Expression of the *Escherichia coli ftsZ* gene: trials and tribulations of gene fusion studies

ALINE ROBIN* AND RICHARD D'ARI
Institut Jacques Monod, C.N.R.S., Université Paris 7, 2, place Jussieu, 75251 Paris Cedex 05, France

(*Received 17 July 1992*)

Summary

The *ftsZ* gene of *Escherichia coli*, which codes for an essential cell division protein, is subjected to multiple regulation, as shown in part with studies using an *ftsZ::lacZ* operon fusion located on phage λJFL100. Using this same fusion, we sought to isolate regulatory mutants overexpressing *ftsZ* by selecting mutants able to grow on lactose. One Lac^+ mutant was obtained which overexpressed the *ftsZ::lacZ* fusion 70-fold. The mutation responsible for the overexpression lies in a new gene, *cot*, located near 56 min on the *E. coli* genetic map. The *cot* mutation probably affects the transcription of a chromosomal open reading frame, ORF1, lying downstream of the *bioA* gene and adjacent to the *ftzZ::lacZ* fusion of the λJFL100 prophage integrated at att^{λ}. Using an *ftsZ84*(Ts) strain, in which there was a double selection for overexpression of both *ftsZ::lacZ* and $ftsZ^+$, no Lac^+Tr mutants were obtained from $3{\cdot}6 \times 10^{10}$ bacteria; the introduction of a *mutL* allele, increasing spontaneous base substitution mutation rates 75-fold, did not permit us to isolate such a mutant. We conclude that Lac^+ *ftsZ*-constitutive mutations cannot be obtained in λJFL100 lysogens by a single base substitution.

1. Introduction

Gene fusions, which place the structural gene of a 'reporter' enzyme under control of a foreign promoter whose expression is to be studied, have proved to be a powerful tool. Operon (or transcriptional) fusions can yield quantitative data on the regulation of genes whose products are not readily assayed, they can be used to locate genes obeying particular regulatory patterns, and they can also be used to identify regulatory genes and select regulatory mutants. Protein fusions produce hybrid proteins which can provide information on cellular location and can be purified on the basis of properties conferred by the reporter moiety, then used to characterize the unknown protein or to raise antibodies against it. Such studies have provided a wealth of information on a large number of different operons and regulons in bacteria, yeast, and higher eukaryotes (Silhavy & Beckwith, 1985). However, despite their elegant simplicity, gene fusions can give rise to a number of red herrings which the experimenter must be vigilant to avoid.

The *ftsZ* gene product has been shown to be a key factor in cell division in *Escherichia coli* (Lutkenhaus, 1990), with homologues in other bacterial species, including the distantly related Gram positive strain, *Bacillus subtilis* (Corton, Ward & Lutkenhaus, 1987). The *E. coli* FtsZ protein acts early in the septation process (Walker *et al.* 1975) and has recently been shown to form a ring around the middle of cells at the time when constriction begins (Bi & Lutkenhaus, 1991). We previously used an *ftsZ::lacZ* operon fusion to study the regulation of the *ftsZ* gene during the division cycle and after nutritional shift-up in *Escherichia coli* (Robin, Joseleau-Petit & D'Ari, 1990). Our results, which indicated bilinear expression of the *ftsZ* gene under conditions of synchronous cell division, suggested that this gene was likely to be regulated by one or more *trans*-acting transcriptional factors. Other investigators, using the same fusion, have reported near total shut-off of *ftsZ* expression during most of the cycle (Dewar *et al.* 1989) and regulation by the initiation of DNA replication (Masters *et al.* 1989). This *ftsZ::lacZ* fusion has also been shown to have higher expression at lower growth rates (Dewar *et al.* 1989; Robin *et al.* 1990). To identify specific regulators genetically, we used this *ftsZ::lacZ* fusion to select mutants exhibiting higher expression of the *ftsZ* gene. In the course of the work we discovered a regulator of a previously sequenced gene of unknown function and we isolated several

* Corresponding author.

Table 1. *Bacterial strains*

Strain	Genotype	Source or reference
GC3439	*thr leu pro his arg lacY gal rpsL* (λJFL100)	(Robin *et al.* 1990)
GC3447	As GC3439, *leu*$^+$ *ftsA13*(Ts)	(Robin *et al.* 1990)
GC3448	As GC3439, *leu*$^+$ *ftsQ1*(Ts)	(Robin *et al.* 1990)
GC3560	As GC3439, *lacY*$^+$ *lacZΔM15 lacI*^{q}	Transduction of GC3439, selection on melibiose 42 °C
GC3567	*thr leu pro his arg lacY gal rpsL* (λ*imm*^{21})	Lysogenization of AB1157
GC3575	As GC3439, *lacY*$^+$ *lacZΔM15 lacI*^{q} *cot*	This work
GC3617	*thr leu pro his arg gal rpsL lacIpoZΔ*(*Mlu*) *proC::*Tn*5*	Transduction* of AB1157
GC3668	*thr leu pro his arg lacY gal rpsL cot* (λ$^+$)	Superinfection of GC3575
GC3670	As GC3439, *cot lacIpoZΔ*(*Mlu*) *proC::*Tn*5*	Transduction*
GC3671	As GC3560, *gal*$^+$ *Δ(pgl-att*^{λ}*-bio-uvrB-chlA*)	Transduction†
GC3672	As GC3439, *leu*$^+$ *ftsQ1*(Ts) *nadB::*Tn*10 cot*	Transduction of GC3448
GC3673	As GC3439, *leu*$^+$ *ftsQ1*(Ts) *nadB::*Tn*10 cot*$^+$	Transduction of GC3448
GC3675	*thr pro his arg lacY gal rpsL ftsQ1*(Ts) (λ$^+$)	Superinfection of GC3448
GC3677	As GC3439, *leu*$^+$ *ftsQ1*(Ts) *lacIpoZΔ*(*Mlu*)	Transduction of GC3448
GC3693	*thr pro his arg lacY gal rpsL* (λ*imm*^{21})	Superinfection of GC3668
GC3750	As GC3439, *leu*$^+$ *ftsZ84*(Ts) *lacIpoZΔ*(*Mlu*) *proC::*Tn*5*	Transduction
GC2862	*ftsA8–25 leu::*Tn*10 thi relA araD lacΔU169*	E. Brikman & J. Beckwith
BM1161	*araD Δ(lac-argF)U169 thi rpsL ø(bioA::lacZ)*	(Campbell, Del Campillo-Campbell & Barker, 1978)
BM5076	As BM1161, *bioR206*	(Barker & Campbell, 1980)

* The donor strain was a *proC::*Tn*5* derivative of a *lacIpoZΔ*(*Mlu*) strain (Rasmussen, Møller & Atlung, 1991) kindly provided by T. Atlung.

† The donor strain was SA263 (S. Adhya), obtained from the laboratory of R. Thomas.

false positives resulting from secondary events, the identification and elimination of which may be instructive for others working with gene fusions. We did not, however, find any mutants derepressed for *ftsZ* expression, and our results strongly suggest that no single substitution mutation can confer this phenotype.

2. Materials and methods

(i) *Bacterial and phage strains*

All bacterial strains were *Escherichia coli* K12 derivatives; they are described in Table 1. P1*vir* (for transduction) and λ$^+$ (wild type) were from our laboratory collection. λD69 (Mizusawa & Ward, 1982), called here λ*imm*^{21}, was given to us by E. Maguin. λJFL100 (Masters *et al.* 1989) is described in the text.

(ii) *Media*

Rich medium was LB broth and synthetic medium was M63 (Miller, 1972), to which was added glucose, galactose or lactose (0·4%), required amino acids (100 μg/ml each) and thiamine (1 μg/ml). For filamentation studies, the M63 buffer was diluted twofold. Other supplements were used, as needed, at the following concentrations: Casamino Acids 0·4%, nicotinic acid 5 μg/ml, biotin 1 μg/ml unless otherwise stated, 5-bromo-4-chloro-3-indolyl-β-D-galactofuranoside (X-Gal) 40 μg/ml, tetracycline 10 μg/ml, kanamycin 40 μg/ml.

(iii) *Miscellaneous methods*

Hfr crosses, P1*vir*-mediated transduction and β-galactosidase assays were carried out according to Miller (1972). Heteroimmune curing of λJFL100 lysogens was done by spotting a drop of λ$^+$ onto a lawn of the lysogen, incubating overnight, then picking bacteria from the turbid centre onto X-Gal plates; white colonies were checked for their sensitivity to λJFL100 (*imm*^{21}) and possible lysogenization by λ$^+$.

Sequence comparison was done with CITI2 facilities (Dessen *et al.* 1990) using the program FASTA (Pearson & Lipman, 1988). The data bank release numbers were No. 30 for NBRF and No. 20 for SwissProt (November 1991).

3. Results and discussion

(i) *Selection for ftsZ regulatory mutants*

We wished to isolate regulatory mutants affected in the expression of the *E. coli* cell division gene *ftsZ*. A possible selection was for strains overproducing the FtsZ protein. Such mutants can in principle be selected by using an *ftsZ::lacZ* operon fusion, whose expression of β-galactosidase is relatively low, and selecting for a Lac$^+$ phenotype in a strain whose chromosomal *lacZ* gene has been inactivated.

https://doi.org/10.1017/S0016672300031050 Published online by Cambridge University Press

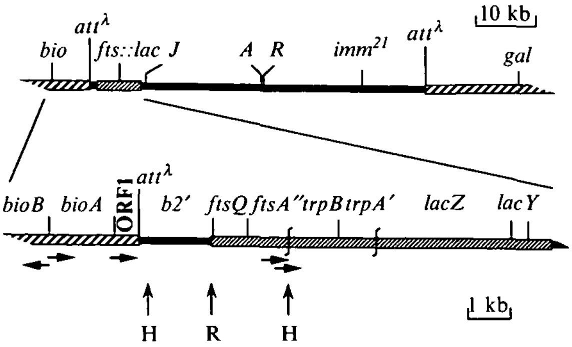

Fig. 1. The structure of a λJFL100 monolysogen. A single λJFL100 prophage is shown integrated at att^{λ}. Note that the orientation is the opposite of that normally used for λ phage. Black lines represent λ DNA and hatched lines *E. coli* DNA sequences; fine hatching is used for *E. coli* DNA cloned in λJFL100. The symbol ∫ represents artificial joints (fusions) within DNA of the same origin. Horizontal arrows indicate the location and orientation of known promoters. H and R are *Hin*dIII and *Eco*RI restriction sites, respectively.

The phage λJFL100, constructed by J. F. Lutkenhaus, is a λimm^{21} vector which carries the *lacZ* gene lacking its own promoter and fused to two of the promoters of the *ftsZ* gene (Masters *et al.* 1989). The fusion replaces part of the *b2* region of the phage genome, without inactivating the attachment site (cf. Fig. 1). We previously observed that lysogenisation by λJFL100 gives rise to different classes of lysogens, having levels of β-galactosidase varying from 50 to 2000 U/OD (Robin *et al.* 1990). In our earlier work we used a stable lysogen of the class having the lowest level of β-galactosidase, strain GC3439; curing this strain by heteroimmune superinfection produced a derivative whose β-galactosidase level was negligible in the absence of *lac* operon inducers. Strain GC3439 thus seems to be monolysogenic for λJFL100, integrated at the normal λ attachment site, as shown in Fig. 1.

The description of the construction of λJFL100 in the references cited in Masters *et al.* (1989) does not give sufficient information to determine whether the *lacY* gene is intact. Since the *lacY* gene product, lactose permease, is required for rapid growth on lactose, we introduced a chromosomal $lacY^{+}$ gene into strain GC3439, together with the non-polar deletion *lacZΔM15*, making strain GC3560 (cf. Table 1).

(ii) *Isolation of the* Lac^{+} *mutant GC3575*

The level of β-galactosidase in strain GC3560, 50 U/OD in minimal glucose medium, permits only extremely slow growth on lactose, with small colonies appearing in about three days at 37 °C, whereas true Lac^{+} strains form colonies in 24 h. To isolate spontaneous Lac^{+} mutants, a saturated LB culture of GC3560 was centrifuged, washed in phosphate buffer and plated at 37 °C on minimal lactose medium containing 2×10^{-4} M IPTG (to ensure expression of *lacY*) and 40 μg/ml of the indicator X-Gal. Clones overproducing β-galactosidase form dark blue colonies on a thin, pale blue lawn of slow growing bacteria. From a total of about 10^{9} cells, 26 such clones were purified on the same medium. Of these, 22 proved to be true Lac^{+} mutants. They were spread on minimal glucose plates containing X-Gal. On this medium, which permits repression of *lac* operon expression, 10 clones remained dark blue (Lac-constitutive phenotype) whereas the other 12 were very pale blue. Quantitative β-galactosidase assays with one pale blue clone revealed a specific activity of about 50 U/OD in glucose medium compared to 2000 in lactose medium, confirming the acquisition of an inducible lac^{+} genotype, presumably through homologous recombination between the *lac* regions in the chromosome ($lacp^{+}Z^{-}Y^{+}$) and in the genome of the prophage or a superinfecting phage ($lacp^{-}Z^{+}Y$?); the 12 pale blue clones were discarded.

The remaining 10 clones clearly expressed β-galactosidase from a foreign promoter since there was no repression in glucose medium. For 9 of these, the enzyme level was specifically lowered in the presence of tryptophan. Quantitative assays with one clone revealed a specific activity of 500 U/OD in the absence of tryptophan, dropping to about 150 U/OD in the presence of tryptophan. In fact, the *ftsZ::lacZ* fusion used carries a short fragment of the *trp* operon between the *ftsZ* promoters and the *lacZ* structural gene (Fig. 1). These 9 clones presumably arose through homologous recombination between the *trp* regions in the chromosome and in the genome of the prophage or a superinfecting phage. Such recombination would place the *lacZ* gene of λJFL100 under control of the chromosomal *trp* promoter. These 9 *trp::lac* fusions were discarded.

It is likely that the different classes of lysogens previously reported with λJFL100 (Robin *et al.* 1990) include single or multiple integrations at the *lac*, *trp*, and λatt^{λ} loci, explaining the widely varying β-galactosidase levels observed.

The final clone, strain GC3575, was the only one whose level of β-galactosidase expression was high and independent of both lactose and tryptophan. It was characterized further as a potential *ftsZ* regulatory mutant.

(iii) *Analysis of the* Lac^{+} *mutant GC3575*

The differential rate of β-galactosidase synthesis in the mutant strain GC3575 during exponential growth in minimal glucose medium at 37 °C is close to 3500 U/OD. This is 70-fold higher than that of the parent strain GC3439 under the same conditions. A similar level of derepression was observed in LB broth.

Curing of strain GC3575 by heteroimmune superinfection completely abolished β-galactosidase ex-

https://doi.org/10.1017/S0016672300031050 Published online by Cambridge University Press

pression, indicating that the only source of enzyme is the λJFL100 prophage integrated at att^{λ}. We also observed that the plaque morphology of λ^{+} was normal on lawns of the mutant GC3575.

We next asked whether the mutation responsible for the Lac^{+} phenotype of strain GC3575 lay within the prophage. We tested this in two ways: first, by replacing the prophage of strain GC3575 with a wild type λJFL100, and second, by moving the prophage of strain GC3575 into a wild type background. To avoid having to interpret the different levels of β-galactosidase found in different lysogens, we constructed our strains by P1 transduction, taking care to have a λimm^{21} prophage present at all times to avoid zygotic induction (λJFL100 is imm^{21}). As selection, we took advantage of the nearby *gal* locus, which is 10 to 14% cotransducible with λ. In the first transduction, strain GC3575 was transduced to gal^{+} with a P1 stock grown on a gal^{+}(λJFL100) lysogen. Of 96 transductants tested, all remained Lac^{+}, suggesting that the prophage in GC3575 is not mutated. This result was confirmed by the reciprocal transduction, in which the prophage of strain GC3575 was transduced into a wild type strain. A P1 stock was grown on a gal^{+} transductant of GC3575 and used to transduce the $gal^{-}(\lambda imm^{21})$ strain GC3567 to Gal^{+}. Fourteen percent of the transductants made pale blue colonies on plates containing X-Gal, indicating that they had acquired the donor λJFL100 prophage. Quantitative assays of β-galactosidase in these transductants showed that they all had a specific activity around 50 U/OD, the same as the wild type λJFL100 lysogen GC3439 and 70-fold lower than the donor strain GC3575.

These results show conclusively that the mutation conferring a Lac^{+} phenotype on strain GC3575 is not linked to the λJFL100 prophage and therefore must be chromosomal. We next mapped the mutation.

The wild type allele, restoring a Lac^{-} phenotype to strain GC3575, was found to be injected by Hfr PK19 (PO 42·5 min, CW) and by Hfr KL16 (PO 58·5 min, CCW), but not by Hfr KL98 (PO 51 min, CCW), placing the mutation between 51 and 58·5 min on the *E. coli* genetic map. By P1 transduction, we were able to locate the mutation between 55·5 and 56 min, 31% cotransducible with the marker *nadB*::Tn*10* and 38% cotransducible with *ung-152*::Tn*10*. The mutation was transduced into the parental lysogen, GC3439, using as donor a Lac^{+} *nadB*::Tn*10* transductant of GC3575. The cotransduction frequency was only 7% in this direction, but the level of β-galactosidase activity was as high in the cotransductants as in the original mutant GC3575. These results show that a single locus near 56 min is responsible for the Lac^{+} phenotype of the mutant GC3575; we call this gene *cot*.

(iv) *Effects of the* cot *mutation*

The high level of β-galactosidase in the mutant GC3575 raised a certain number of questions. Does the overexpression of *lacZ* reflect a high level of transcription from the *ftsZ* promoters present in the λJFL100 prophage? Is the $ftsZ^{+}$ gene at 2 min similarly overexpressed? Ward and Lutkenhaus (1985) have shown that 12-fold overproduction of FtsZ protein is lethal for the bacterium; is 70-fold overexpression tolerable? The same authors found that overproduction of FtsZ two- to sevenfold, while not lethal, resulted in minicell formation. Observation of our mutant GC3575 in the phase contrast microscope revealed no detectable minicells, nor any other morphological abnormality.

It was clearly important to determine whether the primary effect of the *cot* mutation was on the *ftsZ* promoters. The presence in λJFL100 of the entire coding sequence of the *ftsQ* gene lacking its promoters (cf. Fig. 1) provided a tool for answering this question. Our previous work (Robin *et al.* 1990) established that the basal level of expression of the *ftsQ* gene from a λJFL100 prophage is too low to prevent filamentation in an *ftsQ1*(λJFL100) lysogen, despite the fact that only very low levels of FtsQ are required for septation (Carson, Barondess & Beckwith, 1991). We therefore tested the filamentation of an *ftsQ1 cot* (λJFL100) strain, since overexpression from the *ftsZ* promoters within the *ftsA* gene should not affect expression of the upstream *ftsQ* gene.

The *cot* mutation was transduced into strain GC3448, an *ftsQ1* derivative of the parental strain GC3439. The transductants exhibit the same high β-galactosidase levels in glucose minimal medium at 30 °C as GC3575 at 37 °C. Their cell division pattern was followed in liquid culture at nonpermissive temperature, using the media and growth conditions previously described (Robin *et al.* 1990). Whereas the cot^{+} strain (GC3673) formed long filaments at 42 °C, the isogenic *cot* mutant (GC3672), after some 45 min division inhibition, resumed dividing normally, and cell size returned to that of the wild type strain. If in the *ftsQ1 cot* strain the λJFL100 prophage is replaced by wild type λ, no suppression of filamentation is seen. We conclude that the *cot* mutation increases the expression of the *ftsQ* gene of the λJFL100 prophage. This overproduction of FtsQ did not cause any observable division defect in *cot* (λJFL100) lysogens (*ftsQ1* or $ftsQ^{+}$) cultivated in minimal glucose medium at 30 or 37 °C, unlike that observed by Carson, Barondess and Beckwith (Carson *et al.* 1991). The *cot* mutation, similarly transduced into *ftsA13*(Ts) and *ftsZ84*(Ts) derivatives of the parental strain GC3439, did not suppress the filamentation observed at 42 °C.

The above observations show that the *cot* mutation increases transcription at some promoter upstream of the *ftsQ* coding sequence. Since the bacterial DNA insert in λJFL100 ends with the 5′ portion of the *ftsQ* gene (cf. Fig. 1), we conclude that the target promoter whose activity is increased in the *cot* mutant is not genetically linked to the *ftsZ* gene in the 2 min region of the chromosome but must lie in or near the λJFL100 prophage. Adjacent to the *ftsZ::lacZ* gene fusion is a portion of the λ *b2* region followed by the *att*$^{\lambda}$ site; shortly beyond lies the *bio* operon, coding for the biotin biosynthetic enzymes (Fig. 1).

The *b2* region of wild type λ has been screened for promoter activity and no leftward promoters have been reported in the portion present in λJFL100 (Kravchenko, Vasilenko & Grachev, 1979; Rosenvold *et al.* 1980), but, since the experiments were carried out in *cot*$^{+}$ strains, the level may have been undetectable. To test whether the *cot* mutation activated a normally cryptic promoter in this part of the *b2* region, we carried out a superinfection experiment to measure the level of β-galactosidase expression from a non-integrated λJFL100 phage, which is not connected to chromosomal promoters. We used the *cot* strain GC3693, in which the λJFL100 prophage has been replaced by a λ*imm*^{21} prophage, to remove the highly expressed *lacZ* gene while maintaining repression of lytic growth of the infecting λJFL100; the *cot*$^{+}$ control strain was a λ*imm*^{21} lysogenic derivative of GC3617. Cultures were grown to exponential phase at 37 °C in LB broth containing 0·2% maltose, which induces the λ receptor (Schwartz, 1987). They were concentrated to $2{\cdot}5 \times 10^{8}$ cells/ml and infected with λJFL100 at a multiplicity of 1. After 15 min adsorption, the cultures were diluted 10-fold and incubated at 37 °C for 1 h. Samples were assayed for β-galactosidase activity after centrifugation and washing to remove the enzyme introduced with the phage. The enzyme activity was the same in the two strains. Thus, the *cot* mutation does not affect *ftsZ::lacZ* expression from a non-integrated λJFL100. It is perhaps worth pointing out that, even if a fraction of the superinfecting phage integrated into the chromosome, in the absence of integrase this would occur by homologous recombination, placing the *ftsZ::lacZ* fusion between two λ prophages and not near the left end as in the monolysogen shown in Fig. 1.

This result strongly suggests that the target of the *cot* gene product is not within the *b2* region of the λJFL100 phage. We conclude that it lies in a nearby region of the chromosome, upstream of the prophage *ftsZ::lacZ* fusion.

The divergent *bioA* and *bioBFCD* genes are known to be regulated by a repressor, product of the *birA* (or *bioR*) gene at 89·7 min on the genetic map (Barker & Campbell, 1980). The promoter of the *bioA* gene, oriented toward *att*$^{\lambda}$, was a candidate for the target promoter affected by the *cot* mutation (cf. Fig. 1). Mutations in *birA* derepress this operon about 70-fold, the same extent as the *cot* mutation. Although the *cot* gene, by its genetic location, is clearly different from *birA*, its product could be involved in repression of the *bio* operon. We tested this hypothesis by studying the effect of the *cot* mutation on expression of a *bioA::lacZ* fusion. The *bioA::lacZ* fusion from strain BM5076, carried on a λ prophage, was transduced into strain GC3668, a derivative of the *cot* strain GC3575 in which the λJFL100 prophage was replaced with a λ^{+} prophage; this substitution avoids zygotic induction during the transduction and removes the highly expressed *lacZ* gene of the λJFL100 prophage. Gal^{+} transductants which had received the λ*bioA::lacZ* prophage, detected by their blue colour on X-Gal plates, were assayed quantitatively for β-galactosidase activity in mimimal glucose media containing biotin at 1·6 nM and 8·2 μM, concentrations which respectively induce and repress the *bio* operon in wild type strains (Campbell *et al.* 1978; Barker & Campbell, 1980), and compared with the reference strains BM1161 (*bioR*$^{+}$) and BM5076 (*bioR206*). The transductants were indistinguishable from the wild type control: high expression at 1·6 nM biotin and complete repression at 8·2 μM biotin (data not shown); the *bioR206* control strain had high expression at both concentrations, as previously reported (Barker & Campbell, 1980). We conclude that the *cot* mutation does not affect transcription from the *bioA* promoter.

This conclusion leaves few possibilities for the target of the *cot* mutation. Nucleotide sequence data from the 17 min region (Otsuka *et al.* 1988) established that there are 577 base pairs between *att*$^{\lambda}$ and the 3′ end of the *bioA* gene, containing a single open reading frame, ORF1, of unknown function, oriented towards *att*$^{\lambda}$, followed by a potential Rho-independent transcription terminator and potentially coding for a protein of molecular weight 17 kD (cf. Fig. 1). The *cot* gene, at 56 min, is unlinked to the 17 min region, yet the *cot* mutant seems to increase significantly the rate of transcription from the ORF1 promoter through *att*$^{\lambda}$. We propose that the *cot* gene codes for a *trans*-acting regulator, either increasing transcription initiation or decreasing transcription termination of ORF1 (control of *O*RF1 *t*ranscription). We compared the ORF1 sequence with the NBRF and SwissProt data banks but did not find any significant similarities with other known sequences. The *cot* mutation leads to 70-fold overexpression of the adjacent *ftsZ::lacZ* fusion. Such a strong level of regulation suggests that the product of ORF1 is needed only under certain conditions. The λJFL100 construction and *cot* mutation should provide useful tools for those interested in determining the normal physiological role of the ORF1 product.

(v) *Attempts to integrate phage λJFL100 in the 2 min region*

If the λJFL100 prophage were integrated within the *fts* region at 2 min by homologous recombination, its *lac* operon would be under the control not only of the two *ftsZ* promoters shown in Fig. 1 but also of additional promoters which have been identified

https://doi.org/10.1017/S0016672300031050 Published online by Cambridge University Press

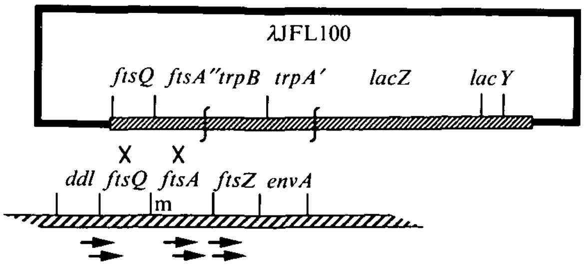

Fig. 2. Integration of phage λJFL100 in the 2 min region. A circularized λJFL100 phage is shown aligned with the homologous chromosomal DNA from the 2 min (*fts*) region. Integration can take place *via* crossing over to the left or right of the *ftsA8–25* mutation (m). A crossover to the right of the mutation would generate an $ftsA^+$ allele to the right of the integrated λJFL100 prophage. The black line represents λ DNA (not drawn to scale) and the hatched lines *E. coli* DNA sequences, cloned (fine hatching) or chromosomal.

upstream of the *ftsQ* gene and downstream of the *Hin*dIII site within the *ftsA* gene (cf. Fig. 2). These latter promoters make only a minor contribution to *ftsZ* expression *in vivo* (Ghelardini *et al.* 1991), so one would expect such a strain to have approximately the same level of *lac* expression as the lysogens in which the λJFL100 prophage is integrated at att^{λ}, *viz.* about 50 U/OD in minimal medium, and thus to have a Lac^- phenotype. On the other hand, such a lysogen should be able to mutate to Lac^+ by regulatory mutations affecting any of the promoters governing expression of the chromosomal *ftsZ* gene, including those not present on the λJFL100 phage, some of which are known to be regulated. The downstream promoter within the *ddl* gene (not present on the λJFL100 phage) has been shown to be metabolically regulated, with lower expression at fast growth rates (Aldea *et al.* 1990); this is presumably different from the metabolic regulation of the λJFL100 *ftsZ::lacZ* fusion (Dewar *et al.* 1989; Robin *et al.* 1990) and may depend on the presumptive sigma factor RpoS (Vicente *et al.* 1991). The upstream promoter in *ddl* (also absent in λJFL100) has similarly been shown to be regulated by the product of the newly discovered *sdiA* gene (Wang, de Boer & Rothfield, 1991). Expression of *ftsZ* is also affected by *gyrB* mutations (Ruberti *et al.* 1991) and by overproduction of the *rcsB* and *relA* gene products (Gervais, Phoenix & Drapeau, 1992), although the target promoters of these regulators have not been identified.

Since integration at att^{λ}, catalyzed by phage integrase and integration host factor, is an efficient process, we first sought to prevent this event by using strain GC3671, a Δatt^{λ} (and therefore nonlysogenic) derivative of strain GC3670, used above. Lysogens were detected as blue colonies on minimal glucose plates containing X-Gal. As expected, their frequency among bacteria growing within a plaque of λJFL100 formed on a lawn of strain GC3671 was considerably lower than that found with an att^+ lawn. We purified 96 blue clones. All were found to be extremely heterogeneous, some colonies displaying dark blue and white sectors, others being pure white. This appearance results from phage growth within a nonlysogenic colony. We were thus unable to isolate a stable λJFL100 lysogen in the Δatt^{λ} background. Since it is relatively easy to integrate a λ*sfiA::lacZ* phage, lacking the phage attachment site and integrase, by homologous recombination (Huisman *et al.* 1983), the failure to recover JFL100 lysogens in the Δatt^{λ} strain may result from the deletion of 0·5 min of chromosomal DNA surrounding att^{λ}.

Since phage λJFL100 carries the entire *ftsQ* gene and the 5′ part of the *ftsA* gene, it is possible in principle to select directly for integration in the 2 min region in an att^+ background by using an *ftsQ*(Ts) or *ftsA*(Ts) strain whose thermosensitivity is not corrected by a λJFL100 integrated at att^{λ}; if the wild type allele is present on the phage, an fts^+ gene can be reconstituted by homologous recombination on the appropriate side of the mutation (cf. Fig. 2).

We first wished to try this trick with an *ftsQ*(Ts) mutant. The λJFL100 prophage integrated at att^{λ} does not prevent filamentation in the *ftsQ1* (λJFL100) lysogens GC3448 and GC3673 (Robin *et al.* 1990; cf. above). However, using a low ionic strength medium to maximize the temperature sensitivity of the strain (Robin *et al.* 1990), we found an efficiency of plating of 10^{-1} for this lysogen, compared to less than 10^{-7} for the isogenic *ftsQ1* strain in which λJFL100 is replaced by λ^+. Thus, although expression of the promoterless *ftsQ* gene from the λJFL100 prophage at att^{λ} is insufficient to restore normal cell division to the *ftsQ1* mutant in this medium at 42 °C, it nevertheless permits relatively efficient colony formation on plates at 42 °C. The cells in the colonies include many filaments, and they are unable to grow further at 42 °C. The high frequency of temperature resistant clones clearly makes it impossible to select for integration in the 2 min region using the *ftsQ1* mutant. We therefore turned our attention to *ftsA*(Ts) strains.

Since λJFL100 carries only part of the *ftsA* gene, we first had to find an *ftsA* mutation which lies within this fragment and leaves enough homology on the downstream side to permit the recombination event needed to reconstitute an $ftsA^+$ gene (cf. Fig. 1). The *ftsA13* allele has recently been sequenced and shown to lie outside the fragment carried by λJFL100; the *ftsA8–25* allele, however, should lie within λJFL100 (Robinson, Begg & Donachie, 1988). We confirmed this by a marker rescue test. λJFL100 was able to donate the wild type allele to the mutant *ftsA8–25* mutant GC2862: the efficiency of plating on LB medium at 42 °C was less than 10^{-9} but rose to 10^{-4} after infection with λJFL100.

We infected the *ftsA8–25* mutant GC2862 with λJFL100 at a multiplicity of infection near 1 and plated the cells at 42 °C on LB medium containing X-Gal. The survival frequency was again 10^{-4}, and about 10% of these colonies were blue, suggesting that they might have a λJFL100 integrated in the 2 min region. Genetic analysis, however, revealed that these clones were in fact the result of two events, marker rescue of an *ftsA*$^+$ gene in the 2 min region, with concomitant elimination of the *ftsA*(Ts) allele, and integration of a λJFL100 prophage elsewhere, presumably at *att*$^\lambda$. These lysogens did not arise from the integration of λJFL100 in the 2 min region. This is in agreement with a previous report that integration of λ16–2 (*ddl-ftsQ-ftsA-ftsZ-envA*) in the 2 min region is lethal in the absence of an *ftsZ*$^+$ plasmid (Dai & Lutkenhaus, 1991).

(vi) *λJFL100 carries an intact* lacY *gene*

We took advantage of the ability of the *cot* mutant to turn on the *lacZ* gene of a λJFL100 prophage to determine whether this phage carries an intact *lacY* gene. For this, we introduced into the *cot* mutant GC3575 a *ΔlacIpoZ* deletion which does not have a functional *lacY* gene and plated the resulting strain (GC3670) on minimal lactose medium. It grew as well as the parental strain GC3575, which has a functional *lacY*$^+$ gene. This experiment demonstrates that phage λJFL100 carries an intact *lacY* gene.

(vii) *New selection for Lac*$^+$ *mutants*

Using the information accumulated in the above unfruitful search for an *ftsZ* regulatory mutant, we set up a new selection designed to avoid or eliminate the three types of spurious Lac$^+$ clones isolated so far. Knowing that λJFL100 carries the entire *lacY* gene, we used a *ΔlacIpoZ* deletion, thereby avoiding the generation of *lac*$^+$ recombinants. Recombinants with *trp::lac* fusions can be detected rapidly by streaking on X-Gal plates with and without tryptophan. We also included the *ftsQ1*(Ts) mutation in the strain in order to test Lac$^+$ mutants quickly for derepression of the prophage *ftsQ* gene, upstream of the *ftsZ* promoters; this permits rapid detection of *cot*-like mutants, which affect promoters outside the cloned *fts* fragment. The resulting strain, GC3677, was used for the selection, which was carried out on X-Gal lactose plates at 30 °C, a permissive temperature for the *ftsQ1* mutant.

As before, about 10^9 bacteria from a saturated LB culture were washed and plated directly on selective medium. No clones were found capable of faster growth than the underlying lawn, even after several days' incubation at 30 °C. We also tried enriching for fast-growing mutants by incubating first in liquid lactose medium, then spreading on selective plates. In this way we isolated some dozen clones which seemed to outgrow the lawn, but after purification all turned out to be slow growers, with no increase in their β-galactosidase level. Curiously, in this series of selections, we recovered no spurious Lac$^+$ clones due to *trp::lac* recombinants. If these recombinants are formed principally *via* superinfecting phage, then the failure to find them may reflect the fact that incubation was at 30 °C, at which temperature λ growth is poor, making for fewer free phage in the culture and therefore less superinfection of the lysogens.

(viii) *Double selection for both Lac*$^+$ *and increased FtsZ levels*

The failure to recover Lac$^+$ mutants with an increased level of *ftsZ* transcription is perhaps due in part to the marginality of the selection: the basal level is not absolutely Lac$^-$, and too high a level of FtsZ is likely to be lethal. To increase the power of the selection, we added a second selective factor, in addition to rapid growth on lactose: we introduced the *ftsZ84*(Ts) mutation into the strain (this is the only known temperature sensitive *ftsZ* allele) and selected for growth on lactose at 42 °C, at which temperature the normal quantity of mutant FtsZ protein does not permit septation. It has been shown that overexpression of this mutant FtsZ protein restores temperature resistant growth (Ghelardini *et al.* 1991; Wang *et al.* 1991). Furthermore, since the mutant protein has lower activity than the wild type protein at 42 °C, higher levels of derepression should be tolerable. This system permitted simultaneous selection for over-expression of both the λJFL100 *ftsZ::lacZ* fusion (Lac$^+$ phenotype) and the chromosomal *ftsZ* gene (temperature resistant phenotype). Spurious Lac$^+$ recombinants which do not overexpress FtsZ protein, such as the *trp::lac* fusions isolated in the first selection, would be unable to form colonies under these conditions.

For the new selection, we constructed an *ftsZ84 ΔlacIpoZ* (λJFL100) lysogen, GC3750. This strain is unable to form colonies on minimal glucose medium at 42 °C ($< 10^{-7}$ efficiency of plating compared to 30 °C). In all, $3{\cdot}6 \times 10^{10}$ bacteria were plated on selective medium at 42 °C. No colonies appeared.

To increase the chances of finding the desired mutant, we transduced a *mutL*::Tn*10* allele into strain GC3750. This mutation abolishes the post-replicative mismatch correction system, thereby increasing the spontaneous rate of base substitution mutations by about 100-fold (Glickman & Radman, 1980). For 12 transductants, the frequency of valine resistant mutants, measured at 30 °C on glucose plates containing 80 μg/ml valine, was shown to be an average of 75 times higher than the *mutL*$^+$ control. From a total of 10^9 *mutL* bacteria plated on minimal lactose plates at 42 °C, two clones were isolated after six days' incubation. Purified on the same medium at 42 °C, they continued to grow slowly. The bacteria in the colonies were of normal size, showing that these two clones had reverted or suppressed the *ftsZ84*(Ts) mutation. The slow growth therefore indicates that they had not derepressed the *lac* operon of their λJFL100 prophage.

We conclude that the Lac$^+$ temperature resistant mutant we were seeking cannot occur by a single base substitution mutation.

We are grateful to Drs Allan Campbell, Emmanuelle Maguin, Danièle Touati and Tove Atlung for the generous gift of strains, to Jean-Claude Liébart and Aline Jaffé for stimulating discussions, and to Philippe Bouloc for carrying out the sequence comparison. We thank our colleagues Danièle Joseleau-Petit and Daniel Vinella for general moral support. This work was financed in part by the Association pour la Recherche sur le Cancer (contract No. 6696).

References

Aldea, M., Garrido, T., Pla, J. & Vicente, M. (1990). Division genes in *Escherichia coli* are expressed coordinately to cell septum requirements by gearbox promoters. *EMBO Journal* **9**, 3787–3794.

Barker, D. F. & Campbell, A. M. (1980). Use of *bio-lac* fusion strains to study regulation of biotin biosynthesis in *Escherichia coli*. *Journal of Bacteriology* **143**, 789–800.

Bi, E. & Lutkenhaus, J. (1991). FtsZ ring structure associated with division in *Escherichia coli*. *Nature* **354**, 161–164.

Campbell, A., Del Campillo-Campbell, A. & Barker, D. (1978). Repression of biotin biosynthesis in *Escherichia coli* during growth on biotin vitamers. *Journal of Bacteriology* **135**, 90–98.

Carson, M. J., Barondess, J. & Beckwith, J. (1991). The FtsQ protein of *Escherichia coli*: membrane topology, abundance, and cell division phenotypes due to overproduction and insertion mutations. *Journal of Bacteriology* **173**, 2187–2195.

Corton, J. C., Ward, J. E. & Lutkenhaus, J. (1987). Analysis of cell division gene *ftsZ*(*sulB*) from gram-negative and gram-positive bacteria. *Journal of Bacteriology* **169**, 1–7.

Dai, K. & Lutkenhaus, J. (1991). *ftsZ* is an essential cell division gene in *Escherichia coli*. *Journal of Bacteriology* **173**, 3500–3506.

Dessen, P., Fondrat, C., Valencia, C. & Mugnier, C. (1990). BISANCE: a French service for access to biomolecules. *Computer Applications in Biosciences* **6**, 355–356.

Dewar, S. J., Kagan-Zur, V., Begg, K. J. & Donachie, W. D. (1989). Transcriptional regulation of cell division genes in *Escherichia coli*. *Molecular Microbiology* **3**, 1371–1377.

Gervais, F. G., Phoenix, P. & Drapeau, G. R. (1992). The *rcsB* gene, a positive regulator of colanic acid biosynthesis in *Escherichia coli*, is also an activator of *ftsZ* expression. *Journal of Bacteriology* **174**, 3964–3971.

Ghelardini, P., Lauri, P., Ruberti, I., Orlando, V. & Paolozzi, L. (1991). Synchronous division induced in *Escherichia coli* K12 by phage Mu: analysis of DNA topology and gene expression during the cell cycle. *Research in Microbiology* **142**, 259–267.

Glickman, B. W. & Radman, M. (1980). *Escherichia coli* mutator mutants deficient in methylation instructed DNA mismatch correction. *Proceedings of the National Academy of Sciences, USA* **77**, 1063–1067.

Huisman, O., Jacques, M., D'Ari, R. & Caro, L. (1983). Role of the *sfiA*-dependent cell division regulation system in *Escherichia coli*. *Journal of Bacteriology* **153**, 1072–1074.

Kravchenko, V. V., Vassilenko, S. K. & Grachev, M. A. (1979). A rightward promoter to the left of the *att* site of λ phage DNA: possible participant in site-specific recombination. *Gene* **7**, 181–195.

Lutkenhaus, J. (1990). Regulation of cell division in *E. coli*. *Trends in Genetics* **6**, 22–25.

Masters, M., Paterson, T., Popplewell, A. G., Owen-Hughes, T., Pringle, J. H. & Begg, K. J. (1989). The effect of DnaA protein levels and rate of initiation at *oriC* on transcription originating in the *ftsQ* and *ftsA* genes: in vivo experiments. *Molecular & General Genetics* **216**, 475–483.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory; Cold Spring Harbor, N.Y.

Mizusawa, S. & Ward, D. F. (1982). A bacteriophage lambda vector for cloning with *Bam*HI and *Sau*3A. *Gene* **20**, 317–322.

Otsuka, A. J., Buoncristiani, M. R., Howard, P. K., Flamm, J., Johnson, C., Yamamoto, R., Uchida, K., Cook, C., Ruppert, J. & Matsuzaki, J. (1988). The *Escherichia coli* biotin biosynthetic enzyme sequences predicted from the nucleotide sequence of the *bio* operon. *Journal of Biological Chemistry* **263**, 19577–19585.

Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences, USA* **85**, 2444–2448.

Rasmussen, L. J., Møller, P. L. & Atlung, T. (1991). Carbon metabolism regulates expression of the *pfl* (pyruvate formate-lyase) gene in *Escherichia coli*. *Journal of Bacteriology* **173**, 6390–6397.

Robin, A., Joseleau-Petit, D. & D'Ari, R. (1990). Transcription of the *ftsZ* gene and cell division in *Escherichia coli*. *Journal of Bacteriology* **172**, 1392–1399.

Robinson, A. C., Begg, K. J. & Donachie, W. D. (1988). Mapping and characterization of mutants of the *Escherichia coli* cell division gene, *ftsA*. *Molecular Microbiology* **2**, 581–588.

Rosenveld, E. C., Calva, E., Burgess, R. R. & Szybalski, W. (1980). *In vitro* transcription from the *b2* region of bacteriophage λ. *Virology* **107**, 476–487.

Ruberti, I., Crescenzi, F., Paolozzi, L. & Ghelardini, P. (1991). A class of *gyrB* mutants, substantially unaffected in DNA topology, suppresses the *Escherichia coli* K12 *ftsZ*$_{84}$ mutation. *Molecular Microbiology* **5**, 1065–1072.

Schwartz, M. (1987). The maltose regulon. In *Escherichia coli and Salmonella typhimurium: cellular and molecular biology* (ed. F. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger), pp. 1482–1502. Washington, D.C.: American Society for Microbiology.

Silhavy, T. J. & Beckwith, J. R. (1985). Use of *lac* fusions for the study of biological problems. *Microbiological Reviews* **49**, 398–418.

Vicente, M., Kushner, S. R., Garrido, T. & Aldea, M. (1991). The role of the 'gearbox' in the transcription of essential genes. *Molecular Microbiology* **5**, 2085–2091.

Walker, J. R., Kovarik, J. S., Allen, J. S. & Gustafson, R. A. (1975). Regulation of bacterial cell division: temperature-sensitive mutants of *Escherichia coli* that are defective in septum formation. *Journal of Bacteriology* **123**, 693–703.

Wang, X., de Boer, P. A. J. & Rothfield, L. I. (1991). A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of *Escherichia coli*. *EMBO Journal* **10**, 3363–3372.

Ward, J. E., Jr. & Lutkenhaus, J. (1985). Overproduction of FtsZ induces minicell formation in E. coli. *Cell* **42**, 941–949.