The EMBO Journal vol.10 no.10 pp.3113-3122, 1991

A single base change in the acceptor stem of $tRNA_3^{Leu}$ confers resistance upon *Escherichia coli* to the calmodulin inhibitor, 48/80

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Communicated by M.Salas

We have isolated several classes of spontaneous mutants resistant to the calmodulin inhibitor 48/80 which inhibits cell division in *Escherichia coli* K12. Several mutants were also temperature sensitive for growth and this property was exploited to clone a DNA fragment from an *E.coli* gene library restoring growth at 42°C and drug sensitivity at 30°C in one such mutant. Physical and genetic mapping confirmed that both the mutation and the cloned DNA were located at 15.5 min on the *E.coli* chromosome at a locus designated *fee*B. By subcloning, complementation analysis and sequencing, the *fee*B locus was identified as identical to the $tRNA_{CUA}^{Leu}$ gene. When the mutant locus was isolated and sequenced, the mutation was confirmed as a single base change, C to A, at position 77 in the acceptor stem of this rare Leu tRNA. In other studies we obtained evidence that this mutant tRNA, recognizing the rare Leu codon, CUA, was defective in translation at both permissive and non-permissive temperatures. The *fee*B1 mutant is defective in division and shows a reduced growth rate at non-permissive temperature. We discuss the possibility that the mutant $tRNA_3^{Leu}$ is limiting for the synthesis of a polypeptide(s), requiring several CUA codons for translation which in turn regulates in some way the level or activity of the drug target, a putative cell cycle protein.

Key words: calmodulin/cell cycle/*E.coli*/tRNA/48/80

Introduction

Considerable evidence exists that calcium plays a key role in higher eukaryotes in the regulation of cell cycle events, in particular through fluctuations in the concentration of free Ca^{2+} in the cytoplasm (Whitaker and Patel, 1990). The intracellular concentration of this cation is tightly regulated through the action of Ca^{2+} transport systems including voltage gated channels acting at the cell surface or to effect mobilization of intracellular stores. The triggering of cell cycle events following a calcium flux is then mediated through, for example, the action of calmodulin, which in turn regulates the activities of target proteins, including kinases (Klee, 1988; Means, 1988). Indeed, calmodulin itself may be limiting for the triggering of DNA synthesis since Rasmussen and Means (1987, 1989) have shown that the length of the G_1 period in mouse C127 cell lines is inversely proportional to the level of calmodulin expressed from a recombinant vector.

A calcium flux during the cell cycle has also been reported in *Saccharomyces cerevisiae* (Saavedra-Molina *et al.*, 1983) and recently Iida *et al.* (1990) have shown that calcium starved yeast cells arrest primarily at the G_2/M boundary, although entry into the DNA synthesis (S) phase was also delayed. Other studies indicate that Ca^{2+} is also required for bud formation in yeast (Ohya *et al.*, 1986) and a gene encoding a calcium binding protein specifically required for duplication of the spindle pole body has been identified (Baum *et al.*, 1986). In addition, a calmodulin gene has been isolated and shown to be essential for growth in both *S.cerevisiae* and *Schizosaccharomyces pombe* (Davies *et al.*, 1986; Takeda and Yamamoto, 1987).

We have previously proposed (Norris *et al.*, 1988; Holland *et al.*, 1990) that the cell cycle in bacteria may also be controlled by a single calcium flux, mediated by calmodulin dependent protein kinases and contractile proteins in order to effect specific events such as initiation of DNA synthesis, nucleoid segregation and invagination of surface layers at division.

In *E.coli*, the average concentration of free intracellular calcium appears to be identical to that of higher organisms (10^{-7} M; Gangola and Rosen, 1987) and the level of total cytoplasmic calcium has been reported to increase substantially during division (Chang *et al.*, 1986). In addition, proteins with calmodulin-like activities have been described in bacilli and in cyanobacteria (Fry *et al.*, 1986, 1991; Shyu and Foegeding, 1989; Pettersen and Bergman, 1989) and a calmodulin-like gene has been sequenced from *Streptomyces erythraeus* (Swan *et al.*, 1987). In recent years, many examples of regulation of protein activity in bacteria through changes in phosphorylation of specific amino acid residues have been described (Saier *et al.*, 1990) including that of DnaK (Cegielska and Georgopoulos, 1989), a heat shock protein implicated in the initiation of DNA replication (Sakakibara, 1988). In addition, we have described a myosin-like protein in *E.coli* (Casarégola *et al.*, 1990) and Hiraga and co-workers have identified a dynamin-like protein which they have shown is specifically involved in the normal segregation of nucleoids in *E.coli* (Niki *et al.*, 1991). All these observations provide considerable support for our hypothesis that highly conserved protein functions control cell cycle events in bacteria. Our current studies have been therefore to investigate whether a calmodulin-like protein might play a key role in the regulation of some aspects of the cell cycle in *E.coli*.

Previous studies have shown that calmodulin inhibitors can be used to isolate mutants with altered calmodulins in higher

eukaryotes (Kurn and Sela, 1981; Speaker *et al.*, 1983). In this study therefore, our approach was first to show that *E.coli* is sensitive to many calmodulin inhibitors, including 48/80. Subsequently, this allowed us to clone a gene conferring sensitivity to the drug through the isolation of mutants resistant to 48/80 at 30°C and defective for growth and division at 42°C. We have shown that this gene in fact encodes the rare $tRNA^{Leu}_{CUA}$ and that the mutation conferring resistance to 48/80 is a C to A change at position 77 in the acceptor stem of the tRNA. The relationship between resistance to 48/80 and altered tRNA may be explained in terms of regulation of the level of synthesis or activity of the drug target protein through translation of rare CUA, Leu codons, by the rare $tRNA^{Leu}_3$.

Results

Effect of 48/80 on growth, cell division and DNA synthesis in E.coli

We tested a range of calmodulin inhibitors for inhibition of growth in *E.coli* and 48/80 was found to have the lowest minimal inhibitory concentration (5 μg/ml). This drug was selected for further study and additional experiments were then carried out to establish whether the drug had any specific effects on cell division or DNA replication. For these experiments we used strain N43 (*acr*$^-$) which displays increased sensitivity to several drugs (Nakamura and Suganuma, 1972) including 48/80, particularly in minimal glucose medium.

An exponentially growing culture of *E.coli* N43, in M9 medium (Helmstetter, 1967) supplemented with casamino acids (M9CAA), was treated with varying concentrations of 48/80. Growth of the culture (measured as A_{450}) was inhibited over the concentration range of 3−10 μg/ml without any detectable lysis, as described in Figure 1A. As shown in Figure 1B, cell division was also inhibited by the drug, resulting in the formation of many short filaments.

Inhibition of cell division in *E.coli* can result from the induction of the SOS response. Thus, following DNA damage, the action of the endogenous inhibitor, SfiA, whose synthesis is induced under these conditions, specifically blocks division (Walker, 1985). In order to test whether 48/80 induced the synthesis of the SfiA inhibitor, we analysed the effect of the drug on β-galactosidase activity in strain N43, carrying a λ prophage containing an *sfiA−lacZ* fusion. No induction of β-galactosidase synthesis was obtained upon treatment of this strain with 48/80 whilst, in contrast, nalidixic acid induced high levels of β-galactosidase synthesis (data not shown). Therefore, 48/80 does not appear to induce the SOS response and the drug may affect septum formation directly or, alternatively, indirectly by delaying termination of DNA replication.

E.coli N43 in M63 minimal medium was grown for at least twenty generations in the presence of ^{3}H-labelled thymidine as described in Materials and methods. The drug, 48/80, was added and subsequent changes in growth and in the accumulation of DNA were measured (Figure 2). Growth (A_{450}) was progressively reduced in the presence of the drug. The accumulation of DNA was blocked before growth was fully inhibited. The final level of radiolabelled DNA was consistent with the drug blocking initiation of further rounds of DNA replication, rather than having any significant effect upon ongoing replication. Indeed, the residual increase in the amount of DNA synthesized (72%) following the addition of 20 μg/ml of 48/80 was identical to that obtained when initiation of DNA synthesis in strain N43 was blocked by the addition of rifamycin (Figure 2C).

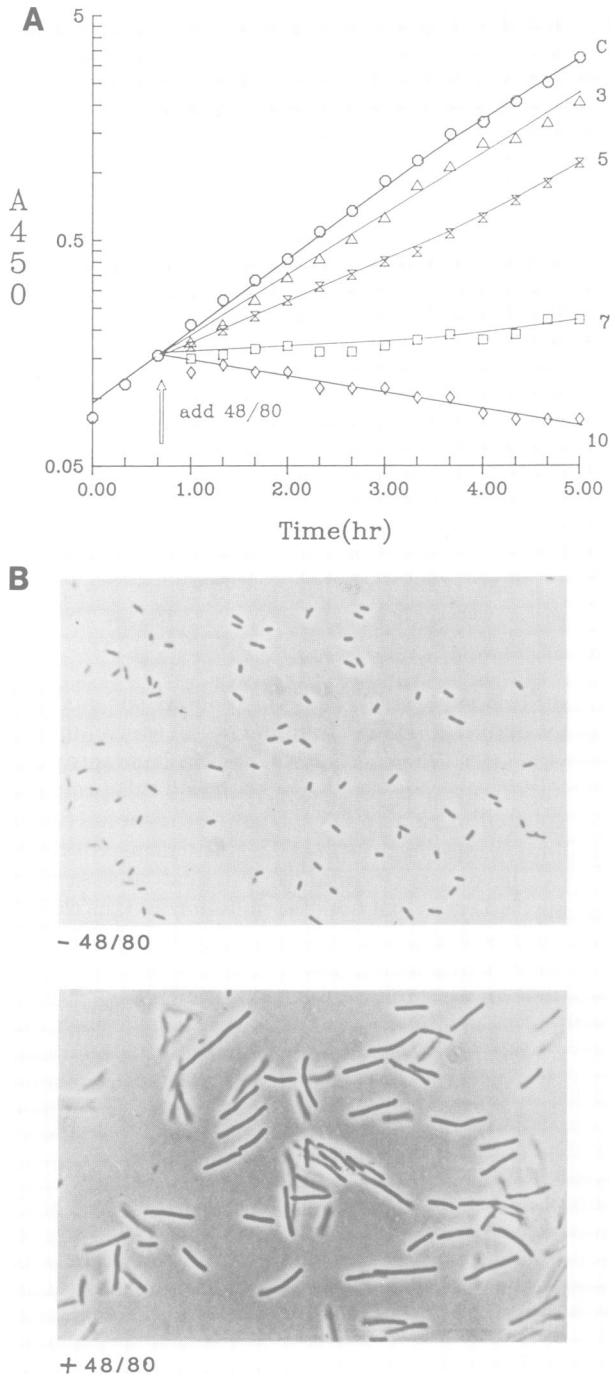


Fig. 1. Effect of 48/80 on growth and cell division of *E.coli*. Cells of the *E.coli* strain N43 were grown exponentially in M9CAA medium at 30°C. At an A_{450} of 0.2, the culture was treated with 48/80, 3(△), 5(X), 7(□), 10 (◇) μg/ml, or left untreated (○). **A.** The mass increase was monitored by measuring A_{450}. **B.** Photographs were taken under phase contrast in the absence of the drug or after 3 h of exposure to the drug (20 μg/ml) in nutrient broth.

Properties of a fee mutant resistant to 48/80

Spontaneous mutants of strain N43 were isolated as described elsewhere (M.Chen, N.Bouquin, S.Bernard, S.J.Seror and I.B.Holland, in preparation), which were selected as resis-

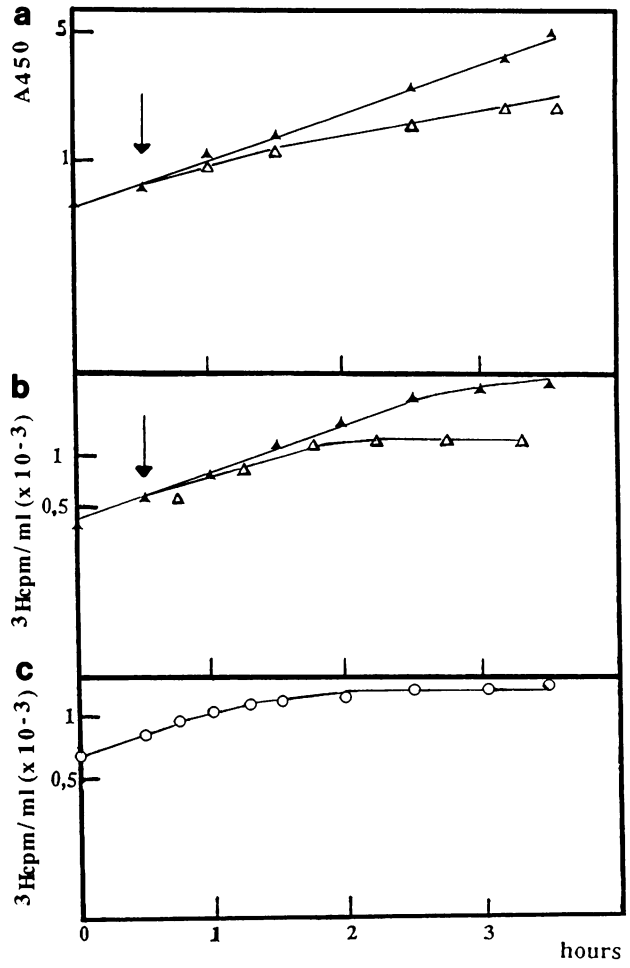


Fig. 2. Effect of 48/80 on DNA synthesis. *E.coli* N43 was grown exponentially in M63 medium previously labelled for at least 20 generations with [^{3}H]thymidine. The drug was added at time indicated by arrow and accumulation of labelled DNA in the cells was measured at intervals. **Panel a**, A_{450}; **panel b**, DNA accumulation; **panel c**, DNA accumulation after addition of rifamycin (200 μg/ml). Open symbols + drug, closed symbols, untreated control.

tant to the drug at 30°C but also unable to grow at 42°C (in the absence of the drug). This indicated that such mutants were defective in an essential gene. Genetic analysis of the mutants indicated that the mutations mapped to a number of different sites on the *E.coli* chromosome. The growth of one such mutant, *fee*B1, is shown in Figure 3A. Growth of the mutant at 30°C was reduced compared with that of the wild type parent and, although the growth rate initially increased after a shift to 42°C, it then continued at a substantially reduced rate at the non-permissive temperature. As shown in Figure 3B, despite the slow rate of mass increase, the inhibition of cell division at 42°C can be detected with the progressive appearance of short filaments.

When the accumulation of DNA in the mutant was measured after temperature shift, no differential reduction of DNA synthesis, when compared with growth (A_{450}), could be detected. Similarly, no significant change in the C time (time for a round of DNA replication) or the DNA:mass ratio could be detected in the mutant at 30°C (data not shown). The *fee*B1 mutant therefore shows reduced growth and drug resistance at 30°C and growth and cell division inhibition after temperature shift, but apparently no specific effect was observed on DNA replication.

The *fee*B1 mutant was tested at 30°C for increased resistance to SDS, several antibiotics, and to verapamil, a calcium channel inhibitor, but was found to remain just as sensitive as the wild type to these compounds. This indicated that the mutant was not generally more resistant to drugs, through for example, changes in envelope permeability, but was specifically resistant to 48/80.

Isolation and physical location of an E.coli gene which restores temperature resistance and 48/80 sensitivity in the feeB1 mutant

As described in Materials and methods, a recombinant plasmid, pLG695, (see Figure 4) was isolated from an *E.coli* gene bank constructed in the low copy number vector, pLG339 (Stoker *et al.*, 1982), which restored the growth of N43 *fee*B1 at 42°C. Moreover, in the presence of this recombinant plasmid the mutant strain N43 *fee*B1 was fully sensitive to 48/80 at 30°C (data not shown).

The 3.5 kb insert from pLG695 was labelled *in vitro* with [^{32}P]ATP as described in Materials and methods and was used to probe, by Southern blotting, total *E.coli* DNA digested with eight different restriction enzymes. The results obtained enabled us to map the clone at 720 kb on the Kohara physical map of the *E.coli* chromosome, equivalent to 15.5 min (Kohara *et al.*, 1987). This result agreed with the data from DNA sequencing of *fee*B DNA described below.

Genetic analysis of the feeB1 mutant

In order to determine whether the *fee*B mutation itself also mapped to the 15.5 min region on the chromosome, P1 transduction experiments were carried out. For this purpose bacteriophage P1 was grown on strain SP238 which carries Tn*10* inserted close to *lip* and *rodA* at 15 min on the *E.coli* chromosome. Tetracycline resistant transductants of strain N43 *fee*B1 were selected at 30°C and five out of 169 transductants (3%) were found to be thermoresistant and sensitive to 48/80 at 30°C. This result clearly indicated that *fee*B is indeed closely linked to the same 15.5 min region on the *E.coli* map as the cloned DNA.

This conclusion was tested further by the introduction by conjugation of an F′ plasmid, F-152, covering the 13−17 min region of the *E.coli* chromosome, with selection for *gal*K$^+$ exconjugants of N43 *fee*B1 at 30°C. Of 500 exconjugants analysed, all were, as expected, now sensitive to 48/80. However, when these partial diploids were screened at 42°C, all except 19 failed to grow. This result indicated that under these conditions at least, temperature sensitivity was dominant, with the 19 exceptions probably due to recombinational exchange of a copy of the wild type allele from an F′ plasmid to the chromosome (homogenization).

This result, however, also raised the possibility that resistance to 48/80 and temperature sensitivity might be due to separate mutations. In order to test this, 300 temperature resistant revertants of N43 *fee*B1 were isolated and all were found to be sensitive to 48/80. Thus, we conclude that the resistance to 48/80 and temperature sensitivity of the mutant were due to a single mutation.

Fine structural analysis of the feeB1 locus

In order to localize further the *fee*B gene in the recombinant plasmid pLG695 carrying the wild type locus, the clone was partially digested with *Sau*3A and the recombinant

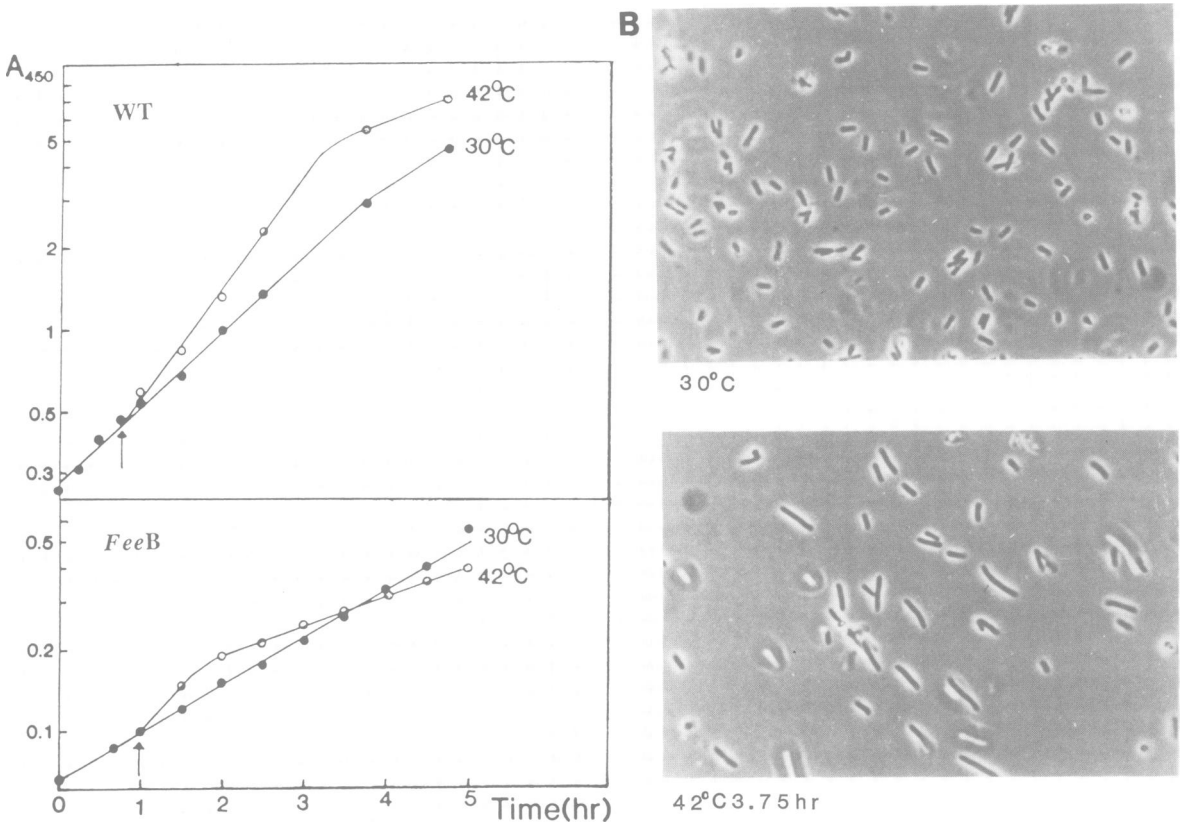


Fig. 3. Effect of temperature shift on growth and cell division of a *fee*B mutant. The wild type (N43) and the *fee*B1 mutant were grown at 30°C in M9CAA medium and transferred to 42°C (indicated by the arrow). Growth was followed by measuring the A_{450}. **A.** A_{450} at 30°C and at 42°C in the wild type (top panel). A_{450} at 30°C and at 42°C in *fee*B1 mutant (bottom panel); **B.** Photographs were taken under phase contrast at 30°C and at 1.25, 2.5 and 3.75 h after transfer to 42°C.

plasmid, pLG696 (see Figure 4), which carried a smaller 1.8 kb insert, was isolated. This plasmid nevertheless still restored temperature resistance in N43 *fee*B1. Several additional subclones and deletions from pLG696 were then constructed utilizing existing restriction sites and tested for their ability to restore temperature resistance in the mutant. The results are summarized in Figure 5, which also shows the detailed restriction map of the Sau3A cloned insert of pLG696. The data clearly indicated that the wild type gene, restoring both temperature resistance and drug sensitivity, was located to the left of the *Kpn*I(a) site and to the right of the *Hinc*II(a) site, encompassing the *Acc*I site.

Sequence analysis and the identification of the feeB locus as tRNA$_3^{Leu}$

The DNA fragments from pLG696 carrying the *fee*B region indicated in Figure 6 were subcloned into M13 and sequenced as described in Materials and methods. The resulting data when compared with sequences in the EMBL database, were identical to the *sup*B-E tRNA operon previously sequenced by Inokuchi *et al.* (1979) and mapped in the 15.5 min region of the chromosome. This result allowed us to identify a *leu* tRNA gene mapping immediately to the left of the *Kpn*I sites. However, as shown in Figure 5, this region is also predicted to encode a short open reading frame (designated ORF 4) on the opposite strand to the *leu* tRNA gene. ORF6, in contrast, contains the *Kpn*I(a) site and is truncated in the *Kpn*I−*Kpn*I deletion (Figure 5) but complementation was still observed.

In order to determine whether the *fee*B product was a tRNA or a polypeptide, a nonsense (UAG) codon was introduced into ORF4 (between *Hinc*II and *Acc*I, see Figure 6) by site directed mutagenesis as described in Materials and methods. This mutation was also designed to generate a unique *Sty*I site upstream of the *leu* tRNA gene. When the derivative of pLG696 carrying this nonsense mutation was introduced into N43 *fee*B1, temperature resistance was still restored and we concluded therefore that *fee*B was in all probability identical to the *leu* tRNA (data not shown). However, although not indicated in the published reference to the *E.coli* strain used in this study (Nakamura and Suganuma, 1972), N43 may in fact carry the nonsense suppressor, *supE*. (B.Bachmann, personal communication). Additionally therefore, taking advantage of the inserted *Sty*I site, sequences upstream of this site, including part of ORF4, were deleted as described in Figure 7. In the resulting construct, pLG697, the intact *leu* tRNA gene is now downstream of the plasmid *tet* promoter. This plasmid still restored temperature resistance, confirming that *fee*B and the tRNA$_3^{Leu}$ recognizing the codon CUA were identical.

Cloning and sequencing of the feeB1 mutation

In order to determine the nature of the mutation at the *fee*B locus, advantage was taken of the known restriction endonuclease sites flanking *fee*B. Chromosomal DNA of the N43 *fee*B1 mutant was therefore digested with *Bgl*II and *Kpn*I (see also Figure 5), fragments were selected in the size range 0.7−0.8 kb and cloned into M13mp18 as described in Materials and methods. When 72 of these clones were analysed, two were found to have a *Hinc*II and an *Acc*I site

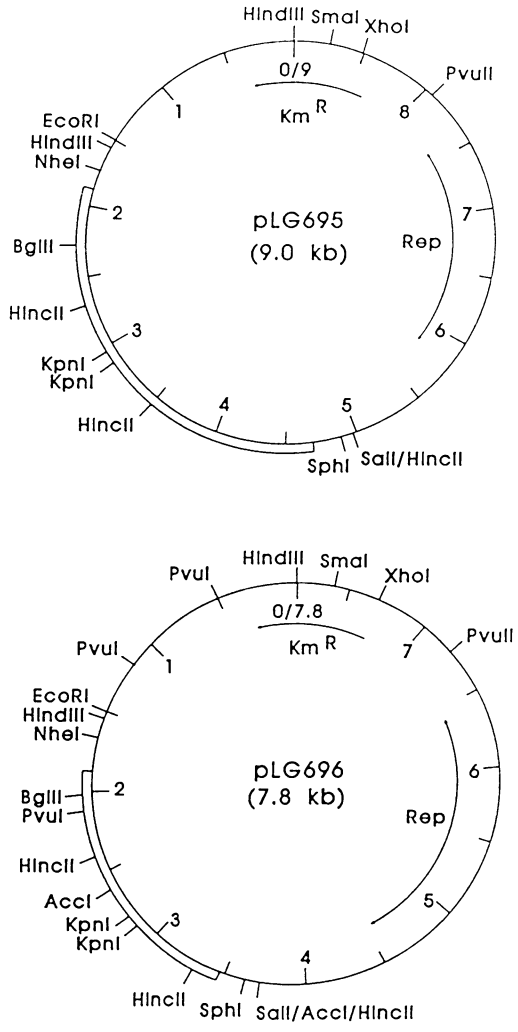


Fig. 4. Map of pLG695 carrying the initially isolated *fee*B region (3.5 kb) and its derivative pLG696 carrying a smaller, 1.8 kb, insert after *Sau*3A digestion.

at the same positions within the *Bgl*II−*Kpn*I fragment as those of the wild type clone, pLG696. Sequencing of the *fee*B region from both the wild type and the mutant was therefore carried out in parallel and the results demonstrated that *fee*B1 contained a single base change, C to A at position 77 in $tRNA^{Leu}_{CUA}$ (Figure 8). This change is in the fifth base pair from the terminus of the acceptor stem as predicted by the sequence of Nakajima *et al.* (1982). This mutational change therefore should disrupt the base paired acceptor stem even at 30°C. However, although this region has been clearly implicated in the specificity for charging by the corresponding amino acyl tRNA synthetase in certain tRNAs (Hou *et al.*, 1989), the basis for this specificity has not been established for $tRNA^{Leu}$.

Analysis of the function of $tRNA^{Leu}_3$ in a wild type strain and in N43 carrying the fee B1 mutation

$tRNA^{Leu}_{CUA}$ is a rare tRNA (Ikemura, 1981) encoding one of the rarest codons, CUA. The relationship between resistance to the calmodulin inhibitor, 48/80, and an altered $tRNA^{Leu}_3$ might be explained by the presence of a specific polypeptide which controls directly or indirectly the response to the drug and whose synthesis from mRNA is strongly dependent upon this tRNA. It was therefore important to determine whether the synthesis of specific proteins were perturbed by this mutational change, particularly at permissive temperature where the mutant is drug resistant.

Fortunately, the coding sequence of β-galactosidase contains six CUA codons whereas that for the LacI repressor contains none, thus providing a convenient assay for the activity of the $tRNA^{Leu}_3$. The level of β-galactosidase (expressed as units per A_{450}) was therefore measured in both the mutant *fee*B1 and its wild type parent, N43 at both 30°C and 42°C and before and after induction. As shown in Figure 9A, the basal level of β-galactosidase in glycerol grown cultures at 30°C is several-fold higher in the wild type than in the mutant strain. Similarly, at 30°C even when high levels of *lac*Z mRNA are induced by the addition of IPTG, the synthesis of β-galactosidase is significantly higher in the wild type strain (Figure 9B). At 42°C the induced levels of β-galactosidase are dramatically reduced in the mutant *fee*B strain (Figure 9C), indicating that the $tRNA^{Leu}$ is now largely inactive.

These results strongly suggest that at 30°C the synthesis of a polypeptide requiring translation of CUA codons is indeed limited in the mutant strain by the availability of an active $tRNA^{Leu}_3$. Moreover, when the wild type tRNA gene is overexpressed from a multicopy plasmid in the mutant strain at 30°C, β-galactosidase synthesis was enhanced compared with the wild type, strongly suggesting the limiting effect of this tRNA even under normal conditions (data not shown). The almost complete loss of $tRNA^{Leu}_3$ activity at 42°C in the mutant (as measured by β-galactosidase synthesis) is most probably a major cause of the temperature sensitivity of this strain, since the synthesis of several key polypeptides should be affected. Interestingly, the synthesis of β-galactosidase at 42°C in the mutant was not fully restored by the introduction of the $tRNA^{Leu}_3$ gene on a multicopy plasmid (data not shown). This result confirms the dominant effect of the mutation at 42°C described above and is consistent with a direct toxic effect of the defective tRNA upon the protein synthesizing machinery under these conditions.

Discussion

In this study we have demonstrated that the drug hyperpermeable strain, N43, is sensitive to low levels (5−20 μg/ml) of the highly specific calmodulin inhibitor 48/80, resulting in observable inhibition of cell division. In addition, in other studies, we found *E.coli* N43 to be sensitive, although at higher concentrations, to other quite different calmodulin inhibitors, including chlorpromazine (Mandi *et al.*, 1976; Molnar *et al.*, 1977), W-7, W-13, W-5, calmidazolium and trifluoperazine (TFP) (Casarégola *et al.*, 1991; M.Chen and N.Bouquin, unpublished data).

Compound 48/80 has a monomer molecular weight of 318 Da but exists in a range of oligomeric forms from trimers up to dodecamers. The drug is a condensation product of *N*-methyl-*p*-methoxyphenylethylamine and formaldehyde, with cationic amphiphilic properties which promote its extremely high specificity for calmodulin (Gietzen, 1983). Although all forms are active against calmodulin, the hexameric and heptameric fraction of 48/80 has been reported to have the greatest potency to induce a second effect of the drug, the induction of histamine release from mast cells (Adamszyk-Engelmann and Gietzen, 1989).

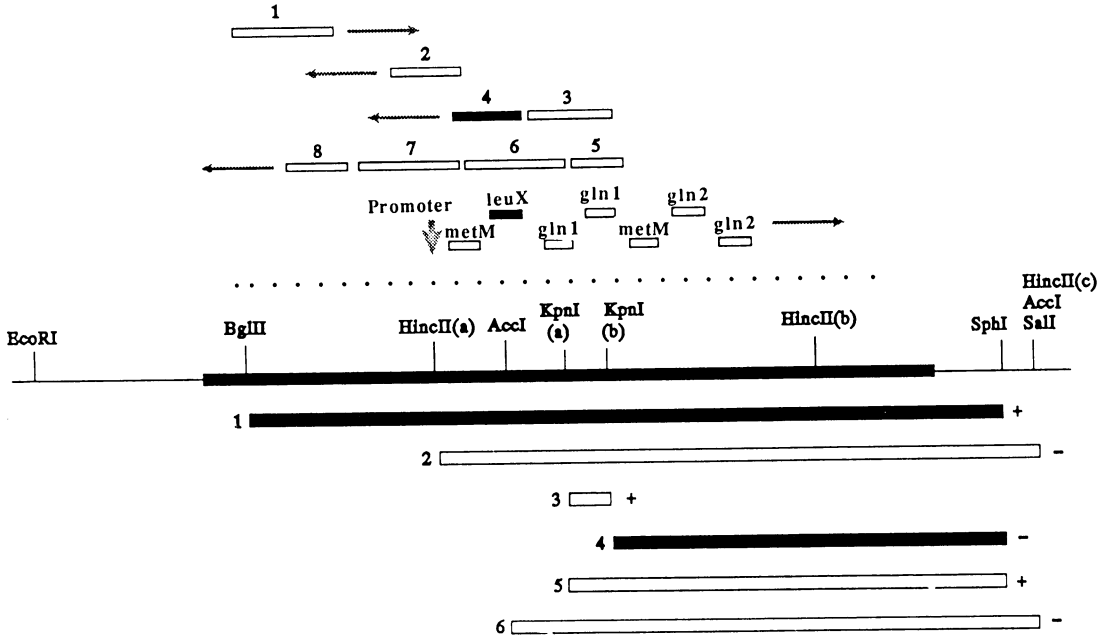


Fig. 5. Localization of the *fee*B gene by complementation, deletion analysis of pLG696. Deletions and subclones of the *fee*B region present in pLG696 were constructed and analysed at 42°C for complementation of the temperature sensitive phenotype and at 30°C for drug sensitivity. The constructions indicated in the lower half of the Figure as filled boxes (subclones) or open boxes (deletions) are as follows: (1) *Bgl*II−*Sph*I fragment subcloned into *Bam*HI and *Sph*I sites of pLG339; (2) *Hinc*II(a)−*Hinc*II(c) deletion; (3) *Kpn*I(a)−*Kpn*I(b) deletion; (4) *Kpn*I(b)−*Sph*I fragment subcloned into *Sma*I site of pLG339; (5) *Kpn*I(a)−*Sph*I deletion; (6) *Acc*I−*Acc*I deletion; +: complementing the temperature sensitivity of the mutant; −: not complementing the temperature sensitivity of the mutant. In the top part of the figure, the positions of individual tRNAs are indicated together with the eight predicted open reading frames (open boxes except ORF4) on both strands (taken from the data of Inokuchi *et al.*, 1979).

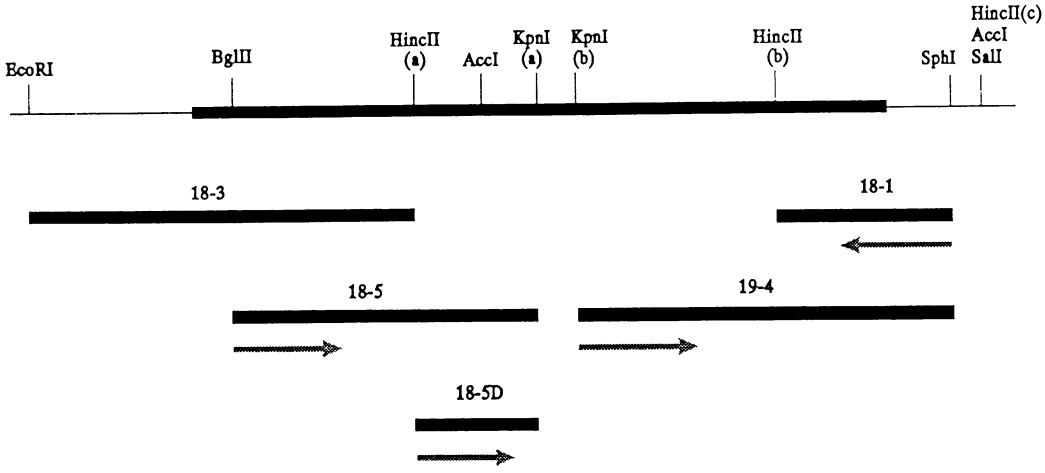


Fig. 6. Cloning the *fee*B region present in pLG696 for sequencing. The figure shows the restriction map of the 1.8 kb fragment containing the *fee*B$^+$ region (solid bar, top line) cloned into pLG696. The fragments indicated were cloned into M13mp18 and M13mp19 in the orientation indicated and sequenced. M13 18-1; *Hinc*II(b)−*Sph*I fragment into *Hinc*II and *Sph*I sites of M13mp18; M13 18-3: *Eco*RI−*Hinc*II(a) fragment into the *Eco*RI and *Hinc*II sites of M13mp18; M13 18-5: *Bgl*II−*Kpn*I(a) fragment into the *Bam*HI and *Kpn*I sites of M13mp18; M13 18-5D: *Hinc*II−*Hinc*II deletion in the 18-5 construct resulting in the fragment of *Hinc*II(a)−*Kpn*I(a) of pLG696 in the *Hinc*II and *Kpn*I sites of M13mp18; M13 19-4: *Kpn*I(b)−*Sph*I fragment into a *Kpn*I and *Sph*I digested M13mp19.

This effect of the drug may be primarily exerted through its lipophilic properties and consequent binding to the cell surface (Douglas and Nemeth, 1982).

St Léger *et al.* (1989) showed that depletion of intracellular Ca^{2+} or treatment with calmodulin inhibitors, including 48/80, specifically inhibited the germination of conidia of the fungus *Metarhizium anisopliae*. Exogenous 48/80, on the other hand, stimulated the phosphorylation of two intracellular polypeptides in the mycelium phase, an effect which could be overcome by Ca^{2+} depletion. The results were interpreted to indicate a role for 48/80 sensitive calmodulin(s) in germination or for a calmodulin dependent phosphatase active in mycelia.

The effect of 48/80 upon cell division in *E.coli* observed in this study suggested that an essential cell cycle function, perhaps a target calcium binding protein, was being inhibited by the drug. The inhibition of cell division at the non-permissive temperature in the *fee*B mutant, resulting in the formation of short filaments, was also consistent with an essential cell cycle function as the target of the drug. It was

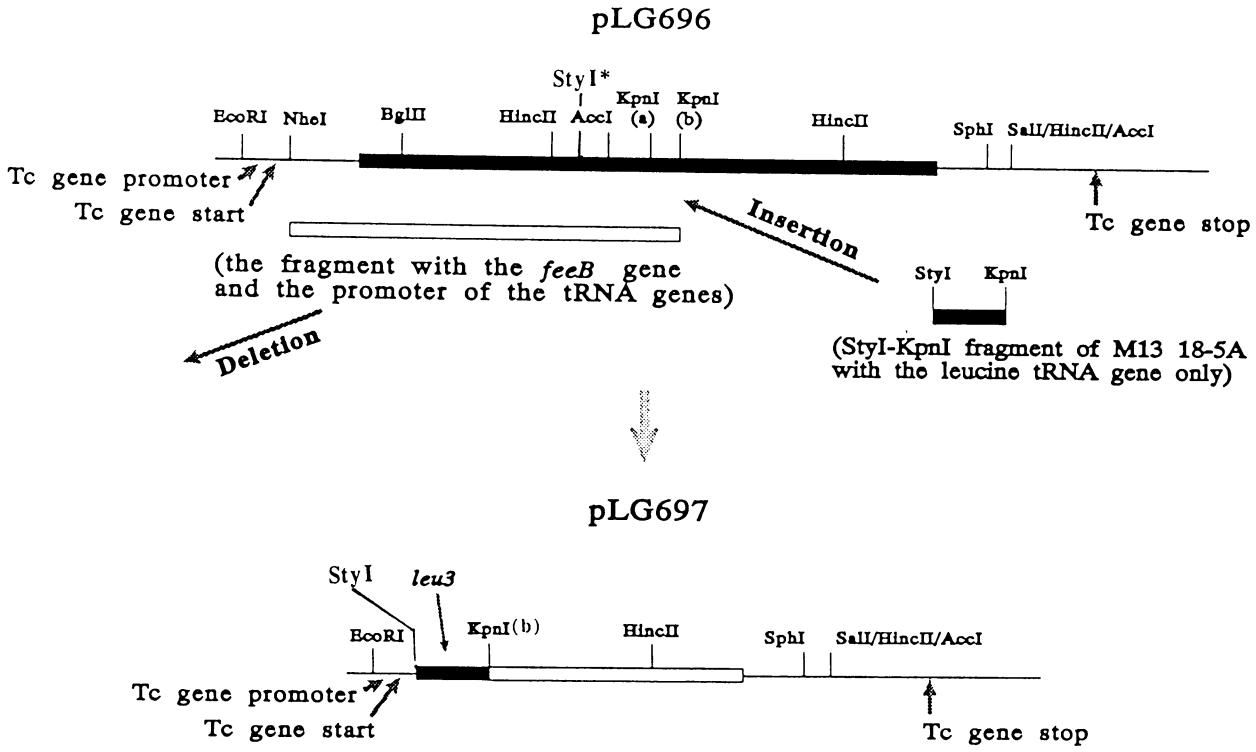


Fig. 7. Identification of the $tRNA_3^{Leu}$ gene as *fee*B. In order to confirm the identity of the $tRNA_3^{Leu}$ with the *fee*B locus, the following construction was made. In the first step, the region from *Nhe*I to the second *Kpn*I site of pLG696, which contains the *fee*B gene together with the promoter for the tRNA genes, was deleted and then the *Sty*I−*Kpn*I fragment encoding the $tRNA_3^{Leu}$, previously cloned into M13, was inserted instead of the deleted fragment (see text for other details). This figure also shows the position of the *Sty*I site (*Sty*I*) introduced into pLG696 by site directed mutagenesis.

consequently surprising to identify the *fee*B gene as encoding a specific tRNA which is unlikely to be the target of the drug. We propose instead that the mutant $tRNA_3^{Leu}$, leads to reduced translation of the rare CUA codon and a consequent reduction in the activity or the level of the specific intracellular target for 48/80 (see below). The $tRNA_3^{Leu}$ is a minor tRNA in *E.coli* which translates the rare CUA codon relatively slowly, compared with other, both rare and abundant codons (Bonekamp *et al.*, 1989) and these properties may allow it to exercise some additional role in gene expression. In fact, recent studies in quite diverse systems have indicated that tRNAs may play vital regulatory roles in cellular metabolism or development in addition to their classical function in mRNA translation. Thus, *E.coli* carrying mutations in an *arg* tRNA (*dna*Y), or in *ser* tRNAs were shown to be defective in the initiation of DNA replication or cell division respectively (Garcia *et al.*, 1986; Tamura *et al.*, 1984; Leclerc *et al.*, 1989). In the case of the *dna*Y mutation, recent data have shown that the mutant tRNA is indeed defective in mRNA translation (Spanjaard *et al.*, 1990). In order to explain their results, the authors proposed in each case that the *arg* or *ser* tRNA mutants were defective in the synthesis of a specific protein or subset of proteins essential for a particular cell cycle event. In addition, Lawlor *et al.* (1987), have shown that *Streptomyces coelicolor bld*A mutants which are defective in antibiotic and aerial hyphae production, have an altered, rare $tRNA^{Leu}$. These authors have proposed that this tRNA facilitates an important developmental control mechanism also at the level of translation. The finding described in this study that the synthesis of β-galactosidase (requiring translation of six CUA codons) is reduced in the *fee*B mutant even at 30°C is

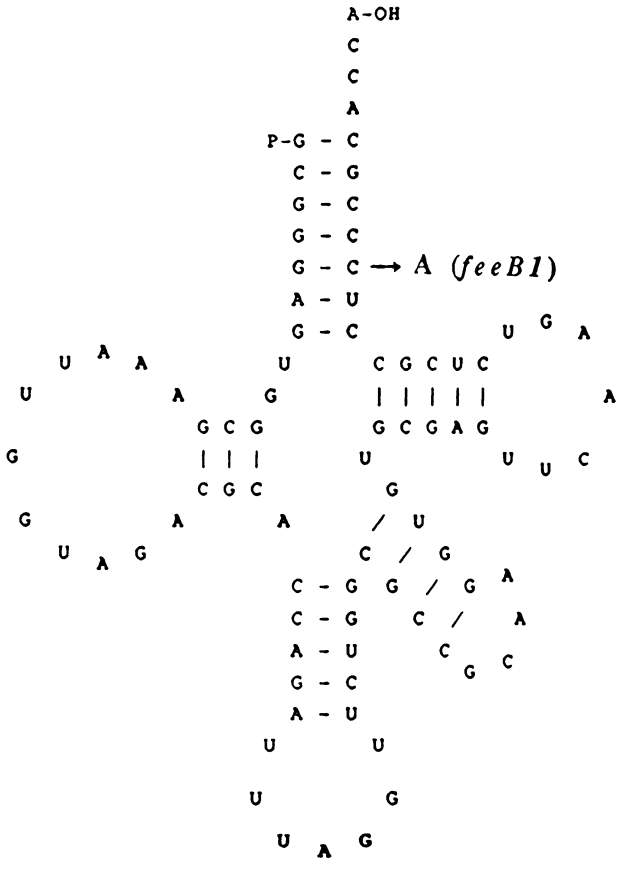
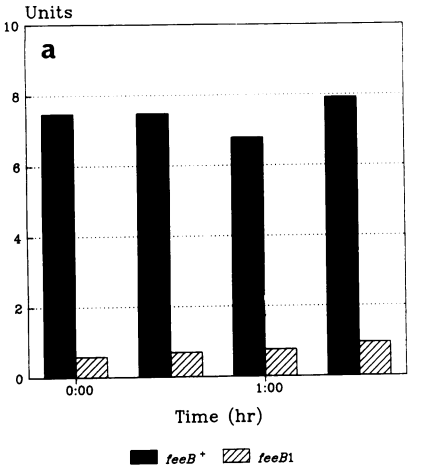
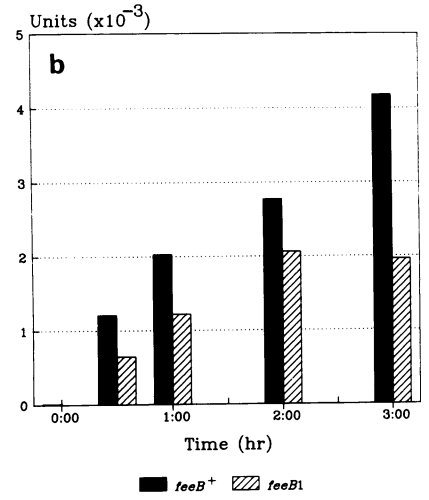


Fig. 8. Structure of the mutant $tRNA_3^{Leu}$. The single base change C to A, the *fee*B1 mutation, is indicated.





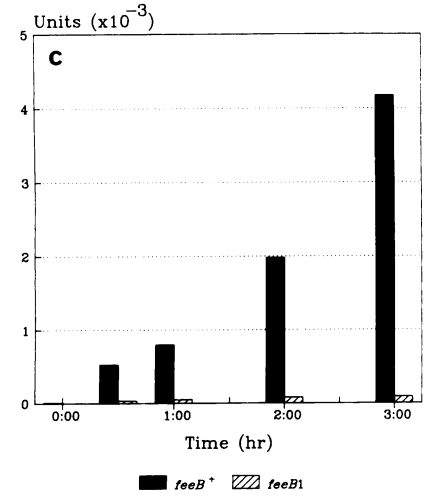


Fig. 9. Synthesis of β-galactosidase in the *fee*B1 mutant at 30°C and 42°C. The *lac*$^+$ derivative of the *fee*B1 mutant and the *lac*$^+$ derivative of the wild type parent were grown exponentially in M9 glycerol medium at 30°C. IPTG (1 mM) was added to the cultures at time 0 and one portion was kept at 30°C whilst the other portion was transferred to 42°C. Samples from the different cultures were taken at 0, 0.5, 1, 2 and 3 h after induction. The enzyme activity was measured according to the method of Miller (1972) and expressed as units per A_{450} unit of culture. **a**. 30°C in the absence of IPTG; **b**. 30°C in the presence of IPTG; **c**. 42°C in the presence of IPTG.

consistent with the regulation of the synthesis of a specific subset of proteins in *E.coli*, for example involved in cell cycle controls, by the levels of rare $tRNA^{Leu}_{CUA}$.

The presence of an unpaired G-A in the acceptor stem of the mutant tRNA strongly indicates that such an RNA will be defective in translation. This is not an unequivocal conclusion, however, since the basis of $tRNA^{Leu}$ identity has not, to our knowledge, been established (see Hou *et al.*, 1989). At 42°C in the *fee*B mutant, growth and in particular the synthesis of β-galactosidase were found clearly to be defective. Whilst this result may lend stronger support for the conclusion that the mutant *fee*B tRNA is defective in translation, the interpretation is complicated by the relative dominance of the mutation at non-permissive temperature, suggesting that defective tRNA molecules may still bind irreversibly to ribosomes or tRNA synthetase producing a poisoning effect. In this respect it is important to note that $tRNA^{Leu}_3$ also normally recognizes the abundant Leu codon, CUG, in mRNA (Emilsson and Kurland, 1990).

On the basis of the studies reported here we propose that the specific $tRNA^{Leu}_{CUA}$ mutation leads to reduced translation of a particular mRNA(s) rich in CUA codons, encoding a polypeptide(s) which in turn controls the level or the activity of the actual target for the drug 48/80. Alternatively, we could envisage that the mutation might affect instead the level of intracellular calcium and hence the response to the drug. It remains a moot point whether the level of the wild type $tRNA^{Leu}_{CUA}$ normally modulates the expression of a key subset of minor proteins, and we are aware of the arguments which have been raised against such regulatory mechanisms in general. Andersson and Kurland (1990) have reviewed the evidence that the availability of tRNAs and therefore variations in the rate of translation of mRNAs by ribosomes, should not normally influence the expression level of a given polypeptide. Nevertheless, it is conceded by these authors that rare codons and consequently their cognate tRNAs might indeed exert such control, if for example, slow codons such as CUA were clustered at the beginning of an mRNA. Such a regulatory mechanism could then be envisaged to act in a number of possible ways: at the level of transcription (Bonekamp *et al.*, 1989), by controlling mRNA stability or by premature termination of translation, for example, by modulation of the stability of mRNA initiation complexes (see Chen and Inouye, 1990).

Materials and methods

Bacterial strains and plasmids

E.coli N43 (*acr*A1, *ara*14, Δ*lac*-85, *gal*K2, *rps*L97, *mal*A1, *xyl*5, *mt*l1) a drug hypersensitive strain was described by Nakamura and Suganuma (1972). pLG339, a low copy number plasmid was described by Stoker *et al.* (1982); DH5 (*end*A1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *thi*1, *rec*A1, *gyr*A96, *rel*A1, λ$^-$); MC4100 (*ara*D139, [Δ*arg*F-*lac*,U169] *rpst*150, *flb*B5301, *pts*F25, *deo*C1, *rbs*R, *rel*A1, *thi*A; TG1 (Δ[*lac-pro*] *thi*1, *hsd*, *sup*E44, F′ (*tra*D36, *pro*A$^+$B$^+$, *lac*I^q, *lac*ZΔM15); NM522 (Δ[*lac-pro*AB], *thi*1, *sup*E44, *hsd*R17, F′*tra*D36, *pro*A$^+$B$^+$); SP238 (*pro*A, *pur*B, *his*, *thi*, *rod*A52, *zbe*::Tn*10*, *mtl*, *xyl*, *gal*K, *lac*Y, *str*) were kindly provided by B.Spratt; KL718 [*pyr*D34, *trp*45, *his*68, *tyr*A2, *rec*A1, *thi*1, *gal*K35, *mal*A1 (λ^r), *xyl*7, *mtl*2, *rps*L118, F′152 (*fep-gal*)] were obtained from B.Bachmann.

Conditions of growth

Liquid cultures were grown with vigorous shaking in nutrient broth (Oxoid no. 2), Luria Broth, M9 minimal salts (Helmstetter, 1967) or M9CAA, M9 supplemented with 0.1% (w/v) casamino acids (Difco). Cell mass was monitored by measuring absorbance (A_{450}) in a Gilford microsample

spectrophotometer −300N. For solid medium, media were supplemented with 1.5% (w/v) agar.

Determination of drug and SDS sensitivity

The minimal inhibitory concentration (m.i.c.) for various drugs was determined by incubation overnight of 5 ml of culture medium inoculated with ~ 10^5 cells/ml in the presence of a serial dilution of a particular drug. The concentration of drug giving minimal inhibition of growth of bacteria was then determined. For determining the sensitivity of strains to SDS, cells were patched on nutrient agar containing 0.3% (w/v) SDS.

Isolation of 48/80 resistant mutants

Full details of these procedures will be published elsewhere (Chen *et al.*, in preparation). Briefly, *E.coli* N43 cells grown in M9CAA medium were plated on the same agar medium containing 120 μg/ml of 48/80 dissolved in water. Mutants, arising at a frequency of ~5 × 10^{-8} were purified and screened for conditional lethal mutants which failed to grow at 42°C in the absence of the drug.

Cloning of the feeB1 mutant gene

A *Sau*3A digest of chromosomal DNA of the wild type strain MC4100 was fractionated on a sucrose velocity gradient and fragments of 3−4 kb were ligated into the *Bam*HI site of the low copy number vector pLG339 (Stoker *et al.*, 1982) in order to prepare a gene library. This library was then amplified by transformation of strain DH5 (*rec*A) which has a high transformation frequency (10^6 transformants/μg DNA) using the RbCl method (Hanahan, 1983). The *fee*B1 mutant was then transformed with this amplified library, using the PEG−DMSO procedure (Chung and Miller, 1988), with selection for Kan-resistance and thermoresistance at 42°C. Colonies growing at 42°C were purified, the recombinant plasmid was extracted and used to re-transform the *fee*B1 mutant at 30°C, in this case with selection for kanamycin resistance only. All these transformants then grew at 42°C without selection confirming that the recombinant plasmid complemented the temperature sensitive character of the mutant.

Isolation of lac$^+$ derivatives of strain N43 and N43 (feeB1)

E.coli N43 is *lac*$^-$, *mal*$^-$(λ^r) and for some purposes had to be converted to *lac*$^+$. This was achieved either by P1 transduction, selecting for growth on lactose or by lysogenization with the λ(*sfi*A::*lac*) phage following P1 transduction to *mal*$^+$ (λ^s) by selecting for growth on maltose.

Site directed mutagenesis

Site directed mutagenesis was carried out in M13 using the Amersham kit exactly as described by the manufacturer. In order to introduce a *Sty*I site into pLG696, a *Bgl*II−*Kpn*I(a) fragment was cloned into M13mp18. The mutated fragment was then removed and pLG696 reconstructed with the new *Sty*I site.

DNA sequencing

DNA sequencing was carried out by the dideoxy method with specific DNA fragments cloned into M13mp18 or M13mp19 as described by Yanisch-Perron *et al.* (1985).

Measurement of β-galactosidase activity

β-galactosidase activity in *lac*$^+$ strains was measured with or without the addition of IPTG (1 mM) by the method described by Miller (1972). 0.1 to 0.5 ml of cells were treated with toluene, incubated with ONPG (800 μg/ml) for appropriate periods and the absorbance measured at A_{420} and A_{550} with a Cecil CE-272 spectrophotometer and the β-galactosidase activity calculated and expressed per unit of A_{450} cells.

P1 transduction and conjugation analysis

P1 transduction and conjugation analysis were carried out as described in Miller (1972).

Measurement of DNA synthesis

DNA was uniformly labelled by cultivation at 30°C in M63 for at least 20 generations in the presence of [^{3}H]thymidine (CEA, France; specific activity 48 Ci/mmol) at a concentration of 2.5 μCi per ml and uridine (340 μg/ml). Accumulation of labelled DNA was determined after precipitation with 10% (w/v) trichloroacetic acid (TCA).

Preparation and labelling of DNA probe for Southern blotting

The pLG695 plasmid was digested with *Eco*RI and *Bam*HI and the 3.5 kb insert was purified from a 0.6% low melting point agarose gel (BRL). The insert was then labelled *in vitro* with [^{32}P]dCTP (Amersham, specific activity 3000 Ci/mmol), using the 'Random Primer Labelling' kit from Amersham. The radiolabelled probe was then used to probe by Southern blotting (Sambrook *et al.*, 1989) total *E.coli* DNA digested with *Bam*HI, *Hin*dIII, *Eco*RI, *Eco*RV, *Bgl*I, *Kpn*I, *Pst*I or *Pvu*II.

DNA manipulations

In other DNA manipulations, restriction enzymes, ligase, phosphorylase and DNA polymerase were used as described by the manufacturer. Other procedures were as described by Sambrook *et al.* (1989).

Acknowledgements

We are very grateful to Françoise Vannier for her excellent assistance. We are also grateful to Professor Brian Spratt and Dr Barbara Bachmann for strains. I.B.Holland is pleased to acknowledge the receipt of MRC project grants G8705318CB, G8815458CB, the Wellcome Trust, 15316/5 and to M.X.Chen by the Government of the Republic of China for support of the work in Leicester. Simone J.Séror is pleased to acknowledge the support of the Institut National de la Santé et de la Recherche Médicale (Grant no. 861024), the Association pour la Recherche sur le Cancer, and the Fondation pour la Recherche Médicale Française. Finally, we appreciate the support of the Cell Cycle Trust.

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Received on June 4, 1991; revised on July 1, 1991