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Studies on Selected Aspects of the Stringent Response in *Escherichia coli*

By

Xiaoming Yang
B.Sc., Hangzhou University, 1991
M.Sc., Chinese Academy of Sciences, 1996

A Dissertation Submitted in Partial Fulfillment of the
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We accept this dissertation as conforming
to the required standard

Dr. E. E. Ishiguro, Supervisor (Department of Biochemistry and Microbiology)

Dr. F. E. Nano, Departmental Member (Department of Biochemistry and Microbiology)

Dr. C. Upton, Departmental Member (Department of Biochemistry and Microbiology)

Dr. W. E. Hintz, Outside Member (Department of Biology)

Dr. H. E. Schellhorn, External Examiner (McMaster University)

Supervisor: Edward E. Ishiguro

ABSTRACT

Amino acid deprivation of *Escherichia coli* results in the accumulation of guanosine 5'-triphosphate 3'-diphosphate and guanosine 3', 5'-bispyrophosphate, collectively designated (p)ppGpp. These nucleotides are synthesized by a ribosome-associated enzyme encoded by the *relA* gene and are thought to represent starvation stress signal molecules. They may mediate the global reorganization of cellular metabolism, known as the stringent response, that is characteristic of starving bacteria and which apparently represents a survival strategy. In this dissertation, the following aspects of the stringent response are characterized: (i) the temperature phenotypes of *relA* mutants; (ii) the C-terminal domain of RelA; and (iii) the role of RelC (ribosomal protein L11) in the regulation of RelA.

All three of the commonly used *relA* mutant alleles of *E. coli*, *relA1*, *relA2*, and *ΔrelA251::kan*, conferred temperature-sensitive (ts) phenotypes. The temperature sensitivity was associated with decreased thermotolerance, and *relA* mutants were killed at temperatures as low as 42°C. The ts phenotypes were suppressed by increasing the osmolarity of growth media and by certain mutant alleles of *rpoB*, the gene encoding the β-subunit of RNA polymerase, suggesting a defect in transcription. DNA in heat-shocked wild type bacteria was initially relaxed but the normal level of negative supercoiling was restored within 10 min after heat shock. In contrast, DNA in heat-shocked *relA* mutants remained relaxed. This *relA*-associated defect in DNA negative supercoiling was suppressed by increased medium osmolarity. Furthermore, the *relA*-mediated ts phenotype was suppressed by low concentrations of novobiocin, a specific inhibitor of the B subunit of DNA gyrase. Moreover, low concentrations of novobiocin restored DNA negative supercoiling in the *relA* mutant at high temperature. Based on previous reports, it is proposed that low concentrations of novobiocin induce the synthesis of the DNA gyrase A and B subunits, and the

resulting increase in DNA gyrase activity restores normal supercoiling at high temperature. Collectively, the data suggest that *relA* mutants are unable to efficiently transcribe key genes required for thermotolerance, and this defect is related to their inability to restore negative supercoiling of DNA at higher temperatures. In addition, the proposed defect in transcription may be related to the observation that ppGpp binds to the β-subunit of RNA polymerase.

The portion of *relA* encoding the C-terminal half of RelA (starting at amino acid 455), designated 'RelA, was subcloned. Overexpression of 'RelA relaxed the stringent response by inhibiting (p)ppGpp synthesis during amino acid deprivation. 'RelA represented the ribosome-binding domain, and when overexpressed, 'RelA somehow replaced RelA on ribosomes. The 'RelA ribosome-binding domain was further localized to a region between amino acids 455 to 682 with the main binding activity in a fragment extending from amino acids 560 to 682. Several criteria were used to establish the fact that 'RelA also mediated the formation of homodimers. These included co-purification of RelA and 'RelA, glutaraldehyde protein crosslinking, and analysis by nondenaturing polyacrylamide gel electrophoresis. The dimerization domain overlapped with the ribosome-binding domain. Affinity blotting assays using 'RelA as a probe revealed RelA and 'RelA as the only proteins in crude cell extracts that bound 'RelA. Therefore, these studies failed to identify the ribosomal components that interact with RelA.

Amino acid-deprived *rplK* (previously known as *relC*) mutants of *E. coli* cannot activate ribosome-bound RelA and consequently exhibit relaxed phenotypes. The *rplK* gene encodes ribosomal protein L11, suggesting that L11 is involved in regulating the activity of RelA. The overexpression of derivatives of *rplK* that contained short N-terminal deletions that eliminated the proline-rich helix resulted in relaxed phenotypes. In contrast, bacteria overexpressing normal L11 exhibited a typical stringent response. The L11 mutant proteins were incorporated into ribosomes. A derivative in which Pro22 was changed to Leu22 was constructed by site-directed mutagenesis. This amino acid substitution was sufficient to confer a relaxed phenotype when it was overexpressed. A variety of

methods were used in attempts to demonstrate a direct interaction between L11 and RelA, but all yielded negative results. These results indicate that the N-terminal proline-rich helix, and Pro22 in particular, is directly involved in activating RelA activity during amino acid deprivation. The mechanism apparently does not involve a direct interaction between RelA and L11 and is presumably mediated by another ribosomal component.

Examiners:

Dr. E. E. Ishiguro, Supervisor (Department of Biochemistry and Microbiology)

Dr. F. E. Nano, Departmental Member (Department of Biochemistry and Microbiology)

Dr. C. Upton, Departmental Member (Department of Biochemistry and Microbiology)

Dr. W. E. Hintz, Outside Member (Department of Biology)

Dr. H. E. Schellhorn, External Examiner (McMaster University)

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AD	yeast GAL4 transcriptional activity domain
Ap^r	ampicillin-resistant
Cm^r	chloramphenicol-resistant
DB	yeast GAL4 DNA binding domain
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GST	glutathione S-transferase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopyropyl-β-D-thiogalatoside
Kan^r	kanamycin-resistant
Kb	kilobase pairs
KDa	kilodalton
LB	Luria broth
MW	molecular weight
NB	nutrient broth
ONPG	o-nitrophenyl-β-D-galctopyranoside
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
ppGpp	guanosine 5'-diphosphate 3'-diphosphate
pppGpp	guanosine 5'-triphosphate 3'-diphosphate
PSI	(p)ppGpp synthetase I

PSII	(p)ppGpp synthetase II
SD	synthetic dropout
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TAE	Tris-acetate/EDTA electrophoresis buffer
TBE	Tris-borate/EDTA electrophoresis buffer
TCA	trichloroacetic acid
TLC	thin-layer chromatography
Tris	Tris-(hydroxymethyl) aminomethane
ts	temperature sensitive
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
3-AT	3-amino-1,2,4-triazole

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Chapter 1: Introduction

The stringent response

Bacteria are always exposed to a wide variety of stressful conditions in their natural habitats, such as heat, cold, oxidative stress, acid stress, and nutrient deprivation. Laboratory studies have indicated that bacteria have developed specific responses to these adverse conditions to maximize their chances of survival. In bacteria such as *Escherichia coli*, deprivation of amino acids results in the arrest of stable RNA (rRNA and tRNA) synthesis (Cashel *et al.*, 1996). This physiological response is known as the stringent response. The stringent response actually represents changes in a diverse group of metabolic activities in addition to stable RNA synthesis. It would appear that the stringent response is designed to minimize energy consumption and to promote survival during periods of starvation. Mutations in certain genes, *e.g.*, *relA* and *relC*, give rise to a defective stringent response known as the relaxed phenotype. Such relaxed mutants, for example, continue to accumulate stable RNA during amino acid starvation.

Amino acid starvation causes a rapid accumulation of two unusual nucleotides, identified as guanosine 5'-triphosphate, 3'-diphosphate (pppGpp) and guanosine 3' 5'-bispyrophosphate (ppGpp), in wild type stains but not in *relA* mutants (Cashel and Gallant, 1969). The synthesis of these nucleotides, collectively designated (p)ppGpp, is catalyzed by an enzyme known as ppGpp synthetase I (PSI) which is encoded by the *relA* gene. The level of ppGpp

increases within a few seconds after amino acid starvation and peaks after 10-15 min (Lund and Kjeldgaard, 1972). The level of ppGpp then drops to a new steady state value that is 10- to 20-fold above the basal level found in normal growing bacteria. During this period, the cellular level of GTP decreases to 50% of the original level in proportion to the increase in ppGpp (Fiil *et al.*, 1977). The various metabolic phenomena comprising the stringent response may be mediated by ppGpp.

Fig. 1.1 summarizes the metabolism of (p)ppGpp in *E. coli*. RelA catalyzes an ATP:GTP pyrophosphoryl transferase reaction. The half-life of the product of this reaction, pppGpp, is about 6 seconds and is rapidly converted to ppGpp by pppGpp 5'-phosphohydrolase (Gpp). This likely explains why ppGpp rather than pppGpp is the major signal molecule detected during the stringent response. The product of the *spoT* gene is a Mn^{++}-dependent (p)ppGpp 5'-pyrophosphohydrolase. The GDP resulting from this reaction can be converted to GTP by nucleoside 5'-diphosphate kinase (Ndk). Mutations in *spoT* cause the accumulation of ppGpp but not pppGpp, indicating that pppGpp is hydrolyzed by another enzyme that has not yet been identified (Somerville and Ahmed, 1979; Heinemeyer and Richter, 1978). In summary, the intracellular concentration of ppGpp is dependent on the relative synthetic and hydrolytic activities of RelA and SpoT, respectively.

GDP and GTP serve equally well as substrates for RelA *in vitro* (the Km values for GTP and GDP are about 0.5 mM). However, pppGpp, and not ppGpp, is the initial product of the stringent response *in vivo*. This is probably based on

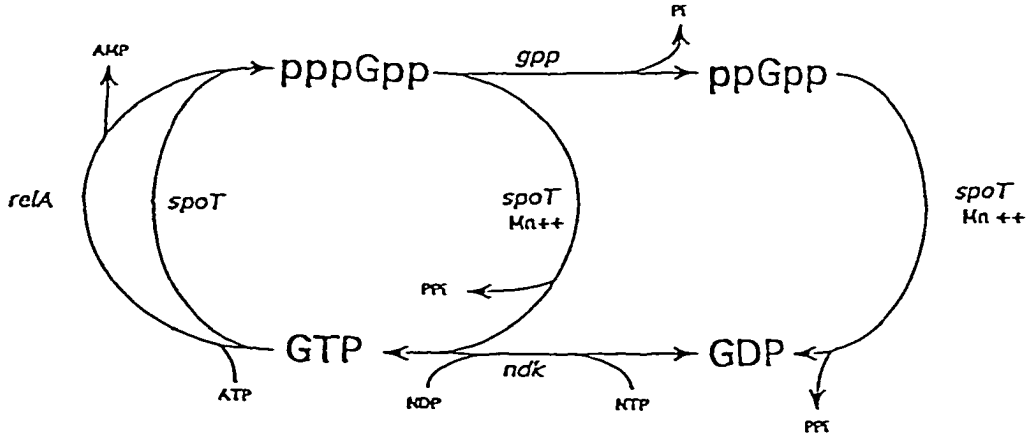

Fig. 1.1. Cellular routes of (p)ppGpp metabolism in *E.coli*

The enzymes shown as their gene names are: (p)ppGpp synthetase I or PSI (*relA*), (p)ppGpp synthetase II (*spoT*), (p)ppGpp 3'-pyrophosphohydrolase (*spoT* Mn^{++}), (p)ppGpp 5'-pyrophosphohydrolase (*gpp*) and nucleoside 5'-diphosphate kinase (*ndk*). (modified from Cashel 1996)

the fact that the physiological level of GDP is significantly lower than that of GTP. (Pedersen and Kjeldgaard, 1977).

RelA is exclusively associated with ribosomes *in vivo*. In *in vitro* experiments, RelA is activated by the codon-specified binding of uncharged tRNA to the A site on translating ribosomes (Haseltine and Block, 1973). Therefore, the specific signal for the stringent response appears to be the limitation of aminoacylated tRNA.

The ribosome-independent pathway for ppGpp synthesis

In *E.coli*, the accumulation of (p)ppGpp occurs not only during amino acid starvation but also during energy source limitation and other stressful conditions. In these cases, (p)ppGpp synthesis is independent of *relA* because these nucleotides accumulate in both wild type and *relA* mutants (Stamminger and Lazzarini, 1974). Therefore, it was hypothesized that there were two functionally discrete mechanisms for (p)ppGpp synthesis. This was confirmed with the identification of (p)ppGpp synthetase II (SPII) as the product of the *spoT* gene (Hernandez and Bremer, 1991). Thus, SpoT is a bifunctional enzyme that exhibits (p)ppGppase as well as (p)ppGpp synthetase activities as summarized in Fig. 1.1 .

Major components of the stringent response

a) *relA*

The *E. coli relA* gene encodes a protein composed of 744 amino acid

residues with a molecular mass of 83.5 kDa (Metzger *et al.*, 1988). Interestingly, *relA* ends with an amber codon, and the suppression of this amber codon results in a slight elongation of RelA by 27 amino acids. This modification of the carboxyl terminus inactivates (p)ppGpp synthetase activity. However, other features of RelA such as ribosomal association are not impaired (Breeden *et al*, 1980). It is unknown whether this modification serves a regulatory function.

RelA is normally bound to the 50S ribosomal subunit and can be removed by a 0.5 M NH_4Cl wash (Haseltine *et al.*, 1972). Purified RelA catalyzes *in vitro* (p)ppGpp synthesis in either a ribosome-dependent or a ribosome-independent reaction. The ribosome-dependent reaction requires mRNA and uncharged tRNA. As indicated above, the uncharged tRNA must recognize the mRNA codon that occupies the ribosome A site in order to activate RelA (Haseltine and Block, 1973). The ribosome-independent activity of RelA is observed in a reaction that contains only buffer, salts and substrates. However, this reaction is dependent on specific conditions such as low temperature, the presence of 20% methanol, or the addition of certain acidic proteins such as the 50S ribosomal proteins, L7 and L12 (Block and Haseltine, 1975). These results suggest that the activation of RelA requires a specific conformational change triggered by the ribosome or by other conditions.

RelA is a low abundance protein which occurs at about 110 molecules per cell in cultures growing in glucose minimal medium (1 copy per 200 ribosomes) (Pedersen and Kjeldgaard, 1977). The expression of RelA is obviously regulated under different growth conditions because even limited overexpression of *relA*

causes the production of growth inhibitory levels of ppGpp. However, little is known about how *relA* is regulated. The overexpression of a truncated RelA protein representing the N-terminal 455 amino acids results in elevated ppGpp level that is almost equivalent to induction of whole *relA* gene (Schreiber *et al.*, 1991). This constitutive (p)ppGpp synthetase activity is apparently ribosome-independent, but it has not been directly demonstrated that this truncated RelA is not ribosome-bound. On the other hand, this shows that the C-terminal domain is necessary for normal RelA control. The truncated RelA protein is metabolically unstable and exhibits a half-life of about 7.5 min or less. In comparison, the full-length protein has a half-life of more than 2 hours (Schreiber *et al.*, 1991).

The most widely used *E.coli* relaxed mutant alleles are *relA1*, *relA2*, and *ΔrelA251::kan*. The *relA1* allele consists of an IS2 insertion between the 85th and 86th codons of *relA* (Metzger *et al.*, 1989a). The IS2 element apparently provides a ribosome binding sequence together with an ATG start codon and 8 additional codons to support the expression the carboxyl-terminal portion of RelA (which represents about 75% of the normal protein). Therefore, the expression of the *relA1* gene results in the production of two peptides, designated α and β which represent the N-terminal and C-terminal portions of RelA, respectively. The *relA1* mutant exhibits little or no RelA activity and has a relaxed phenotype. However, ppGpp synthetic activity is restored if *relA1* is presented in multicopy. These results suggest that the α and β peptides of RelA1 may complement each other *in trans* (Metzger *et al.*, 1989a). Strains carrying the *ΔrelA251::kan* null allele do not accumulate (p)ppGpp during amino acid deprivation. However,

these strains accumulate (p)ppGpp through SpoT when subjected to energy source starvation (Metzger *et al.*, 1989a).

b) *relC*

The ribosome-dependent ppGpp synthetic activity of RelA protein suggests that some components of ribosome have regulatory effects on ppGpp synthesis. Mutations in a gene originally designated *relC* have since been shown to be alleles of *rplK*, the gene encoding ribosomal protein L11 (Parker *et al.*, 1976). L11 is an important protein in the ribosomal complex. It binds to 23S rRNA within the ribosomal GTPase centre which regulates GTP hydrolysis by ribosomal elongation factors (Egebjerg *et al.*, 1990; Said *et al.*, 1988). Thiostrepton is an antibiotic that inhibits protein synthesis by binding to the L11-23S rRNA complex. This interaction results in the inhibition of ribosome-dependent (p)ppGpp accumulation *in vitro* (Sy, 1974). The *relC* mutation eliminates thiostrepton binding. Although the binding of RelA to the ribosome is apparently not affected, it is inefficiently activated during amino acid deprivation (Friesen *et al.*, 1974). To date, L11 is the only ribosomal protein known to be involved in the regulation of (p)ppGpp synthesis. L11 protein may play a role in monitoring the status of ribosomes and in signaling the conformational change in RelA that is essential for its (p)ppGpp synthetic activity.

c) *spoT*

SpoT was initially characterized as a Mn^{++}-requiring enzyme that degraded ppGpp to GDP and inorganic pyrophosphate. *E. coli* strains with mutations in *spoT* show a modest increase in ppGpp basal levels and a slight inhibition of growth rate under normal growth conditions (Sarubbi *et al.*, 1988). They also exhibit an elevated level of ppGpp, as compared to wild type strains, during the stringent response and a slower rate of ppGpp disappearance when the stringent response is reversed (Sy, 1980). As noted above, SpoT is also proposed to be (p)ppGpp synthetase II (PSII). Although this PSII activity has not been demonstrated directly, the homologous enzyme from *Streptococcus equisimilis* exhibits both ppGpp synthetic and hydrolytic activity *in vitro* (Mechold *et al.*, 1996). The *E. coli* SpoT protein has a molecular mass of 79.3KDa. SpoT and RelA share amino acid sequence homology (Metzger *et al.*, 1989b). Mutational analysis of the *E.coli spoT* gene has identified distinct but overlapping regions involved in ppGpp synthesis and degradation (Gentry and Cashel, 1996). The first 203 amino acids of the SpoT protein contain the site responsible for ppGpp degradation while residues 85-375 are required for ppGpp synthesis. The existence of overlapping fragments between the two domains suggests that they may share common functional features. The phenotypes of SpoT C-terminal deletion mutants indicate that the C-terminus plays a role in stabilizing or regulating ppGppase or PSII activity. A ppGppase-defective 1-58 deletion mutant strain fails to synthesize ppGpp in response to glucose starvation. This raises the possibility that the SpoT PSII activity does not increase in response to energy

starvation; instead, ppGpp accumulation may result from the inhibition of the SpoT ppGppase activity (Gentry and Cashel, 1996). In fact, during glucose starvation, the rates of both ppGpp synthesis and degradation decrease. However, the rate of degradation decreases more, and this apparently causes the accumulation of ppGpp. During amino acid starvation, the SpoT ppGppase activity may be controlled by the concentration of uncharged tRNA in the cell because uncharged tRNA inhibits purified SpoT hydrolase *in vitro* (Murray and Bremer, 1996). However, the mechanism which determines whether SpoT exhibits ppGpase or ppGpp synthetase activity is not understood. It is interesting that the PSII activity appears to be unstable and cannot be detected in extracts of *E.coli relA* null mutants (Murray and Bremer, 1996). Furthermore, PSII-generated ppGpp disappears to undetectable levels when protein synthesis is inhibited by chloramphenicol. Experiments like this indicate that the average functional lifetime of PSII is about 40 seconds or less (Murray and Bremer, 1996).

d) Toxin-antitoxin system

In *E. coli*, the *relA* gene is part of an operon which has two genes called *mazE* and *mazF* located downstream of the *relA* gene which encode proteins of 9.4 and 12.1 kDa, respectively (Aizenman *et al.*, 1996). MazF is a stable toxic protein with half-life over 4 hours. MazE is a labile protein with a half-life of about 30 min and which is degraded by the ClpPA serine protease. MazE is an essential protein which serves as an antitoxin that neutralizes the toxic activity of MazF. This toxin-antitoxin system seems to be involved in the stringent

response because the expression of *mazEF* is regulated by the cellular levels of ppGpp. In the *relA* operon, there is a promoter designated P1 just upstream of *relA* gene and two promoters, P2 and P3, between the *relA* gene and *mazEF* genes. Although P2 and P3 are both active *in vitro*, only P2 is active in exponentially growing cells. The transcription from P2 is inhibited by high levels of cellular ppGpp which is produced by the product of upstream *relA* gene. Therefore, the maintenance of an adequate level of MazE is possible only at low cellular levels of ppGpp (Aizenman *et al.,* 1996). During amino acid starvation or energy source limitation, ppGpp inhibits the coexpression of *mazE* and *mazF*. The concentration of MazE consequently drops more quickly than the concentration of MazF. MazF eventually exerts its toxicity to cause cell death. This may explain why ppGpp causes a decrease in cell viability and why a deletion of *mazEF* or a mutation in *clpP* has a protective effect against the toxicity of ppGpp. This system may serve as a mechanism for altruistic cell suicide. According to this view, the survival of part of a nutritionally starved population is ensured by the death of other starved cells which serve as a source of nutrients.

Other conditions that cause (p)ppGpp accumulation

The accumulation of (p)ppGpp is not restricted to starvation stress and also occurs during exposure to high temperature, high osmolarity or to some antibiotics. How these different environmental conditions regulate (p)ppGpp metabolism is unclear. However, since RelA is a ribosome-associated enzyme, it

has been proposed that the ribosome itself may serve as an environmental sensor (VanBogelen and Neidhardt, 1990). High temperature may increase the speed of protein synthesis which, in turn, could result in vacancy of the ribosomal A site. Antibiotics like kanamycin and streptomycin also empty the A site by interacting with ribosomes. Both conditions mimic the signal of the stringent response, *i.e.*, a deficiency in aminoacyl-tRNA. In contrast, (p)ppGpp levels are decreased by exposure to low temperature which has a tendency to slow down the movement of ribosmes. A similar effect is seen during treatment with antibiotics like chloramphenicol and fusidic acid that block the ribosomal A site.

Pleiotropic effects of (p)ppGpp

As already mentioned, (p)ppGpp affects many aspects of *E. coli* metabolism. It is beyond the scope of this review to cover every aspect, and only a few examples, emphasizing macromolecular synthesis, are given here.

a) Initiation of DNA replication

The initiation of DNA replication is regulated by (p)ppGpp. DNA replication is initiated at a specific nucleotide sequence, termed *oriC*. The timing as well as the frequency of initiation is determined primarily by DnaA, a protein that binds to specific sequences located in *oriC*. DnaA accumulates to threshold levels at specific times in the cell cycle (Herrick *et al.*, 1996). The expression of *dnaA* is regulated at the level of transcription from two promoters, *dnaA*P1 and *dnaA*P2. During amino acid starvation, the levels of *dnaA*P1 and *dnaA*P2

transcripts decrease, suggesting that (p)ppGpp regulates initiation by controlling the activities of promoters of *dnaA* . However, in cells where DnaA has accumulated to a high level, induction of the stringent response still inhibits the initiation of DNA replication, indicating that other unknown factors are involved in the stringently controlled DNA replication (Chiaramello and Zyskind, 1990).

b) Stable RNA synthesis and transcription from stringent promoters

The inhibition of stable RNA synthesis is the best known feature of the stringent response. In *E.coli*, all seven of the *rRNA* operons (*rrn*) contain two promoters. The upstream and downstream promoters, designated P1 and P2, respectively, are spaced about 120bp apart. The transcriptional activity from P1 activity is inversely proportional to ppGpp concentration whereas the P2 promoter is only weakly affected (Sarubbi *et al.*, 1988). The persistence of the P2 promoter activity may serve to ensure that at least some rRNA are always synthesized, even during starvation.

How (p)ppGpp exerts its regulatory effect on stable RNA synthesis has been hotly debated for some time. The so-called stringently regulated promoters, such as the *rrn* P1 promoters, are sensitive to (p)ppGpp. It has been proposed that the binding of ppGpp to RNA polymerase changes its specificity such that it is unable to interact with stringent promoters but is still capable of initiating transcription from mRNA promoters (Ryals *et al.*, 1982). Therefore, the intracellular concentration of ppGpp determines the relative fraction of transcriptional activities devoted to either stable or messenger RNA synthesis. In

support of this promoter selection model, the direct binding of ppGpp to RpoB, the β-subunit of RNA polymerase, has been demonstrated (Reddy *et al.*, 1995). Furthermore, certain *rpoB* mutants exhibit insensitivity to ppGpp.

There are several models for the stringent regulation of transcription of stable RNA and stringent promoters in general. The only model which is relevant to this dissertation is discussed here. The mechanism of stringent control has been studied *in vivo* with a plasmid-encoded copy of the *E.coli rrnB* operon (Gourse *et al.*, 1983). The DNA sequence required for stringent regulation is around the P1 promoter, and deletion of P2 does not weaken the stringent control of transcripts from P1. A comparison of promoters from stringently controlled genes indicates that they share certain structural features including the presence of a key G-C rich sequence between the –10 box and the transcription initiation site. This element is necessary for the stringent regulation of the $tRNA^{Tyr}$ gene and the *rrnB* operon (Kingston *et al*, 1981; Kingston and Chamberlin,1981). It has been proposed that ppGpp acts at the level of transcription by preventing the DNA melting required for the formation of the open complex. Experiments on the stringently regulated *hisR* gene in *Salmonella typhimurium* support this proposal (Shand *et al.*, 1989). Some mutations that impaired the transcription of *hisR* are located in DNA gyrase subunit genes (*gyrA* and *gyrB*), suggesting that the *hisR* promoter requires negative DNA supercoiling for optimal activity (Toone *et al.*, 1992). Furthermore, the *hisR* regulatory defect of a *gyrB* mutant could be suppressed by changing G-C to A-T within the *hisR* promoter. These results and the results of other studies (Ohlsen and Gralla,1992;Figueroa-Bossi *et al.*, 1998),

suggest that stringent promoters are refractory to melting due to the high G-C content in the promoter and need the torsional energy of negative supercoiled DNA to help open the promoter. Therefore, changes in DNA supercoiling may be involved in the stringent response.

c) Positive control of transcription by ppGpp

Transcription of certain genes has been reported to be positively regulated by ppGpp, but the mechanism is far from clear. It is possible that this may involve the direct interaction of ppGpp with the β-subunit of RNA polymerase as already noted. The positively regulated genes include those encoding proteins involved in proteolysis and amino acid biosynthesis (Cashel *et al.*, 1996). Under starvation conditions, the expression of these genes presumably reflects the need to replenish amino acid supplies through protein turnover and through biosynthesis. In addition, the gene for RpoS (σ^S) is positively regulated by ppGpp (Eichel *et al.,* 1999). RpoS is a sigma factor of RNA polymerase which controls the expression of more than 30 genes (Hengge-Aronis, 1996). The *rpoS* gene is induced during stationary phase or during various stressful conditions such as increased osmolarity in a manner that is apparently dependent on ppGpp. Recently, it was reported that ppGpp is actually essential for the expression of RpoS-controlled genes (Kvint *et al.*, 2000). These genes encode proteins confer a general protective function against certain stresses, *e.g.*, osmotic and oxidative stress.

In summary, Fig. 1.2 shows the proposed roles of ppGpp as a regulator

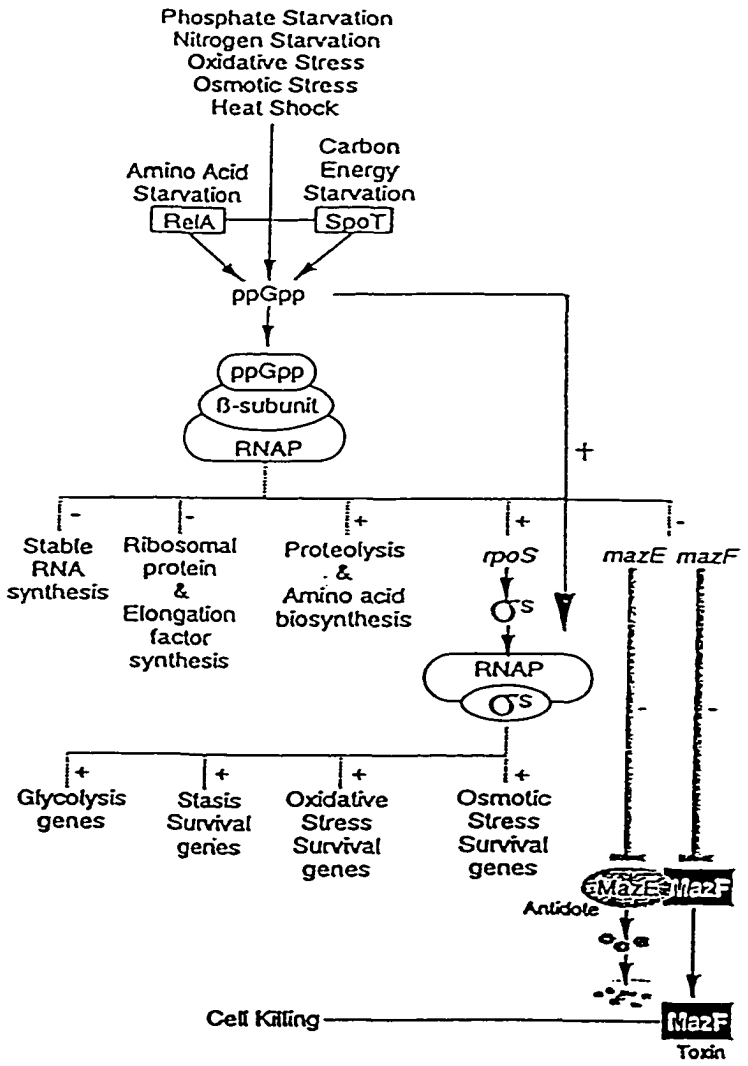

Fig. 1.2. Transcriptional control by ppGpp

Nutrient starvation and other stressful conditions result in the synthesis of ppGpp by RelA or SpoT. Gene expression is both negatively and positively regulated by ppGpp. It is proposed that this involves the binding of ppGpp to the β-subunit of RNA polymerase. The expression of genes under the control of σ^s has also been shown to require ppGpp, possibly through direct interaction with RNA polymerase (modified from Nystrom,1998).

of gene expression during starvation and other stresses.

d) Protein synthesis

The overexpression of RelA in the absence of amino acid starvation inhibits protein synthesis, suggesting that ppGpp directly inhibits translation (Svitil *et al.*, 1993). Although high levels of ppGpp inhibit ribosomal RNA synthesis, the magnitude of the observed inhibition of protein synthesis cannot be explained by the diminished cellular content of ribosomes. It has therefore been proposed that ppGpp competes with GTP at the level of GTP-dependent steps in initiation, elongation, and termination of protein synthesis

The (p)ppGpp^0 phenotype

Strains carrying null alleles of both the *relA* and *spoT* genes are incapable of synthesizing (p)ppGpp (Xiao *et al.*, 1991). Although they are viable, they are clearly compromised and exhibit a pleiotropic phenotype, known as (p)ppGpp^0. This phenotype resembles that of *rpoS* mutants in some respects. In addition, (p)ppGpp^0 strains maintain high level expression of several ribosomal proteins during starvation and appear to exhibit significantly decreased translational fidelity as demonstrated by the unusual heterogeneity in isoelectric points of several proteins and the failure to express higher molecular weight proteins during starvation (Nystom, 1994). The (p)ppGpp^0 strains are also multiauxotrophic and will not grow on minimal medium unless they are provided with at least 9 amino acids (Arg, Gly,His, Leu, Met, Phe, Ser, Thr, and Val) (Xiao

et al., 1991; Cashel *et al.*, 1996) . This multiauxotrophic character is suppressed by certain mutant alleles of *rpoB*, the gene encoding the β-subunit of RNA polymerase. Although this suggests a defect in transcription, no such defect has been directly demonstrated.

In addition, the *relA spoT* double mutant strains are found to express high levels of cold shock proteins and these cells behave as if they are cold adapted. They continue to grow exponentially when shifted to 10°C rather than exhibiting 2-hour lag displayed by wild-type strains (Jones *et al.*, 1992).

Objectives and organization of this dissertation

Chapter 2 describes the materials and methods used in this study. In Chapter 3, it is reported, for the first time, that mutations in *relA* confer a temperature-sensitive phenotype. Temperature sensitivity is shown to be suppressed by certain *rpoB* mutant alleles as well as by high osmolarity, and a correlation to negative supercoiling of DNA is demonstrated. In Chapter 4, the functions of the C-terminus of RelA are examined. It is shown that the RelA C-terminus confers, not only ribosome-binding, but also dimerization. In Chapter 5, the relationship of ribosomal protein L11 protein to RelA is investigated. It is shown that L11 does not form a direct contact with RelA. However, the N-terminal domain of L11 is essential for the activation of RelA during the amino acid starvation.

Chapter 2: Methods and Materials

Bacterial strains and plasmids

The bacterial strains and recombinant plasmids used in this study are listed in Table 2.1 and Table 2.2 respectively. Plasmids were electroporated into bacteria with a Bio-Rad Gene Pulser. Bacterial strains were constructed for this study by P1*vir*-mediated transduction according to Miller (Miller, 1972).

Strain W3110 was a prototrophic derivative of *E.coli* K-12 from our laboratory collection. Strain VC6216 was a derivative of W3110 which was lysogenized with phage λDE3. The λDE3 prophage contains the gene encoding phage T7 RNA polymerase under the control of the *lac* promoter. VC6216 was specifically constructed in this laboratory for the purpose of expressing genes controlled by the T7 promoter. Strains carrying the *ΔrelA251*::*kan* and *ΔspoT207*::*cat* alleles were constructed by directly selecting for kanamycin-resistant and chloramphenicol-resistant transductants, respectively. Strains carrying the *relA1* and *relA2* alleles were constructed by using the closely linked *zei348*::Tn*5* insertion as a selective marker. In these constructions, the *relA* genotype of the kanamycin-resistant transductants were determined by screening for sensitivity to a combination of serine, methionine, and glycine (Uzan and Danchin, 1976), and for sensitivity to 3- amino-1,2,4-triazole (Rudd *et al.*, 1985). The *relA* genotypes of the transductants were confirmed by measuring the incorporation of [5,6-^{3}H]uracil into stable RNA in amino acid-deprived cultures as described previously (Ishiguro and Ramey, 1976). For the

experiments discussed in Chapter 3, two strains carrying each of the three *relA* alleles that were independently constructed were tested for temperature sensitivity in preliminary experiments. The duplicate strains exhibited identical temperature-sensitive phenotypes, and one representative from each set, strains VC6129 (*ΔrelA251*::*kan*), VC6133 (*relA2*), and VC6141 (*relA1*), were used in the experiments described here.

Derivatives of VC6129 carrying the various *rpoB* alleles were constructed using the linked *btuB*::Tn*10* insertion as a selective marker. All of the mutant *rpoB* alleles conferred resistance to rifampicin, and this property was used to identify the *rpoB* transductants.

Derivatives of strain VC6141 carrying *spoT202*, *spoT203*, and *spoT204* were constructed essentially as described by Sarubbi *et al.* (Sarubbi *et al.*, 1988). Briefly, the procedure was as follows. In the first step, the linked markers *pyrE60* and *zib563*::Tn*10* were cotransduced from CF5034 into VC6141 by selection for tetracycline resistance to create strain VC7238. Strain VC7237 was a tetracycline-resistant transductant that did not co-inherit *pyrE60*. In the second step, the various *spoT* alleles from *pyrE*$^+$ donors were transduced into VC7238 and selecting for *pyrE*$^+$ transductants. The *spoT* derivatives were obtained by screening the *pyrE*$^+$ transductants that formed small colonies for tetracycline sensitivity.

In some cases, to express proteins for purification, *E.coli* strain BL21(DE3) and BL21(DE3)*ΔrelA* which was constructed by inserting a kanamycin resistance gene into the chromosomal location of *relA*, were used.

Media and growth conditions

Bacteria were routinely grown in nutrient broth or nutrient agar (Difco) unless indicated otherwise. Other media used during the course of this study were M9 minimal medium (Ishiguro and Ramey, 1976), Davis minimal medium (Difco), modified M53 low phosphate medium (Bell, 1974). Luria broth or agar Miller (Difco), tryptic soy agar (Difco), LB broth or agar Lennox (Difco), and LB broth or agar Miller (Difco). Broth cultures were grown in gyrotory waterbath shakers (New Brunswick Scientific Co.), and culture turbidity was measured with a Beckman DU-64 spectrophotometer at 600 nm. To induce the stringent response, cultures were grown in M9 minimal medium with 0.4% glucose, and serine hydroxamate or L-valine, at 500µg per ml was added to cause amino acid deprivation. When antibiotics were required, they were added at the following concentrations: ampicillin, 100µg/ml; kanamycin 50µg/ml; chloramphenicol, 50µg/ml; tetracycline 20µg/ml.

Determination of temperature sensitivity and antibiotic sensitivity

The effect of incubation temperature on colony formation is expressed as plating efficiency. Cultures which were 1 to 2 hours into stationary phase were serially diluted in sterile saline. Each dilution was plated in quadruplicate on nutrient agar, and two plates for each dilution were incubated at 30°C and at 42°C or other indicated temperatures for 36 hours before counting. The plating efficiency is defined as the ratio of the colony count at the higher temperature to the colony count at 30°C.

To determine the sensitivity to the antibiotics, similar experiments were performed. Platting efficiency was determined by ratio of colony count in media containing the indicated concentration of antibiotic to the colony count in media lacking the drug.

Bacterial survival at 55°C

Bacteria were grown in nutrient broth at 30°C and harvested by centrifugation either during exponential phase (optical density of 0.5) or after 1 hour in stationary phase. The cells were resuspended in sterile saline to a density of approximately 2×10^9 cells per ml. A 1-ml suspension was incubated in a water bath set at 55°C. Samples (50 µl) were removed at the indicated times, diluted, and plated on nutrient agar. The survivors of the heat treatment were determined from the plate counts after 36 hours of incubation at 30°C.

Gene expression studies using *E. coli* DNA arrays

Commercial DNA arrays (Panorama *E. coli* Gene Array, Sigma-Genosys Biotechnologies, Inc., Woodland, Texas) containing the 4,290 *E. coli* open reading frames, in duplicate, were used to compare gene expression patterns of *relA*$^+$ and *relA*$^-$ strains during heat shock by procedures recommended by the manufacturer. The bacterial culture was grown in 20 ml of nutrient broth at 30°C to an optical density of 0.5. At this point, half of the culture was shifted to 42°C. After 30 min, the 30°C and 42°C cultures were harvested, and RNA fractions were extracted from the cells using the Qiagen Rneasy mini kit. The synthesis of

^{33}P-labeled cDNAs and their hybridization to the DNA arrays were according the protocols provided by Sigma-Genosys Biotechnologies. The arrays were exposed to phosphorimager screens which were scanned on a Molecular Dynamics Storm 840 Phosphorimager. The data were analyzed as described by Tao *et al.* (Tao *et al.*, 1999).

Measurement of superhelical density of plasmid DNA

DNA supercoiling was measured by methods described by Goldstein and Drlica (Goldstein and Drlica, 1984) with minor modifications. Plasmid pUC18 was used as a reporter. Stationary phase cultures of bacteria harboring pUC18 were diluted 1:100 into fresh medium as indicated in the text (usually nutrient broth). Cultures were incubated in waterbath shakers at indicated temperatures. At the indicated times, samples were removed from the cultures and quickly chilled by adding crushed ice to the medium. The cells were collected by centrifugation and washed with ice-cold water. Plasmid DNA was extracted from the cells with a QIAprep Spin Miniprep Kit (QIAGEN). Plasmid preparations were analyzed by 1% agarose gel electrophoresis in the presence of 3µg/ml chloroquine (Sigma). Electrophoresis was carried out under a constant voltage (2V/cm) at room temperature for 15 hours in TBE buffer (90 mM Tris-Borate, 2 mM EDTA). Under these conditions, the more negatively supercoiled topoisomers migrated faster. After electrophoresis, the chloroquine was washed from the gel by soaking in distilled water for 4 to 8 hours. Gels were then stained with ethidium bromide and photographed under UV light.

General recombinant DNA techniques

The procedures for plasmid and DNA purification, restriction endonuclease digestion, DNA ligation, and PCR amplification were those described by Sambrook *et al.* (Sambrook *et al.*, 1989). Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs Inc. Constructions of specific plasmids are described in each chapter.

Construction of DNA library

Genomic DNA from *E. coli* strain W3110 was prepared as described (Ausubel *et al.*, 1994). The DNA was then partially digested by *Sau3*AI. DNA fragments from 2 Kbp to 6 Kbp were purified from agarose gel and cloned into vector pGADT7 digested by *Bam*HI and treated with alkaline phosphatase. The library was amplified in *E. coli* before it was transformed into the yeast strain.

PCR amplification of *relA* gene

PCR amplication of the wild-type *relA* gene was performed with the 5' primer, 5'GGAGAGGACCATGGTTGCGG3', and the 3' primer, 5'ATTGAGCGCCTGCATTAACGTAGCC3'. The reaction contained 1 unit of *Taq* DNA polymerase, 1X Reaction Buffer (Pharmacia) supplied with 20 pMol of each primer, 400μM dNTP and 1.5mM $MgCl_2$ in a total of 50μl. The plasmid pALS10 was used as the template. Thermocycling` consisted of an initial denaturation at 95^{O}C for 5 min, followed by 30 cycles with denaturation at 95^{O}C for 1 min, annealing at 55^{O}C for 1 min and extension at 72^{O}C for 2 min.

PCR amplification of *rplK* gene

PCR amplification of *rplK* gene was performed with the 5' primer, 5'GGGGGATCCTAATGGCTAAGAAAGTACAAGCCTA3', and the 3' primer, 5'TGCGTCGACTTTCTCGCGGATAACACGC3'. The reaction contained 1 unit of Vent DNA polymerase and 1X ThermoPol Reaction Buffer (New England BioLabs Inc.) supplied with 100 pMol of each primer, 200μM dNTP and 4mM $MgSO_4$ in a total of 100 μl. Genomic DNA from *E.coli* strain W3110 was used as the template. Thermocycling consisted of an initial denaturation at 95^{O}C for 5 min, followed by 25 cycles with denaturation at 95^{O}C for 1 min, annealing at 52^{O}C for 1 min and extension at 72^{O}C for 1 min.

Site-directed mutagenesis of *rplK* gene

A primer, 5'GGCTAACCCGAGTCTGCCAGTAGG 3', in which the codon for proline 22 was changed to leucine 22 was made. PCR amplification was carried out by using this primer and the 3'end primer for the wild-type *rplK* gene under the same conditions. The PCR product was treated by *Ava*I and *Sal*I. Then the *Ava*I - *Sal*I fragment of the wild-type *rplK* was replaced by this PCR product to generate a derivative of *rplK, mrplK.*

Expression and purification of His-tag recombinant proteins

L11, 'L11, RelA and 'RelA are recombinant proteins derived from pET cloning vectors. They contain both His-tag and S-tag elements. The methods for expression and purification of these proteins were according to procedures

described in the pET System Manual (Novagen). In summary, exponential phase cultures of VC6216 or BL21(DE3) carrying either pXY41, pXY51, pXY38 or pXY64 grown in LB were induced by adding 0.5 mM isopropyl β-D-galactoside (IPTG). After 3 hours of incubation at 30°C, bacteria were harvested by centrifugation and broken by sonication. The recombinant His-tag fusion proteins in the crude extracts were purified by His-tag affinity chromatography (His-Bind Resin, Novagen).

Assay of stable RNA synthesis

An exponential phase culture of VC6216, carrying a recombinant plasmid of interest, was grown in M9 minimal medium until it reached a density of about 2 x 10^8 cells per ml. The culture was divided into two portions, and 0.5 mM IPTG was added to one of these to induce the synthesis of the recombinant protein encoded on the plasmid. After 40 min, [5,6-^{3}H]uracil (Amersham Corp.) was added to each of the two cultures at a final concentration of 1 μg per ml (0.5 μCi per ml). After an additional 20 min of incubation, each culture was further subdivided as indicated and portions were subjected to amino acid deprivation in the presence and absence of IPTG. From this point on, the 100-μl samples were removed from the cultures at the indicated intervals, and the incorporation of [5,6-^{3}H]uracil into RNA was determined by measuring the radioactivity in cold trichloroacetic acid (TCA) insoluble fractions as described previously (Ishiguro and Ramey, 1976). Amino acid deprivation was achieved by the addition of either L-valine or serine hydroxymate to cultures at 500 μg per ml. In addition to

the amino acid-deprived cultures, an untreated control culture and a culture that was treated with IPTG alone were included in all experiments.

Measurement of (p)ppGpp

The synthesis of (p)ppGpp was measured in VC6216 carrying either plasmid pXY38 or plasmid pXY41 grown in the modified M56LP medium of Bell (Bell, 1974). As described in the text, plasmids pXY38 and pXY41 encode 'RelA and 'L11, respectively. $^{32}P_i$ (New England Nuclear, 40 µCi per ml) was added to an exponential phase culture containing 2 x 10^8 cells per ml. One hour later, the culture was divided into 4 portions as described in the text. One portion was a control that did not receive any further treatment for the duration of the experiment. Two of the portions received IPTG at 0.5 mM to induce the expression of proteins from plamids. After 1 additional hour of incubation, the fourth portion and one of the IPTG-treated portions were subjected to amino acid deprivation by adding L-valine at 500 µg per ml. Ten min later, 200 µl samples were removed from each of the 4 cultures. Each sample was extracted with 20µl of 11 M formic acid on ice for 30 minutes. The samples were centrifuged to remove cell debris, and 10µl aliquots of the extracts were applied to a polyethyleneimine cellulose thin-layer chromatography plate (Aldrich Chemical Company Inc.). The chromatogram was developed in 1.5 M KH_2PO_4. The developed chromatogram was visualized by autoradiography or scanned on a Molecular Dynamics Storm phosphoimager.

Ribosome preparation

Ribosomes were prepared by the methods of Gentry and Cashel (Gentry and Cashel, 1995) and Homann and Nierhaus (Homann and Nierhaus, 1971) with minor modifications. Briefly, bacteria were collected by centrifugation and washed once with Ribosome Buffer (10 mM Tris-HCl, pH 7.5; 14 mM $MgCl_2$; 10 mM KAc; 1 mM DTT). The washed cells were resuspended in Ribosome Buffer and disrupted by sonication. Cell debris was removed by centrifugation at 20,000 x *g* for 30 min. The ribosomes were then recovered by centrifugation at 160,000 x *g* for 3 hours. The ribosome pellets were resuspended in Ribosome Buffer and repurified in a two-step process. The crude ribosomes were first subjected to low speed centrifugation (14,000 x *g* for 30 min), and the ribosomes in the supernatant from this step were pelleted by high speed centrifugation (160,000 x *g* for 150 min). The concentration of ribosomes was estimated by measuring the absorbance of the preparation at 260 nm.

***In vitro* ribosome-binding experiment**

Ribosomes from strain W3110 with the overexpression of pALS10 were prepared as described above. Constant amounts of ribosomes were incubated with varying amounts of purified 'RelA protein in ribosome buffer at room temperature for 30 minutes. The ribosomes were repurified, and the amounts of ribosome-bound RelA and 'RelA were then determined by immunoblotting.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), non-denaturing gel electrophoresis, and Western blotting

SDS-polyacrylamide gel electrophoresis was carried out according to the protocol described by Ausubel *et al* (Ausubel *et al* 1994). Proteins were separated by 10% separating gel and 4% stacking gel, using Mini-PROTEAN system (Bio Rad). Samples were run at a constant voltage (200 V) for about 45 min. For non-denaturing gels, SDS and DTT were omitted from all buffers, and the electrophoresis was carried out at a lower voltage for a longer time to avoid causing high temperature. The gel was either stained with Coomassie blue or transferred to a nitrocellulose membrane for Western blot analysis. Western blots were developed with polyclonal rabbit antibodies prepared against 'L11 or 'RelA. Alternatively, a monoclonal antibody specific for the S-tag (alkaline phosphatase-conjugated anti-S-Tag antibody; Novagen) was used.

Yeast two-hybrid techniques

The yeast two-hybrid technique was used to determine protein-protein interaction. The technology was based on the Matchmaker Two-Hybrid System 3 (Clonetech). The 2 cloning vectors, pGBKT7 and pGADT7, contain the GAL4 DNA-binding and activation domains, respectively. Plasmids pGBKT7-53 and pGADT7-T are positive controls encoding murine p53 fused to the *Gal4* DNA-binding domain and SV40 T-antigen fused to the *GAL4* activation domain, respectively; these two proteins are known to interact with each other.

Saccharomyces cerevisiae (*MATa*, *trp1-901*, *leu2-3*, *112*, *ura3-52*, *his3-*

200, *gal4*, *gal80*, *LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$HIS3*, *GAL2$_{UAS}$-GAL1$_{TATA}$-ADE2*, *ura3::MEL$_{UAS}$-MEL1$_{TATA}$-lacZ*) was used as a host for the two-hybrid plasmids. Yeast cells were grown at 30°C in either YPD or SD synthetic media as described in the Clontech User Manual for the Matchmaker System. In this system, a positive yeast two-hybrid was indicated by growth on SD medium lacking histidine, adenine, leucine, and tryptophan as well as by a high level of β-galactosidase activity from a reporter gene as measured by the method of Miller (Miller, 1972).

DNA sequencing

Manual DNA sequencing was carried out with a Sequenase Version 2.0 sequencing kit (Amersham). Double-stranded plasmid DNA was used as a template. The 3'-AD sequencing primer from the yeast two-hybrid system kit (Clontech) was used to sequence the cloned insert in the positive clones of yeast two-hybrid library.

Affinity chromatography using GST fusion protein: demonstration of RelA dimerization

Cultures of strain W3110 carrying either plasmid pGEX-5X-1, encoding glutathione S-transferase (GST), or plasmid pXY37, encoding a GST-'RelA fusion, were grown at 30°C until the absorbance of the culture reached 1.0. IPTG was added to a concentration of 0.1 mM, and incubation was continued for 2 hours. Cells were harvested and broken by sonication. The GST or GST-

'RelA proteins in the extracts were adsorbed to columns containing Glutathione Sepharose 4B according to the protocol provided by Pharmacia. After the columns had been washed with 10 bed volumes of PBS, GST or GST-'RelA were the sole proteins adsorbed to the column. To demonstrate the specific dimerization of GST- 'RelA and wild type RelA, a crude extract prepared from cells that had overexpressed RelA from plasmid pALS10 was incubated with either GST- or GST-'RelA- bound beads for 30 min at room temperature. The beads were then washed extensively by PBS. Proteins that were bound to the beads were eluted by Glutathione Elution Buffer (Pharmacia) and detected by immunoblotting.

Glutaraldehyde Crosslinking to demonstrate RelA dimerization

Purified 'RelA was dissolved in 100 mM Tris-Cl pH7.5 containing 10% glycerol. Protein crosslinking was carried out by adding glutaraldehyde to the final concentration of 0.2%. The sample was incubated at room temperature for 15 minutes and then dialyzed against distilled water at 4°C for 20 hours. The sample was concentrated by lyophilization and analyzed by immunobloting.

Affinity blotting assay

Proteins were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The filter was incubated in HEPES (20mM HEPES pH 7.9, 20% glycerol, 0.1mM EDTA, 0.1m KCl, 10Mm $MgCl_2$) with 5% of skim milk for 2-5 days at 4°C to block the membrane as well as to

renature proteins bound to the membrane. After being rinsed with HEPES two times, the filter was incubated with partially purified His-tag-'RelA overnight at 4°C. The filter was then incubated with AP-conjugated anti-S-protein antibody (Novagen) for 1-2 hours at room temperature and followed by a wash and development.

Table 2.1. Bacterial strains

Bacterial Strains	Relevant Genotype and Description	Source
CF1693	*ΔrelA251::kanΔspoT207::cat*	Cashel laboratory
CF1736	*pyrE*$^+$ *spoT202*	Cashel laboratory
CF1738	*pyrE*$^+$ *spoT203*	Cashel laboratory
CF1740	*pyrE*$^+$ *spoT204*	Cashel laboratory
CF1969	*btuB*::Tn*10* *rpoB3443*	Cashel laboratory
CF1970	*btuB*::Tn*10* *rpoB3449*	Cashel laboratory
CF1971	*btuB*::Tn*10* *rpoB114*	Cashel laboratory
CF1972	*btuB*::Tn*10* *rpoB337*	Cashel laboratory
CF4268	*btuB*::Tn*10* *rpoB3445*	Cashel laboratory
CF4293	*btuB*::Tn*10* *rpoB8*	Cashel laboratory
CF4297	*btuB*::Tn*10* *rpoB3401*	Cashel laboratory
CF5034	*pyrE60 zib563::Tn*10	Cashel laboratory
CP79	*relA2*	Ishiguro laboratory
MC4100	*relA1*	Ishiguro laboratory
VC348	*zei348*::Tn*5*	Ishiguro laboratory
VC6129	W3110 *ΔrelA251::kan* (W3110 X CF1693)	This study
VC6130	VC6129 *ΔspoT207::cat* (VC6129 X CF1693)	This study
VC6132	W3110 *zei348*::Tn*5* *relA*$^+$	This study
VC6133	W3110 *zei348*::Tn*5* *relA2*	This study
VC6141	W3110 *zei348*::Tn*5* *relA1*	This study
VC6159	VC6129 *btuB*::Tn*10* *rpoB*$^+$ (VC6129 X CF1970)	This study
VC6158	VC6129 *btuB*::Tn*10* *rpoB3449* (VC6129 X CF1970)	This study
VC6160	VC6129 *btuB*::Tn*10* *rpoB3401* (VC6129 X CF4297)	This study
VC6166	VC6129 *btuB*::Tn*10* *rpoB3445* (VC6129 X CF4268)	This study
VC7262	VC6129 *btuB*::Tn*10* *rpoB8* (VC6129 X CF4293)	This study
VC6191	VC6129 *btuB*::Tn*10* *rpoB114* (VC6129 X CF1971)	This study
VC6192	VC6129 *btuB*::Tn*10* *rpoB3443* (VC6129 X CF1969)	This study
VC6193	VC6129 *btuB*::Tn*10* *rpoB3370* (VC6129 X CF1972)	This study
VC7237	VC6141 *zib563*::Tn*10*	This study
VC7238	VC6141 *pyrE60 zib563*::Tn*10* (VC6141 X CF5034)	This study
VC7239	VC6141 *spoT202* (VC6141 X CF1736)	This study
VC7240	VC6141 *spoT203* (VC6141 X CF1738)	This study
VC7241	VC6141 *spoT204* (VC6141 X CF1740)	This study
VC6216	W3110 with DE3 lysogen	This study
W3110	prototrophic	laboratory collection
BL21(DE3)	$r^- m^-$; an *E.coli* B strain with DE3	laboratory collection
BL21(DE3)*ΔrelA*	BL21(DE3)*ΔrelA251::kan*	This study

Table 2.2. Plasmids

Plasmids	Relevant Genotype and Description	Source
pALS10	Ap^r, *E. coli relA* clone	Svitil et al 1993
pALS13	Ap^r, C-terminal deleted *E. coli relA* clone	Svitil et al 1993
pALS14	Ap^r, truncated, non-active *E. coli relA* clone	Svitil et al 1993
pDJJ12	Ap^r, colE1 derivative carrying *E. coli rpoBC*	Jin and Gross 1988
pDS1	Cm^r, *E. coli rpoH* in pACYC184	C. Gross, UCSF
pXY36	Cm^r, N-terminal deleted *E. coli relA* clone (*'relA*)	This study
pXY37	Ap^r, *E. coli 'relA* in pGEX-5X-1	This study
pXY38	Kan^r, *E. coli 'relA* in pET30(a)	This study
pXY38-1	Kan^r, Fragment 1 of *'relA* in pET30(a)	This study
pXY38-2	Kan^r, Fragment 2 of *'relA* in pET30(a)	This study
pXY38-3	Kan^r, Fragment 3 of *'relA* in pET30(a)	This study
pXY41	Kan^r, *E.coli 'rplK* in pET30(a)	This study
pXY48	Ap^r, *E.coli rplK* in pGADT7	This study
PXY49	Kan^r, *E.coli rplK* in pGBKT7	This study
pXY51	Kan^r, *E.coli rplK* in pET30(C)	This study
pXY54	Kan^r, *E.coli mrplK* in pET30(C)	This study
pXY62	Kan^r, *E. coli 'relA* in pGBKT7	This study
pXY62-1	Kan^r, Fragment 1 of *'relA* in pGBKT7	This study
pXY62-2	Kan^r, Fragment 2 of *'relA* in pGBKT7	This study
pXY62-3	Kan^r, Fragment 3 of *'relA* in pGBKT7	This study
pXY63	Ap^r, *E. coli 'relA* clone in pGADT7	This study
pXY64	Kan^r, *E. coli relA* clone in pET30(a)	This study
pXY65	Kan^r, *E. coli relA* clone in pGBKT7	This study
pXY66	Ap^r, *E. coli relA* clone in pGADT7	This study
pBCSK(-)	Cm^r, clone vector	Stragene
pET30(a)	Kan^r, His-S-tag fusion protein expression vector	Novagen
pET30(c)	Kan^r, His-S-tag fusion protein expression vector	Novagen
pGEX-5X-1	Ap^r, GST fusion protein expression vector	Pharmacia
pGBKT7	Kan^r, Yeast Two-hybrid vector (GAL4 DB)	Clontech
pGADT7	Ap^r, Yeast Two-hybrid vector (GAL4 AD)	Clontech
pGBKT7-53	Kan^r, a DNA-BD/murine p53 fusion protein in pGBKT7	Clontech
pGADT7-T	Ap^r, an AD/SV40 T-antigen fusion protein in pGADT7	Clontech
pUC18	Ap^r, clone vector	laboratory collection

Chapter 3: Temperature sensitivity of *relA* mutants

Results

Temperature-sensitive growth of *relA* mutants

This investigation was initiated when it was fortuitously observed that several key *E. coli* strains carrying mutations in *relA* exhibited temperature-sensitive growth on certain media. For example, strains CF1693 (□*ΔrelA251*::*kan ΔspoT207*::*cat*), CP79 (*relA2*), and MC4100 (*relA1*) all failed to produce colonies on nutrient agar at 42°C. A more extensive survey of our culture collection revealed that temperature-sensitive growth was a common phenotype associated with all *relA* mutants tested. In an effort to establish the relationship between mutations in *relA* and temperature sensitivity, a set of isogenic derivatives of strain W3110 carrying the *relA1*, *relA2*, and *relA251*::*kan* alleles were constructed by phage P1-mediated transduction (Table 2.1). Fig.3.1 compares the plating efficiencies of these strains with the plating efficiency of a *relA*$^+$ strain, VC6132, on nutrient agar as a function of incubation temperature. All of the *relA* mutants had near normal plating efficiencies at temperatures as high as 39°C to 40°C but lost colony-forming capability at higher temperatures. Temperature sensitivity was especially notable in strain VC6129 (*ΔrelA251*::*kan*) and in a derivative of VC6129, strain VC6130, carrying the *ΔspoT207*::*cat* allele (Fig. 3.1, b and c, respectively). Both strains exhibited temperature-sensitive growth at 40°C and higher. In comparison, strains VC6133 and VC6141, which carry the

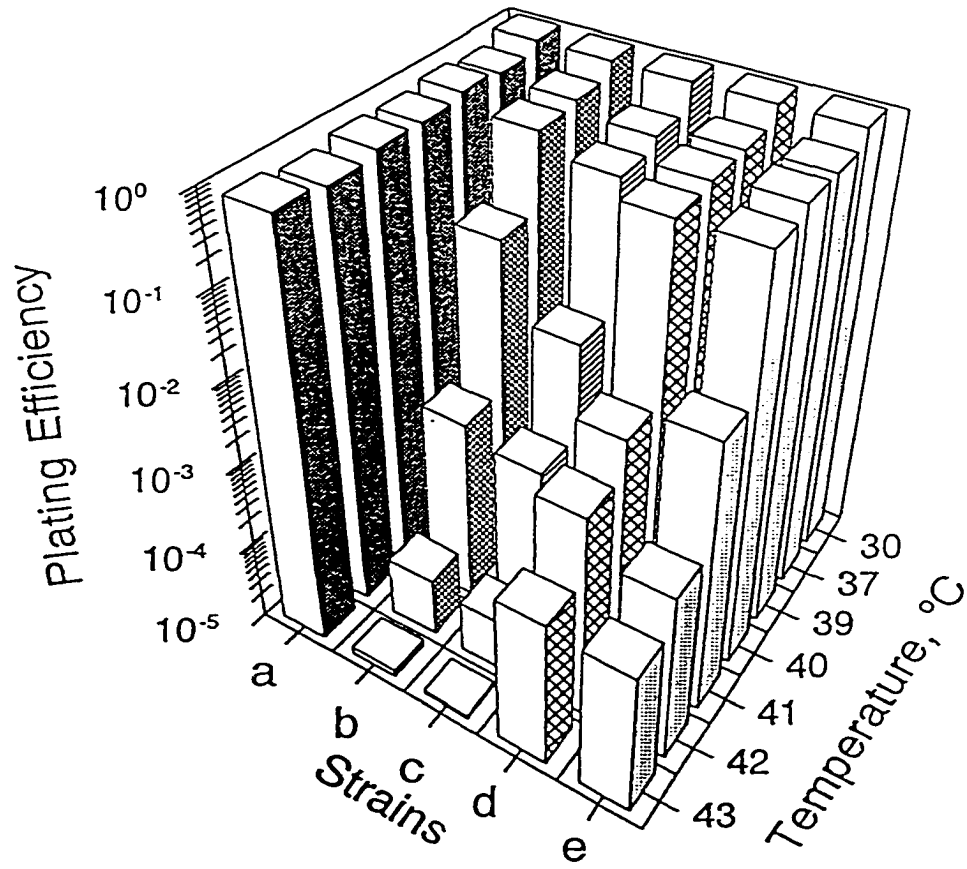

Fig. 3.1. Temperature-sensitive growth of *relA* mutants

A set of isogenic strains carrying 3 common *relA* mutant alleles were constructed. Growth of these strains were compared on nutrient agar at temperatures ranging from 30° to 43°C. Growth is expressed as plating efficiency (plate counts at the high temperature/plate counts at 30°C). a, strain VC6132 (wild type); b, strain VC6129 (Δ*relA251*::*kan*); c, strain VC6130 (Δ*relA251*::*kan* Δ*spoT207*::*cat*); d, strain VC6133 (*relA2*); e, strain VC6141 (*relA1*).

relA1 and *relA2* alleles, respectively, showed normal colony formation at 40°C but were temperature-sensitive at higher temperatures (Fig. 3.1, d and e, respectively). More extensive comparative studies indicated that the plating efficiencies of strains carrying the *relA1* and *relA2* alleles, such as VC6133 and VC6141 (Fig. 3.1, d and e, respectively), were consistently over 10-fold higher than those of strains carrying the *ΔrelA251::kan* allele. Although the plating efficiencies of strains VC6129 and VC6130 were roughly similar, data presented below indicate that VC6130 was less thermotolerant than VC6129. This suggests that the *ΔspoT207::cat* allele exacerbated the temperature-sensitive phenotype associated with *ΔrelA251::kan*.

Temperature sensitivity was also demonstrated in nutrient broth. As shown in Fig. 3.2A, all strains grew at 30°C with doubling times ranging from 110 to 114 min. On the other hand, only the wild type strain, VC6132, grew at 42°C (Fig. 3.2B). Cultures of the *relA* mutants, VC6129, VC6133, and VC6141, stopped growing after less than 2 doublings at the restrictive temperature.

The temperature-sensitive phenotypes of strains VC6129, VC6133, and VC6141 on nutrient agar or in nutrient broth were eliminated by the introduction of plasmid pALS10 (*relA*$^+$) but not by the introduction of plasmid pALS14 (truncated inactive *relA*). These results indicate that the temperature-sensitive growth of these strains was directly attributable to the mutations in *relA*.

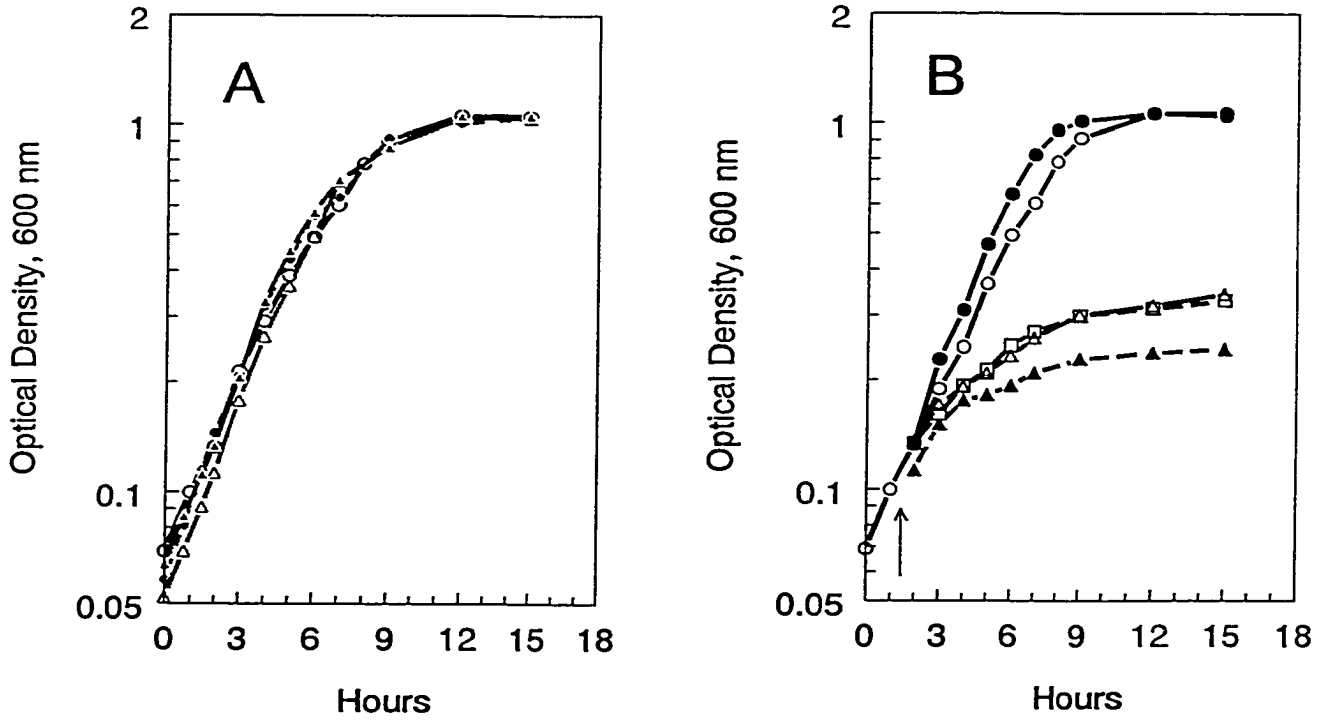

Fig. 3.2. Growth in Nutrient Broth at 30°C (A) and at 42° C (B)

Symbols: strains VC6132 (wild type), ●; VC6129 (Δ*rela251*::*kan*), △; VC6133 (*relA2*), ▲; VC6141 (*relA1*), □. In panel B, cultures growing at 30° C were shifted to 42°C at 1.5 h (arrow). For comparative purposes, the growth of a culture of VC6132 (wild type) at 30°C (O) is shown.

Osmoremediality of *relA*-mediated temperature-sensitive growth

As noted above, the *relA*-associated temperature sensitivity was dependent on the growth medium. We have routinely used nutrient agar or broth for most experiments. However, all *relA* mutant strains were temperature-resistant on most common complex media such as tryptic soy medium. Moreover, it is noteworthy that Luria broth or agar, and the various formulations of LB media supported colony formation of *relA* mutants at 42°C.

The observed medium dependence probably reflected the osmolarity of the growth medium because the *relA*-associated temperature-sensitive phenotype was osmoremedial. The ability of *relA* mutants to form colonies on nutrient agar at temperatures as high as 43°C was restored significantly by the addition of NaCl (0.3M), KCl (0.1M), or sucrose (0.35M) to the medium. Fig. 3.3 shows the effects of adding different concentrations of NaCl to nutrient agar on the plating efficiency of strain VC6129. A final concentration of 0.3 to 0.8M NaCl completely restored colony forming ability at 42°C.

Suppression of *relA*-mediated temperature-sensitive growth by mutations in *rpoB*

E. coli strains that cannot synthesize ppGpp, *e.g.*, VC6130 (*ΔrelA251*::*kan ΔspoT207*::*cat*), possess pleiotropic phenotypes. For example, they cannot

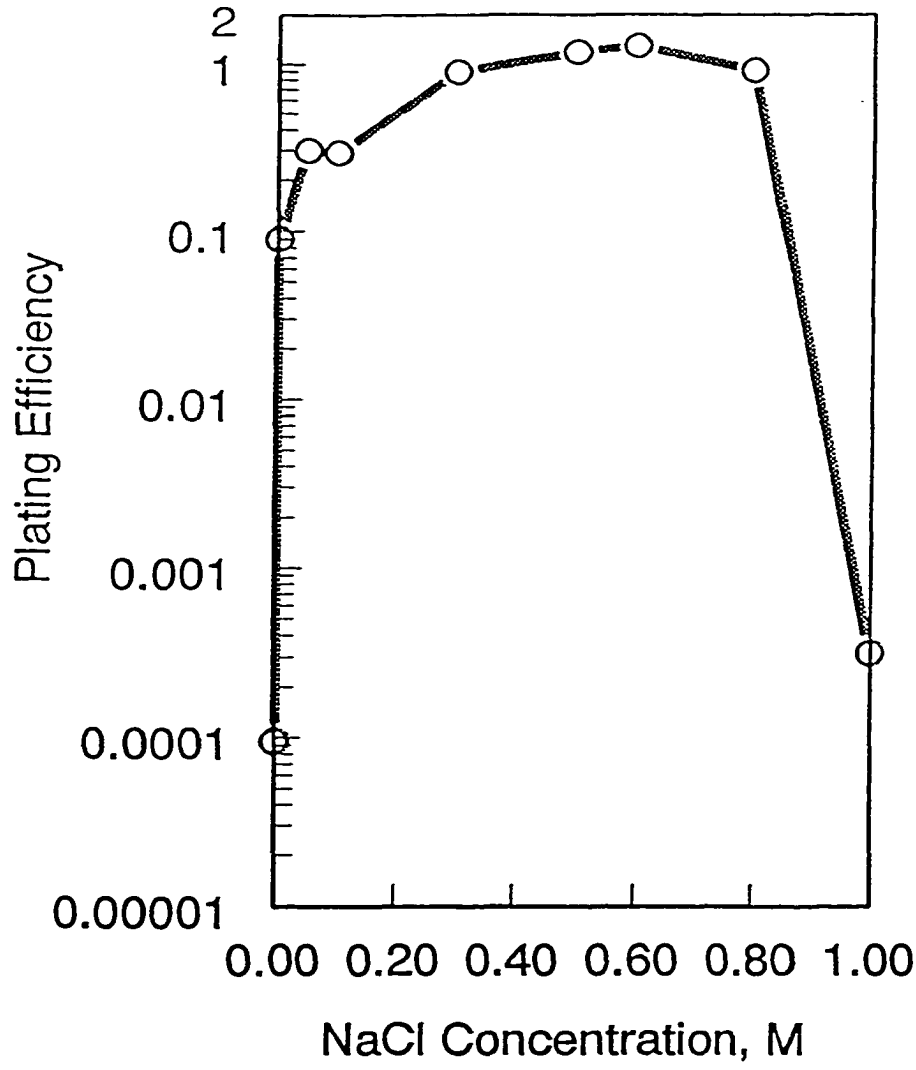

Fig. 3.3. The effect of medium osmolarity on colony formation by strain VC6129(*ΔrelA251::kan*)

Nutrient agar plates containing the indicated amounts of NaCl were inoculated with serial dilution series of an exponential phase culture of VC6129. Duplicate sets of plates were prepared for each medium with one set being incubated at 30°C and the other set at 42°C. Colonies were counted after 36 hours of incubation, and plating efficiencies (colony count at 42°C / by colony count at 30°C) were calculated for each type of medium.

Table 3.1. Suppression of thermosensitive phenotype of strain VC6129 (*ΔrelA251::kan)* mutants by mutations in the *rpoB* gene

Strain	Allele	Mutation in RpoB	Plating Efficiency
VC6159	*rpoB*$^{+}$	None	4.4×10^{-5}
VC6158	*rpoB3449*	532A	0.91
VC6160	*rpoB3401*	R529C	6.2×10^{-5}
VC6166	*rpoB3445*	(507-511)V	3.7×10^{-5}
VC7262	*rpoB8*	Q513P	2.4×10^{-5}
VC6191	*rpoB114*	S531F	0.94
VC6192	*rpoB3443*	L533P	0.92
VC6193	*rpoB3370*	T563P	0.82

grow on minimal medium unless it is supplemented with a mixture of amino acids (Xiao *et al.*, 1991). This multi-auxotrophic phenotype is suppressed by certain mutant alleles of *rpoB* (Cashel *et al.*, 1996). A representative group of *rpoB* alleles were transduced into VC6129, and their effects on temperature sensitivity were determined. As shown in Table 3.1, 4 of the 7 alleles tested restored temperature resistance. Interestingly, the 4 suppressor alleles also suppressed the multi-auxotrophic phenotype of the *relA spoT* null mutant whereas the 3 nonsuppressors did not (Cashel, unpublished data). Identical results were obtained with derivatives of VC6130 carrying these *rpoB* alleles (data not shown). In addition to these experiments, the effect of the wild type *rpoBC* genes as multicopy suppressors of the *relA*-mediated temperature sensitivity was tested by transforming plasmid pDJJ12 into strains VC6129, VC6130, VC6133, and VC6141. Multicopy suppression by pDJJ12 was not observed in any of these strains.

Suppression of temperature-sensitive phenotype of VC6141 by *spoT* mutant alleles

Sarrubi *et al.* (Sarubbi *et al.*, 1988) have described *spoT* mutant alleles that raise the basal levels of ppGpp in *relA1* backgrounds. Derivatives of strain VC6141 (*relA1*) carrying 3 such *spoT* alleles were constructed. Table 3.2 shows that these *spoT* alleles significantly restored colony formation in the *relA1* mutant at 42°C. These results suggest that the temperature sensitivity exhibited by the *relA* mutants was directly related to intracellular ppGpp levels.

Table 3.2. Suppression of *relA1*-mediated temperature sensitivity by *spoT* mutant alleles

Strain	Relevant Genotype	Plating Efficiency
VC7237	*relA1 spoT*$^{+}$	2.3 x 10^{-3}
VC7239	*relA1 spoT202*	0.12
VC7240	*relA1 spoT203*	0.34
VC7241	*relA1 spoT204*	0.46

Decreased thermotolerance of *relA* mutants

The *relA* mutants were killed during incubation at 42°C. This is demonstrated in Fig. 3.4 with strain VC6129. Identical sets of plates, inoculated with a serial dilution of VC6129, were incubated at 42°C beginning at 0 hour. At the indicated times, sets of plates were removed and incubated further at 30°C to determine the number of bacteria that had survived the incubation period at 42°C. In the case of VC6129, less than 50% of the original cells were able to form colonies at 30°C after a 7-hour incubation period at 42°C. Moreover, there was less than 0.1% survival after 10 hours.

The survival of a set of strains carrying the *ΔrelA251*::*kan* allele were compared at 55°C. Fig. 3.5 represents data obtained with exponential phase cells. Virtually identical results were obtained with stationary phase bacteria. The results clearly show that the *ΔrelA251*::*kan* strain, VC6129, was significantly less thermotolerant than the *relA*$^+$ strain, VC6132. Moreover, strain VC6130 was killed at a faster rate at 55°C than strain VC6129, indicating that the presence of the *ΔspoT207*::*cat* mutation further reduced the thermotolerance associated with the *ΔrelA251*::*kan* allele. Interestingly, derivatives of strain VC6129 carrying *rpoB* suppressor mutations, *e.g.*, strain VC6158 with *rpoB3449*, were significantly more thermoresistant, although their levels of thermoresistance were still not restored to the wild type level. Collectively, the results described here suggest that the temperature-sensitive growth exhibited by the *relA* mutants was associated with decreased thermotolerance.

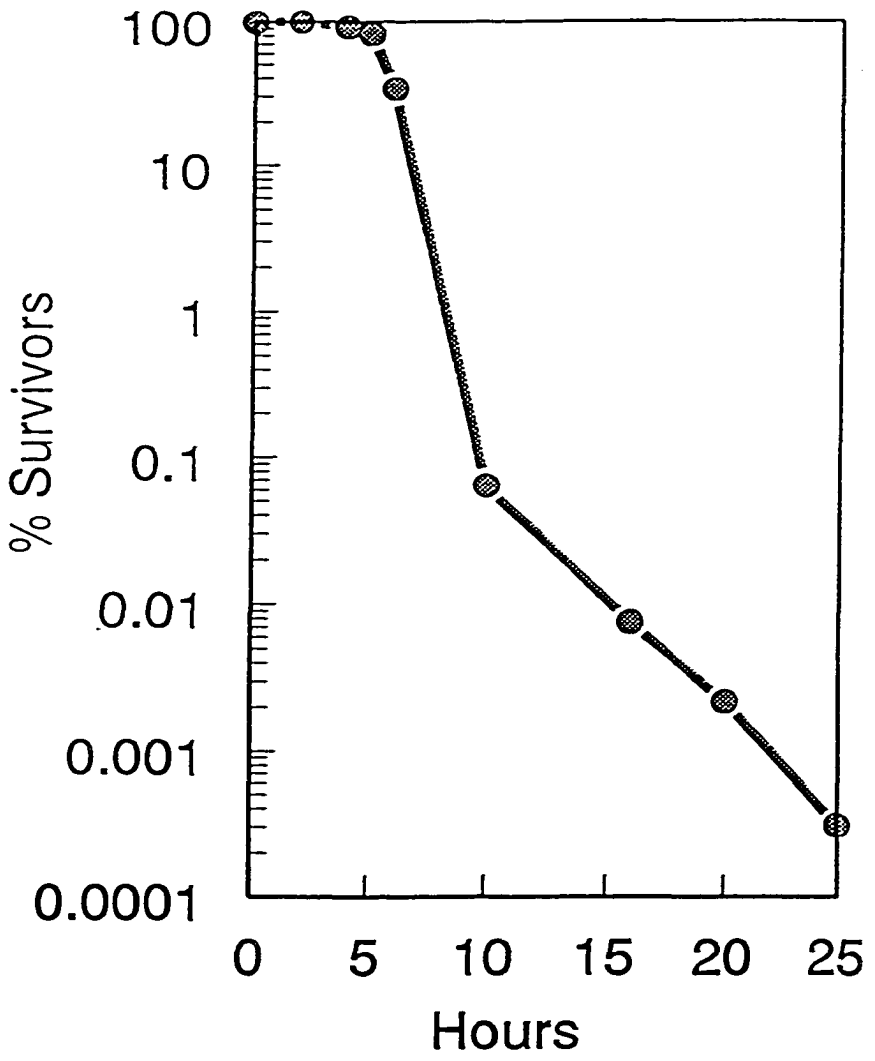

Fig. 3.4. Killing of strain VC6129 (Δ*relA251::kan*) at 42°C

Identical sets of plates inoculated with serial dilutions of VC6129 were incubated at 42°C beginning at 0 h. At indicated times, sets of plates were removed and incubated further at 30°C to determine the number of survivors of the 42°C treatment.

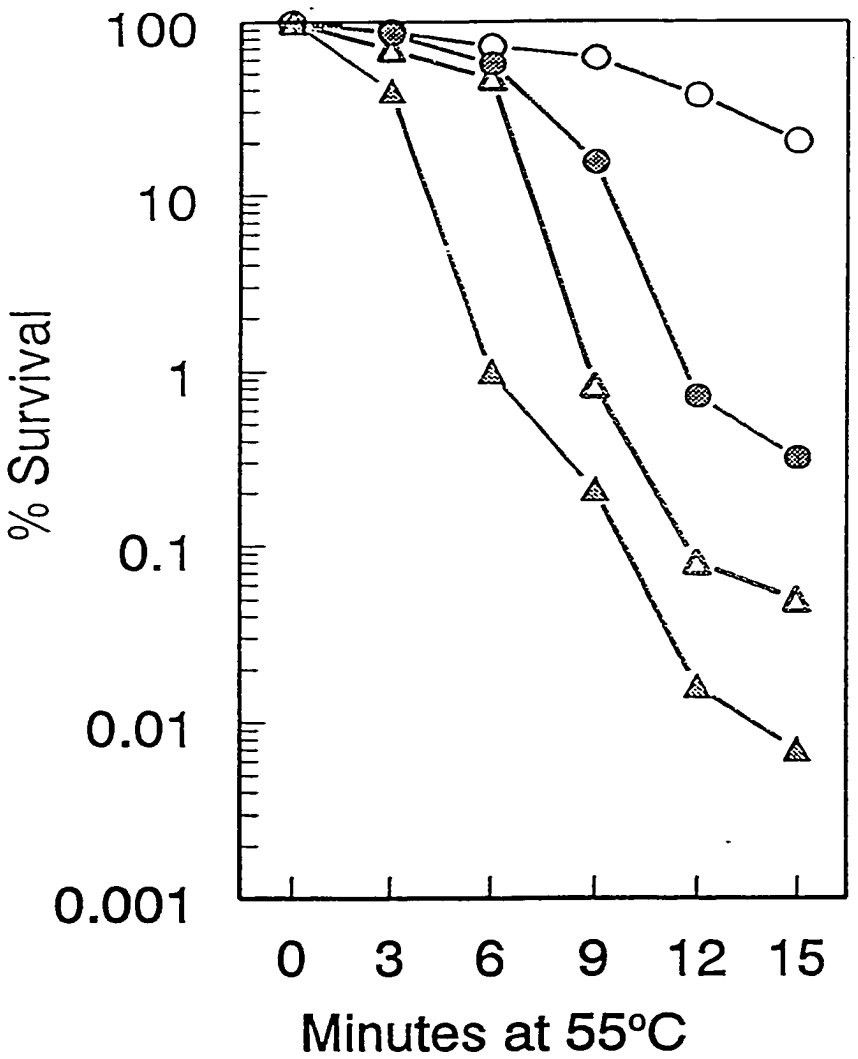

Fig. 3.5. Decreased thermotolerance conferred by *relA* mutation

Suspensions of bacteria in saline were incubated at 55°C. At the indicated times, samples were removed, and the number of survivors were determined. Symbols: VC6132 (wild type), ○; VC6129 (Δ*relA251*::*kan*), △; VC6130 (Δ*relA251*::*kan* Δ*spoT207*::*cat*), ▲; VC6158 (Δ*relA251*::*kan rpoB3449*), ●.

The thermotolerance of VC6129 (*ΔrelA251::kan*) was not improved by growing the cells at 37°C or by a brief 30-minute pre-exposure of the cells to 42°C prior to testing (data not shown).

Preliminary experiments on induction of heat shock gene expression

We employed a commercial DNA array containing 4,290 *E. coli* open reading frames to compare gene expression patterns in *relA*$^+$ and *relA*$^-$ bacteria growing exponentially at 30°C and after heat shock to 42°C. There were no obvious differences between the *relA*$^+$ and *relA*$^-$ strains under these conditions (data not shown). Moreover, the *relA* mutants expressed heat shock genes as efficiently as wild type bacteria. It is also noteworthy that the introduction of pDS1, a recombinant plasmid carrying *rpoH*, which encodes the heat shock transcription factor, σ^{32}, did not restore colony formation by *relA* mutant strains at 42°C, indicating that *rpoH* was not a multicopy suppressor of the *relA*-temperature sensitivity. Therefore, the *relA*-mediated temperature sensitivity was apparently not based on defective heat shock gene expression.

Effect of heat shock on negative supercoiling of DNA

The plasmid pUC18 was used as a reporter to determine whether there was a correlation between the superhelical density of DNA and the temperature sensitivity of *relA* mutants. The basic experiment was to subject an exponential phase culture growing in nutrient broth to a temperature upshift from 30°C to

44°C. Samples of cells were collected at regular intervals, and plasmid DNA extracted from the cells was analyzed by agarose gel electrophoresis in the presence of chloroquine. Fig. 3.6 shows the results of an analysis of pUC18 from the wild type strain, VC6132. The heat shock initially caused a transient relaxation of pUC18, but the superhelical density was restored to the original level within about 10 min after heat shock. This result is consistent with previous reports (Goldsterin and Drlica, 1984 and Mizushima *et al.*, 1993). The same experiment was performed on the isogenic *relA* mutant, VC6129. As shown in Fig. 3.7, the heat shock treatment also caused initial relaxation of pUC18 in VC6129. Although some negative supercoiling was restored after 10 min at 44°C, the normal level of negative supercoiling was never achieved in this case, even after 30 min. The results for the *relA spoT* double mutant, strain VC6130, are shown in Fig. 3.8. As with the other strains, pUC18 was initially relaxed by the heat shock. However, in VC6130, the plasmid DNA was more relaxed than in the case of VC6129, and there was no indication of increased negative supercoiling after 20 min. Therefore, there was an apparent correlation between the ability to synthesize ppGpp and the restoration of DNA negative supercoiling during heat shock, and these results suggest that ppGpp is essential for this process. Furthermore, the inability of *relA* mutants to restore their DNA supercoiling may be related to their temperature sensitivity.

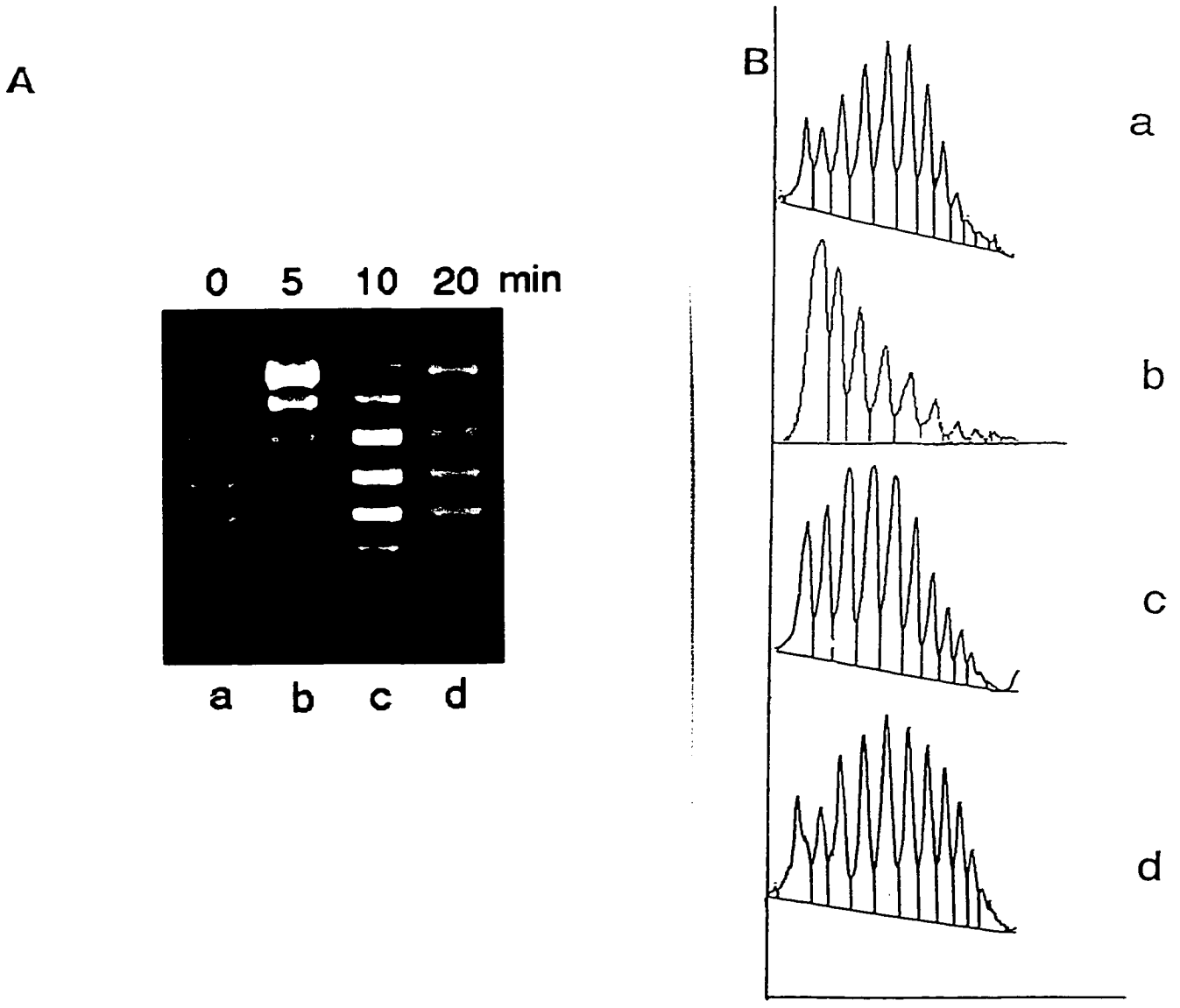

Fig. 3.6. Effect of heat treatment on plasmid supercoiling in the wild type *E.coli* strain

A. *E.coli* strain VC6132 harboring pUC18 DNA was grown in NB medium. Exponentially growning cells at 30°C (a) were shifted to 44°C for 5 min (b), 10 min (c) and 20 min (d). pUC18 DNA was extracted and analyzed by agarose gel electrophoresis in the presence of 3μg/ml chloroquine. More negatively supercoiled topoisomers before electrophoresis migrate more rapidly at this concentration of chloroquine.

B. Densitometer tracing of each lane in Fig. 3.6A. The direction of migration is from left to right.

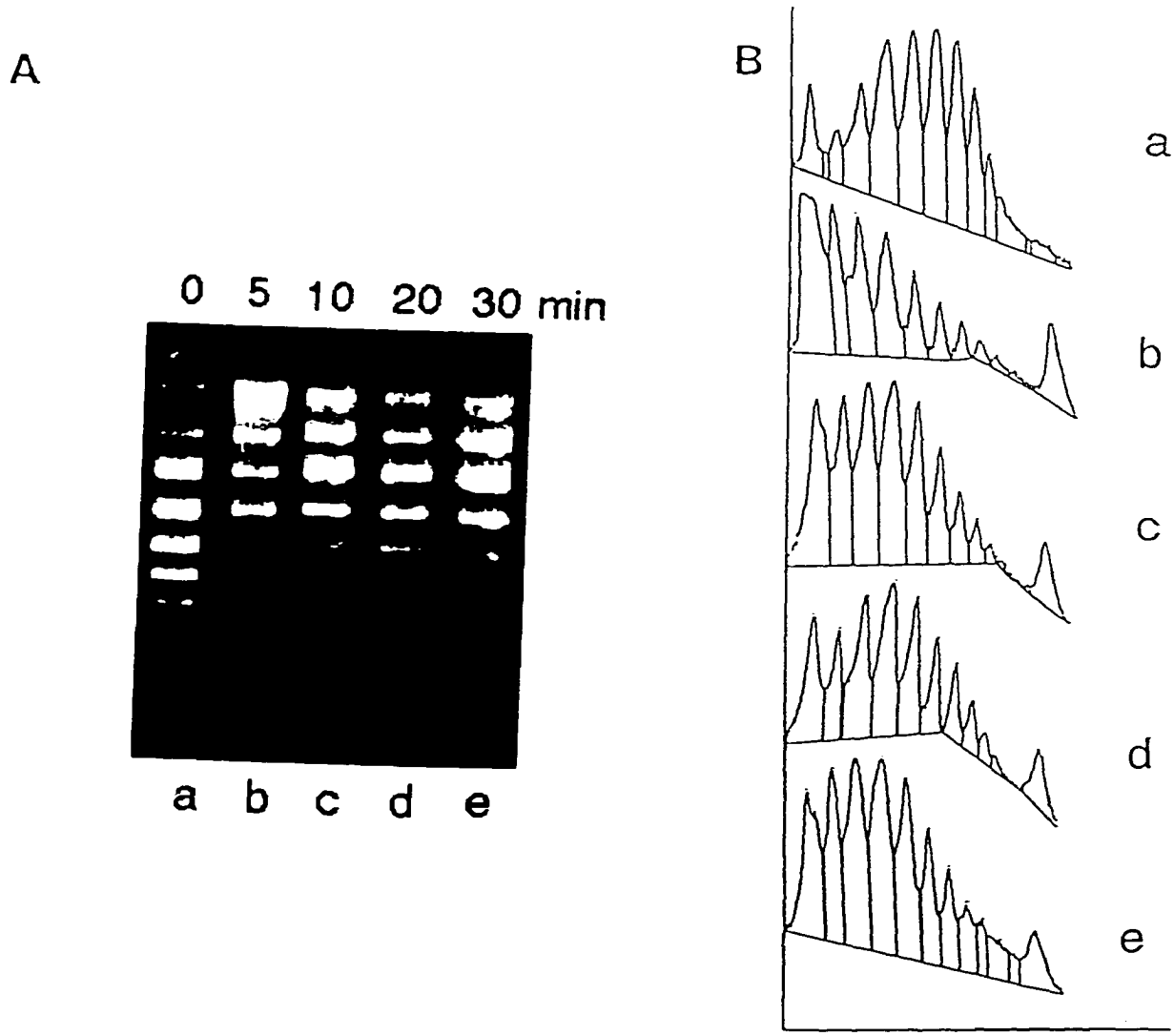

Fig 3.7. Effect of heat treatment on plasmid supercoiling in the *relA* mutant

A. *E.coli* strain VC6129 harboring pUC18 grown in NB at 30°C (a) was shifted to 44°C for 5 min (b), 10 min (c), 20 min (d) and 30 min (e). pUC18 DNA was analyzed under the same conditions as those in Fig. 3.6A.

B. Densitometer tracing of each lane in Fig. 3.7A. The direction of migration is from left to right.

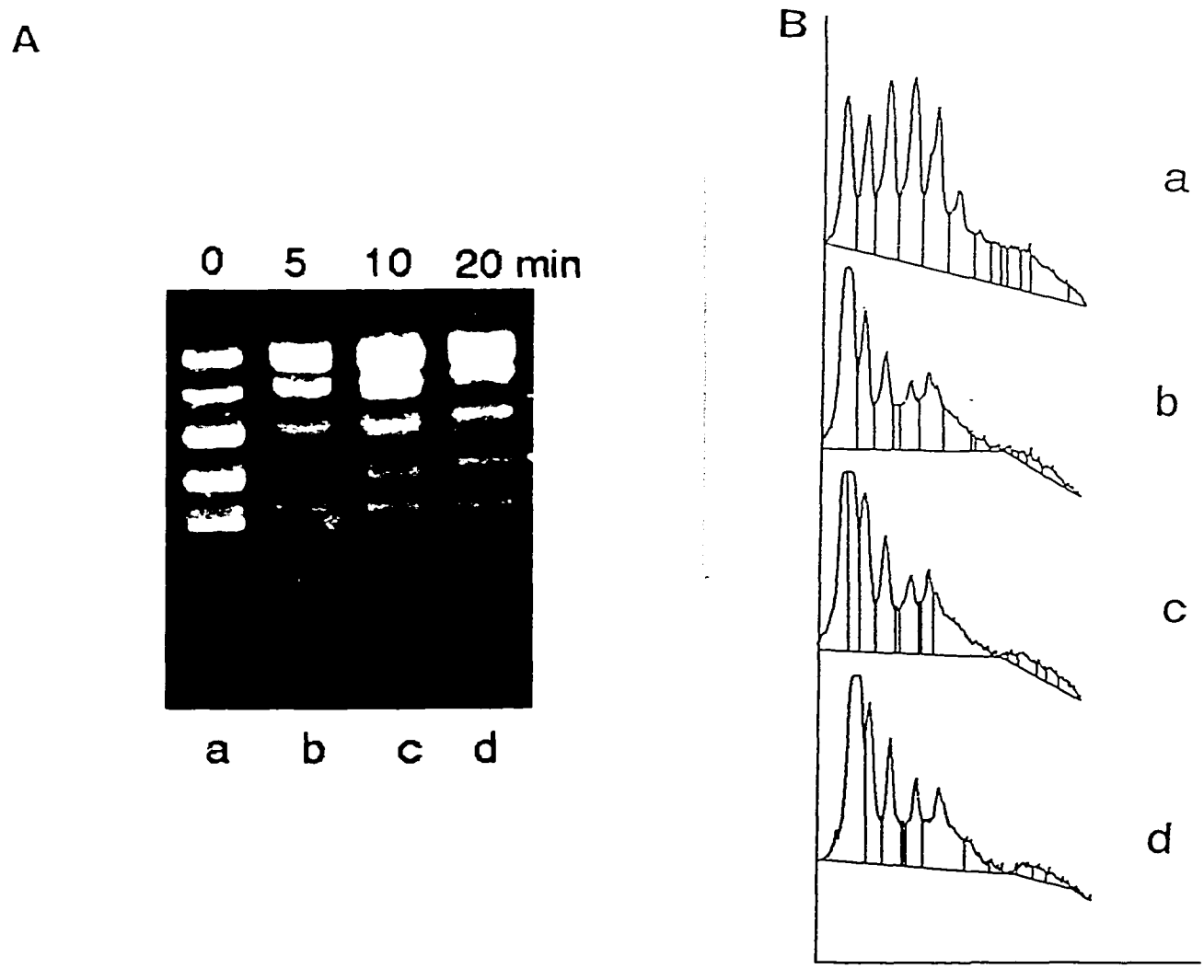

Fig 3.8. Effect of heat treatment on plasmid supercoiling in the *relA spoT* mutant

A. *E.coli* strain VC6130 harboring pUC18 grown in NB at 30°C (a) was shifted to 44°C for 5 min (b), 10 min (c) and 20 min (d). pUC18 DNA was analyzed under the same conditions as those in Fig.3.6A.

B. Densitometer tracing of each lane in Fig.3.8A. The direction of migration is from left to right.

Effect of *rpoB* suppressor mutation on DNA negative supercoiling

As noted above, certain *rpoB* alleles suppress the *relA*-mediated temperature sensitivity. It was of interest to determine the effect of such suppressors on DNA negative supercoiling. For this purpose, strain VC6158 carrying the reporter pUC18 was subjected to a 30°C to 44°C heat shock. Fig. 3.9 shows an analysis of the effect of heat shock on plasmid supercoiling. As in other strains, there was an initial relaxation of plasmid DNA. Although there was trend toward the restoration of negative supercoiling after 10 min, the major topoisomer remained relaxed. Therefore, the *rpoB* allele, at best, only partially suppressed the inability of the *relA* mutant to restore DNA negative supercoiling.

Restoration of DNA supercoiling in *relA* mutant by salt at high temperature

The *relA*-mediated temperature sensitivity was osmoremedial. The effect of medium osmolarity on DNA supercoiling in VC6129 during heat shock was therefore examined. As shown in Fig. 3.10, heat shock of VC6129 in nutrient broth 0.3M NaCl did not result in the relaxation of plasmid supercoiling. In fact, prolonged incubation (15 to 60 min) at 44°C caused a progressive increase in the degree of negative supercoiling. Therefore, there was a correlation between the osmoremediality of the *relA*-mediated temperature sensitivity and the ability of the *relA* mutant to restore DNA negative supercoiling during heat shock.

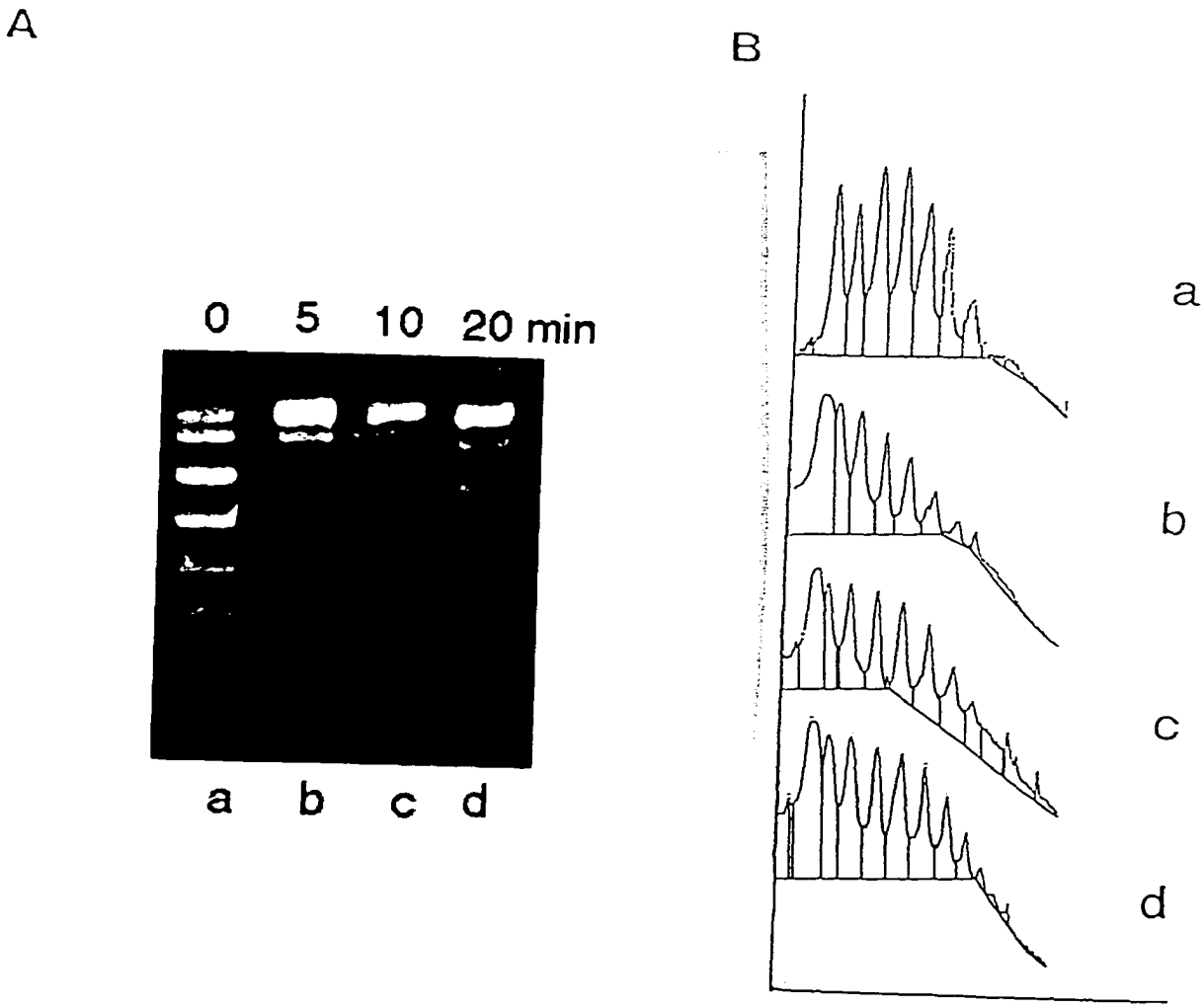

Fig 3.9. DNA supercoiling in temperature resistant *relA rpoB* strain

A. *E.coli* strain VC6158 harboring pUC18 grown in NB at 30°C (a) was shifted to 44°C for 5 min (b), 10 min (c) and 20 min (d). pUC18 DNA was analyzed under the same conditions as those in Fig 3.6A.

B. Densitometer tracing of each lane in Fig 3.9A. The direction of migration is from left to right.

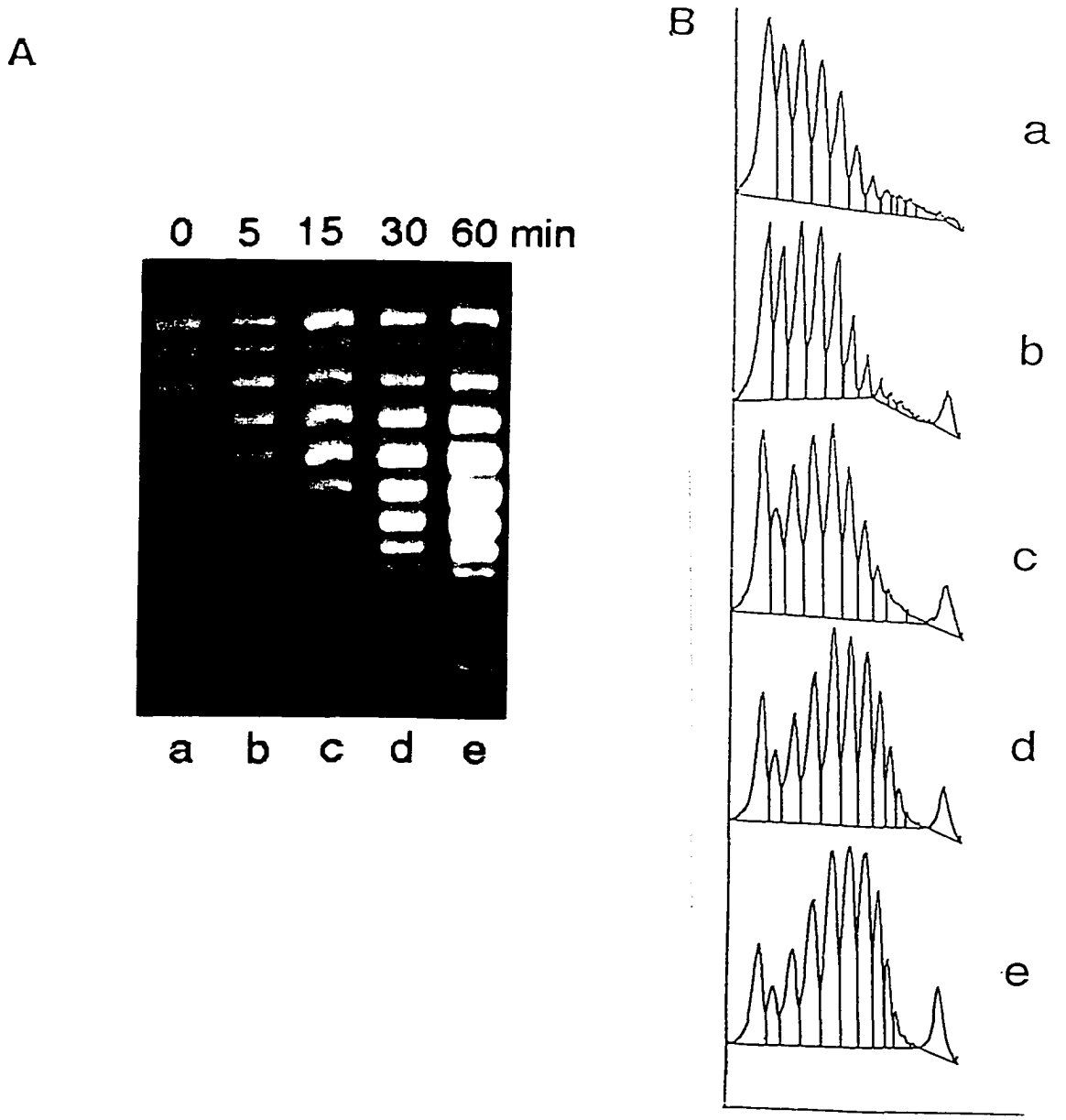

Fig. 3.10. Effect of heat treatment on supercoiling of DNA in *relA* mutant strain under high osmotic condition

A. Strain VC6129 harboring pUC18 was grown in NB containing 0.3M NaCl at 30°C. Exponentially growing cells (a) were exposed to 44°C for 5 min (b), 15 min (c), 30 min (d) and 60 min (e). pUC18 DNA was analyzed under the same conditions as those in Fig.3.6A.

B. Densitometer tracing of each lane in Fig.3.10A. The direction of migration is from left to right.

Hypersensitivity to novobiocin conferred by *relA* mutants

Since the *relA* mutant was unable to restore DNA supercoiling at high temperature, the effects of DNA gyrase inhibitors on the *relA* mutant were determined. Fig. 3.11 compares the sensitivities of strains VC6132 and VC6129 to novobiocin and nalidixic acid on LB agar. As shown in panel A, VC6132 was resistant to novobiocin, an inhibitor of the DNA gyrase B subunit, at a concentration of 200 µg/ml. In contrast, the plating efficiency of VC6129 was reduced by over 90% by 50 µg of novobiocin per ml. Novobiocin resistance could be restored to near the wild type level in VC6129 by introducing the plasmid containing the *relA* gene. The ppGpp^0 mutant, strain VC6130, was even more sensitive to novobiocin than VC6129. Fig. 3.11B shows the effects of nalidixic acid, an inhibitor of the DNA gyrase A subunit. In contrast to the results obtained with novobiocin, there were little or no differences in the sensitivities of strains VC6132, VC6129, and VC6130 to nalidixic acid. Fig. 3.12 shows that novobiocin had the same general effects when nutrient agar was used although the hypersensivity of VC6130 was not as distinct in this case. Furthermore, VC6158 was at least as resistant to novobiocin as the wild type strain, VC6132. This indicates that the *rpoB3449* suppressor allele eliminated the novobiocin hypersensivity associated with the *ΔrelA251::kan* mutation.

In order to examine whether novobiocin causes excessive relaxation of DNA supercoiling in *relA* mutant strains, we compared the superhelical densities of plasmid DNA in VC6132 and VC6129 in the presence of 200µg/ml of novobiocin. Fig 3.13 shows that pUC18 had exactly the same pattern of DNA

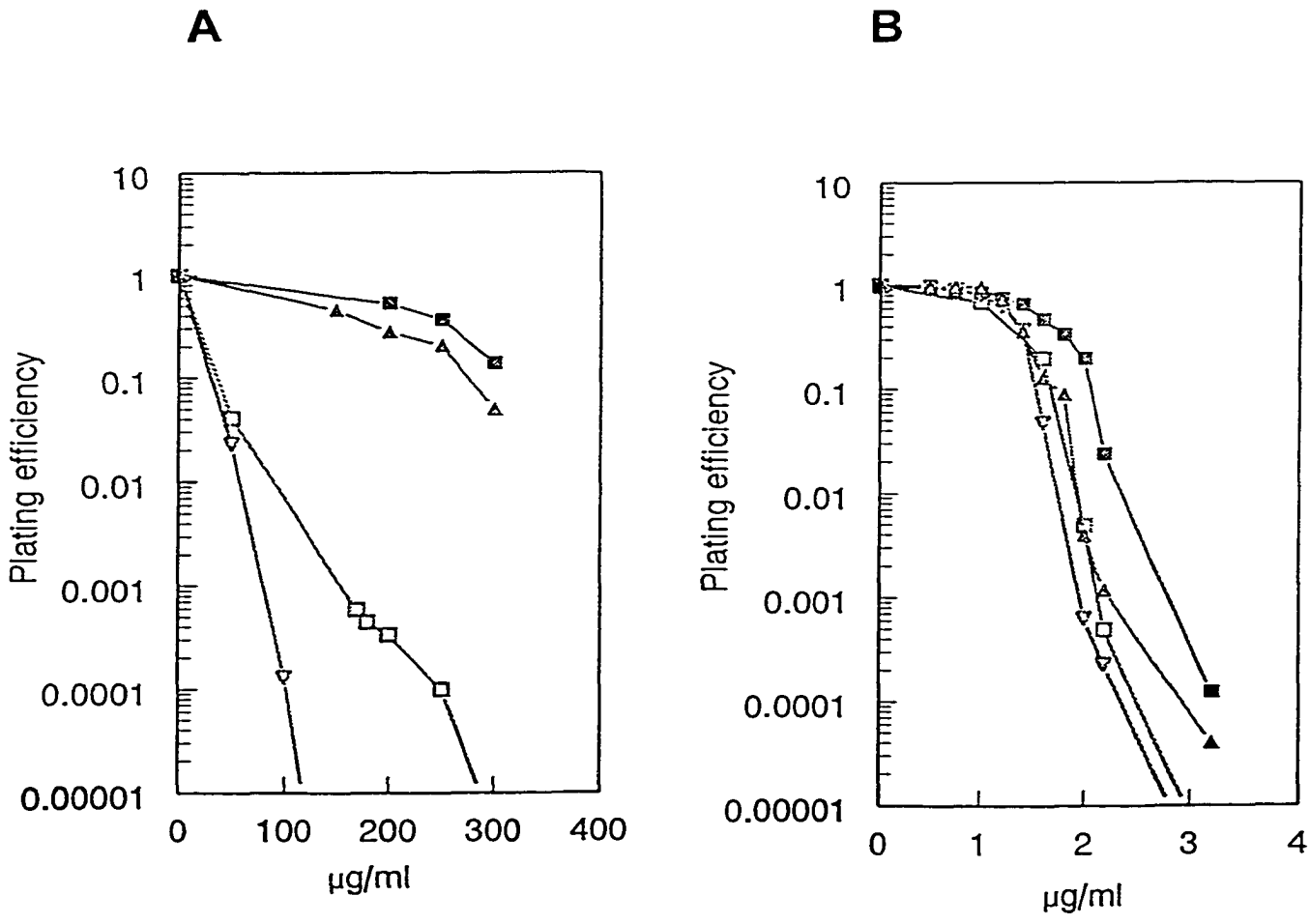

Fig. 3.11. Sensitivity to DNA gyrase inhibitors

Serial dilution series of exponential phase culture of VC6132 (■), VC6129 (□), VC6129/pALS10 (▲) and VC6130 (▽) were plated on LB agar pates containing indicated concentration of novobiocin (panel A) or nalidixic acid (panel B) . Plates were incubated at 30°C for 48 hr, and plating efficiencies were determined.

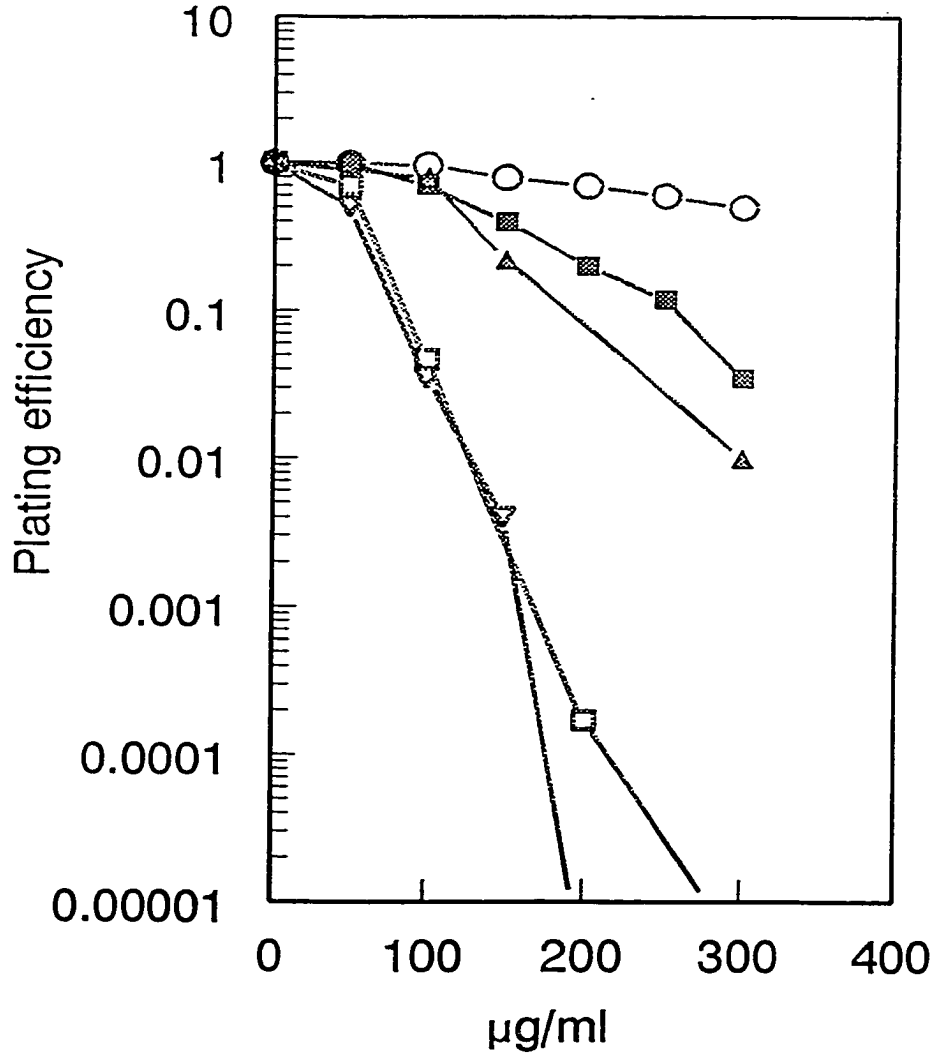

Fig. 3.12. Sensitivity to novobiocin in NB

Serial dilution series of exponential phase culture of VC6132 (■), VC6129 (□), VC6129/pALS10 (▲), VC6130 (▽) and VC6158 (○) were plated on NB agar pates containing indicated concentration of novobiocin. Plates were incubated at 30°C for 48 hr, and plating efficiencies were determined.

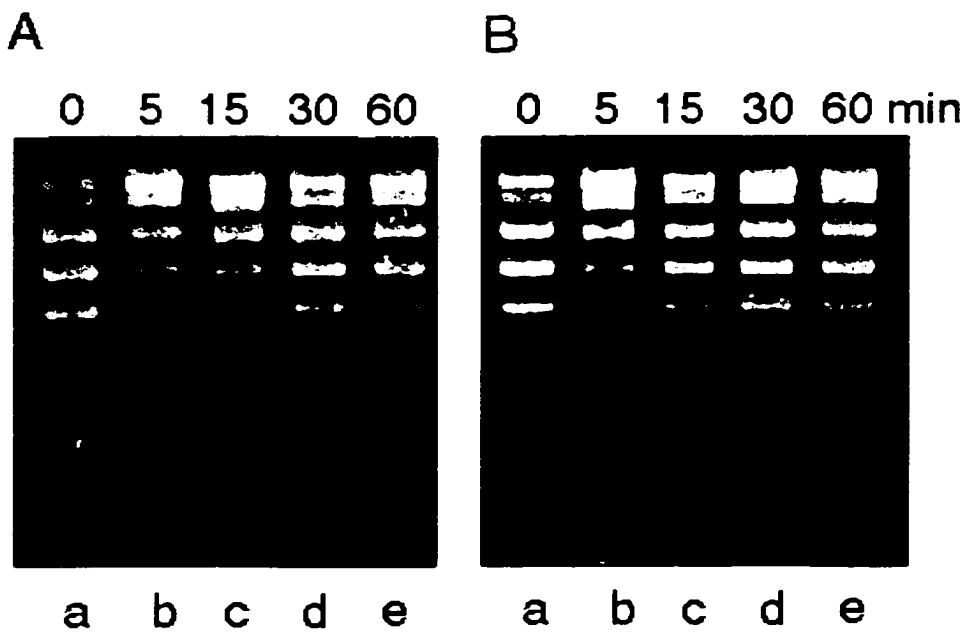

Fig. 3.13. Effect of novobiocin on DNA supercoiling

VC6132 and VC6129 harboring pUC18 were grown in NB at 30°C. Novobiocin was added to the final concentration of 200μl/ml for 0 min (a), 5 min (b), 15 min (d), 30 min (d) and 60 min (e).

pUC18 DNA from VC6132 (A) and VC6129 (B) was extracted and analyzed as described in Fig.3.6A

supercoiling in both strains. DNA was initially rapidly relaxed by novobiocin, and negative supercoiling was gradually restored to a certain degree. This result indicates hypersensitivity to novobiocin of *relA* mutant is not due to an excessive relaxation of DNA.

Supression of the *relA*-mediated temperature sensitivity by novobiocin

The effects of different concentrations of novobiocin in nutrient agar on VC6129 at 30°C and at 42°C were determined. The *relA*-mediated temperature-sensitive phenotype of VC6129 was significantly suppressed by novobiocin. As shown in Fig. 3.14, the plating efficiencies of VC6129 in the presence of novobiocin at 100 and 200 µg per ml were identical at both 30°C and at 42°C. The effects of these low concentrations of novobiocin on the negative supercoiling of pUC18 in strain VC6129 were determined. Panel A in Fig. 3.15 shows that novobiocin at 100 µg per ml initially caused a transient relaxation of pUC18 at 30°C. Negative supercoiling was progressively restored begining at 15 min. In panel B of Fig. 3.15, a culture of VC6129 growing exponentially at 30°C was treated with 100 µg of novobiocin per ml for 5 min and then shifted to 42°C. A sample taken at start of the incubation period at 42°C (lane 1) confirms that novobiocin caused relaxation of pUC18. After 15 min, a progressive restoration of negative supercoiling was observed. Therefore, the partial suppression of temperature sensitivity was related to the ability of low concentrations of novobiocin to restore negative supercoiling of DNA. As discussed below, it is proposed that this seemingly contradictory result is due to the induction of DNA

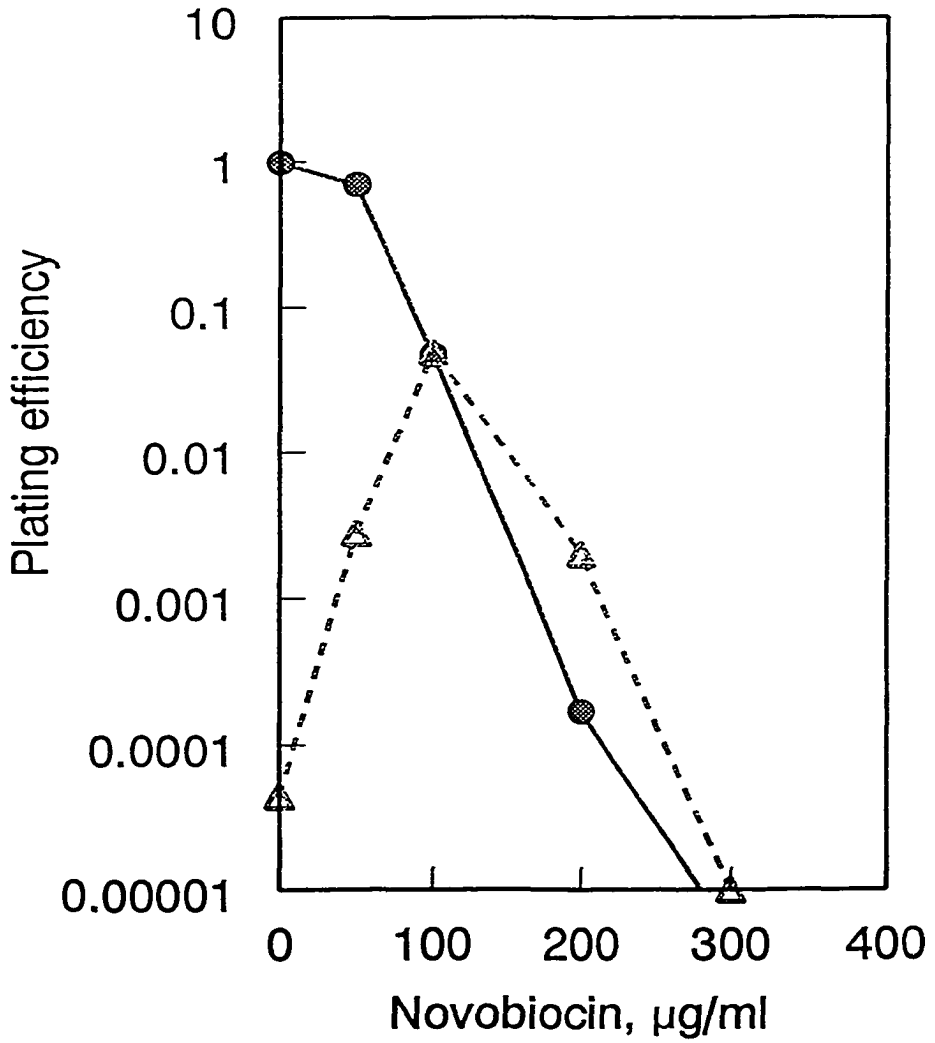

Fig. 3.14. Effect of novobiocin on *relA*-mediated temperature sensitivity

Nutrient agar plates containing the indicated concentration of novobiocin were inoculated with serial dilution series of an exponential phase culture of VC6129. Duplicate sets of plates were prepared for each medium with one set being incubated at 30°C and the other set at 42°C. Colonies were counted after 36 hours of incubation, and plating efficiencies at 30°C (●) and at 42°C (△) were calculated for each type of medium.

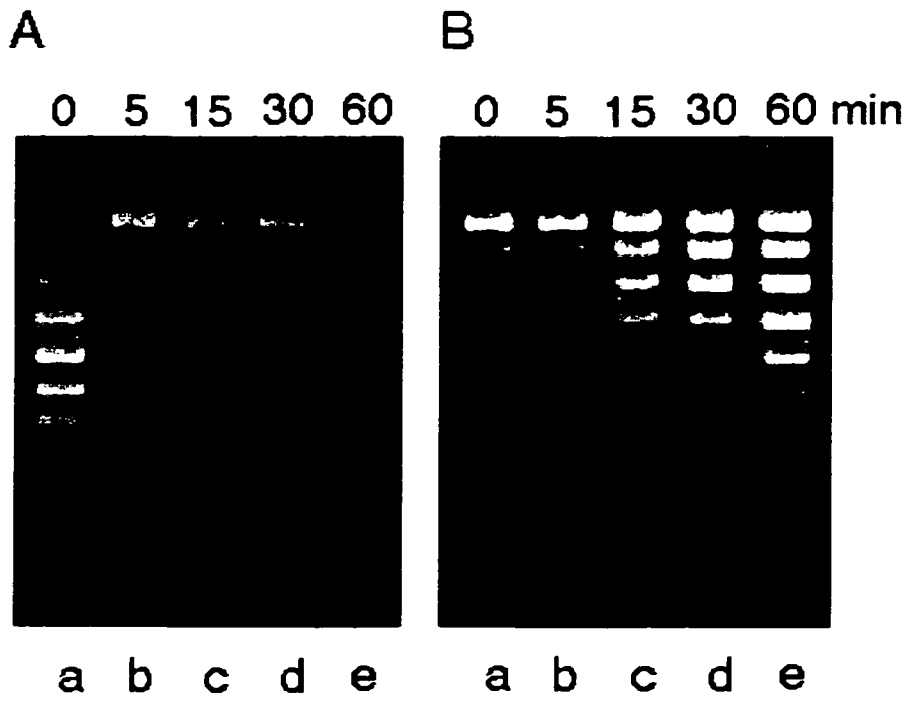

Fig. 3.15. Effect of novobiocin on DNA supecoiling during heat shock in *relA* mutant

A. VC6129 harboring pUC18 was grown in NB at 30°C. Novobiocin (100 μl/ml) was added for 0 min (a), 5 min (b), 15 min (c), 30 min (d) and 60 min (e).

B. Novobiocin (100 μl/ml) was added to VC6129 harboring pUC18 grown in NB at 30°C for 5 min. Then the cultures were shifted to 42°C for 0 min (a), 5 min (b), 15 min (c), 30 min (d) and 60 min (e).

pUC18 DNA was extracted and analyzed as described in Fig. 3.6A.

gyrase synthesis.

It is interesting that nalidixic acid, an inhibitor of the DNA gyrase A subunit, did not suppress the *relA*-mediated temperature sensitivity at a broad range of concentrations starting from subinhibitory levels (data not shown).

Discussion

The three commonly used *relA* mutant alleles conferred temperature-sensitive growth phenotypes. In all cases, the temperature sensitivity was eliminated by the introduction of a recombinant plasmid carrying the wild type *relA* gene. Furthermore, the *spoT* mutant alleles, described by Sarrubbi *et al.* (Sarrubbi *et al.,* 1988), that cause increased basal levels of ppGpp in a *relA1* background suppressed the temperature-sensitive phenotype of the *relA1* mutant strain. The *relA* deletion strain, VC6129, was significantly more temperature-sensitive than strains carrying the *relA1* and *relA2* alleles, and this probably reflects the leakiness of the latter mutant alleles. The temperature sensitivity of the VC6129 was exacerbated by the introduction of the *spoT207*::*cat* mutation (VC6130). Collectively, these results indicate that the observed temperature sensitivity was directly attributable to decreased intracellular levels of ppGpp.

The temperature-sensitive phenotypes of the *relA* mutant strains were osmoremedial and were only evident in media of low osmotic strength. The osmolarities of most common media, *e.g.*, the various formulations of LB, were adequate to support growth at high temperature. The *relA*-mediated temperature sensitivity was suppressed by increasing the osmolarity of nonpermissive growth

media with a variety of solutes, *e.g.*, NaCl, KCl, or sucrose. Osmoremedial temperature-sensitive mutations are apparently quite common but how the increase in external osmolarity suppresses the mutant phenotypes is far from understood (Csonka, 1989). In some instances, it has been proposed that the resulting increase in the intracellular concentration of a compatible solute could lead to the stabilization of a temperature-sensitive mutant protein (Csonka, 1989). This is an unlikely explanation for the *relA*-mediated temperature sensitivity. For example, strains carrying the *relA251*::*kan* mutation did not produce RelA but still exhibited an osmoremedial temperature sensitive phenotype

The *relA*-mediated temperature sensitivity was also suppressed by four of the seven *rpoB* mutant alleles tested. The alleles used in this study have been systematically characterized and shown to possess altered transcriptional termination activities (Jin and Gross, 1988; Jin *et al.*, 1988). The connection between mutations in *rpoB* and ppGpp-dependent phenomena is well documented. The four alleles that suppress *relA*-mediated temperature sensitivity were previously shown to suppress the multiauxotrophic phenotypes of ppGpp-deficient mutants (Cashel *et al.*, 1996). These alleles also enhanced the survival of ppGpp-deficient strains during prolonged stationary phase (Cashel *et al.*, 1996). Mutations in *rpoB* have also been reported to suppress the sensitivity of *relA* mutants to serine, methionine, and glycine (Uzan and Danchin, 1978), and have been shown relieve the growth inhibitory effects of high levels of ppGpp (Tedin and Bremer, 1992). It is intriguing that seemingly distinct phenomena,

e.g., multiauxotrophy and temperature sensitivity, are suppressed by common mechanisms, *i.e.*, mutations in RNA polymerase. These findings suggest that these ppGpp-dependent phenomena may have a transcriptional basis, and it would be interesting to consider the possibility that they, in fact, may have a common basis.

There is evidence for the direct and specific binding of ppGpp to the β-subunit of RNA polymerase (Reddy *et al.*, 1995; Chatterji *et al.*, 1998). The *in vitro* activities of RNA polymerase to which azido-ppGpp had been cross-linked were compared on stringent and non-stringent promoters (Chatterji *et al.*, 1998). The transcription of ribosomal genes was inhibited by azido-ppGpp whereas transcription from the *lac* UV5 promoter was unaffected. However, the relationship of these findings to ppGpp-dependent phenomena such as multiauxotrophy and thermotolerance is unknown.

It is possible that ppGpp may be required for the expression of genes that are necessary for thermotolerance, and an obvious possibility was the involvement of heat shock proteins. The heat shock response is dependent on the concentration of σ^{32}, and heat shock protein synthesis can be induced, even in the absence of heat shock, when σ^{32} is overproduced (Grossman *et al.*, 1987). Our initial experiments showed that the overproduction of σ^{32} did not relieve the *relA*-mediated temperature sensitivity. However, in retrospect, this is perhaps not surprising since VanBogelen *et al.* (VanBogelen *et al.*, 1987a) have shown that the induction of the heat shock regulon by inducing the overproduction of σ^{32} at 28°C, for unknown reasons, was insufficient to confer thermotolerance. More

importantly, no differences in gene expression patterns in *relA*$^+$ and *relA*$^-$ bacteria during heat shock were observed, suggesting that the heat shock response is functional in *relA* mutant strains. Moreover, VanBogelen and Neidhardt (VanBogelen and Neidhardt, 1990) have demonstrated that strain CF1946, a W3110 derivative carrying *relA251*::*kan spoT207*::*cat*, exhibited a heat shock response albeit an altered one. They noted that strain CF1946 normally had a higher basal level of several heat shock proteins when it was grown at 30°C. The heat shock regulon was induced when CF1946 was subjected to a temperature upshift to 42°C, but this induction was 10 min later than that observed in wild type strain, W3110. It has been previously noted that temperature upshifts result in the accumulation of ppGpp (Pao and Gallant, 1978; VanBogelen *et al.*, 1987b), but Van Bogelen and Neidhardt (VanBogelen and Neidhardt, 1990) concluded from their results that ppGpp was neither sufficient nor necessary for the induction of the heat shock regulon. In an earlier study, Grossman *et al.* (Grossman *et al.*, 1985) demonstrated that the expression of heat shock proteins was induced during the stringent response. In their experiments, a temperature-sensitive valyl-tRNA synthetase mutant was shifted from 28°C to 33.5°C, a semipermissive temperature at which protein synthesis was inhibited by about 50% with the concomitant increase in ppGpp levels. The induction of heat shock protein synthesis under these conditions was *relA*$^+$-dependent and did not occur in an isogenic *relA* mutant strain. Curiously, heat shock protein synthesis during the stringent response also occurred in an *rpoH* mutant strain. Furthermore, subsequent studies by VanBogelen *et al.*

(VanBogelen *et al.*, 1987b) indicated that the heat shock response was not induced when the stringent response was invoked by isoleucine deprivation. Therefore, at the present time, it is not clear what role, if any, ppGpp plays in the heat shock response. However, on the basis of the results reported here, it would appear that the expression of heat shock proteins is not sufficient to relieve the temperature sensitivity exhibited by *relA* mutant strains.

The effect of growth temperature on DNA supercoiling has been previously studied using plasmids as reporters (Goldstein and Drlica, 1984; Mizushima *et al.*, 1993). When wild-type *E.coli* is shifted to a low temperature, DNA linkage number decreases, becomes more negatively supercoiled. In contrast, an increase in temperature induces a relaxation in DNA supercoiling. Both effects of growth temperatures have been shown to be transient, because restoration of normal negative DNA supercoiling is essential for the resumption of cell growth. The results presented here confirm the effect of temperature upshift on DNA supercoiling in wild type bacteria. It is further shown that a *relA* mutant is defective in restoring negative supercoiling after heat shock. Moreover, the defect in negative supercoiling at high temperature was even more pronounced in the (p)ppGpp^0 strain, VC6130. The defect in negative supercoiling of DNA in the heat-shocked *relA* mutant was suppressed by increasing the osmolarity of the growth medium. The results presented here are consistent with previous data on the effects of osmolarity on DNA supercoiling (Higgins *et al.*, 1988). On the other hand, the *rpoB* suppressor seemed to, at best, only partially restore negative supercoiling, and its action may not be directly related to restoring DNA

negative supercoiling. Nevertheless, the collective data strongly suggest that the thermosensitivity of the *relA* and *spoT* mutants is directly related to the defect in DNA negative supercoiling.

The key enzyme that catalyzes the negative supercoiling of DNA in *E.coli* is DNA gyrase. Two observations support the proposal that the *relA*-mediated temperature sensitivity is directly related to the restoration of normal negative supercoiling after heat shock. First, the *relA* mutant was hypersensitive to novobiocin, a specific inhibitor of the B subunit of DNA gyrase. Second, low concentrations of novobiocin suppressed the *relA*-mediated temperature sensitivity. In both phenomena, nalidixic acid, a specific inhibitor of the A subunit of DNA gyrase, did not have an effect. This may reflect basic differences in the mechanisms of toxicity of nalidixic acid and novobiocin. The negative supercoiling reaction catalyzed by DNA gyrase is an ATP-driven process involving a double-stranded DNA cleavage and rejoining. Nalidixic acid and other members of the quinolone family of drugs inhibit the DNA gyrase A-mediated DNA rejoining step. The effect is lethal and leaves DNA gyrase A covalently attached to the free ends of the DNA. This occurs at low drug concentrations, under conditions where no DNA relaxation is observed. Therefore, the quinolones, *e.g.*, nalidixic acid, are not considered to be DNA relaxing agents despite their interaction with gyrase A (Drlica and Franco, 1988; Gellert *et al.*, 1977). In contrast, novobiocin is a member of the coumarin family of DNA gyrase B inhibitors. The major feature of the coumarins is that they cause the relaxation of DNA supercoiling by inhibiting the ATPase activity of the B subunit

of DNA gyrase, and this process is not lethal at low drug concentrations (Gellert *et al.*, 1977; Drlica and Franco, 1988).

Ueshima *et al.* (Ueshima *et al.*, 1989) have shown that the transient relaxation of DNA during temperature upshift is necessary for the expression of heat shock genes during the heat shock response. Furthermore, the heat shock proteins are required for restoring the negative supercoiling of DNA during the adaptation stage of the heat shock response, the period during which heat shock genes are down-regulated. To this end, they demonstrate that the heat shock chaperone, DnaK, interacts directly with DNA gyrase after heat shock to facilitate the negative supercoiling of DNA. Therefore, both DNA relaxation and the induction of heat shock proteins are simultaneous and transient phenomena associated with heat shock. The restoration of normal supercoiling of DNA is necessary to inhibit the continued high level expression of the heat shock genes during the adaptation stage of the heat shock response (Ueshima *et al.*, 1989; Camacho-Carranza *et al.*, 1995; Ogata *et al.*, 1996). As noted above, the temperature sensitivity of *relA* mutants is not due to their inability to express the heat shock proteins. In fact, the results from DNA array analysis indicated that after 30 minutes of heat shock, the levels of the heat shock proteins DnaJ, DnaK and GrpE were higher in the *relA* mutant than in the isogenic wild-type strain. Therefore, it is possible that the adaptation stage of the heat shock response is defective in *relA* mutants and that the abnormal high level of the heat shock proteins may be the result of their inability to restore normal negative supercoiling of DNA. If so, the continued abnormally high expression of heat shock proteins

may prevent the bacteria from recovering from heat shock, and this could explain the temperature-sensitive phenotype. These ideas are worth continued investigation.

The apparent role of (p)ppGpp in restoring negative supercoiling of DNA is far from clear. DNA is slightly relaxed during glucose starvation but not during amino acid starvation of wild type bacteria (Ohlsen and Gralla, 1992). The latter observation has been confirmed in this study. Since both of these conditions result in accumulation of (p)ppGpp, it would appear that (p)ppGpp does not directly influence DNA supercoiling.

There is increasing evidence that DNA supercoiling is crucial in facilitating gene expression in response to environmental changes (Pruss and Drlica, 1989). Moreover, a number of environmental conditions including temperature, osmolarity, anaerobiosis, and nutrient deficiency have been reported to alter the level of DNA supercoiling (Balke and Gralla, 1987; Bhriain *et al.*, 1989; Dorman *et al.*, 1989; Ramirez and Villarejo, 1991; Lopez-Garcia and Forterre, 1997), and these conditions, in turn, affect gene expression. As already noted, it appears that the *rpoB* suppressors may not act at the level of restoring negative supercoiling in heat-shocked *relA* mutants. It is possible that they act by rendering RNA polymerase insensitive to the supercoil state of DNA under these conditions to facilitate the expression of certain key genes necessary for thermoresistance.

The ability of low concentrations of novobiocin to suppress the temperature-sensitive growth of the *relA* mutant deserves further comment. It

was shown in Fig. 3.15 that negative supercoiling of DNA was partially restored at high temperature by novobiocin. This seems to be contradictory since, as already noted, novobiocin normally inhibits the formation of negative supercoiling. This result is most likely related to the observation that the expression of both the DNA gyrase A and B genes in *E.coli* are induced by low concentrations of novobiocin or coumermycin A1, two specific inhibitors of gyrase B (Menzel and Gellert, 1983). The rates of syntheses of DNA gyrase A and B may be increased up to10- folds by such treatments, and partial restoration of negative supercoiling of DNA occurs. Therefore, it is proposed that suppressor activity exhibited by low concentrations of novobiocin were due to the induction of *gyrA* and *gyrB*, the genes encoding DNA gyrase.

Finally, it is relevant to address, once more, the question of why the *relA* mutants are apparently vulnerable to the relaxation of DNA supercoiling taking into account the involvement of RNA polymerase and DNA gyrase in the process. The actions of RNA polymerase and DNA gyrase are known to be interrelated. On the one hand, negatively supercoiled DNA introduced by DNA gyrase lowers the energy barrier for melting the DNA duplex, and this possibly facilitates transcription by RNA polymerase. On the other hand, DNA gyrase is required for removing the positive supercoiling generated by RNA polymerase during the elongation of transcripts (Liu and Wang, 1987). There are reports that substantiate a link between RNA polymeràse and DNA gyrase. For example, in *Salmonella typhimurium*, *rpoB* mutants have been obtained by selecting for low-level resistance to DNA gyrase inhibitors (Blanc-Potard *et al.*, 1995). It has also

been reported that some rifampicin-resistant mutations make *E.coli* slightly resistant to DNA gyrase B inhibitors coumermycin A1 (Mirkin *et al.*, 1979). Furthermore, one class of rifampicin-resistant alleles suppressed a temperature sensitive allele, *gyrB41,* in *E. coli* (Filutowicz and Jonczyk, 1983). One result even suggests that RNA polymerase is a determinant of DNA supercoiling because mutations in *rpoB* could partially restore the relaxation of negative supercoiled DNA caused by a mutation in *rho* in *E. coli* (Arnold and Tessman, 1988). It is noted in this report that the hypersensitivity to novobiocin of a *relA* mutant could be completely suppressed by certain mutations in *rpoB*. Therefore, the *relA*-mediated temperature-sensitive phenotype suggests a relationship between ppGpp, RNA polymerase, and DNA gyrase. On the basis of the collective observations, there is an indication that the temperature sensitivity is associated with a defect in negative supercoiling, and my preliminary hypothesis is that when DNA negative supercoiling is lower than normal, ppGpp bound to RNA polymerase may be required for transcription of key, as yet unidentified, genes.

Chapter 4: Functional studies on the C-terminal domain of RelA

Results

Construction of plasmids

Fig 4.1 summarizes the construction of plasmids used in this study. A DNA fragment encoding the C-terminal 289-amino acid portion of RelA (designated 'RelA) was obtained from plasmid pALS10 (Svitil *et al.*, 1993) by digestion with *Pst*I and *Bam*HI. This DNA fragment was subcloned into the vector pBCSK(-) digested with the same restriction enzymes, and the result was plasmid pXY36. Plasmid pXY37 was constructed by subcloning an *Eco*RI-*Not*I fragment containing '*relA* from pXY36 into the vector, pGEX-5X-1. This gave rise to a GST-'RelA fusion protein that was expressed from a P*tac* promoter. The '*relA* fragment from plasmid pXY36, obtained by digestion with *Eco*RI and *Not*I, was also subcloned into the expression vector pET-30a (+). The resulting plasmid, pXY38, encoded 'RelA carrying an N-terminal His-tag and S-tag fusion to facilitate affinity purification and detection of the recombinant protein. As described in Chapter 2, 'RelA was readily expressed and purified in high yield. The yeast two-hybrid plasmids, pXY62 and pXY63, were constructed by subcloning the *Eco*RI-*Bam*HI fragment from pXY36 containing '*relA* into the *Eco*RI-*Bam*HI sites of pGBKT7 and pGADT7 respectively. As a result, the '*relA* gene was fused to the sequences encoding yeast GAL4 DNA-binding domain (DB) and the GAL4 activator domain (AD) in plasmids pXY62 and pXY63, respectively.

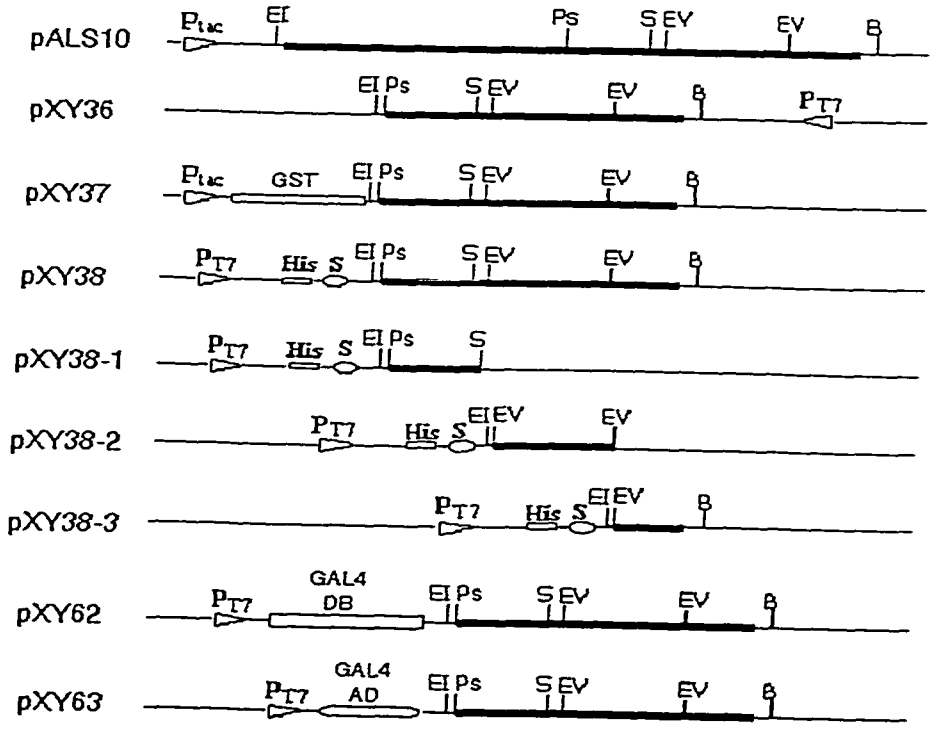

Fig. 4.1. Relevant properties of plasmids containing derivatives of *relA* gene
The *relA* gene and its fragment are shown as a solid box. The plasmid pALS10 contains the intact *relA* gene. The '*relA* gene encoding the 289-amino-acid C-terminal portion of RelA is from *Pst*I site downstream to the end. Abbreviations of restriction enzymes are: B: *BamH*I; EI: *EcoR*I; EV: *EcoR*V; S: *Sal*I and Ps: *Pst*I. Only those enzymes that were involved in the construction of plasmids are shown.

In further functional analyses, the *'relA* gene was digested into three pieces by taking advantage of the restriction endonuclease sites within the gene. Fragment 1 was an *Eco*RI-*Sal*I fragment representing 248 bp of the 5'-end of *'relA*. Fragment 2 is an internal 396-bp segment of *'relA* located between two *Eco*RV sites. Fragment 3 is the 313-bp 3'-end of *'relA* located between *Eco*RV and *Bam*HI sites. The *Eco*RI-*Sal*I Fragment 1 was subcloned into pET30(a) digested with same enzymes to yield plasmid pXY38-1. Fragments 2 and 3 were first subcloned into the vector pBCSK(-) and then subsequently moved into the vector pET30(a) to generate plasmids pXY38-2 and pXY38-3 respectively. The orientation of Fragment 2 was verified by Western blot analysis of the expressed protein using anti-'RelA antibody. The three fragments of *'relA* were also cloned into the yeast two-hybrid system vector pGBKT7. To accomplish this, plasmids pXY38-1, pXY38-2, and pXY38-3 were digested with *Eco*RI and *Sal*I, and the resulting frangments were subcloned into plasmid pGBKT7 digested with the same enzymes to generate pXY62-1, pXY62-2, and pXY62-3, respectively.

C-terminus of RelA contains the domain responsible for the ribosome-binding

RelA is known to be a ribosome-associated protein. To determine whether 'RelA was ribosome-associated the following experiment was performed. Ribosomes purified from various strains were analyzed by SDS-PAGE and Western blotting using anti-'RelA antibody. As expected, RelA was not detected in the ribosomes prepared from *relA* null mutant, VC6129 (Fig. 4.2, lane1).

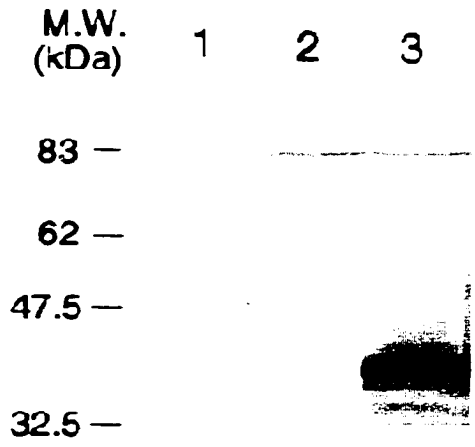

Fig. 4.2. The C-terminus of RelA ('RelA) binds to the ribosome *in vivo*
Purified ribosomes from VC6129 (lane 1), VC6216 (lane 2) and VC6216/pXY38 (lane 3) were analyzed by SDS-PAGE and Western Blotting using anti-'RelA antibody.

A faint band corresponding to RelA, and reflecting the low abundance of this protein, was detected in the ribosomes from the wild-type strain, VC6216 (Fig 4.2, lane 2). Bands corresponding to both RelA and 'RelA occurred in the case of VC6216 carrying plasmid pXY38 (lane 3), indicating that 'RelA contains the ribosome-binding domain of RelA.

Relaxation of the stringent response by overexpression of 'RelA

The effect of 'RelA overexpression on the synthesis of ppGpp was determined in strain VC6216 carrying pXY38. Bacteria were pulse-labeled with [^{32}P]-phosphate, and formic extracts were analyzed by thin layer chromatography followed by autoradiography. As shown in Fig. 4.3, an untreated culture of VC6216/pXY38 as well as one that received IPTG to induce the synthesis of 'RelA showed little or no accumulation of ppGpp (lanes a and c). In contrast, amino acid deprivation caused a large accumulation of ppGpp (lane b), suggesting that VC6216/pXY38 exhibited a typical stringent response. However, the overexpression of 'RelA, induced by IPTG, inhibited the accumulation of ppGpp (lane d). To verify these effects, the synthesis of stable RNA, as measured by the incorporation of [^{3}H]uracil, was determined in these cultures. Fig. 4.4 confirms that VC6216/pXY38 exhibited a normal stringent response in that amino acid deprivation caused an inhibition of stable RNA accumulation. The induction of 'RelA overexpression by IPTG treatment of growing bacteria did not affect normal RNA synthesis. However, RNA synthesis was relaxed during IPTG induction of amino acid-deprived cells. These results confirm that the

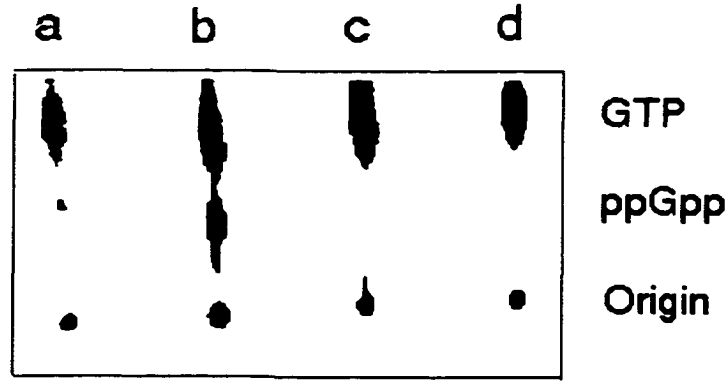

Fig. 4.3. Inhibition of ppGpp accumulation by the overexpression of 'RelA
Formic acid extracts of [^{32}P]-labeled strain VC6216 carrying plasmid pXY38 were fractionated by thin-layer chromatography. Lane a, no treatment; b, amino acid-deprived; c, IPTG induction of 'RelA; and d, amino acid-deprived and IPTG induction of 'RelA.

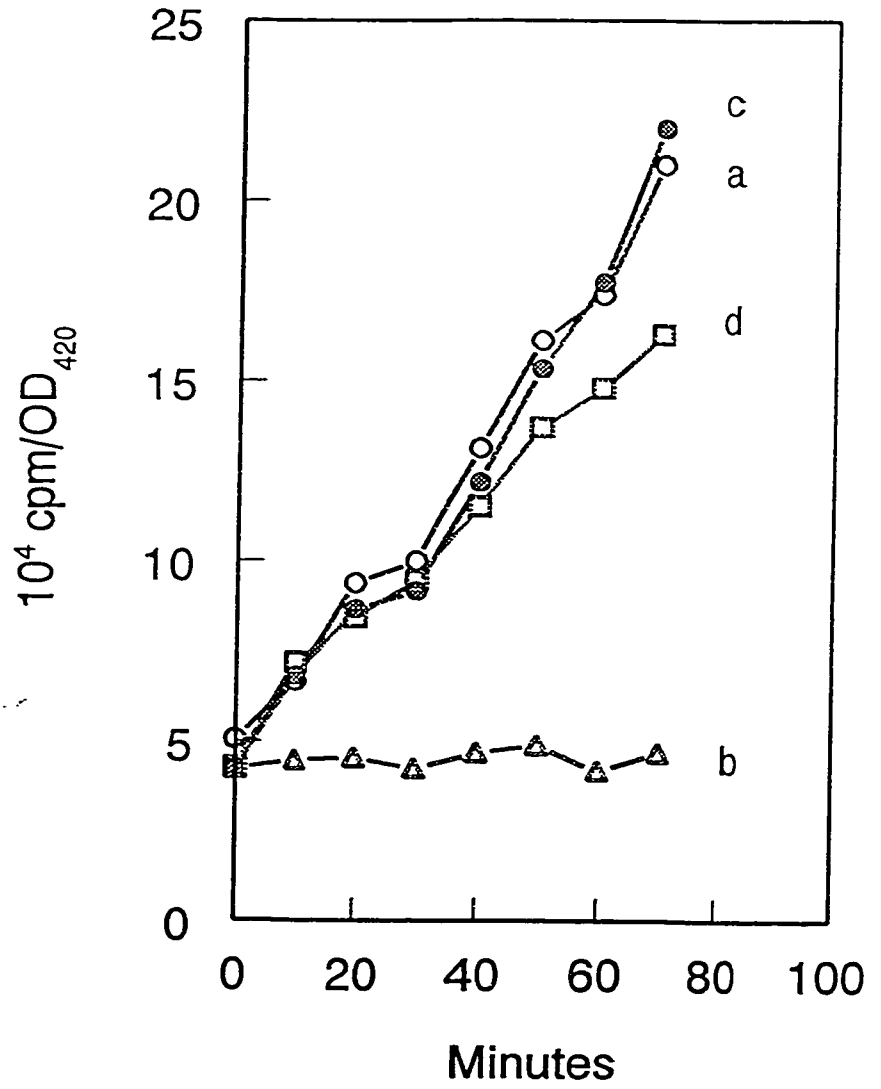

Fig. 4.4. Effect of overexpression of 'RelA on the incorporation of [^{3}H]uracil into stable RNA by *E.coli* strain VC6216 carring the plasmid pXY38

Culture a (○) represents an untreated control. Culture b (△) was amino acid-deprived with 500μg of L-valine per ml. Culture c (●) was treated with 0.5mM of IPTG. Culture d (□) was amino acid-deprived after overexpression of 'RelA from pXY41.

overexpression of 'RelA relaxed the stringent response. The overexpressed 'RelA was localized in both the ribosomal and cytoplasmic fractions of cells. On the other hand, the wild type RelA in these bacteria was not detected in either fraction (data not shown). It should be noted that the cytoplasmic fraction was dilute, and, considering the inherent low copy of RelA, its detection would have been difficult under these conditions. On the other hand, this should not have been the case with the concentrated ribosomal fraction.

Displacement of ribosome-bound RelA by 'RelA *in vitro*

The above results suggested that the overexpression of 'RelA caused the displacement of wild type RelA from ribosomes *in vivo*. The ribosome-binding activity of 'RelA was therefore examined *in vitro*. The overexpression of RelA was induced in strain W3110 carrying plasmid pALS10. Under these conditions, the ribosomes prepared from the culture were saturated with wild type RelA. The purified ribosomes were incubated with different amounts of purified 'RelA. The ribosomes in the mixture were then repurified from the incubation mixtures, and the amounts of RelA and 'RelA in the ribosome pellets and the supernatant fractions were examined by immunoblotting. As shown in Fig 4.5, the amounts of ribosome-associated wild type RelA decreased as the concentration of 'RelA in the incubation mixture was increased. At the same time, there were increases in the amounts of RelA in the supernatants and 'RelA in the ribosome pellets. These results suggest that free 'RelA could displace ribosome-associated RelA *in vitro*.

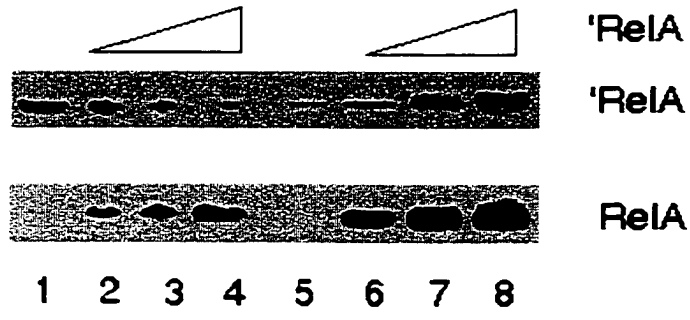

Fig. 4.5. Displacement of ribosome-bound RelA by 'RelA *in vitro*

RelA saturated ribosomes from W3110 were incubated in the absence (lane 1 and 5) or with increasing amounts of purified 'RelA. After ribosomes were repurified, the wild type RelA and 'RelA in the ribosome pellets (lane 1 to 4) and in the supernatants (lane 5 to 8) were measured by immunoblotting using anti-'RelA antibody.

Analysis of 'RelA domains involved in ribosome-binding

As described above, the *'relA* gene was subcloned in 3 segments. The ribosome-binding activities of the peptides encoded by these segments, designated Fragments 1, 2, and 3, were assayed as shown in Fig. 4.6. Cultures of *E. coli* strain VC6216 carrying plasmids pXY38, pXY38-1, pXY38-2, and pXY38-3 were fractionated into ribosome and cytoplasmic fractions. The fractions were analyzed in Western blots probed with anti-S-tag antibody. 'RelA was primarily localized on ribosomes (lane 1), but the excess overexpressed 'RelA was localized in the cytoplasm (lane 5). The peptides from Fragments 1 and 3 were not detected in the ribosome fractions (lanes 2 and 4, respectively) but were localized in the cytoplasm (lanes 6 and 8, respectively). The peptide from Fragment 2 was the only one found on ribosomes (lane 3), but most of this peptide was still localized in the cytoplasm (lane 7). These results indicate the dominant ribosome-binding domain of RelA is located between amino acid residues 550 to 682. On the other hand, other areas of 'RelA may be involved since the binding of this peptide was apparently incomplete.

The ribosome-binding activities of 'RelA were also assessed by determining the effects of the overexpressed Fragments 1, 2, and 3 on the stringent response as measured by the incorporation of [^{3}H]uracil into stable RNA. Fig. 4.7 shows that the overexpression of 'RelA relaxed the stringent response, confirming the result shown in Fig. 4.4. The overexpression of 'RelA Fragment 2 from pXY38-2 also relaxed RNA synthesis although not as effectively as 'RelA. This result is consistent with the observation that Fragment 2 binds to

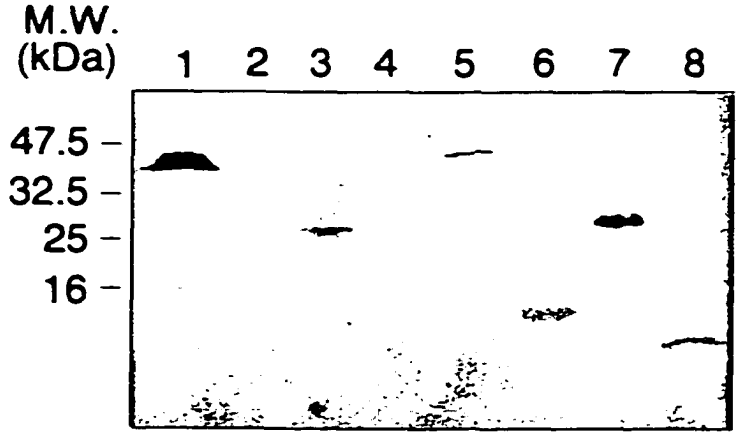

Fig.4.6. Cellular location of three 'RelA segments

Cultures of VC6216 containing pXY38 (lane 1and 5), pXY38-1 (lane 2 and 6), pXY38-2 (lane 3 and 7) or pXY38-3 (lane 4 and 8) were grown in LB at 30°C. Ribosome pellets (lane 1 to 4) and supernatants (lane 5 to 8) from cellular extracts were detected by anti-S-tag antibody

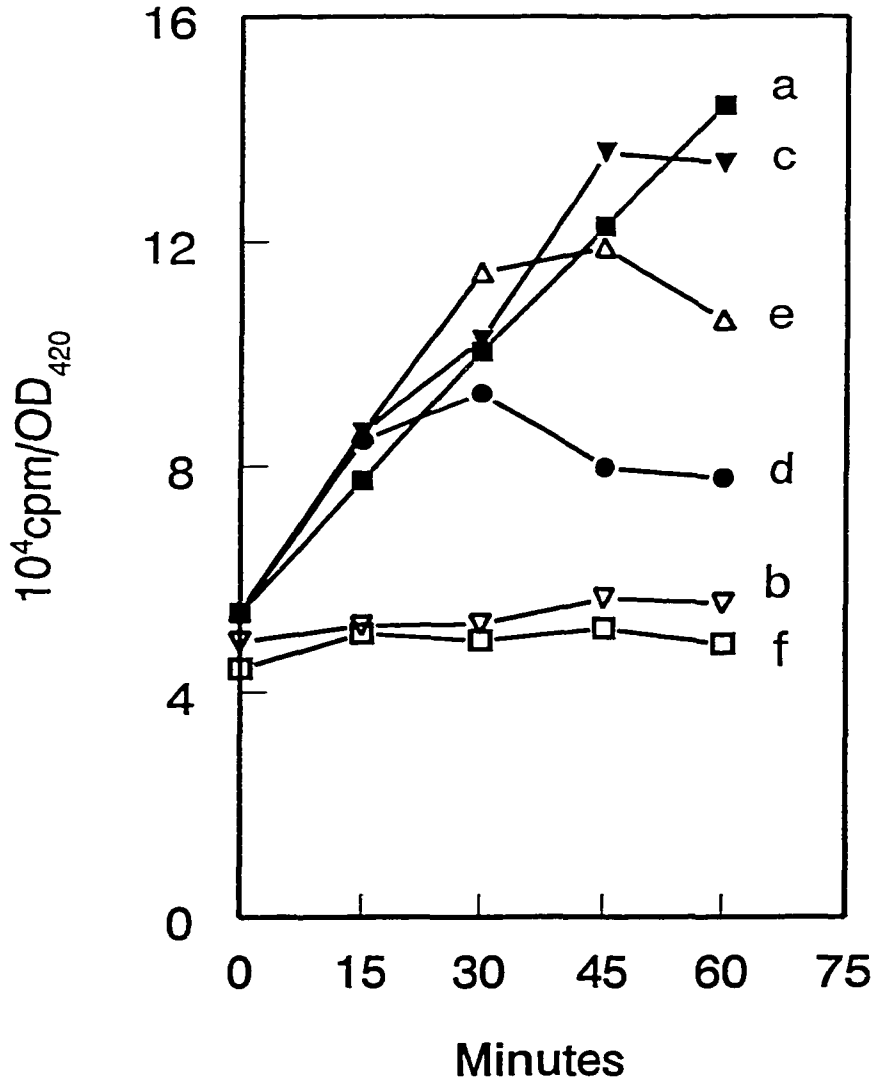

Fig. 4.7. Effect of overexpression of three 'RelA segments on the incorporation of [^{3}H]uracil into stable RNA

Culture a (■) represents an untreated control. The other cultures were amino acid-deprived by the addition of 500 µg of L-valine per ml. Culture b (▽) was treated with amino acid deprivation alone. Culture c (▼) was amino acid deprived after overexpression of 'RelA from pXY38. Culture d (●) was amino acid-deprived after overexpression of Fragment 1 from pXY38-1. Culture e (△) was amino acid-deprived after overexpression of Fragment 2 from pXY38-2. Culture f (□) was amino acid-deprived after overexpression of Fragment 3 from pXY38-3.

ribosomes weakly. The overexpression of Fragment 1 from pXY38-1 partially relaxed the stringent response and was much less effective than Fragment 2 in this regard. This suggests that Fragment 2 may contain information required for ribosome-binding. Fragment 3 had not effect on the stringent response and is apparently not involved in ribosome-binding.

Dimerization of RelA as demonstrated by yeast two-hybrid analysis

The yeast two-hybrid system was employed to search for proteins that interact with RelA. For this purpose, plasmid pXY62, encoding 'RelA, was used as bait to screen an *E. coli* library. After eliminating the false-positive clones, a total of 26 positive clones were obtained. The plasmids from these clones were analyzed and divided into 3 groups according to the lengths of their inserts. Three plasmids, one from each group, were selected at random for detailed study. In all 3 cases, the sequences of the inserts revealed that they were derived from the *relA* gene. These results suggested that RelA interacts with itself.

The interaction of RelA with itself was tested further in a yeast two-hybrid assay. The '*relA* gene fragment was fused in-frame to the transcriptional activation domain of GAL4 in pGADT7 to generate the plasmid, pXY63 (Fig 4.1). The yeast strain, AH109, carrying a combination of the control plasmids, pGADT7-T and pGBKT7-53, was able to express the Ade2, His3, and *lacZ* reporter genes whereas AH109 carrying the vectors, pGADT7 and pGBKT7, did not express the reporters. When a combination of either pXY62 and pGADT7 or

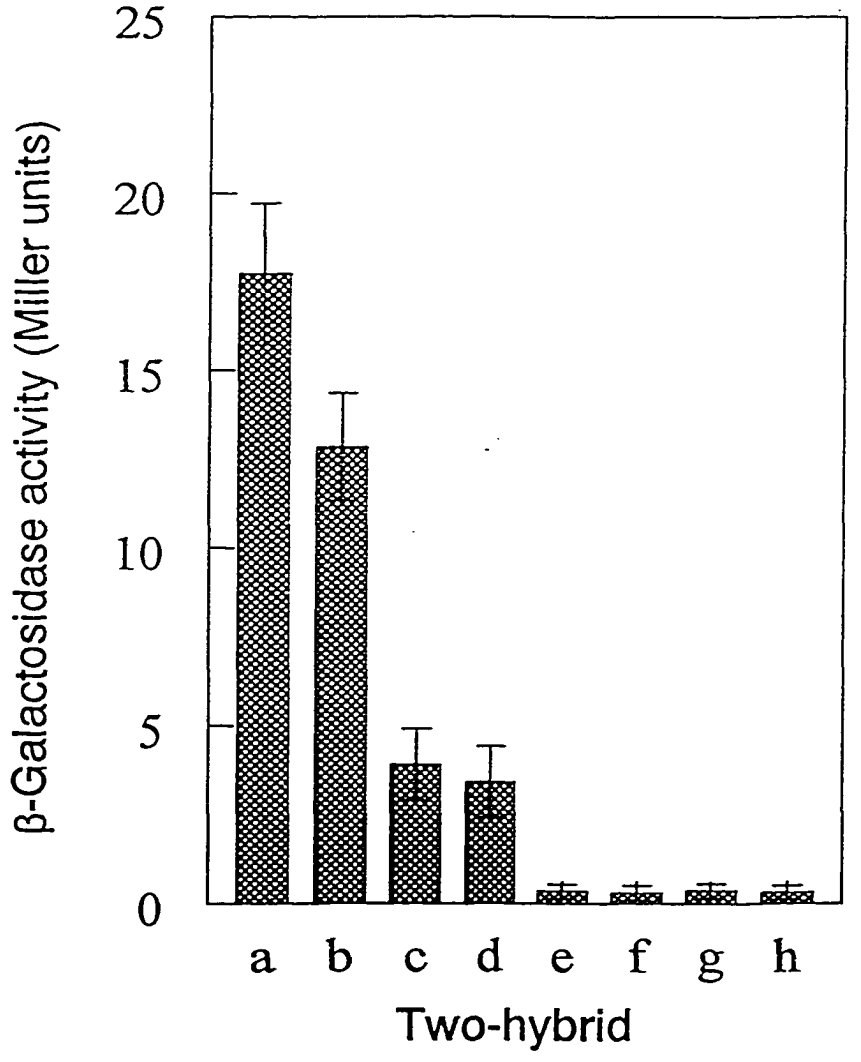

Fig. 4.8. Identification of dimerization domain of 'RelA by yeast two-hybrid system
Yeast strain AH109 transformed with the plasmids (a) pGBKT7-53 and pGADT7-T (positive control), (b) pXY62 and pXY63, (c) pXY62-1 and pXY63, (d) pXY62-1 and pXY63, (e) pXY62-3 and pXY63, (f) pXY62 and pGADT7, (g) pGBKT7 and pXY63, and (h) pGDKT7 and pGADT7 (negative control) grown to mid-log phase in SD media lacking leucine and tryptophan.Extracts were prepared and assayed for β-galactosidase activity, using ONPG as substrate. Values are averages of 3 determinations.

pXY63 and pGBKT7 were used, the yeast two-hybrid assays were negative. However, the combination of pXY62 and pXY63 resulted in the expression of Ade2, His3, and *lacZ*, confirming that 'RelA dimerizes *in vivo*. The quantification of β-galactosidase activities in yeast cells carrying various combinations of plasmids indicated that the interaction of 'RelA with itself was relatively strong (Fig. 4.8, compare lanes a and b).

Copurification of wild-type RelA and 'RelA.

The following experiment was designed to demonstrate the copurification of RelA and 'RelA. *E. coli* BL21(DE3) carrying a combination of plasmids pALS10 and pXY38 which contain the entire *relA* gene and the His tag-'*relA* gene, respectively, was used. RelA and His tag-'RelA were coexpressed, and the His tag-'RelA protein in the cell extract was then purified by Ni^{++} affinity chromatography. The same procedure was applied to BL21 (DE3) carrying only pALS10 as a control. The material specifically eluted from the Ni^{++} affinity columns in the 2 experiments were analyzed by SDS-PAGE. As shown in lane 1 of Fig 4.9A, no protein was detected in the case of BL21(DE3) expressing only pALS10. In contrast, two proteins were copurified from extracts of BL21(DE3) expressing both pALS10 and pXY38 (lane 2). The major protein corresponded to His-tag'RelA, and the minor protein had a molecular mass of 83 kDa which was consistent with that of RelA. The two proteins were analyzed by Western blotting using polyclonal antiserum specific for 'RelA. Fig. 4.9B shows that both proteins were recognized by anti-'RelA (lane 2). Moreover, the 83 kDa protein

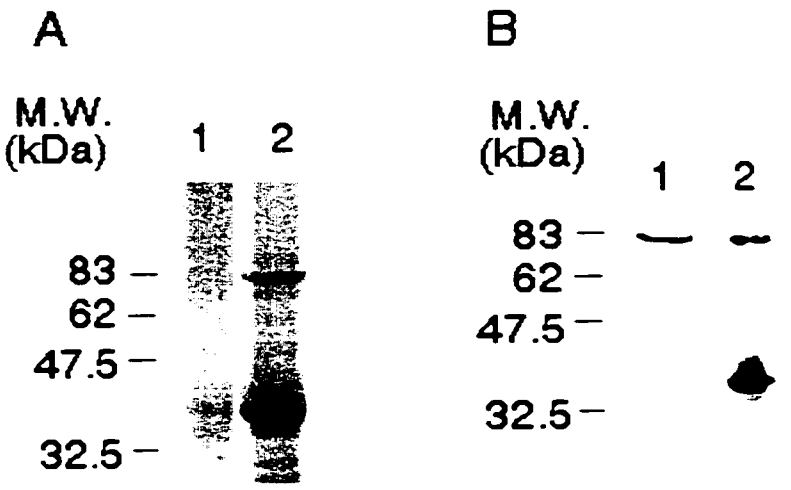

Fig. 4.9. Copurification of wild type RelA with His-tag-'RelA

Cultures of *E.coli* BL21(DE3) carrying pALS10 alone or in combination with pXY38 were induced with IPTG. Extracts prepared from the cells were subjected to N^{++} affinity chromatography. A: Coomassie blue-stained SDS-PAGE gel. Lane 1: extract from cells overexpressing RelA alone; Lane 2: extract from cells overexpressing both RelA and His-tag-'RelA. B. Western blot probed with anti-'RelA antibody. Lane 1: purified wild type RelA (control). Lane 2: copurified RelA and His tag-'RelA.

co-migrated with the purified RelA protein control (lane 1). These results indicated that 'RelA interacted with RelA *in vivo*. The predominance of 'RelA in this sample may reflect differences in the relative amounts of 'RelA and RelA in the extract, with 'RelA being more abundant.

Dimerization of 'RelA *in vitro*.

In order to examine the dimerization of 'RelA *in vitro*, purified 'RelA protein was separated by electrophoresis on nondenaturing polyacrylamide gels. As shown in Fig. 4.10, a sample that was boiled for 5 min to denature the protein before it was analyzed contained a single major protein band (lane b). In contrast, the major band in the native sample migrated much slower (lane a). This band represented a multimeric form of 'RelA, but it was not possible to determine whether it was a dimer by this method. 'RelA was conclusively shown to form dimers by analysis of glutaraldehyde-crosslinked 'RelA as shown in Fig. 4.11.

Analysis of 'RelA interactions by affinity blotting assay

An affinity blotting assay was employed in an attempt to identify other proteins that could interact with 'RelA. Total cellular extracts from various bacterial strains were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was then probed with the purified 'RelA followed by detection of the 'RelA probe with anti-S-tag antibody. As shown in Fig. 4.12, 'RelA did not bind to any proteins in the extract from

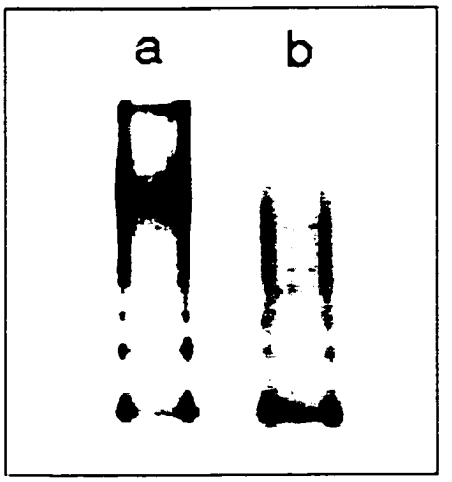

Fig. 4.10. Analysis of purified 'RelA by nondenaturing gel

Purified His-tag-'RelA, without treatment (a) or boiled for 5 min (b), was separated by nondenaturing polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue.

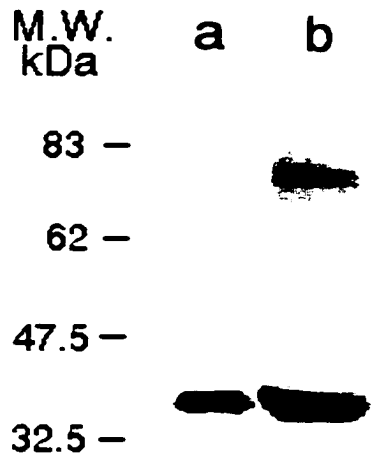

Fig. 4.11. Glutaraldehyde crosslinking of purified 'RelA

Purified His-tag-'RelA received no treatment (a) or 0.2% glutaraldehyde for 15 min (b). The samples were analyzed by SDS-PAGE and immunoblotting.

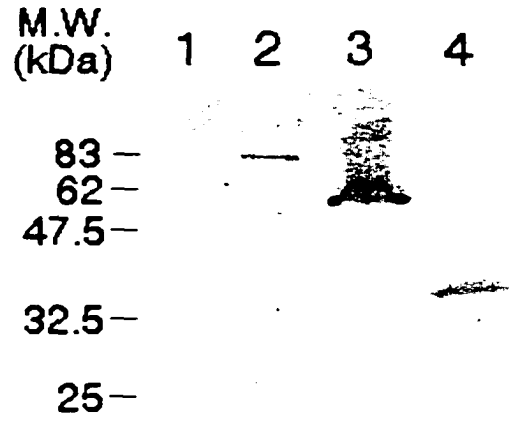

Fig. 4.12. Affinity blotting assay of the interaction between RelA and 'RelA
Cellular extracts from BL21(DE3)*ΔrelA* (lane 1), BL21(DE3)/pALS10 (lane 3) and BL21(DE3)/pXY37 (lane 3) were transferred to nitrocellulose member after the proteins were separated by SDS-PAGE. The membrane was probed with His-tag-'RelA followed by detection with anti-S-tag antibody. As a control, lane 4 was cellular extracts from strain BL21(DE3)/pXY38.

BL21(DE3)*ΔrelA* (lane 1). 'RelA bound a component in BL21(DE3)/pALS10 with a molecular mass of 83 kDa (lane 2). 'RelA also bound to components with molecular masses of 60 kDa and 37 kDa in strain BL21(DE3) carrying plasmids pXY37 (lane 3) and pXY38 (lane 4), respectively. The molecular masses of the components bound by 'RelA in lanes 2, 3, and 4 are consistent with RelA, GST-'RelA, and His tag-'RelA, respectively. These results confirm that 'RelA forms homodimers. Moreover, 'RelA apparently did not bind to other components by this method.

Analysis of 'RelA domains involved in dimerization

The yeast two-hybrid system was used to analyze the domains of 'RelA that are involved dimerization. Derivatives of *S. cerevisiae* AH109 carrying combinations of pXY63 ('RelA fused to GAL4 AD element) and either pXY62-1, pXY62-2 or pXY62-3 were constructed. The latter 3 plasmids contain different Fragments 1, 2, and 3 of *'relA* fused to the GAL4 DB element. The interactions were determined by assaying the strains for β-galactosidase activity as shown in Fig.4.8. Fragments 1 (Fig. 4.8, c) and 2 (Fig. 4.8, d) interacted with 'RelA whereas Fragment 3 (Fig. 4.8, e) did not. The relative strengths of the interactions between Fragments 1 and 2 with 'RelA were approximately equal, but they were only about 30% of the degree of interaction between 'RelA and 'RelA (Fig. 4.8, b). Therefore, the dimerization domain overlapped Fragments 1 and 2.

Discussion

RelA has long been known to be ribosome-associated, and the original objective of this study was to determine the basis of this association. The ribosome association of RelA is clearly an important regulatory factor because the truncated 455-amino acid N-terminus of RelA lacking the ribosome-binding domain exhibits a constitutive ppGpp synthetase activity (Schreiber *et al.*, 1991). Moreover, the purified ribosome-free RelA protein is normally inactive (Sy and Lipmann, 1973).

The 3'-end of *relA* encoding the C-terminus beginning at amino residue 455 ('RelA) was cloned and characterized. 'RelA not only bound to ribosomes, but its overexpression relaxed the stringent response. This phenomenon was related to the absence of detectable RelA in the ribosomal fraction of 'RelA-overexpressing cells. The fate of the original ribosome-associated RelA is uncertain, but it was clear that RelA could not be detected on ribosomes. The *in vitro* ribosome-binding experiment suggests that the ribosome-bound RelA could be displaced by 'RelA, but the basis for this is not understood and requires further study. After these experiments were completed, I became aware of the unpublished data of Glaser (cited by Cashel *et al.*, 1996). Glaser has also shown that the overexpression of the C-terminal portion of RelA relaxes the stringent response.

Initially, the yeast two-hybrid system was used to screen for proteins that interact with 'RelA. Only one interacting protein, identified as RelA by DNA

sequencing, was discovered. The absence of ribosomal proteins was unexpected, and this approach did not yield clues to the nature of the ribosome-RelA interaction. The reason for this is unclear. For example, it is possible that the library was incomplete. Several independent lines of evidence, *e.g.*, copurification, nondenaturing gel electrophoretic analysis, *etc.*, showed unequivocally that 'RelA interacts with itself both *in vitro* as well as *in vivo*. The yeast two-hybrid analysis indicated that the 'RelA interaction was very strong. Glutaraldehyde cross-linking indicated that the product of this interaction was a dimer. At present, it is not known whether dimerization is a prerequisite to ribosome-binding. However, there are interesting consequences if this was the case. For example, the current estimate of the proportion of ribosomes bearing RelA (1%) is based on the assumption that RelA behaves as a monomer . Moreover, this would double the mass of the (p)ppGpp synthetase bound to the ribosome. Consequently, the strategic nature of the proposed location of RelA on the ribosome (see Chapter 5), raises questions about whether the presence RelA may influence ribosome function. It is noteworthy that the affinity blotting experiment, with 'RelA as a probe, revealed only 'RelA and RelA as proteins capable of binding 'RelA, but, again, no evidence for interaction with ribosomal proteins was obtained.

Preliminary attempts have been made to further localize the ribosome-binding and dimerization domains on 'RelA. The dimerization domain was localized to two segments of 'RelA covering amino acid residues 455 to 682. The main ribosome-binding domain was located in one of these two segments,

extending from amino acid 560 to 682. However, this fragment did not bind to ribosomes as efficiently as 'RelA, suggesting that additional sequences were required. Although it was not possible to demonstrate direct ribosome-binding of Fragment 1, which covers amino acids 455 to 559, it is likely that the additional information for ribosome-binding resides in this segment because the overexpression of this peptide relaxed the stringent response.

In summary, the C-terminus of RelA was shown to contain information for ribosome-binding as well as for the formation of homodimers. The domains for these two functions overlap and cover the regions spanning amino acids 455 to 682.

Chapter 5: Involvement of the N-terminus of Ribosomal Protein L11 in the Regulation of the RelA Protein of *Escherichia coli*

Results

Cloning and characterization of the *E.coli rplK* and '*rplK* genes

Fig. 5.1 summarizes the relevant features of recombinant plasmids used in this study. Plasmid pXY51 was derivative of of the vector, pET30c(+), containing the complete *rplK* gene which was PCR-amplified from *E. coli* genomic DNA.

A derivative of *rplK* containing a deletion that removed the first 36 amino acids on the N-terminus of L11 was constructed by by subcloning an *Eco*RI-*Sal*I fragment from plasmid pXY51 into pET30a(+) to generate plasmid pXY41. The subcloned gene and its product are designated '*rplK* and 'L11, respectively.

Fig 5.2A shows that the expression of *rplK* in pXY51 yielded a protein of approximately 21 kDa which is consistent with the expected molecular weight of L11 fused to His-tag and S-tag sequences (lanes 4 and 5). When '*rplK* was expressed from pXY41, a protein of 17 kDa was produced (Fig. 5.2A, lanes 1 and 2). Both 'L11 and L11 were readily purified by affinity chromatography (lanes 3 and 6, respectively). The identities of these proteins were verified in Western blots developed with anti-S-tag antibodies (Fig. 5.2B). The Western blot indicated that both *rplK* and '*rplK* genes were expressed at low levels in the absence of IPTG (lanes 1 and 4).

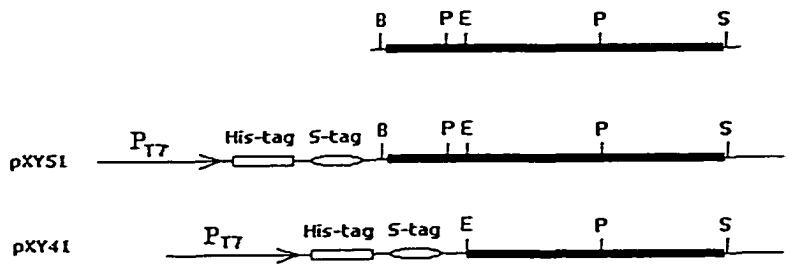

Fig. 5.1. Relevant properties of plasmids pXY41 and pXY51

The complete *rplK* gene encoding ribosomal protein L11 is represented by the solid box at the top. Plasmid pXY51 is a derivative of pET30c(+) and contains the complete *rplK* gene. Plasmid pXY41 is a derivative of pET30a(+) and contains a mutant *rplK* gene, designated *'rplK,* with a deletion extending from the 5' terminus to the *Eco*RI site (nucleotide 109). In both cases, the genes were fused to His-tag and S-tag sequences on their 5' ends and were under the control of T7 promoters (these elements are not drawn to scale). Abbreviations: B, *Bam*HI; E, *Eco*RI; S, *Sal*I; P, *Pst*I.

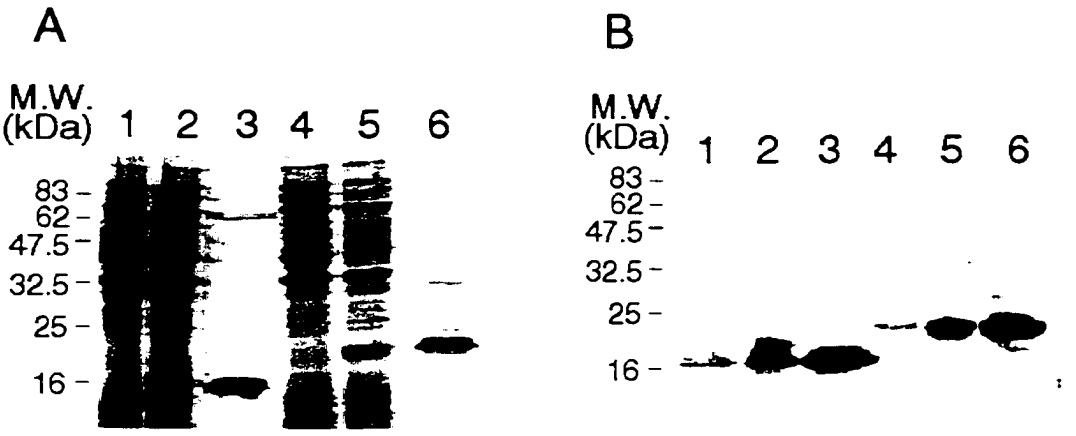

Fig. 5.2. Expression and purification of L11 and 'L11proteins

Panel A represents a Coomassie blue-stained SDS-PAGE gel and panel B is a Western blot developed with anti-S-Tag antibody. Lane 1, crude extract from VC6216/PXY41 uninduced cells; lane 2, crude extract from VC6216/PXY41 induced with 0.5 mM IPTG at 30°C for 2 hr; lane 3, His-tag affinity column purified protein from induced VC6216/pXY41; lane 4, crude extract from VC6216/PXY51 uninduced cells; lane 5, crude extract from VC6216/PXY51 induced with 0.5mM IPTG at 30°C for 2 hr; lane 6, His-tag affinity column purified protein from induced VC6216/pXY51.

Effects of L11 and 'L11 on the stringent response

Fig.5.3 shows the effect of the overproduction of L11 on the incorporation of [^{3}H]uracil into RNA. Strain VC6216 carrying plasmid pXY51 exhibited a normal stringent response. Amino acid deprivation inhibited the accumulation of stable RNA (compare curves a and b). Moreover, RNA synthesis was relaxed when the amino acid-deprived bacteria were treated with chloramphenicol (curve c). The addition of IPTG to induce the *rplK* gene on pXY51 did not affect the stringent response (curve d) and RNA synthesis in growing bacteria (curve e).

Fig.5.4 shows that the stringent response in strain VC6216 carrying plasmid pXY41 was also normal, and RNA synthesis was inhibited during amino acid deprivation (compare curves a and b). When IPTG was added to a growing culture to induce *'rplK*, RNA synthesis was actually stimulated (curve c). Furthermore, RNA synthesis was completely relaxed for at least 80 minutes when the IPTG-treated culture was subjected to amino acid deprivation (curve d). The intracellular levels of ppGpp under these various conditions were examined by thin layer chromatography as shown in Fig.5.5. As expected, a large amount of ppGpp, along with a small amount of pppGpp, accumulated within 10 minutes after the onset of amino acid deprivation (compare lanes a and b). The addition of IPTG to the growing culture actually caused a decrease in the basal level of ppGpp (compare lanes a and c), and this likely explains the stimulatory effect that IPTG had on RNA synthesis in growing bacteria. Finally, the treatment with IPTG significantly inhibited ppGpp accumulation by amino acid-deprived bacteria (compare lanes b and d). These results suggest that the overproduction of 'L11

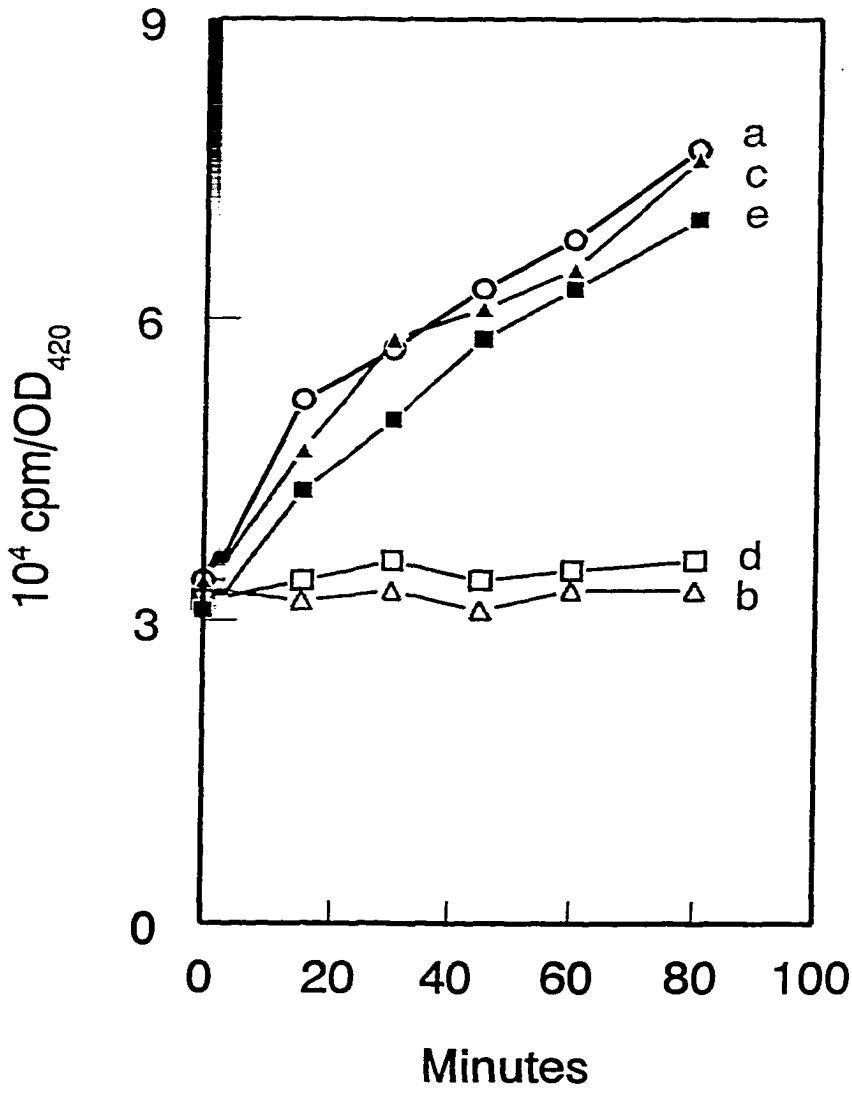

Fig 5.3. Effect of overexpression of wild type L11 on the incorporation of [^{3}H]uracil into stable RNA by strain VC6216 carrying the plasmid pXY51

Culture a (○) was an untreated control. Culture b (△) was amino acid-deprived by the addition of 500μg of serine hydroxamate per ml. Culture c (■) treated with 0.5 mM IPTG to induce L11 expression. Culture d (□) was amino acid-deprived and treated with 0.5 mM IPTG. Culture e (▲) was amino acid-deprived and treated with 100μg/ml of chloramphenicol to relax the stringent response.

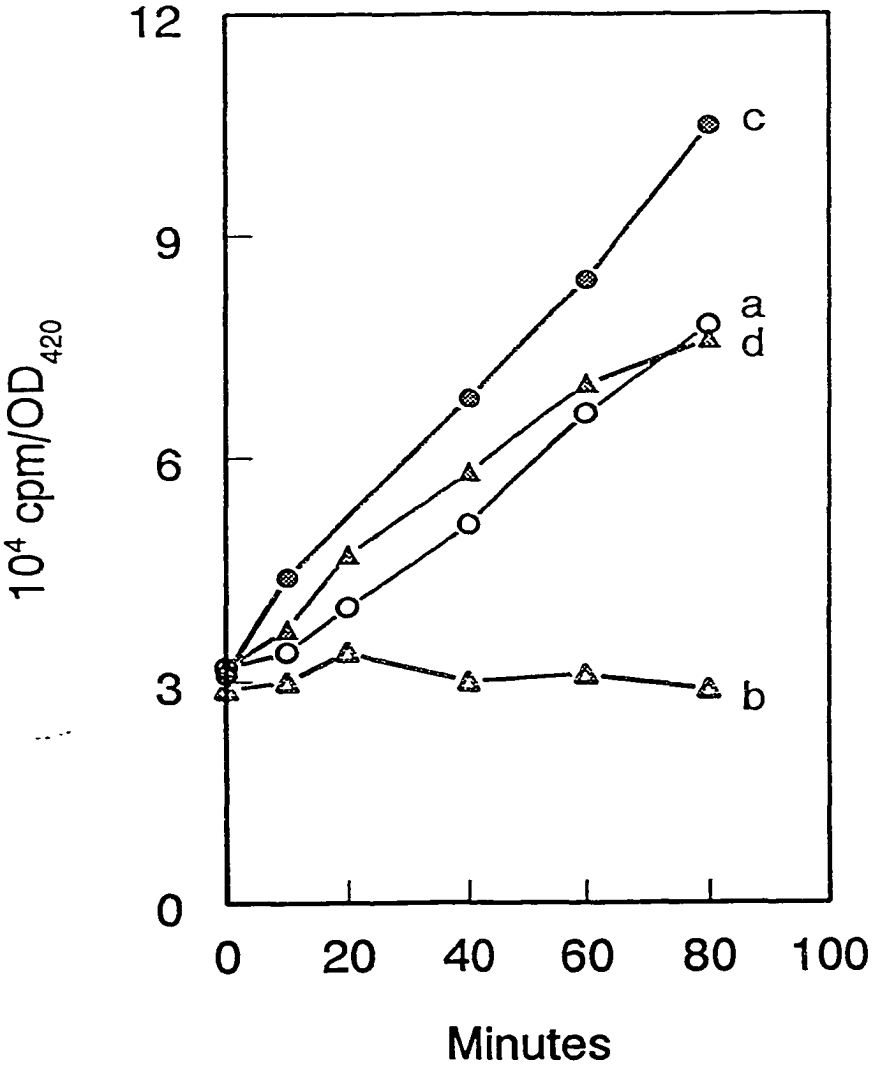

Fig. 5.4. Effect of overexpression of 'L11 on the incorporation of [^{3}H]uracil into stable RNA by strain VC6216 carrying the plasmid pXY41

Culture a (○) was an untreated control. Culture b (△) was amino acid-deprived by adding 500 μg of serine hydroxamate per ml. Culture c (●) was treated with 0.5 mM IPTG to induce 'L11. Culture d (▲) was amino acid-deprived and treated with 0.5 mM IPTG.

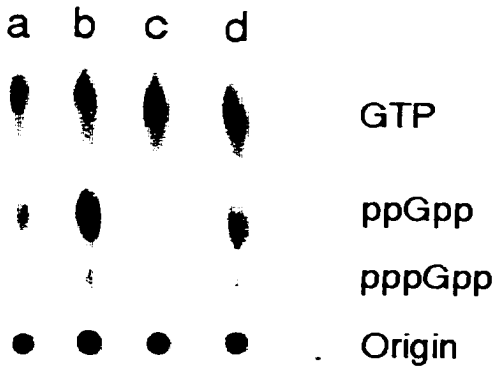

Fig. 5.5. Effect of overexpression of 'L11 on ppGpp accumulation
Formic acid extracts of [^{32}P]-labeled strain VC6216 carrying plasmid pXY41 were fractionated by thin-layer chromatography. Lanes a, no treatment; b, amino acid-deprived; c, IPTG induction of 'L11; and d, amino acid-deprived and IPTG induction of 'L11.

interferes with the activity of RelA. It is interesting that the intact L11 protein displayed no such effect. Therefore, somewhere in the 36-amino acid N-terminal portion that was deleted to create 'L11 is essential for RelA activation.

Incorporation of L11 and 'L11 into ribosomes

Ribosomes were purified from strain VC6216 carrying either pXY41or pXY51. They were used to confirm that the recombinant L11 protein was incorporated into ribosomes and to determine whether the N-terminal deletion in 'L11 prevented this from happening. The ribosomal proteins from these preparations were analyzed in Western blots using anti-S-Tag antibodies to detect the recombinant L11 proteins. Both L11 and 'L11 were readily detected (data not shown; see Fig. 5.6A). These results indicate that the N-terminal deletion in L11 did not affect its incorporation into ribosomes.

Effect of 'L11 on RelA-ribosome interaction

The effect of 'L11 on the interaction of RelA with ribosomes was investigated. Cultures of strain VC6216 carrying pXY41 were induced with concentrations of IPTG ranging from 0 to 100 μM for 30 min. Ribosomes prepared from these cultures were then analyzed for 'L11 and RelA by Western blotting. As shown in Fig. 5.6A, increasing amounts of 'L11 were incorporated into ribosomes with increasing concentrations of IPTG. On the other hand, no differences were observed in the amounts of RelA bound to these ribosomes

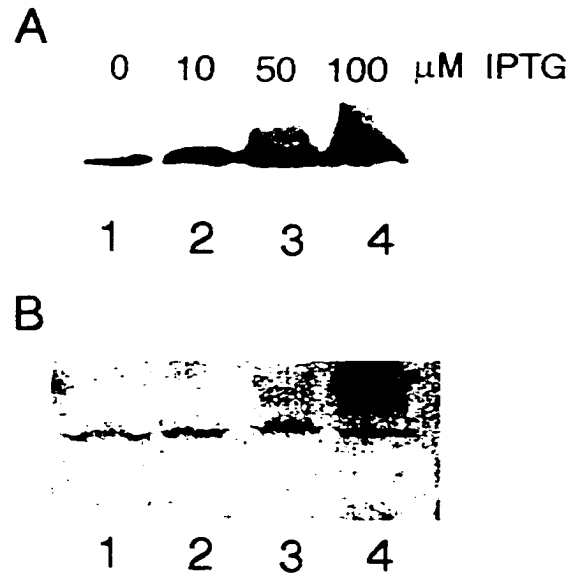

Fig. 5.6. Effect of 'L11 on RelA-ribosome interaction

(A) Incorporation of 'L11 into ribosomes. Cultures of strain VC6216 carrying plasmid pXY41 were induced with different concentrations of IPTG for 30 min. Equal amounts of ribosomes from these cultures were analyzed for 'L11 in a Western blot developed with anti S-Tag antibody. (B) RelA-ribosome interaction. The same ribosomes preparations were analyzed for RelA in a Western blot developed with anti-RelA antibody. The concentrations of IPTG (μM) were: lanes 1, 0; 2, 10; 3, 50; and 4, 100.

samples (Fig. 5.6B). These results indicate that the presence of 'L11 in ribosomes had no apparent effect on RelA-ribosome interaction. Therefore, the inhibition of ppGpp accumulation by amino acid-deprived bacteria overproducing 'L11 was not due to the inability of RelA to bind ribosomes.

Influence of proline-rich helix of L11 on RelA regulation

The deletion 'L11 includes a key conserved region known as the proline-rich helix which includes Pro22 and Pro23. To determine whether this region was involved in RelA regulation, Pro22 was changed to Leu22 by site-directed mutagenesis to create plasmid pXY54. The effect of this mutation, called mL11, on the stringent response was tested as described in Fig. 5.7. As already described, the overexpression of L11 by amino acid-deprived strain VC6216 did not affect the stringent response (curve a). In contrast, the overexpression of either 'L11 (curve b) or mL11 (curve c) relaxed the stringent response. These results unequivocally show that Pro22 plays an essential role in regulating the activity of RelA.

Yeast two-hybrid analysis of L11-RelA interaction

Table 5.1 summarizes attempts to demonstrate the interaction of L11 with RelA. Transformant 1 was a negative control which carried the cloning vectors. Transformant 2 was a positive control carrying two-hybrid plasmids encoding 2 proteins, murine p53 and SV40 T antigen, that are known to interact with each other. Transformant 3 carried plasmids encoding RelA fused to the *GAL4* DNA-

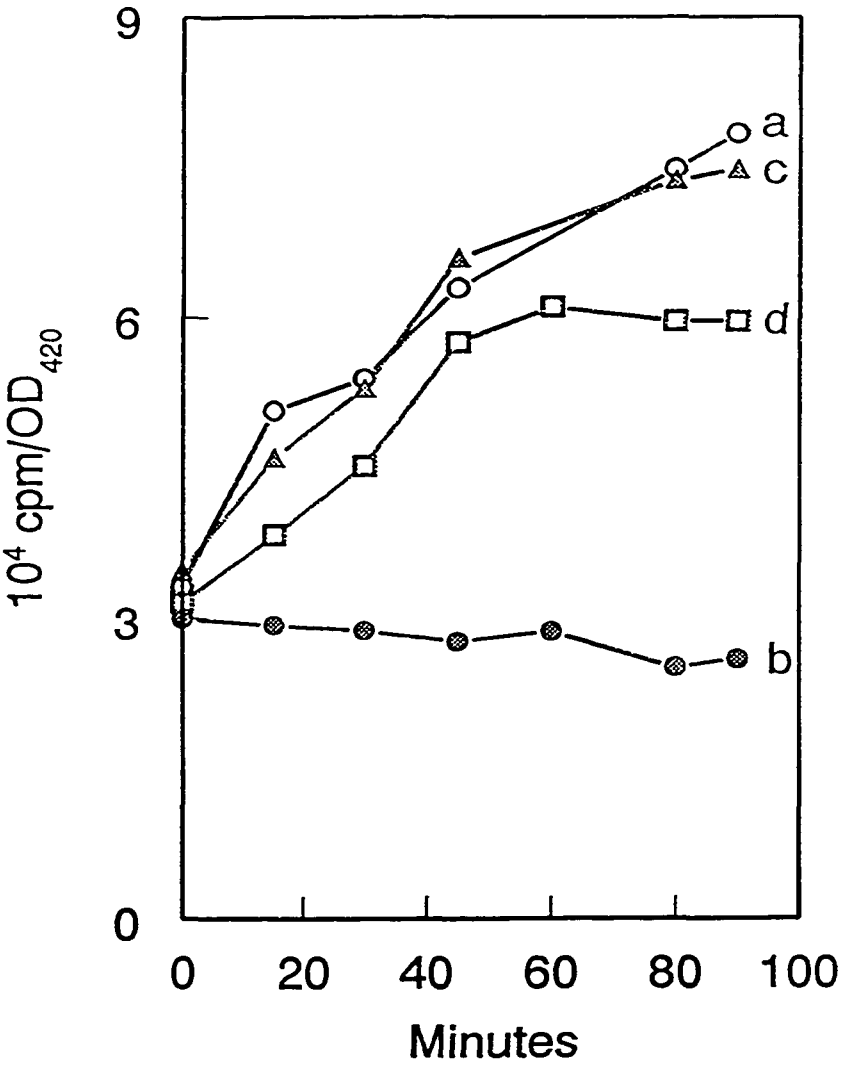

Fig 5.7. Effect of overexpression of L11, 'L11 and mL11 on the incorporation of [^{3}H]uracil into stable RNA by strain VC6216

Culture a (○) represents an untreated control. The other cultures were amino acid-deprived by the addition of 500 µg of L-valine per ml. Culture b (●) was amino acid-deprived after overexpression of the wild-type L11 from pXY51. Culture c (▲) was amino acid-deprived after overexpression of 'L11 from pXY41. Culture d (□) was amino acid-deprived after overexpression of mL11 from pXY54.

Table 5.1. Analysis of L11-RelA interaction in yeast two-hybrid system

Transformant	*GAL4* (DB) plasmid	*GAL4* (AD) plasmid	Growth on SD selection medium	β-galactosidase activity (Miller units)[1]
1	pGBKT7	pGADT7	-	0.33
2	pGBKT7-53	pGADT7-T	+	18.77
3	pXY65	pXY48	-	0.59
4	pXY49	pXY66	-	0.45

[1]Average of 3 independent determinations

binding domain and L11 fused to the *GAL4* activation domain. This transformant clearly exhibited a negative two-hybrid test. As a check, the roles of the proteins were reversed in transformant 4. In this case, the L11 protein was fused to the *GAL4* DNA-binding domain, and the RelA protein was fused to the *GAL4* activation domain. The results for this transformant were also negative. Therefore, L11 apparently does not bind to RelA.

Attempts to demonstrate L11-RelA interaction *in vitro*

Two other approaches were used to test whether L11 interacts with RelA. The first was an affinity blotting experiment. An extract of W3110 containing the complete RelA protein, overexpressed from plasmid pALS10, was fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. The RelA protein was readily detected using anti-RelA antiserum, but when the membrane was incubated with purified His-tag L11, no interaction between L11 and RelA was observed (data not shown). The second approach was affinity chromatography. Purified His-tag L11 was passed through a column containing GST-'RelA immobilized on glutathione Sepharose 4B. Again, L11 did not bind to 'RelA.

Discussion

The removal of the C-terminal ribosome-binding domain of RelA results in a metabolically unstable constitutive ppGpp synthetase (Schreiber *et al.*, 1991; Svitil *et al.*, 1993). This clearly demonstrates the crucial role the ribosome plays

in regulating RelA-dependent (p)ppGpp synthesis. The existence of *rplK* (*relC*) mutants has implicated ribosomal protein L11 in the regulation of RelA activity during the stringent response in *E. coli* (Friesen *et al.*, 1974; Parker *et al.*, 1976). In fact, L11 has so far been the only ribosomal protein demonstrated to be involved in the regulation of RelA. However, the basis for the relaxed phenotype of the *rplK* (*relC*) mutants is still unclear. Although the nucleotide sequences of L11 relaxed mutations have not been reported, it is clear that they affect the activity of RelA which, *in vitro,* was less than 10% of the activity exhibited by the wild type. It is notable that the *relC* mutation apparently did not affect the RelA association with ribosomes (Friesen *et al.*, 1974).

The new findings reported here indicate that the overexpression of a mutant L11 protein lacking its N-terminus resulted in a relaxed phenotype. In contrast, a normal stringent response was observed when the wild type L11 protein was overexpressed. The overexpressed 'L11 was incorporated into ribosomes. Site-directed mutagenesis of L11 indicated that a single amino acid substitution, Pro22 to Leu22, was sufficient to confer a relaxed phenotype. Pro22 is part of the so-called proline-rich helix region of L11. Using several *in vivo* and *in vitro* approaches, we were unable to demonstrate that RelA forms a direct contact with wild type L11.

The involvement of L11 in the regulation of RelA activity is especially interesting in view of its important functional role in the bacterial ribosome. L11 is complexed to a 58-nucleotide segment (nucleotides 1051 to 1108) of 23S rRNA (Porse and Garrett, 1999). In addition to its involvement in regulating RelA

activity, the L11-rRNA complex controls several key processes in translation including (i) the binding of N-formyl-methionyl-tRNA mediated by initiation factor 2; (ii) the binding of aminoacyl-tRNA mediated by elongation factor EF-Tu; (iii) translocation mediated by elongation factor EF-G; and (iv) termination mediated by release factor (Porse and Garrett, 1999). Since most of these protein factors are GTPases, the L11-rRNA complex has been referred to as the GTPase-associated site (Cundliffe, 1986). The crystal structure of this highly conserved complex has recently been solved at 2.8 angstrom and 2.6 angstrom resolution by Conn *et al.* (Conn *et al.*, 1999) and by Wimberly *et al.* (Wimberly *et al.*, 1999), respectively. The rRNA domain is precisely folded, and the crystal structure suggests that the primary role of L11 is to stabilize the tertiary structure of the rRNA (Conn *et al.*, 1999). Two domains have been characterized in L11 (Xing and Draper, 1996). A C-terminal domain is tightly bound to the tertiary structure of RNA and is responsible for stabilizing its conformation. An N-terminal domain is involved in the cooperative binding of thiopeptide antibiotics such as thiostrepton and micrococcin. There are only limited contacts between the RNA and the N-terminal domain of L11.

Evidence for an association between RelA and the thiostrepton-micrococcin-binding site on the ribosome has come from both *in vivo* and *in vitro* experiments. For example, thiostrepton inhibits *in vitro* RelA-dependent (p)ppGpp synthesis (Haseltine *et al.*, 1972; Sy, 1974). Moreover, thiopeptin, an analogue of thiostrepton, inhibits the RelA-dependent synthesis of ppGpp in *Streptomyces lividans* (Ochi, 1989), and thiostrepton-resistant mutants of

Bacillus subtilis exhibit relaxed phenotypes (Smith *et al.*, 1978; Smith *et al.*, 1980). Thiostrepton does not bind to L11 alone and binds weakly to 23S rRNA (Thompson *et al.*, 1979). High affinity binding of thiostrepton requires the L11-rRNA complex. A mutation in either nucleotide A1067 or A1095 confers resistance to thiostrepton indicating that these residues form part of the antibiotic-binding site on 23S rRNA (Rosendahl and Douthwaite, 1994). A1067 and A1095 are located at the ends of adjacent stem-loops, and the crystal structure indicates that they are located very close to each other (Wimberly *et al.*, 1999). The N-terminal domain of L11 also plays a crucial role in high affinity-binding of thiostrepton (Xing and Draper, 1996). In this regard, the proline-rich helix is particularly important (Porse *et al.*, 1998). For example, a mutation in Pro22, the same residue that affects RelA regulation, results in thiostrepton resistance. Many of these observations can be explained through the recently solved crystal structure of the L11-RNA complex (Wimberly *et al.*, 1999). The proline-rich helix is located close to 23S rRNA nucleotides A1067 and A1095. A cleft formed between the RNA and the proline-rich helix has been proposed to represent the binding site for thiostrepton and micrococcin The antibiotics are hypothesized to interact specifically with A1067 and A1095 on one side and with the proline-rich region of the L11 N-terminal domain on the other side. The crystal structure predicts that the removal of the N-terminal domain of L11 would greatly compromise the binding of thiostrepton. Mechanistically, it is thought that the thiazole groups on the thiopeptide antibiotics could mimic the proline residues in the N-terminal domain of L11 and, in this way, could facilitate the binding of the

antibiotics to RNA (Wimberly *et al.*, 1999). The data presented here implicating the involvement of Pro22 in RelA regulation is significant because they are consistent with two previous findings: (i) the correlation between thiostrepton resistance and relaxation of the stringent response, and (ii) the identification of the thiostrepton interaction site on the N-terminus of L11.

Thiostrepton inhibits the function of EF-G, and it has been proposed that the antibiotic bound to the GTPase associated site could prevent a conformational change within L11 necessary for the elongation process (Cundliffe, 1986; Porse *et al.*, 1998). Based on their crystal structure, Wimberly *et al.* (Wimberly *et al.*, 1999) hypothesize that the N-terminal domain of L11 may represent a molecular switch that alternates between RNA-bound and RNA-free states during the elongation cycle. This switching mechanism is proposed to be controlled by the binding of elongation factors. It is noteworthy that, despite the apparent critical function of L11 in translation, *E. coli* mutants that completely lack this protein are viable (Dabbs, 1979; Stoffler *et al.*, 1980). To account for this, Wimberly *et al.* (Wimberly *et al.*, 1999) suggest that the putative N-terminal switch may function by regulating the conformation or the accessibility of the RNA in the GTPase-associated site.

It has long been hypothesized that the activity of RelA is controlled by conformational changes in the ribosome. Based on the available data, it would appear that this conformational change involves the GTPase-associated site. In this regard, our results indicate that RelA does not directly interact with L11. Therefore, the relaxed phenotype of the *rplK* mutants must be based on an

indirect effect of L11 on the activity of RelA. The involvement of Pro22 suggests that the proposed L11 N-terminal switch domain may promote the conformational change necessary to activate RelA during amino acid deprivation. The switch could mediate this process through either a neighboring ribosomal protein or 23S rRNA. To test this idea further, the precise location of RelA on the ribosome must be determined.

References:

Aizenman, E., Engelberg-Kulka, H. and Glaser, G. (1996) An *Escherichia coli* chromosomal "addiction module" regulated by guanosine [corrected] 3',5'-bispyrophosphate: a model for programmed bacterial cell death [published erratum appears in *Proc Natl Acad Sci U S A* 1996 Sep 3;93(18):9991]. *Proc Natl Acad Sci U S A.* 93:6059-63.

Arnold, G. F., and Tessman, I. (1988) Regulation of DNA superhelicity by *rpoB* mutations that suppress defective Rho-mediated transcription termination in *Escherichia coli. J Bacteriol.* 170:4266-71.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1994) Current protocols in molecular biology. Greene Publishing Associates & Wiley Interscience, New York.

Autret, S., Levine, A., Vannier, F., Fujita, Y. and Seror, S. J. (1999) The replication checkpoint control in *Bacillus subtilis*: identification of a novel RTP-binding sequence essential for the replication fork arrest after induction of the stringent response. *Mol Microbiol.* 31:1665-79.

Balke, V. L., and Gralla, J. D. (1987) Changes in the linking number of supercoiled DNA accompany growth transitions in *Escherichia coli. J Bacteriol.* 169:4499-506.

Bell, R.M. (1974) Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: macromolecular synthesis in an sn-glycerol 3-phosphate acyltranferase Km mutant. *J Bacteriol.* 117(3):1065-76

Bhriain, N. N., Dorman, C. J. and Higgins, C. F. (1989) An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. *Mol Microbiol.* 3:933-42.

Blanc-Potard, A. B., Gari, E. Spirito, F. Figueroa-Bossi, N. and Bossi, L. (1995) RNA polymerase (*rpoB*) mutants selected for increased resistance to gyrase inhibitors in *Salmonella typhimurium. Mol Gen Genet.* 247:680-92.

Block, R., and Haseltine, A. W. (1975) Purification and properties of stringent factor. *J Biol Chem.* 250:1212-7.

Breeden, L., Yarus, M. and S. Cline. (1980) A cloned suppressor tRNA gene relaxes stringent response. *Mol Gen Genet* 179(1):125-33.

Camacho-Carranza, R., Membrillo-Hernandez, J., Ramirez-Santos, J., Castro-Dorantes, J., Chagoya de Sanchez, V. and . Gomez-Eichelmann, M. C. (1995) Topoisomerase activity during the heat shock response in *Escherichia coli* K-12. *J Bacteriol.* 177:3619-22.

Cashel, M., Gentry, D.R., Hernandez, V. J. and Vinella, D. (1996) The Stringent Response, p.1458-1496 *In Escherichia coli* and *Salmonella.* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), ASM Press, Washington, D.C.

Cashel, M., and Gallant, J. (1969) Two compounds implicated in the function of the RC gene of *Escherichia coli. Nature.* 221:838-41.

Chatterji, D., Fujita, N. and Ishihama, A. (1998) The mediator for stringent control, ppGpp, binds to the beta-subunit of *Escherichia coli* RNA polymerase. *Genes Cells.* 3:279-87.

Chiaramello, A. E., and Zyskind, J. W. (1990) Coupling of DNA replication to growth rate in *Escherichia coli*: a possible role for guanosine tetraphosphate. *J Bacteriol.* 172:2013-9.

Conn, G. L., Draper, D. E., Lattman, E. E. and .Gittis, A. G (1999) Crystal structure of a conserved ribosomal protein-RNA complex. *Science.* 284:1171-4.

Csonka, L. N. (1989) Osmoregulation in Bacteria. *Microbiological Reviews.* 53:121-147.

Cundliffe, E. (1986) Involvement of specific portions of ribosomal RNA in defined ribosomal functions: a study utilizing antibiotics. *In* Strcuture, function, and genetics of ribosomes. B. Hardesty and G. Kramer, editors. Springer-Verlag, New Yorlk. 586-604.

Dabbs, E. R. (1979) Selection for *Escherichia coli* mutants with proteins missing from the ribosome. *J Bacteriol.* 140:734-7.

Dorman, C. J., Lynch, A. S., Bhriain, N. N.and Higgins, C. F. (1989) DNA supercoiling in *Escherichia coli*: *topA* mutations can be suppressed by DNA amplifications involving the *tolC* locus. *Mol Microbiol.* 3:531-40.

Drlica, K., and Franco, R. J. (1988) Inhibitors of DNA topoisomerases. *Biochemistry.* 27:2253-9.

Egebjerg, J., Doutheait, S.R. , Liljas, A. and Garrett, R.A. (1990) Characterization of the binding sites of protein L11 and L10(l12)4 pentameric complex in the GTPase domain of 30 S ribosomal RNA from *Escherichia coli. J Mol Biol.* 213(2):275-88.

Eichel, J., Chang, Y.Y., Riesenber, D. and Cronan,J.E. Jr. (1999) Effect of ppGpp on *Escherichia coli* cyclopropane fatty acid synthesis is mediated through the RpoS sigma factor (sigma S). *J Bacteriol.* 181(2):572-6.

Figueroa-Bossi, N., Guerin, M., Rahmouni, R., Leng, M. and Bossi, L. (1998) The supercoiling sensitivity of a bacterial tRNA promoter parallels its responsiveness to stringent control. *Embo J.* 17:2359-67.

Fiil, N.P., Willumsen, B.M. and Meyenberg, K. (1977) Interaction of alleles of the *relA, relC* and *spoT* genes in *Escherichia coli*: analysis of the interconversion of GTP, ppGpp and pppGpp. *Mol Gen Genet.*150(1): 87-101.

Filutowicz, M., and Jonczyk, P. (1983) The *gyrB* gene product functions in both initiation and chain polymerization of *Escherichia coli* chromosome replication: suppression of the initiation deficiency in *gyrB*-ts mutants by a class of *rpoB* mutations. *Mol Gen Genet.* 191:282-7.

Friesen, J. D., Fiil, N. P., Parker, J. M. and Haseltine, W. A. (1974) A new relaxed mutant of *Escherichia coli* with an altered 50S ribosomal subunit. *Proc Natl Acad Sci U S A.* 71:3465-9.

Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T. and Tomizawa, J. I. (1977) Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. *Proc Natl Acad Sci U S A.* 74:4772-6.

Gentry, D. R., and Cashel, M. (1995) Cellular localization of the *Escherichia coli* SpoT protein. *J Bacteriol.* 177:3890-3.

Gentry, D. R., and Cashel, M. (1996) Mutational analysis of the *Escherichia coli spoT* gene identifies distinct but overlapping regions involved in ppGpp synthesis and degradation. *Mol Microbiol.* 19:1373-84.

Goldman, E., and Jakubowski, H. (1990) Uncharged tRNA, protein synthesis, and the bacterial stringent response. *Mol Microbiol.* 4:2035-40.

Goldstein, E., and Drlica, K. (1984) Regulation of bacterial DNA supercoiling: plasmid linking numbers vary with growth temperature. *Proc Natl Acad Sci U S A.* 81:4046-50.

Grossman, A. D., Taylor, W. E., Burton, Z. F., Burgess, R. R.and Gross, C. A. (1985) Stringent response in *Escherichia coli* induces expression of heat shock proteins. *J Mol Biol.* 186:357-65.

Grossman, A. D., Straus, D. B., Walter, W. A. and Gross, C. A. (1987) Sigma 32 synthesis can regulate the synthesis of heat shock proteins in *Escherichia coli. Genes Dev.* 1:179-84.

Haseltine, W. A., Block, R., Gilbert, W. and Weber, K. (1972) MSI and MSII made on ribosome in idling step of protein synthesis. *Nature.* 238:381-4.

Haseltine, W. A. and Block, R. (1973) Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. *Proc Natl Acad Sci U S A.* 70(50):1564-8.

Heinemeyer, E.A. and Richter, D. (1978) Mechanism of the in vitro breakdown of guanosine 5'-diphosphate 3'-diphosphate in *Escherichia coli*. *Proc Natl Acad Sci U S A.* 75(9):4180-3.

Hengge-Aronis, R. (1996) Regulation of gene expression during entry into starionary phase, p. 1497-1512. *In Escherichia coli* and *Salmonella*. F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), ASM Press, Washington, D.C.

Hernandez, V. J., and Bremer, H. (1991) *Escherichia coli* ppGpp synthetase II activity requires *spoT*. *J Biol Chem.* 266:5991-9.

Herrick, J., Kohiyama, M., Atlung, T. and Hansen, F. G. (1996) The initiation mess? *Mol Microbiol.* 19:659-66.

Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G. and Bremer, E. (1988) A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell.* 52:569-84.

Homann, H. E., and Nierhaus, K. H. (1971) Ribosomal proteins. Protein compositions of biosynthetic precursors and artifical subparticles from ribosomal subunits in *Escherichia coli* K 12. *Eur J Biochem.* 20:249-57.

Ishiguro, E. E., and Ramey, W. D. (1976) Stringent control of peptidoglycan biosynthesis in *Escherichia coli* K- 12. *J Bacteriol.* 127:1119-26.

Jin, D. J., and Gross, C. A. (1988) Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. *J Mol Biol.* 202:45-58.

Jin, D. J., Walter, W. A. and Gross, C. A. (1988) Characterization of the termination phenotypes of rifampicin-resistant mutants. *J Mol Biol.* 202:245-53.

Jones, P. G., Cashel, M., Glaser, G. and Neidhardt, F. C. (1992) Function of a relaxed-like state following temperature downshifts in *Escherichia coli*. *J Bacteriol.* 174:3903-14.

Kingston, R.E. and Chamberlin, M. J. (1981) Pausing and attenuation of *in vitro* transcription in the *rrnB* operon of *E.coli*. *Cell* 27(3 Pt 2):523-31.

Kingston, R.E., Gutell, R.R., Taylor, A.R. and Chamberlin, M.J. (1981) Tanscriptionalmapping of plasmid pKK3535. Quantitation of the effect of guanosine tetraphosphate on binding to the *rrnB* promoters an a lambda promoter with sequence homologies in the CII binding region. *J Mol Biol.*146(4):433-49.

Kvint, K., Farewell, A. and Nystrom, T. (2000) RpoS-dependent promoters requires guanosine tetraphosphate for induction even in the presence of high level of σ^S. *J Biol Chem.* 275(20):14795-98.

Lagosky, P. A., and Chang, F. N. (1980) Influence of amino acid starvation on guanosine 5'-diphosphate 3'-diphosphate basal-level synthesis in *Escherichia coli*. *J Bacteriol.* 144:499-508.

Liu, L.F. and Wang. J.C. (1987) Supercoiling of the DNA temperlate during transcription. *Proc Natl Acad Sci U S A.* 84(20):7024-7.

Lopez-Garcia, P., and Forterre, P. (1997) DNA topology in hyperthermophilic archaea: reference states and their variation with growth phase, growth temperature, and temperature stresses. *Mol Microbiol.* 23:1267-79.

Lund,E. and Kjeldgaard, N.O. (1972) Metabolism of guanosine tetraphosphate in *Escherichia coli. Eur J Biochem.* 28(3):313-26.

Mechold, U., Cashel, M., Steiner, K., Gentry D.and Malke, H. (1996) Functional analysis of a *relA/spoT* gene homolog from *Streptococcus . J Bacteriol.* 178(5):1401-11.

Menzel, R., and Gellert, M. (1983) Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. *Cell.* 34:105-13.

Metzger, S., Dror, I. B., Aizenman, E.,Schreiber, G., Toone, M., Friesen, J. D., Cashel, M. and Glaser, G. (1988) The nucleotide sequence and characterization of the *relA* gene of *Escherichia coli. J Biol Chem.* 263:15699-704.

Metzger, S., Schreiber, G., Aizenman, E., Cashel, M. and Glaser. G., (1989a) Characterization of the *relA1* mutation and a comparison of *relA1* with new *relA* null alleles in *Escherichia coli. J Biol Chem.* 264:21146-52.

Metzger, S., Sarubbi, E., Glaser, G. and Cashel, M. (1989b) Protein sequences encoded by the *relA* and *spoT* genes of *Escherichia coli* are interrelated. *J Biol Chem.* 264:9122-25.

Miller, J. H. (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mirkin, S. M., Bogdanova, E. S., Gorlenko, Z. M., Gragerov, A. I. and Lariono, O. A. (1979) DNA supercoiling and transcription in *Escherichia coli*: influence of RNA polymerase mutations. *Mol Gen Genet.* 177:169-75.

Mizushima, T., Natori, S. and Sekimizu, K. (1993) Relaxation of supercoiled DNA associated with induction of heat shock proteins in *Escherichia coli. Mol Gen Genet.* 238:1-5.

Murray, K. D., and Bremer, H. (1996) Control of *spoT*-dependent ppGpp synthesis and degradation in *Escherichia coli. J Mol Biol.* 259:41-57.

Nystrom, T. (1994) Role of guanosine tetraphosphate in gene expression and the survival of glucose or seryl-tRNA starved cells of *Escherichia coli* K12. *Mol Gen Genet.* 245:355-62.

Nystrom, T. (1998) To be or not to be: the ultimate desicision of the grwoth-arrested bacterial cell. *FEMS Microbiol Rev.* 21:283-90.

Ochi, K. (1989) The tsr gene-coding plasmid pIJ702 prevents thiopeptin from inhibiting ppGpp synthesis in *Streptomyces lividans. FEMS Microbiol Lett.* 52:219-23.

Ogata, Y., Inoue, R., Mizushima, T., Kano, Y., Miki, T. and Sekimizu, K. (1997) Heat shock-induced excessive relaxation of DNA in *Escherichia coli* mutants lacking the histone-like protein HU. *Biochim Biophys Acta.* 1353:298-306.

Ogata, Y., Mizushima, T., Kataoka, K., Kita, K., Miki, T. and Sekimizu, K. (1996) DnaK heat shock protein of *Escherichia coli* maintains the negative supercoiling of DNA against thermal stress. *J Biol Chem.* 271:29407-14.

Ohlsen, K. L., and Gralla, J. D. (1992) Interrelated effects of DNA supercoiling, ppGpp, and low salt on melting within the *Escherichia coli* ribosomal RNA *rrnB* P1 promoter. *Mol Microbiol.* 6:2243-51.

Pao, C. C., and Gallant, J. (1978) A gene involved in the metabolic control of ppGpp synthesis. *Mol Gen Genet.* 158:271-7.

Parker, J., Watson, R. J. and Friesen, J. D. (1976) A relaxed mutant with an altered ribosomal protein L11. *Mol Gen Genet.* 144:111-4.

Pedersen, F. S., and Kjeldgaard, N. O. (1977) Analysis of the *relA* gene product of *Escherichia coli*. *Eur J Biochem.* 76:91-7.

Porse, B. T., and Garrett, R. A. (1999) Ribosomal mechanics, antibiotics, and GTP hydrolysis. *Cell.* 97:423-6.

Porse, B. T., Leviev, I., Mankin, A. S. and Garrett, R. A. (1998) The antibiotic thiostrepton inhibits a functional transition within protein L11 at the ribosomal GTPase centre. *J Mol Biol.* 276:391-404.

Pruss, G. J., and Drlica, K. (1989) DNA supercoiling and prokaryotic transcription. *Cell.* 56:521-3.

Ramirez, R. M., and Villarejo, M. (1991) Osmotic signal transduction to proU is independent of DNA supercoiling in *Escherichia coli*. *J Bacteriol.* 173:879-85.

Reddy, P. S., Raghavan, A. and Chatterji, D. (1995) Evidence for a ppGpp-binding site on *Escherichia coli* RNA polymerase: proximity relationship with the rifampicin-binding domain. *Mol Microbiol.* 15:255-65.

Rosendahl, G., and Douthwaite, S. (1994) The antibiotics micrococcin and thiostrepton interact directly with 23S rRNA nucleotides 1067A and 1095A. *Nucleic Acids Res.* 22:357-63.

Rudd, K. E., . Bochner, B. R, Cashel, M. and Roth, J. R. (1985) Mutations in the *spoT* gene of *Salmonella typhimurium*: effects on *his* operon expression. *J Bacteriol.* 163:534-42.

Ryals, J., Little, R. and Bremer, H.(1982) Control of RNA synthesis in *Escherichia coli* after a shift to higher temperature. *J Bacteriol.*151:1425-32.

Said, B., Cole, J.B. and Nomura, M.(1988) Mutational analysis of the L11 binding site of 23S rRNA in *Escherichia coli. Nucleic Acids Res.* 16(22):10529-45.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sarubbi, E., Rudd, K. E. and Cashel, M. (1988) Basal ppGpp level adjustment shown by new *spoT* mutants affect steady state growth rates and *rrnA* ribosomal promoter regulation in *Escherichia coli. Mol Gen Genet.* 213:214-22.

Sarubbi, E., Rudd, K. E., Xiao, H., Ikehara, K., Kalman, M. and Cashel, M. (1989) Characterization of the *spoT* gene of *Escherichia coli. J Biol Chem.* 264:15074-82.

Schreiber, G., Metzger, S., Aizenman, E., Roza, S., Cashel, M. and Glaser, G. (1991) Overexpression of the *relA* gene in *Escherichia coli. J Biol Chem.* 266:3760-7.

Shand, R.F., Blum, P.H., Holzschu, D.L., Urdea M.S. and Artz, S.W. (1989) Mutational analysis of the histidine operon promoter of *Salmonella typhimurium. J Bacteriol.*171(11):6330-7.

Smith, I., Paress, P. and Pestka, S. (1978) Thiostrepton-resistant mutants exhibit relaxed synthesis of RNA. *Proc Natl Acad Sci U S A.* 75:5993-7.

Smith, I., Paress, P., Cabane, K. and Dubnau, E. (1980) Genetics and physiology of the rel system of *Bacillus subtilis. Mol Gen Genet.* 178:271-9.

Stamminger, G. and Lazzarini, R.A. (1974): Analysis of the tRNA of detective VSV particles. *Cell.* 3(1):85-93.

Somerville,C.R. and Ahmed, A. (1979) Mutants of *Escherichia coli* defective in the degradation of guanosine 5'-triphosphate, 3'-diphosphate (ppGpp). *Mol Gen Genet.* 169(3):315-23.

Sorensen, M. A., Jensen, K. F. and Pedersen, S. (1994) High concentrations of ppGpp decrease the RNA chain growth rate. Implications for protein synthesis and translational fidelity during amino acid starvation in *Escherichia coli. J Mol Biol.* 236:441-54.

Stoffler, G., Cundliffe, E., Stoffler-Meilicke, M. and Dabbs, E. R. (1980) Mutants of *Escherichia coli* lacking ribosomal protein L11. *J Biol Chem.* 255:10517-22.

Svitil, A. L., Cashel, M. and Zyskind, J. W. (1993) Guanosine tetraphosphate inhibits protein synthesis in vivo. A possible protective mechanism for starvation stress in *Escherichia coli. J Biol Chem.* 268:2307-11.

Sy, J. (1974) Reversibility of the pyrophosphoryl transfer from ATP to GTP by *Escherichia coli* stringent factor. *Proc Natl Acad Sci U S A.* 71:3470-3.

Sy, J. (1980) Activation of ppGpp-3'-pyrophosphohydrolase by a supernatant factor and ATP. *J Biol Chem.* 255:10056-9.

Sy, J., and Lipmann, F. (1973) Identification of the synthesis of guanosine tetraphosphate (MS I) as insertion of a pyrophosphoryl group into the 3'-position in guanosine 5'-diphosphate. *Proc Natl Acad Sci U S A.* 70:306-9.

Tao, H., Bausch, C., Richmond, C., Blattner, F. R. and Conway, T. (1999) Functional genomics: expression analysis of *Escherichia coli* growing on minimal and rich media. *J Bacteriol.* 181:6425-40.

Tedin, K., and Bremer, H. (1992) Toxic effects of high levels of ppGpp in *Escherichia coli* are relieved by *rpoB* mutations. *J Biol Chem.* 267:2337-44.

Thompson, J., Cundliffe, E. and Stark, M. (1979) Binding of thiostrepton to a complex of 23-S rRNA with ribosomal protein L11. *Eur J Biochem.* 98:261-5.

Toone, W.M., Rudd, K.E. and Friesen, J.D. (1992) Mutations causing aminotriazole resistance and temperature sensitivity reside in *gyrB*, which encodes the B subunit of DNA gyrase. *J Bacteriol.* 174(16):5479-81.

Ueshima, R., Fujita, N. and Ishihama, A. (1989) DNA supercoiling and temperature shift affect the promoter activity of the *Escherichia coli rpoH* gene encoding the heat-shock sigma subunit of RNA polymerase. *Mol Gen Genet.* 215:185-9.

Uzan, M., and Danchin, A. (1976) A rapid test for the *rel A* mutation in *E. coli. Biochem Biophys Res Commun.* 69:751-8.

VanBogelen, R. A., Acton, M. A. and Neidhardt, F. C. (1987a) Induction of the heat shock regulon does not produce thermotolerance in *Escherichia coli. Genes Dev.* 1:525-31.

VanBogelen, R. A., Kelley, P. M. and Neidhardt, F. C. (1987b) Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli. J Bacteriol.* 169:26-32.

VanBogelen, R. A., and Neidhardt, F. C. (1990) Ribosomes as sensors of heat and cold shock in *Escherichia coli. Proc Natl Acad Sci U S A.* 87:5589-93.

Wimberly, B. T., Guymon, R., McCutcheon, J. P., White, S. W. and Ramakrishnan, V. (1999) A detailed view of a ribosomal active site: the structure of the L11-RNA complex [see comments]. *Cell.* 97:491-502.

Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G. and Cashel, M. (1991) Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. *J Biol Chem.* 266:5980-90.

Xing, Y., and Draper, D. E. (1996) Cooperative interactions of RNA and thiostrepton antibiotic with two domains of ribosomal protein L11. *Biochemistry.* 35:1581-8.