics, as summarized in Table 1. There is no doubt that further accumulation of physiological data, using more mutants, will shed more light on the nature of the corresponding functions. However, the ultimate understanding of the mechanism of cell division will remain difficult until our knowledge of the structure and functioning of cell envelope progresses further.

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