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Penicillin binding protein 2 is dispensable in *Escherichia coli* when ppGpp synthesis is induced

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Mecillinam, a β-lactam antibiotic which specifically inactivates penicillin binding protein 2 (PBP2) in *Escherichia coli*, prevents lateral cell wall elongation, inducing spherical morphology and cell death. Two mecillinam resistant mutants, *lov-1* and *lovB*, both able to dispense entirely with PBP2, are shown here to be affected in the aminoacyl-tRNA synthetase genes *argS* and *alaS*, respectively. Although the *argS* and *alaS* mutants grow slowly, we show that there is no correlation between mecillinam resistance and either growth rate or translation speed. A role of the ribosomes in mecillinam sensitivity, suggested by our earlier report that the *lov-1* mutation is suppressed by certain *rpsL*(Str^R) alleles affecting ribosomal protein S12, is supported by the present observation that a pseudo-streptomycin dependent mutant is mecillinam resistant in the presence of streptomycin. The *argS* and *alaS* mutants have high pools of the nucleotide ppGpp (effector of the stringent response) and the mecillinam resistance of both mutations is suppressed by a *relA* mutation, inactivating the ribosome-associated ppGpp synthetase and preventing ppGpp synthesis in response to aminoacyl-tRNA starvation. Furthermore, a p^{tac}*relA'* multicopy plasmid makes a wild type strain mecillinam resistant. The effect of ppGpp is probably mediated by RNA polymerase, since sublethal doses of the polymerase inhibitor rifampicin suppress mecillinam resistance in *argS*, *alaS* and p^{tac}*relA'*-bearing strains. We conclude that ppGpp regulates the transcription of a gene whose product is involved in mecillinam sensitivity, possibly as part of a chain of interacting elements which coordinate ribosomal activity with that of the PBPs.

Key words: aminoacyl-tRNA synthetases/mecillinam/PBP2/S12/stringent response

Introduction

Escherichia coli cell shape is maintained by the rigid layer of peptidoglycan, a highly cross-linked macromolecule lying between the inner, or cytoplasmic, membrane and the outer membrane. Penicillin binding proteins (PSPs) are integral proteins of the cytoplasmic membrane which catalyse the assembly of disaccharide units into the peptidoglycan (Nanninga, 1991; Waxman and Strominger, 1983). *E.coli* morphogenesis depends on the activities of PBP2 for cell wall elongation into a rod shape and of PBP3 for septation, or cross wall formation (Spratt, 1975). Cellular localization and regulation of the activity of these PBPs determine the balance between elongation and septation and may be driven by the cellular clock to bring about the proper timing of cell division (Lleo *et al.*, 1990).

The coordination of surface growth with mass increase in rod-shaped bacteria poses a problem. The rigid peptidoglycan layer must maintain rod shape and turgor pressure, whereas its growth requires hydrolysis of pre-existing cross-links to permit the insertion of new glycan chains. Koch's surface stress theory (Koch, 1988) proposes that the turgor pressure itself, which results from mass increase, governs the rate of hydrolysis of the appropriate bonds, an increase in turgor permitting more rapid hydrolysis. This essentially passive model requires no other coupling between mass increase (protein synthesis) and envelope synthesis. However, several observations suggest that such coupling mechanisms may in fact exist, directly linking envelope synthesis and cell shape to protein synthesis and ribosomes. For example, particular alterations in ribosomal proteins S4 or S5 have been reported to result in the *E.coli* membrane becoming extremely fragile, associated with loss of specific membrane proteins and the insertion in the membrane of the soluble translation elongation factor Tu (EF-Tu). The ribosomal alterations confer resistance to spectinomycin, but only in media of high osmolarity: at low osmolarity, spectinomycin does not inhibit translation but causes the mutants either to filament or to lose their shape completely, forming monsters (Miyoshi and Yamagata, 1976; Mizuno *et al.*, 1976). These observations strongly suggest that the ribosomes exert a direct influence on envelope synthesis and cell shape, although the molecular details of the coupling remain to be elucidated.

A second hint of a direct connection between ribosomes and PBPs came from studies of the mecillinam resistant *lov-1* mutant (Bouloc *et al.*, 1989). Mecillinam (Lund and Tybring, 1972) is a β-lactam antibiotic which specifically inhibits PBP2 activity (Spratt, 1977; Spratt and Pardee, 1975), preventing cell wall elongation and resulting in the formation of spherical cells which ultimately stop dividing and die (James *et al.*, 1975; Matsuhashi *et al.*, 1974; Park and Burman, 1973). To analyse the role of PBP2 in the cell cycle and possible interactions of PBP2 with other components, mecillinam resistant mutants have been analysed. The first mecillinam resistant mutants described grew as spheres in the presence or absence of mecillinam. Some of these were affected in the *pbpA* gene, coding for PBP2, the mecillinam target, and had no detectable PBP2. Others were affected in the adjacent *rodA* gene (Iwaya *et al.*, 1978; Spratt *et al.*, 1980; Tamaki *et al.*, 1980); PBP2, a transpeptidase, acquires transglycosylase activity in the presence of RodA (Ishino *et al.*, 1986). These mutants, however, probably carry additional mutations, since, in some cases at least, introduction of the *pbpA*$^+$ *rodA*$^+$ region does not restore mecillinam sensitivity (Spratt, 1977; A.Jaffé,

personal communication). Morphologically similar mutants have been isolated and shown to be affected in the *mre* operon, also involved in cell shape determination and possibly regulating the expression of certain PBPs (Wachi *et al.*, 1987; Westling-Häggström and Normark, 1975). A second class of mecillinam resistant mutants, although spherical in the presence of mecillinam, have a normal rod shape when grown in its absence. This phenotype can result from inactivation of the cAMP–CAP complex (Aono *et al.*, 1979; D'Ari *et al.*, 1988; Jaffé *et al.*, 1983), a transcriptional regulator of a large number of operons (Ullmann and Danchin, 1983). The cAMP–CAP complex may affect the transcription of genes involved in mecillinam sensitivity. The *lov-1* mutant has a similar phenotype (Bouloc *et al.*, 1988, 1989). It grows slowly, even in rich media. We previously showed that certain alterations in the ribosomal protein S12 suppress the *lov-1* mutation, restoring both rapid growth and mecillinam sensitivity. The allele specificity of the suppression suggested a direct interaction of the *lov* gene product with the ribosomes. These observations led us to speculate that the Lov protein might be involved in a chain of interacting elements connecting the ribosomes and the PBPs in order to coordinate mass and envelope synthesis (Bouloc *et al.*, 1989). The recent observation (Bylund *et al.*, 1991) that *lov-1* mutant cells have significantly lower buoyant density than wild type cells may reflect the coordinating role of the Lov protein.

PBP2 is an essential protein: its inactivation by mecillinam or by genetic deletions is lethal in wild type strains, although tolerable in mecillinam resistant mutants such as *lov-1* (Ogura *et al.*, 1989). Its vital role, however, does not seem to be cell wall elongation, as indicated by the isolation of viable *pbpA* mutants which grow as spheres, with no lateral elongation, but which are still sensitive to mecillinam (Ogura *et al.*, 1989). We have suggested that PBP2, in addition to its (non-essential) ability to catalyse elongation, has a second (essential) function, possibly related to the coordination of septation with mass increase (Ogura *et al.*, 1989). According to this point of view, mutants which, like *lov-1*, are able to grow and divide in the complete absence of PBP2 may define elements which determine cell shape and coordinate mass and envelope synthesis in *E.coli*.

In the present report we show that the *lov-1* mutation and a new mutation conferring mecillinam resistance, *lovB*, lie in genes coding for aminoacyl-tRNA synthetases. These mutants have an elevated level of ppGpp, which is essential for their mecillinam resistance. Furthermore, wild type cells in which the ppGpp level is artificially high due to over-production of a hyperactive form of the RelA protein are also mecillinam resistant. We conclude that ppGpp regulates a key determinant of mecillinam sensitivity.

Results

Mutant isolation

An unmutagenized overnight culture of the wild type *E.coli* strain GC2700 was plated at 37°C on rich medium containing 50 μg/ml mecillinam. After 48 h, resistant colonies, which appeared at a frequency of 10^{-4}, were purified on the same medium. Cells grown in the absence of mecillinam were examined under the light microscope Rod-shaped mutants were tested for a functional cAMP–CAP transcriptional regulator, as judged by its ability to ferment maltose. Two mutants were chosen for study, the previously described *lov-1* mutant (GC2702) (Bouloc *et al.*, 1988, 1989) and a second mutant, *lovB* (GC2701).

The lov gene is argS

The recessive *lov-1* mutation, located at 40.8 min on the *E.coli* map (Bachmann, 1990), confers mecillinam resistance and slow growth (Bouloc *et al.*, 1988, 1989). Lambda phage numbers 337 and 338 from the collection of Kohara *et al.* (1987) conferred more rapid growth and mecillinam sensitivity on the *lov-1* mutant. These two phage carry chromosomal sequences having a 7.2 kb overlap. A 7.3 kb *Hin*dIII–*Kpn*I fragment, located at kb 1970 (40.4 min) on the *E.coli* chromosome (Kohara *et al.*, 1987), was subcloned from λ 337 into the low copy plasmid pLG338 and into the multicopy cloning vector pHSG399. Transformation of *lov-1* derivatives with both constructs conferred fast growth and mecillinam sensitivity, confirming the presence of the entire *lov* gene. The fragment was further subcloned in plasmid pHSG399, using as host strains GC2702 (*lov-1*) and GC3381 (*lov-1 recA*). Plasmids pGC201 and pGC202 (Figure 1) conferred a Lov^+ phenotype on both strains, indicating that the 2.7 kb *Sma*I–*Hin*dIII DNA fragment contains the entire *lov* gene. Transformation with plasmids pGC204, pGC205, pGC206 and pGC209 gave rise to both rapidly and slowly growing transformants in GC2702 (*lov-1*) but only to slowly growing transformants in the *recA* derivative GC3381, suggesting that the 0.6 kb *Ban*I–*Sal*I fragment permits RecA-dependent marker rescue but does not contain the entire *lov* gene. Other fragments were negative in both hosts (Figure 1 and unpublished results).

To determine the nucleotide sequence of the *lov* gene, we started to sequence the *Sma*I–*Hin*dIII fragment of pGC202. Comparison of 960 sequenced nucleotides with the EMBL data bank sequences revealed total homology with the recently sequenced *argS* gene (Eriani *et al.*, 1989), which codes for arginyl-tRNA synthetase and has been localized in the 40 min region (Cooper *et al.*, 1969).

Analysis of the *argS* sequence revealed an open reading frame (ORF) present on the strand complementary to that encoding *argS*, entirely within the *argS* coding sequence and potentially coding for a 53 kDa protein. If this protein is expressed, then appropriate mutations could alter the products of both genes, *argS* and the complementary ORF. To detemine which alteration accounted for the Lov^- phenotype, we constructed a plasmid, pGC209, carrying the *Ban*I–*Hin*dIII fragment, lacking the *argS* promoter but carrying the entire complementary ORF (see Figure 1). This plasmid did not correct the *lov-1* mutation in strain GC3381 (*lov-1 recA*).

This result shows that the Lov^+ phenotype requires the presence of an $argS^+$ gene and that the Lov^- phenotype of slow growth and mecillinam resistance is due to a mutation in the *argS* gene. Furthermore, the marker rescue results suggest that the *lov-1* mutation lies in the *Ban*I–*Hin*dIII interval, entirely within the *argS* coding region, implying that the mutation results in an altered arginyl-tRNA synthethase.

The lovB gene is alaS

A second mecillinam resistant mutant, GC2701, exhibited the same phenotype as GC2702 (*lov-1*), namely mecillinam

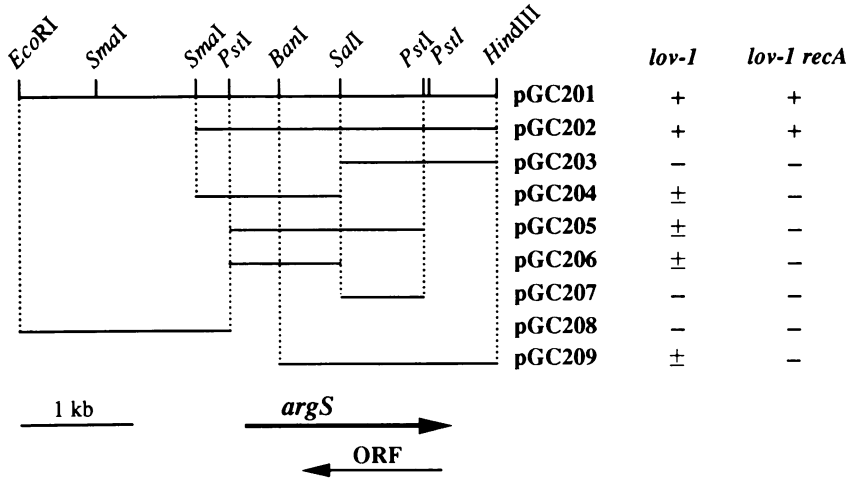

Fig. 1. Location of the *argS* (*lov*) gene. The extent of the inserts of several plasmids is shown, together with the results of functional tests in strains GC2702 (*lov-1*) and GC3381 (*lov-1 recA*) carrying each plasmid. '+' indicates large, mecillinam sensitive colonies (Lov^+ phenotype) and '–' indicates small, mecillinam resistant colonies (Lov^- phenotype). '±' indicates heterogeneous colony size, consisting of a mixture of large Mec^S and small Mec^R clones, presumably due to marker rescue (see text).

Table I. Plating efficiency of *rpsL* derivatives on mecillinam

Strain	Without streptomycin		With streptomycin	
	Generation time (min)	Plating efficiency	Generation time (min)	Plating efficiency
SØ3829 (Sm^S)	20	6.9×10^{-4}	–	$< 10^{-8}$
SØ3830 (Sm^R)	22	1.5×10^{-4}	22	5.3×10^{-4}
SØ3831 (Sm^P)	[a]	5.3×10^{-5}	30	0.79

[a]The pseudo-dependent strain SØ3831 exhibited a gradually decreasing growth rate after removal of streptomycin; steady state was not reached in 7 h at 37°C.

resistance, slow growth and rod-shaped cells. Like the other mecillinam resistant strains tested, GC2701 tolerated the complete inactivation of *pbpA* (strain GC3540). P1-mediated transduction showed that the mutation was not at the *lov-1* locus, and the new mutation was named *lovB*. By conjugation and transduction we mapped the *lovB* mutation very close to the *recA* gene (see Materials and methods). A *lovB*/F'*lovB*$^+$ merodiploid strain (GC3591) recovered a normal growth rate and mecillinam sensitivity, showing that the *lovB* mutation, like *lov-1*, is recessive.

The *alaS* gene, coding for alanyl-tRNA synthetase, is adjacent to *recA* (Bachmann, 1990; Putney *et al.*, 1981). Since the *lov-1* mutation lies in *argS*, an aminoacyl-tRNA synthetase gene, we tested the possibiliy that *lovB* lies in *alaS*. Transformation of GC2701 (*lovB*) with plasmid pTAC875N, carrying the *alaS* gene, restored a wild type growth rate. Furthermore, an independent *alaS* mutant (GC764) was found to be resistant to mecillinam.

These studies show that the *lovB* allele lies in the *alaS* gene. The *lov-1* and *lovB* mutations will subsequently be referred to as *argS201* and *alaS21*, respectively.

The fact that the two mecillinam resistant mutants analysed were affected in aminoacyl-tRNA synthetases led us to examine other aminoacyl-tRNA synthetase mutants which had not been selected for mecillinam resistance. Strains carrying a *glnS*(Ts), *pheS*(Ts), *leuS*(Ts) or *thrS* mutation were all resistant to mecillinam on LB plates. In the case of the *leuS*(Ts) mutant, which is unable to form colonies at 42°C, mecillinam resistance was observed at 37°C but not at 30°C. The phenotype was further confirmed by the ability of this strain to accept a $\Delta(pbpA-rodA)$::Km^R deletion–insertion at 37°C but not at 30°C; the *leuS*(Ts) $\Delta(pbpA-rodA)$::Km^R double mutant (GC3744), constructed at 37°C, was unable to form colonies at 30°C (D.Vinella, R.D'Ari and P.Bouloc, in preparation). Thus mecillinam resistance appears when aminoacyl-tRNA synthetase activity becomes limiting for cell growth.

These observations led us to pose the general question: how can modifications of aminoacyl-tRNA synthetases cause mecillinam resistance? In the following sections we examine possible explanations.

Mecillinam resistance is not due to slow growth

Mecillinam resistance is often associated with a low growth rate, and a wild type strain was reported to be phenotypically resistant to mecillinam when growing on minimal glucose plates (Barbour *et al.*, 1981). The *argS201* and *alaS21* mutations cause slow growth. We therefore looked for a possible relationship between growth rate and mecillinam resistance. Wild type strains FB8, C600 and PB103 grow with generation times ranging from 21 to 160 min in different laboratory media. The plating efficiency in the presence of 10 μg/ml mecillinam was about 10^{-4} on all of these media, whereas the *argS201* mutant GC2702, whose growth rate varied from 60 to 190 min on these same media, had a plating efficiency of 100%. The mecillinam resistant clones of the wild type strains obtained on poor media were resistant to mecillinam even on rich media, indicating that the resistance was genetic rather than phenotypic.

Similarly, the *alaS21 relA* double mutant GC3708 had the

same low growth rate as the *alaS21 relA*$^+$ single mutant GC3702, doubling in 54 min in LB broth, but it was sensitive to mecillinam (see below). Strain GC3738, on the other hand, carrying plasmid pGC401 (*relA'*), grew with a doubling time of 36 min in LB broth but was mecillinam resistant (see below).

These results demonstrate that slow growth *per se* is neither necessary nor sufficient to confer mecillinam resistance on wild type *E.coli*.

Mecillinam resistance is not due to slow translation

Mutations which lower translation efficiency by affecting the ribosomes or aminoacyl-tRNA synthetases (including an *alaS* mutation) have been shown to be partial suppressors of a *secA*(Ts) thermosensitive secretion mutation (Lee and Beckwith, 1986). Suppression of *secA*(Ts) was also observed in the presence of sublethal concentrations of the translation inhibitor chloramphenicol, indicating that suppression results from decreased translation efficiency.

The *argS201* and *alaS21* alleles, which cause mecillinam resistance, presumably lower translation efficiency. By analogy with the *secA*(Ts) system, it was possible that mecillinam resistance might be correlated with slow growth resulting from lowered translation efficiency, given that it is not caused by slow growth due simply to metabolically poor media. We therefore looked to see whether the effects of the *argS201* and *alaS21* mutations could be mimicked by sublethal concentrations of chloramphenicol. The addition of chloramphenicol (1−3 μg/ml) or tetracycline (0.5−2 μg/ml) to solid LB medium slowed growth of the wild type strain GC2553 but did not confer resistance to mecillinam. Twenty clones arising on mecillinam−chloramphenicol plates were tested and found to have 100% plating efficiency on mecillinam plates without chloramphenicol, showing that their resistance was genetic, not phenotypic.

Although this observation already suggested that slow translation in itself does not result in mecillinam resistance, a second test was carried out. We examined the effects of three alleles of the *rpsL* gene, coding for the ribosomal protein S12 and characterized for their translation speed: (i) a streptomycin sensitive (wild type) *rpsL*$^+$ allele (strain SØ3829), which permits an incorporation rate of 15 amino acids per second per ribosome, (ii) a typical streptomycin resistant *rpsL* allele (strain SØ3830), which permits an incorporation rate of 9 or 11 amino acids per second per ribosome, according to whether streptomycin is present or not, respectively and (iii) a pseudo-streptomycin dependent allele, *rpsL*(Sm^P) (strain SØ3831), which permits an incorporation rate of 10 amino acids per second per ribosome in the presence of streptomycin but only five in its absence (Jensen, 1988). The plating efficiency of these strains was measured in LB plates with or without streptomycin (200 μg/ml) and mecillinam (10 μg/ml). In the absence of streptomycin, all three strains were sensitive to mecillinam, including the slow-translating pseudo-dependent mutant (Table I).

These results show unambiguously that slow translation is not sufficient to produce mecillinam resistance; indeed, there seems to be no simple relationship between translation speed and mecillinam resistance.

In the course of the above experiments we found, unexpectedly, that in the presence of streptomycin, the pseudo-dependent mutant SØ3831 became resistant to mecillinam. Thus, mecillinam sensitivity must somehow involve the translation apparatus: the change in ribosome conformation produced by the addition of streptomycin results in mecillinam resistance in the *rspL*(Sm^P) mutant. This is reminiscent of our previous observation that the changes in ribosome conformation produced by certain *rpsL* alleles abolish mecillinam resistance in the *argS201* (*lov-1*) mutant (Bouloc *et al.*, 1989).

Resistance to mecillinam results from increased ppGpp levels

The *argS* and *alaS* genes code for aminoacyl-tRNA synthetases, which activate arginine and alanine and charge the cognate tRNAs with the amino acid. The mutants that we selected probably lower the efficiency of the corresponding enzymes, leading to partial aminoacyl-tRNA starvation and slow growth. A decrease in the charge ratio of a tRNA species activates the stringent response. This occurs by the interaction of an uncharged tRNA with the mRNA codon present in the ribosome recognition site. Under these conditions, the *relA* gene product, or stringent factor, catalyses the synthesis of ppGpp, which is an RNA polymerase effector causing shut-off of the transcription of rRNA and tRNA operons (Cashel and Rudd, 1987). We would thus expect our *argS201* and *alaS21* mutants to be partially induced for the stringent response.

To examine the role of the ppGpp pool in mecillinam resistance, we constructed the double mutant strains *argS201 relA* (GC3706) and *alaS21 relA* (GC3708) and tested their plating efficiency on LB mecillinam plates. Both double mutants were sensitive to mecillinam (Table II). In the case of the *argS201 relA* strain, the growth rate was also increased (Table II), and colony size on LB plates was heterogeneous. Reintroduction of the *relA*$^+$ allele into both double mutant strains restored mecillinam resistance and slow growth. The mecillinam resistance of the *argS201* and *alaS21* mutants is therefore likely to be due to elevated ppGpp levels. Direct HPLC measurements of the ppGpp pools in the *argS201* and *alaS21* mutants confirmed that both had higher levels than the wild type (Figure 2 and data not shown). The *alaS21 relA* double mutant, on the other hand, had a low level, similar to that of the wild type or *relA* strains.

We next attempted to mimic the effect of aminoacyl-tRNA synthetase mutations in a wild type strain by artificially increasing the ppGpp level. To do this, we took advantage of plasmid pSM11(*relA'*), constructed by Schreiber *et al.* (1991), in which the *relA* gene, deleted at its 3' end, is cloned downstream of a *tac* promoter. The product of this truncated

Table II. Influence of the stringent response on mecillinam resistance

Strain	Plating efficiency[a]	Generation time (min)
GC2553	4.8×10^{-5}	21
GC3698 (*relA*)	4.4×10^{-6}	25
GC3700 (*argS201*)	1	60
GC3702 (*alaS21*)	1	54
GC3706 (*argS201 relA*)	1×10^{-5}	34−45[b]
GC3708 (*alaS21 relA*)	6.7×10^{-6}	54
GC3738 (F'*lacI*^{q}/pGC401)	0.65	36

[a]Ratio of titre on LB plates containing 10 μg/ml mecillinam to that on LB plates.
[b]The growth rate of *argS201 relA* strains varied from day to day.

gene is more active than the wild type RelA protein, causing cells to overproduce ppGpp even without amino acid starvation. We introduced a Km^R cassette within the *bla* gene of pSM11 to inactivate the β-lactamase, which hydrolyses mecillinam (see Materials and methods), producing plasmid pGC401(*relA'*). We then constructed strain GC3741, of genotype GC2553/F'*lacI*^{q}/pGC401. Even in the absence of the *lac* operon inducer IPTG, plasmid pGC401 in a *relA*//F'*lacI*^{q} strain confers a Rel^+ phenotype (data not shown), and in strain GC3741 it confers mecillinam resistance (Table II). (In the presence of 10^{-3} M IPTG, strain GC3741 is unable to form colonies.) The mecillinam resistance was further confirmed by the ability of a pSM11-bearing strain to accept a Δ(*pbpA*−*rodA*)::Km^R deletion−insertion (strain GC3745).

The above results, taken together, show a striking correlation between high ppGpp levels and mecillinam resistance.

High ppGpp causes mecillinam resistance via RNA polymerase

The nucleotide ppGpp is known primarily as an RNA polymerase effector. The observation that a high ppGpp level causes mecillinam resistance naturally led us to ask whether this effect reflects altered transcription of some target operon. Rifampicin specifically inhibits the β subunit of RNA polymerase, and sublethal doses of this antibiotic have been reported to affect certain operons differentially (Blumenthal and Dennis, 1978; Satta and Pardee, 1978). The three mecillinam resistant strains, *argS201* (GC3700), *alaS21* (GC3702) and GC2553/F'*lacI*^{q}/pSM11*relA'* (GC3738), were unable to grow on minimal glucose−mecillinam plates containing 5 μg/ml of rifampicin (Table III).

This observation suggests that the mecillinam resistance observed in the presence of high ppGpp pools is mediated by RNA polymerase.

Discussion

Bacterial penicillin binding proteins are known to catalyse the synthesis of the rigid peptidoglycan layer, or cell wall, which determines cell shape. In *E.coli*, PBP2, the target of the β-lactam antibiotic mecillinam, is required for lateral cell wall elongation and is thus indispensable for the maintenance of rod shape (Spratt, 1975, 1977). We have shown that PBP2 is an essential protein in wild type cells (Ogura *et al.*, 1989). However, its essential function does not seem to be the elongation reaction: *pbpA* mutants have been described which grow as spheres (i.e. no lateral elongation occurs), yet remain sensitive to mecillinam, suggesting that PBP2 is involved in some other vital process (Ogura *et al.*, 1989). Complete inactivation of PBP2, either by mecillinam (James *et al.*, 1975) or by mutation (Ogura *et al.*, 1989), results in perturbed cell division. This observation led us to suggest that PBP2 might play an essential role in the regulation of the septation process. Nevertheless, mecillinam resistant mutants such as *argS201* (*lov-1*) are able to tolerate a deletion of the PBP2 structural gene, *pbpA* (Ogura *et al.*, 1989); the resulting *argS201* Δ*pbpA* cells grow and divide as cocci. The present work was motivated in part

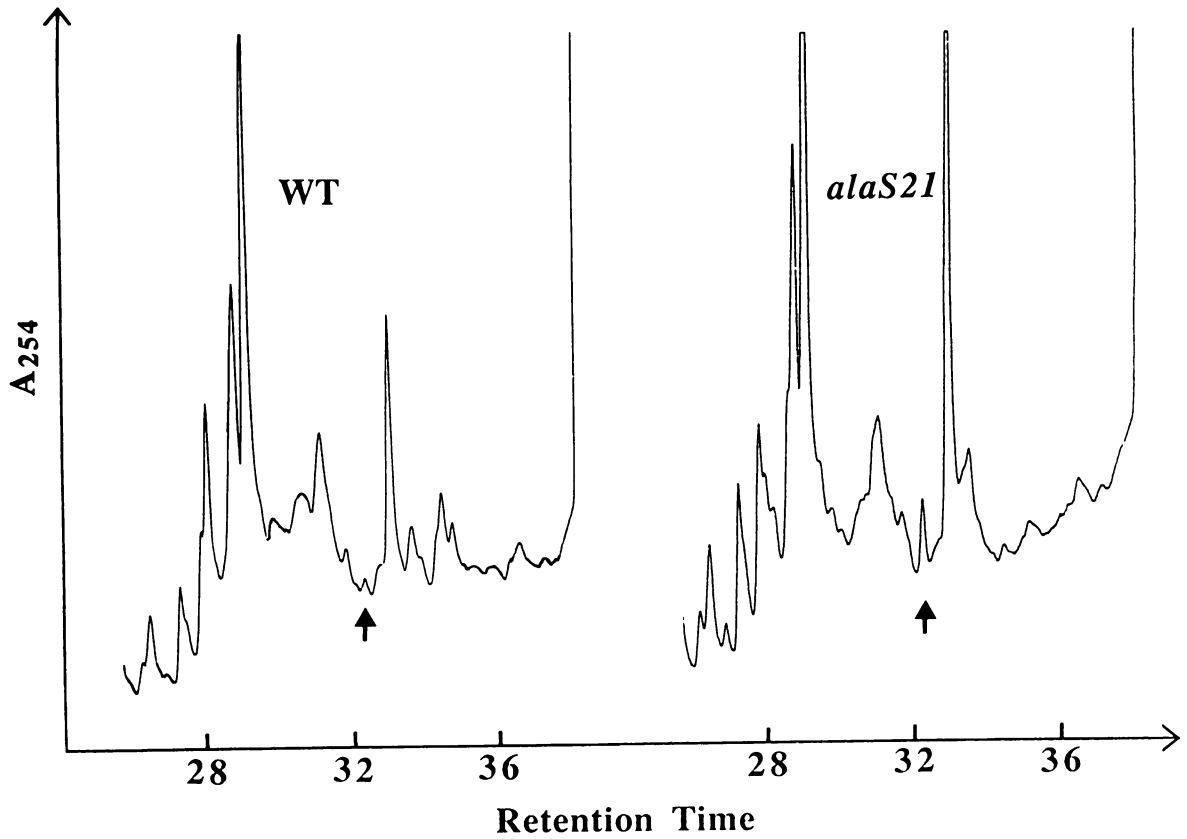

Fig. 2. High ppGpp pool in the *alaS21* mutant. Early exponential cultures of strains GC2553 (WT) and GC3702 (*alaS21*) were extracted and analysed by HPLC as described in Materials and methods. The ordinate represents the absorbance at 254 nm and the abscissa represents the retention time. The ppGpp peak is indicated by an arrow.

Table III. Suppression of mecillinam resistance by rifampicin

Strain	Plating efficiency[a] on		
	Rif[b]	Mec[c]	Rif[b] Mec[c]
GC2553	0.34	4.7×10^{-5}	4.7×10^{-6}
GC3700 (*argS201*)	1.07	0.66	7.6×10^{-6}
GC3702 (*alaS21*)	0.58	1.16	7.1×10^{-6}
GC3738 (F'*lacI*^{q}/pGC401)	0.13	0.50	$<6.7 \times 10^{-6}$

[a]Ratio of colony titre on minimal glucose medium containing antibiotics to that on minimal glucose medium.
[b]5 μg/ml.
[c]10 μg/ml.

by our desire to understand how mutations to mecillinam resistance circumvent the need for PBP2.

High ppGpp pools confer mecillinam resistance

Spratt (1977) reported that wild type *E.coli* is resistant to mecillinam in media supporting slow growth, suggesting that under these conditions the cell's requirement for peptidoglycan is qualitatively or quantitatively different. In the present work we have shown that mecillinam resistance is associated with high intracellular levels of ppGpp. At first sight, this observation is in general agreement with Spratt's, since the ppGpp concentration is generally considered to be inversely related to the growth rate and indeed has been postulated to determine the growth rate (Sarubbi *et al.*, 1988). However, we found that slow growth *per se* is neither necessary nor sufficient to confer mecillinam resistance. This apparent contradiction—correlation of mecillinam resistance with high ppGpp pools but not with low growth rate—may have several explanations. It has recently been shown that operons whose expression is proportional to the growth rate maintain their regulation in the complete absence of ppGpp (Gaal and Gourse, 1990), suggesting that the inverse correlation between ppGpp concentration and growth rate may be fortuitous and not determinant. Furthermore, Schreiber *et al.* (1991) report different growth rates for a given intracellular ppGpp concentration, according to whether the strain contained a *relA* or *relA'* plasmid.

From DNA sequence and functional analysis we discovered that the gene we originally called *lov* is in fact the structural gene for arginyl-tRNA synthetase, *argS*, previously located near the 41 min region and recently sequenced. Analysis of a second mecillinam resistant mutant, provisionally called *lovB*, revealed that it was mutated in the *alaS* gene, coding for alanyl-tRNA synthetase. Other aminoacyl-tRNA synthetase mutants, not selected for mecillinam resistance, also proved to be resistant when plated at temperatures at which the synthetase, although still functional, presumably became limiting for growth. Under such conditions, the charge ratio of the corresponding tRNA species was presumably low. This suggested that the stringent response, turned on by aminoacyl-tRNA starvation, might be involved in mecillinam resistance. The *argS201* (*lov-1*) and *alaS21* (*lovB*) mutants do indeed have a high ppGpp level. The role of ppGpp was confirmed by introducing a *relA* mutation into these strains, inactivating the ribosome-associated ppGpp synthetase. Both the *argS201 relA* and the *alaS21 relA* double mutants once again became sensitive to mecillinam. Thus the mecillinam resistance of the original mutants was a result of their abnormally high ppGpp pool. This situation seems to be general: in wild type strains, the presence of a *p*^{lac}*relA'* plasmid, coding for a hyperactive form of the RelA protein, also confers mecillinam resistance.

The nucleotide ppGpp is an effector of RNA polymerase, leading to turn-off of most operons coding for elements of the protein synthesis system: rRNA, tRNA, ribosomal proteins, translation factors and several aminoacyl-tRNA synthetase operons. It also stimulates the transcription of certain anabolic operons, including those involved in the biosynthesis of several amino acids and of peptidoglycan precursors (Cashel and Rudd, 1987). The mecillinam resistance associated with high ppGpp pools may be mediated by transcription, since sublethal levels of the RNA polymerase inhibitor rifampicin restored mecillinam sensitivity to three resistant strains, *argS201*, *alaS21* and GC2553/F'*lacI*^{q}/pSM11*relA'*. The nucleotide ppGpp must therefore regulate the expression of one or more operons whose products are involved in mecillinam sensitivity.

It has long been known that non-growing bacteria are more resistant to the killing effects of antibiotics than growing cells (Hobby *et al.*, 1942; Tuomanen, 1986; Tuomanen and Tomasz, 1991). Furthermore, increased resistance to β-lactam antibiotics appears within minutes after amino acid deprivation in wild type strains but not in *relA* mutants. This 'phenotypic tolerance' has been attributed to decreased autolysin activity and changes in peptidoglycan composition (Goodell and Tomasz, 1980; Tuomanen and Tomasz, 1991), possibly related to the stringent control of peptidoglycan synthesis (Ishiguro and Ramey, 1976; Vanderwel and Ishiguro, 1984; Kusser and Ishiguro, 1987). Our observation of *relA*$^+$-dependent mecillinam resistance in certain mutants, however, is probably unrelated to phenotypic tolerance. First, our strains are specifically resistant to mecillinam but remain sensitive to other β-lactams (unpublished observations), and second, although the resistance is *relA*$^+$-dependent, it is independent of growth rate.

We have shown here that the growth rate of the *argS201* mutant is increased by introduction of a *relA* mutation, and we have previously shown that certain *rpsL* alleles, affecting ribosomal protein S12, cause a similar growth rate increase (Bouloc *et al.*, 1989). Since none of these mutations would be expected to modify the quality of the mutant arginyl-tRNA synthetase, we conclude that they must affect its quantity. In fact, the *argS* gene is known to be under stringent control, negatively regulated by ppGpp. A simple explanation would be that the suppressing *rpsL* alleles, like the *relA* mutation, lower the ppGpp level in the *argS201* strain. The very slow growth of the *argS201* single mutant still requires some explanation. In fact, since arginyl-tRNA synthetase is under stringent control, the partial starvation of arginyl-tRNA caused by the *argS201* mutation, by stimulating ppGpp synthesis, actually reduces the amount of synthetase, aggravating the problem. The result is a vicious circle: the more ppGpp there is, the more the strain is starved for arginyl-tRNA, resulting in a growth regime in which the stringent response is turned on strongly and *argS* expression is extremely low, limiting growth. In contrast, the growth rate of the *alaS21* mutant was not increased by introduction of a *relA* allele, and in fact the *alaS* gene has not been reported to be under stringent control.

Ribosomes and mecillinam resistance

Models proposed to explain the *E.coli* cell cycle do not generally suggest any direct connection between protein synthesis and cell wall assembly, except insofar as the former process creates the turgor pressure presumed to favour the murein hydrolysis reactions required for insertion of new glycan chains (Koch, 1988). We have evidence for more direct coupling mechanisms connecting peptiglycan synthesis to ribosomal activity. We observed that certain *rpsL*(Str^R) alleles suppress the mecillinam resistance due to the *argS201* mutation; in these *argS201 rpsL* double mutants, PBP2 was once again essential for cell survival (Bouloc *et al.*, 1989). This result was interpreted as indicating that PBP2, and perhaps other PBPs as well, are in some way 'aware' of ribosomal activity. Amongst five well characterized *rpsL* alleles tested, suppression of *argS201* was observed only with the two which are the most accurate with respect to translation. These alleles provide increased accuracy by rejecting many correct aminoacyl-tRNAs during translation. Although these *rpsL* alleles have not been reported to confer a relaxed phenotype, one might speculate that they tend to reject uncharged cognate tRNAs before RelA-dependent ppGpp synthesis is triggered. In any case, it is clear that the essentiality of PBP2 depends on the state of the ribosomes.

In the present work we present a second piece of evidence for a connection between the ribosomes and the PBPs. The pseudo-streptomycin dependent allele *rpsL*(Sm^P) confers mecillinam resistance, but only in the presence of streptomycin. The streptomycin binds to the ribosomes and increases their translational speed from five to 10 amino acids per second, and at the same time, the strain becomes resistant to mecillinam. Here again, the normally essential function of PBP2 becomes dispensable when the ribosomes assume a certain conformation.

Yet another indication that the cell envelope is directly affected by the ribosomes comes from an interesting set of spectinomycin resistant mutants which present morphological defects—filamentation or complete loss of shape—in low osmotic media containing spectinomycin. The mutants were shown to be affected in ribosomal proteins S5 or S4 (or possibly S3 in one case), and they seemed to lack certain membrane proteins. We have recently found that several of these alleles also confer mecillinam resistance (D.Joseleau-Petit and D.Vinella, unpublished observations). The simplest explanation of how an altered ribosomal protein can have such profound effects on the cell membrane is to postulate the existence of a coupling mechanism through which the activity of the PBPs, including cell division and shape maintenance, is directly affected by the conformation of the ribosomes.

One possible link between ribosome activity and the cell envelope may be via the translational elongation factor Tu (EF-Tu). This soluble protein has been found in cell membrane fractions (Jacobson and Rosenbusch, 1976) and also appeared in the outer membrane of the above-mentioned mutants in nonpermissive conditions (Dombou *et al.*, 1981). Its presence in the membrane could serve as a signal to the peptidoglycan synthesizing machinery after particular stresses or, conceivably, at a certain stage of the unperturbed growth cycle. Methylation of membrane-associated EF-Tu has been observed during nutrient depletion and it was suggested that EF-Tu might be a nutritional sensor involved in growth regulation (Young and Bernlohr, 1991).

Cell division control and ppGpp

We have previously argued that PBP2 plays an essential regulatory role in the septation process (Ogura *et al.*, 1989). This implies the existence of elements which transmit information on the state of PBP2 to the septation machinery, such that PBP2 inactivation results in a division arrest. These information transmitters could be proteins or small molecules produced by specific enzymes. Mecillinam resistance may arise via mutations that interrupt the information transmission. The fact that high ppGpp levels confer mecillinam resistance suggests that the gene coding for one transmitter is controlled by this nucleotide. Similarly, since mutants lacking a functional cAMP–CAP complex are also mecillinam resistant, one transmitter may be regulated by CAP. It is possible that a single target gene is controlled by both regulators. In this case, if the transmitter acts in a division promoting capacity, one would expect positive reuglation by ppGpp and negative by CAP, whereas if it acts to inhibit division one would expect negative regulation by ppGpp and positive by CAP. We are currently trying to identify such information transmitters, regulated by ppGpp and CAP and involved in linking the functional state of PBP2 to cell division.

Although the RelA protein is responsible for the massive synthesis of ppGpp observed during the stringent response, it has long been known that *E.coli* possesses a second ppGpp synthetase. This has recently been identified as the *spoT* gene product, and Δ*relA* Δ*spoT* strains have been shown to be viable despite the lack of a detectable level of ppGpp (Hernandez and Bremer, 1991; Xiao *et al.*, 1991). These strains, which can only be grown in relatively rich media, were reported to exhibit a partial inhibition of cell division, suggesting a requirement for ppGpp in order for septation to take place properly (Xiao *et al.*, 1991). We have shown here that an excess of ppGpp, on the contrary, permits the cell to live and divide in the presence of mecillinam, when PBP2 is inhibited and growth occurs by septation alone, with no lateral wall elongation. These observations suggest that ppGpp may be a positive effector of septation.

Materials and methods

Bacterial strains and phages

The strains used in this work are all *E.coli* K12 derivatives; they are described in Table IV. The parental strain for this work is FB8 (also known as UTH1038). This strain was stored in duplicate in our laboratory collection as GC2553 and GC2700. Recently, strain GC2700 exhibited an altered phenotype due to a mutation acquired in the stab: it was resistant to mecillinam on minimal glucose medium, although still sensitive on LB plates, resembling the 'wild type' strain described by Spratt (Barbour *et al.*, 1981; Spratt, 1977). Most of the experiments presented here were done with strains constructed from GC2553, which maintained the mecillinam sensitivity originally described for FB8 (Bouloc *et al.*, 1988, 1989). Lambda phages 337 (20H4) and 338 (12C7), carrying the *lov* gene, were obtained from Y.Kohara (Kohara *et al.*, 1987). We are grateful to Peter Poulsen, Mathias Springer and Yuji Kohara for kindly giving strains.

P1 *vir* mediated transduction, Hfr crosses and F′ crosses were carried out as described by Miller (1972).

Media and growth conditions

Rich medium was LB broth and minimal medium was M63 (Miller, 1972) supplemented with thiamine (1 μg/ml), carbon sources (0.4 or 0.2%) and

Table IV. *Escherichia coli* strains used

C600	*thr leu lac thi tonA*	Laboratory collection
GC764	*alaS*(Ts) *thr leu met thy lac rpsL*	(Ruffler *et al.*, 1974)
GC2553	FB8	Laboratory collection
GC2700	FB8	(Bouloc *et al.*, 1989)
GC2701	*alaS21* (*lovB*)	This work
GC2702	*argS201* (*lov-1*)	(Bouloc *et al.*, 1989)
GC3365	*recA srl*::Tn*10 cysG*::Tn*5*	Laboratory collection
GC3381	As GC2553, *argS201* (*lov-1*) *recA*	This work
GC3540	As GC2553; *alaS21* (*lovB*) Δ*pbpA*::Tn*5*	This work
GC3559	As GC2553, *alaS21* (*lovB*)/pCI857 (Km^R)	This work
GC3591	As GC2553, *alaS21* (*lovB*) *srl*::Tn*10*/F'143	This work
GC3592	As GC2553, *alaS21*/pTAC875N	This work
GC3698	As GC2553, *relA*	This work
GC3700	As GC2553, *argS201* (*lov-1*)	This work
GC3702	As GC2553, *alaS21* (*lovB*)	This work
GC3706	As GC2553, *argS201* (*lov-1*) *relA*	This work
GC3708	As GC2553, *alaS21* (*lovB*) *relA*	This work
GC3736	GC2553/F'*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10*	This work
GC3738	As GC3736, pSM11	This work
GC3741	As GC3736, pGC401	This work
GC3745	As GC3738, Δ(*pbpA*−*rodA*)::Km^R	This work
KL231	*leuS3*(Ts) *thyA6 deoC1? rpsL120*	(Low *et al.*, 1971)
GC3744	As KL231, Δ(*rodA*−*pbpA*)::Km^R	This work
IBPC420	*glnS1 thi-1 argE3* Δ*lacX74 mtl-1 xyl-5 tsx-29 supE44? rpsL argG6 his-4 zbf507*::Tn*10*	Mathias Springer
IBPC1601	*pheS5 thi-1 argE3 his-4 proA2 lacY1 galK2 mtl-1 xyl-5 tsx-29 supE44*	Mathias Springer
IBPC4771	*thrS1029 thi-1 argE3 his-4 proA2 lacY-1 galK2 mtl-1 xyl-5 tsx-29 supE44 rpsL*	Mathias Springer
PB103	*dadR trpE61trpA62 tna-5*	(De Boer *et al.*, 1989)
SØ3829	*thi araD139* Δ*lacU169 relA*	(Jensen, 1988)
SØ3830	*rpsL999 thi araD139* Δ*lacU169 relA*	(Jensen, 1988)
SØ3831	*rpsL thi araD139* Δ*lacU169 relA*	(Jensen, 1988)
XL1-Blue	*endA1 hsdR17* (r_K^-, m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* Δ*lac*/ F'*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10*	(Bullock *et al.*, 1987)

required amino acids (100 μg/ml). Solid media contained 1.5% agar. Antibiotics, when required, were added at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml (unless otherwise indicated); kanamycin, 40 μg/ml; mecillinam, 10 μg/ml; streptomycin, 200 μg/ml; and tetracycline, 10 μg/ml.

All experiments were carried out at 37°C unless otherwise stated.

DNA techniques

Plasmid DNA was extracted and transformation was carried out as described by Sambrook *et al.* (1989). Restriction enzymes were used according to the supplier's instructions (Appligène, France). The *argS* gene was subcloned into the low copy number vector pLG338 (Stroker *et al.*, 1982), kindly provided by Brian Spratt, and into the high copy number vector pHSG399 (Takeshita *et al.*, 1987), obtained from the Japanese Cancer Research Resources Bank. Plasmid pTAC875N, kindly provided by Ya-Ming Hou and Paul Schimmel, was constructed by introducing a *tac* promoter in front of the *alaS* gene in plasmid pMJ301 (Jasin *et al.*, 1984). Plasmid pSM11, overproducing ppGpp synthethase, was kindly provided by Gideon Shreiber and Gad Glaser (Schreiber *et al.*, 1991). We inactivated the *bla* gene (which confers mecillinam resistance) by inserting a kanamycin cassette (*Sma*I−*Hinc*II) in the unique *Pvu*I site, producing pGC401. The plasmid carrying the kanamycin cassette was kindly provided by Philippe Noirot (INRA, Jouy en Josas, France). It is a derivative of plasmid pUC18 in which the 1256 bp *Xho*I−*Hin*dII fragment containing the kanamycin resistance cassette from pJH1 (Trieu-Cuot and Courvalin, 1983) was inserted at the *Xba*I (423) site.

DNA from phage λ was prepared according to a protocol provided by Annette Campbell (University of Edinburgh). Phage stocks (~4 ml) were treated with RNase and DNase (12 μg/ml each, 30 min at 37°C). The phage were then precipitated with polyethylene glycol 6000 (20% w/v) and the pellet resuspended in 0.5 ml SM phage buffer (0.2 M NaCl, 0.02 M Tris, 1 mM $CaCl_2$, 20 mM $MgSO_4$, 0.1% gelatin). DNA was extracted by treating first with 0.5 ml Tris-equilibrated phenol and 0.1 ml TE (10 mM Tris pH 8.0, 1 mM EDTA), then with 0.5 ml phenol−chloroform (1:1). The DNA was cleaned with chloroform, then precipitated with ethanol, washed with 70% ethanol and resuspended in TE. One-tenth volume 3 M sodium acetate was added and the DNA was again precipitated with ethanol, resuspended in 50 μl TE and stored at −20°C. The final concentration was ~200 μg/ml.

The nucleotide sequence of part of the *lov* gene was determined on the 2.7 kb *Eco*RI−*Hin*dIII fragment of pGC202 (Figure 1). The fragment was extracted from an agarose gel by 'Gene Clean' (Bio 101, Inc., USA) and digested with *Sau*IIIA, *Alu*I or *Rsa*I. Fragments were subcloned at random into pBlueScript (Short *et al.*, 1988) and transformed into XL1-Blue (Bullock *et al.*, 1987). Single-stranded DNA was generated with NK107 phage (Vieira and Messing, 1987) and the nucleotide sequence was determined by the dideoxynucleotide method (Sanger *et al.*, 1977), using a multiwell microtitre plate DNA sequencing system (Amersham SA, France).

Mapping of lovB

To map the *lovB* gene, we first crossed strain GC3559 (the *lovB* strain GC2701 carrying a Km^R plasmid) with a collection of Hfr strains, each of which has a proximal Tc^R marker (Wanner, 1986). Exconjugants were selected on LB plates containing kanamycin and tetracycline. The presence of big colonies amongst the exconjugants indicated that the *lovB*$^+$ gene had been introduced by the Hfr. Such *lovB*$^+$ recombinants were obtained with Hfrs PK19 (PO 41 min, CW) and KL16 (PO 61 min, CCW), placing the *lovB* gene between 41 and 61 min of the *E.coli* map. The interval was narrowed down by P1 *vir* mediated transduction with markers in this region, using the original *lovB* strain GC2701 as recipient. With an *srl*::*Tn5* donor strain, large and small Km^R transductants were obtained; the large colony formers were mecillinam sensitive. In a three point cross, using an *srl*::Tn*10* *recA* donor strain (GC3365), the *lovB* mutation was found to be tightly linked to *recA*, possibly lying just beyond it.

Measurement of ppGpp pools

The nucleotide ppGpp was extracted as described by Little and Bremer (1982). Bacterial strains were grown in medium 63 supplemented with glucose and casamino acids to an OD_{600} of 0.2. Twenty millilitres of culture were mixed with 2 ml of 1.9% formamide (v/v) and immediately chilled on ice; the extraction was carried out at 4°C. Cells were pelleted (15 000 *g*, 30 min) and resuspended in 1 ml of 0.1 M KOH. After 30 min, the KOH was neutralized by addition of 5 μl 85% orthophosphoric acid (v/v). Cell debris was removed by centrifugation (17 000 *g*, 40 min) and the supernatant was filtered through 0.2 μm nitrocellulose fibre filters and stored at −70°C for up to two weeks. HPLC analysis was as described by Payne and Ames (1982), using a Waters 510 HPLC and a Beckman Ultrasphere-C18 column (4.6 mm × 250 mm), with no precolumn; 100 μl of supernatant were used per run. We found that resolution was best using 30 mM KH_2PO_4, pH 6.6, and 5 mM tetrabutyl-ammonium as buffer A; buffer B was 100% acetonitrile. The elution gradient was a 60 min linear gradient (no. 6), from 4 to 80% (v/v) of B, at a flow rate of 1 ml/min. The OD_{254} of the eluant was monitored on a Waters LC spectrophotometer and recorded on a Waters 740 data module.

We were unable to find a commercial supplier of ppGpp and are indebted to Paul Boquet (CEA, Saclay, France), who kindly gave us his remaining stock of precious authentic ppGpp, bought over ten years ago and essential for calibration of the column.

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