Temperature-Sensitive Growth and Decreased Thermotolerance Associated with *relA* Mutations in *Escherichia coli*

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The *relA* gene of *Escherichia coli* encodes guanosine 3′,5′-bispyrophosphate (ppGpp) synthetase I, a ribosome-associated enzyme that is activated during amino acid starvation. The stringent response is thought to be mediated by ppGpp. Mutations in *relA* are known to result in pleiotropic phenotypes. We now report that three different *relA* mutant alleles, *relA1*, *relA2*, and *relA251::kan*, conferred temperature-sensitive phenotypes, as demonstrated by reduced plating efficiencies on nutrient agar (Difco) or on Davis minimal agar (Difco) at temperatures above 41°C. The *relA*-mediated temperature sensitivity was osmoremedial and could be completely suppressed, for example, by the addition of NaCl to the medium at a concentration of 0.3 M. The temperature sensitivities of the *relA* mutants were associated with decreased thermotolerance; e.g., *relA* mutants lost viability at 42°C, a temperature that is normally nonlethal. The *spoT* gene encodes a bifunctional enzyme possessing ppGpp synthetase and ppGpp pyrophosphohydrolase activities. The introduction of the *spoT207::cat* allele into a strain bearing the *relA251::kan* mutation completely abolished ppGpp synthesis. This ppGpp null mutant was even more temperature sensitive than the strain carrying the *relA251::kan* mutation alone. The *relA*-mediated thermosensitivity was suppressed by certain mutant alleles of *rpoB* (encoding the β subunit of RNA polymerase) and *spoT* that have been previously reported to suppress other phenotypic characteristics conferred by *relA* mutations. Collectively, these results suggest that ppGpp may be required in some way for the expression of genes involved in thermotolerance.

Amino acid deprivation results in the global readjustment of metabolic activities in *Escherichia coli*. This phenomenon, designated the stringent response, may be designed to promote bacterial survival during periods of starvation (for a review, see reference 1). The hallmark of the stringent response is rapid intracellular accumulation of guanosine 3′,5′-bispyrophosphate (ppGpp). Amino acid starvation activates ppGpp synthetase I, a ribosome-associated enzyme encoded by the *relA* gene that catalyzes the synthesis of ppGpp. The metabolic changes associated with the stringent response are thought to be mediated by ppGpp. Thus, the so-called relaxed (*relA*) mutants of *E. coli* are unable to accumulate ppGpp in response to amino acid deprivation and consequently do not exhibit the stringent response. Alternatively, the stringent response is phenotypically blocked by agents, such as chloramphenicol, that inhibit the activation of RelA. A wide range of metabolic activities is regulated by ppGpp, either positively or negatively, but much remains to be learned about the underlying mechanisms.

The accumulation of ppGpp also occurs in a RelA-independent fashion, for example, during carbon source downshift (1). This reaction is catalyzed by ppGpp synthetase II, the product of the *spoT* gene (8, 30). SpoT also exhibits a 3′-pyrophosphatase activity that is the primary mechanism for ppGpp degradation during recovery from starvation (22).

Mutations affecting ppGpp metabolism result in pleiotropic phenotypes, suggesting that this nucleotide plays a complex role in cellular physiology (1). One relevant example is the so-called (p)ppGpp^0 mutants that carry deletions in both *relA* and *spoT* (30). These (p)ppGpp-deficient mutants have nearly normal growth rates on complex media but exhibit a multiple amino acid auxotrophic phenotype on minimal medium. We report here a new phenotype associated with *relA* mutations. We demonstrate that *relA* mutants exhibit temperature-sensitive growth. This temperature sensitivity was correlated to significantly decreased levels of thermotolerance. The temperature sensitivity was suppressed by certain mutant alleles of *rpoB*, the gene encoding the β subunit of RNA polymerase, by mutations in *spoT* that result in increased basal ppGpp levels, and by high osmolarity.

MATERIALS AND METHODS

Bacterial strains. All of the bacteria used in this study were derivatives of *E. coli* K-12. A list of the strains and recombinant plasmids used in this study is shown in Table 1. Plasmids were electroporated into bacteria with a Bio-Rad Gene Pulser. Bacterial strains were constructed for this study by P1*vir*-mediated transduction as described by Miller (16).

Strains carrying the *relA251::kan* and *spoT207::cat* alleles were constructed by direct selection of kanamycin-resistant and chloramphenicol-resistant transductants, respectively. Strains carrying the *relA1* and *relA2* alleles were constructed by using the closely linked *zei-348*::Tn5 insertion as a selective marker. In these constructions, the *relA* genotypes of the kanamycin-resistant transductants were determined by screening for sensitivity to a combination of serine, methionine, and glycine (25) and for sensitivity to 3-amino-1,2,4-triazole (20). The *relA* genotypes of the transductants were confirmed by measuring the incorporation of [5,6-³H]uracil (Amersham) into stable RNA in amino acid-deprived cultures as described previously (10). 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-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source (reference)
Bacteria		
CF1693	ΔrelA251::kan ΔspoT207::cat	M. Cashel
CF1736	pyrE⁺ spoT202	M. Cashel
CF1738	pyrE⁺ spoT203	M. Cashel
CF1740	pyrE⁺ spoT204	M. Cashel
CF1964	btuB::Tn10 rpoB7	M. Cashel
CF1969	btuB::Tn10 rpoB3443	M. Cashel
CF1970	btuB::Tn10 rpoB3449	M. Cashel
CF1971	btuB::Tn10 rpoB114	M. Cashel
CF1972	btuB::Tn10 rpoB337	M. Cashel
CF4268	btuB::Tn10 rpoB3445	M. Cashel
CF4297	btuB::Tn10 rpoB3401	M. Cashel
CF5034	pyrE60 zib-563::Tn10	M. Cashel
CP79	relA2	Laboratory collection
MC4100	relA1	Laboratory collection
VC348	zei-348::Tn5	Laboratory collection
VC6129	W3110 ΔrelA251::kan (W3110 × CF1693)	This study
VC6130	VC6129 ΔspoT207::cat (VC6129 × CF1693)	This study
VC6132	W3110 zei-348::Tn5 relA⁺	This study
VC6133	W3110 zei-348::Tn5 relA2	This study
VC6141	W3110 zei-348::Tn5 relA1	This study
VC6159	VC6129 btuB::Tn10 rpoB⁺ (VC6129 × CF1970)	This study
VC6158	VC6129 btuB::Tn10 rpoB3449 (VC6129 × CF1970)	This study
VC6160	VC6129 btuB::Tn10 rpoB3401 (VC6129 × CF4297)	This study
VC6166	VC6129 btuB::Tn10 rpoB3445 (VC6129 × CF4268)	This study
VC6173	VC6129 btuB::Tn10 rpoB7 (VC6129 × CF1964)	This study
VC6191	VC6129 btuB::Tn10 rpoB114 (VC6129 × CF1971)	This study
VC6192	VC6129 btuB::Tn10 rpoB3443 (VC6129 × CF1969)	This study
VC6193	VC6129 btuB::Tn10 rpoB3370 (VC6129 × CF1972)	This study
VC7237	VC6141 zib-563::Tn10	This study
VC7238	VC6141 pyrE60 zib-563::Tn10 (VC6141 × CF5034)	This study
VC7239	VC6141 spoT202 (VC6141 × CF1736)	This study
VC7240	VC6141 spoT203 (VC6141 × CF1738)	This study
VC7241	VC6141 spoT204 (VC6141 × CF1740)	This study
W3110	Prototrophic	Laboratory collection
Plasmids		
pALS10	Apʳ; E. coli relA clone	J. Zyskind (23)
pALS13	Apʳ; truncated E. coli relA clone	J. Zyskind (23)
pDJJ12	Apʳ; ColE1 derivative carrying E. coli rpoBC	M. Cashel (11)
pDS1	Cmʳ; E. coli rpoH in pACYC184	C. A. Gross

sensitive phenotypes, and one representative from each set, strains VC6129 (relA251::kan), VC6133 (relA2), and VC6141 (relA1), was used in the experiments described here.

It should be noted that Pao and Gallant (18) have characterized a mutation in a gene they designated relX that decreased intracellular levels of ppGpp and caused temperature-sensitive growth. We deliberately chose the zei-348::Tn5 marker for the construction of relA mutants to minimize the possibility of picking up relX mutations from our donor strains. The zei-348::Tn5 marker is closely linked to cysC and is therefore more closely linked to relA than to relX. In retrospect, we were unable to demonstrate relX in our strains (unpublished data).

Derivatives of VC6129 carrying the various rpoB alleles were constructed by using the linked btuB::Tn10 insertion as a selective marker. All of the mutant rpoB alleles conferred resistance to rifampin, 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. (21). Briefly, the procedure was as follows. In the first step, the linked markers pyrE60 and zib-563::Tn10 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 coinherit pyrE60. In the second step, the various spoT alleles from pyrE⁺ donors were transduced into VC7238 and pyrE⁺ transductants were selected for. The spoT derivatives were obtained by screening the pyrE⁺ transductants that formed small colonies in the presence of tetracycline (M. Cashel, personal communication).

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 (16), Davis minimal medium (Difco), Luria broth or agar as described by Miller (Difco), tryptic soy agar (Difco), LB broth or agar as described by Lennox (Difco), and LB broth or agar as described by Miller (Difco). Broth cultures were grown in Gyrotory water bath shakers (New Brunswick Scientific Co.), and culture turbidity was measured with a Beckman DU-64 spectrophotometer at 600 nm. The effect of the incubation temperature on colony formation is expressed as plating efficiency, which, in most experiments, is defined as the ratio of colony formation at 42°C to colony formation at 30°C, as determined in the following way. Bacteria were serially diluted in sterile saline (0.015 M NaCl). For each dilution, aliquots of 100 μl were plated in quadruplicate on nutrient agar, and two plates for each dilution were incubated at 30°C and at 42°C for 36 h before counting. In Fig. 1, a full range of temperatures was used, and in this case, the plating efficiencies are defined as the ratio of the colony count at the higher temperature to the colony count at 30°C. The experiments described in this report were performed with exponential-phase as well as stationary-phase bacteria. It is noteworthy that the same results were obtained in all cases, and the growth phase apparently did not influence the temperature sensitivity of the relA mutants. Unless indicated otherwise, the data shown were obtained with bacterial cultures that were 1 to 2 h into stationary phase.

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 h in stationary phase. The cells were resuspended in sterile saline to a density of approximately 2×10^9/ml. A 1-ml suspension was incubated in a water bath set at 55°C. Samples (50 μl) were removed at the indicated times,
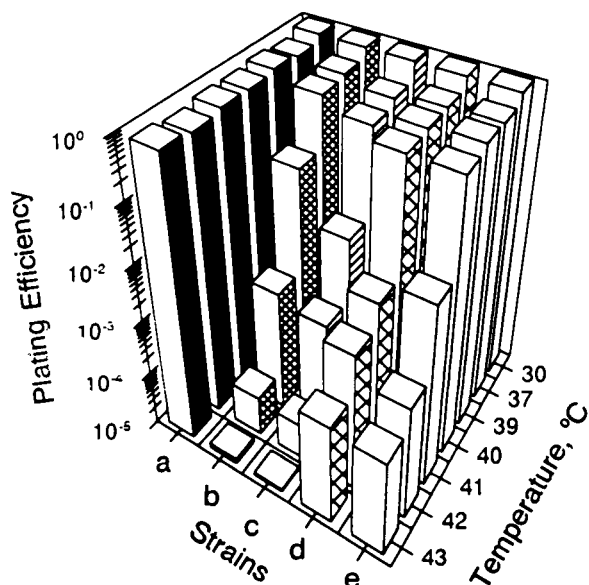
FIG. 1. Temperature-sensitive growth of *relA* mutants. A set of isogenic strains carrying three common *relA* mutant alleles were constructed. These strains were compared for growth on nutrient agar at temperatures ranging from 30 to 43°C. Growth is expressed as plating efficiency (plate counts at the high temperature divided by 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*).

diluted, and plated on nutrient agar. The survivors of the heat treatment were determined from the plate counts after 36 h of incubation at 30°C.

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 (Δ*relA251*::*kan* Δ*spoT207*::*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 is a common phenotype associated with all of the *relA* mutants tested. To establish the relationship between mutations in *relA* and temperature sensitivity, a set of isogenic derivatives of strain W3110 carrying the *relA1*, *relA2*, and Δ*relA251*::*kan* alleles were constructed by phage P1-mediated transduction (Table 1). Figure 1 compares the plating efficiencies of these strains with the plating efficiency of an isogenic *relA*+ strain, VC6132, on nutrient agar as a function of incubation temperature. All of the *relA* mutants had nearly normal plating efficiencies at temperatures as high as 39 to 40°C, but they progressively lost colony-forming capabilities at higher temperatures. Temperature sensitivity was especially notable in strain VC6129 (Δ*relA251*::*kan*) and in a derivative of VC6129, strain VC6130, carrying the Δ*spoT207*::*cat* allele (Fig. 1, strains b and c, respectively). Both strains exhibited temperature-sensitive growth at 40°C and higher temperatures. In comparison, strains VC6133 and VC6141, which carry the *relA1* and *relA2* alleles, respectively, showed normal colony formation at 40°C but were sensitive to higher temperatures (Fig. 1, strains d and e, respectively). Additional

comparative studies indicated that the plating efficiencies of strains carrying the *relA1* and *relA2* alleles, such as VC61333 and VC6141 (Fig. 1, strains d and e, respectively), were consistently more than 10-fold higher than those of strains carrying the Δ*relA251*::*kan* allele. Although the plating efficiencies of strains VC6129 and VC6130 were roughly similar in these experiments, data presented below indicate that VC6130 was less thermotolerant than VC6129. This suggests that the Δ*spoT207*::*cat* allele exacerbated the temperature-sensitive phenotype associated with Δ*relA251*::*kan*.

Temperature sensitivity was also demonstrated in nutrient broth. As shown in Fig. 2A, all of the 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. 2B). Cultures of the *relA* mutants, VC6129, VC6133, and VC6141, stopped growing after less than two doublings at the restrictive temperature.

The temperature sensitivity 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*.

Osmoremediation 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 of our experiments. However, the temperature-sensitive phenotype of *relA* mutants could also be demonstrated on minimal media such as M9 and Davis minimal media (supplemented with a mixture of amino acids in the case of strain VC6130). All *relA* mutant strains were heat 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 Luria-Bertani medium 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 or on Davis minimal agar at temperatures as high as 43°C was restored by the addition of NaCl (0.3 M), KCl (0.1 M), or sucrose (0.35 M) to the medium. Figure 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 0.8 M NaCl completely abolished temperature sensitivity at 42°C. A similar experiment with KCl showed that it was an effective suppressor of temperature sensitivity at 0.1 to 0.3 M (data not shown).

Suppression of *relA*-mediated temperature-sensitive growth by mutations in *rpoB*. As noted above, (p)ppGpp⁰ strains, such as VC6130 (Δ*relA251*::*kan* Δ*spoT207*::*cat*), possess pleiotropic phenotypes. For example, they are multiauxotrophic on minimal medium (30). This multiauxotrophic phenotype is suppressed by certain mutant alleles of *rpoB* (1). A representative collection of *rpoB* alleles were transduced into VC6129 to determine whether they also suppress the *relA*-associated temperature sensitivity. As shown in Table 2, four of the seven *rpoB* alleles tested restored heat resistance. Identical results were obtained with derivatives of VC6130 carrying these *rpoB*
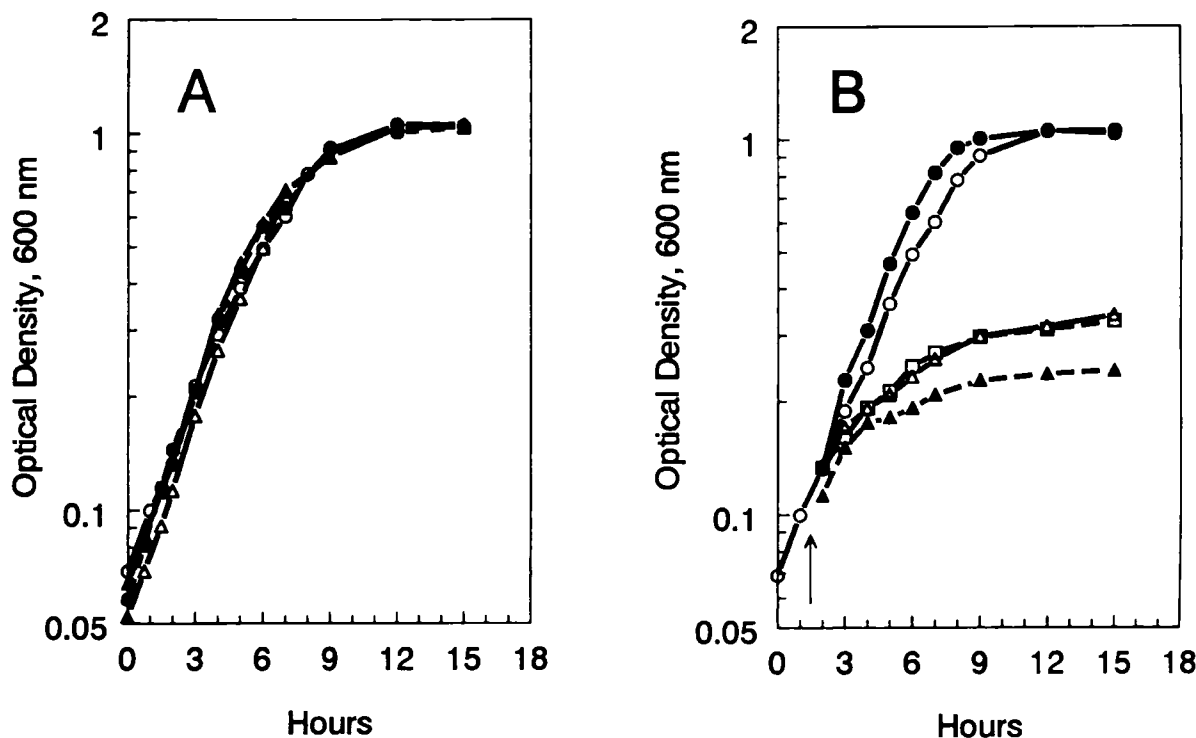
FIG. 2. Growth in nutrient broth at 30°C (A) and at 42°C (B). Symbols: ●, strain VC6132 (wild type); △, strain VC6129 (Δ*relA251::kan*); ▲, strain VC6133 (*relA2*); □, strain VC6141 (*relA1*). In panel B, cultures growing at 30°C were shifted to 42°C at 1.5 h (arrow). For comparison, the growth of a culture of strain VC6132 (wild type) at 30°C (○) is also shown.

alleles (data not shown). Interestingly, these same four alleles were the only ones in the collection capable of suppressing the (p)ppGpp⁰-associated multiauxotrophic phenotype (M. Cashel, personal communication), suggesting that this phenomenon is mechanistically related to *relA*-associated temperature sensitivity.

Suppression of the temperature-sensitive phenotype of VC6141 by *spoT* mutant alleles. Sarubbi et al. (21) have described *spoT* mutant alleles that raise the basal levels of ppGpp in *relA1* backgrounds. Derivatives of strain VC6141 (*relA1*) carrying three such *spoT* alleles were constructed. Table 3 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 is directly related to decreased basal levels of (p)ppGpp.

Decreased thermotolerance of *relA* mutants. Figure 4 shows that incubation at 42°C was lethal to *relA* mutants, e.g., strain VC6129. Identical sets of plates inoculated with a serial dilution of VC6129 were incubated at 42°C beginning at 0 h. 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 period of incubation 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-h period of incubation at 42°C. Moreover, there was less than 0.1% survival after 10 h.

A set of strains carrying the Δ*relA251::kan* allele were compared for survival at 55°C. Figure 5 shows that *relA251::kan* mutant strain VC6129 was significantly less thermotolerant than *relA*⁺ strain VC6132. Thermotolerance was restored to nearly wild-type levels by the introduction of plasmid pALS10

(*relA*⁺) into strain VC6129. Moreover, strain VC6130 was killed at a faster rate at 55°C than strain VC6129, indicating that the presence of the *spoT207::cat* mutation further reduced the thermotolerance associated with the *relA251::kan* allele. 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 *relA*-associated temperature-sensitive growth phenotype was associated with decreased thermotolerance. The results shown in Fig. 5 were obtained with exponential-phase cells. Virtually identical results were obtained when stationary-phase bacteria were used. Moreover, the thermotolerance of strain VC6129 (Δ*relA251::kan*) was not improved by cell growth at 37°C or by a brief (30-min) preexposure of the cells to 42°C prior to testing (data not shown).

DISCUSSION

Here we show that all three commonly used *relA* mutant alleles conferred temperature-sensitive growth phenotypes. In each case, temperature sensitivity was eliminated by the introduction of a recombinant plasmid carrying the wild-type *relA* gene but not by one carrying a truncated *relA* gene. Furthermore, the *spoT* mutant alleles described by Sarubbi et al. (21) that cause increased basal levels of ppGpp in a *relA1* background partially suppressed the temperature-sensitive phenotype of the *relA1* mutant strain. It was also noted that the Δ*relA251::kan* strain was significantly more temperature sensi-
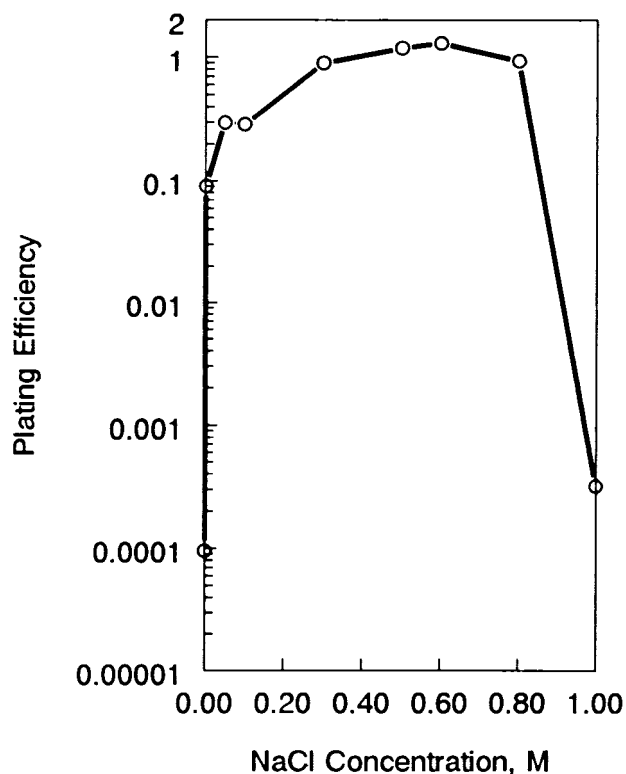
FIG. 3. Effect of medium osmolarity on colony formation by strain VC6129 (Δ*relA251::kan*). Nutrient agar plates containing the indicated amounts of NaCl were inoculated with a series of dilutions of an exponential-phase culture of strain VC6129. Duplicate sets of plates were prepared for each salt concentration, with one set incubated at 30°C and the other incubated at 42°C. Colonies were counted after 36 h of incubation, and plating efficiencies (colony count at 42°C divided by colony count at 30°C) were calculated for each salt concentration.

tive than strains carrying the *relA1* and *relA2* alleles, and this may reflect the leakiness of the latter mutant alleles. Furthermore, the temperature sensitivity of the Δ*relA251::kan* strain was exacerbated by the introduction of the Δ*spoT207::cat* mutation. Collectively, these results indicate that the observed temperature sensitivity was directly attributable to decreased intracellular levels of (p)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 me-

TABLE 2. Suppression of thermosensitive phenotype of strain VC6129 (Δ*relA251::kan*) mutants by mutations in *rpoB* gene

Strain	Allele	Mutation in RpoB	Plating efficiency[a]
VC6159	*rpoB*[+]	None	4.4×10^{-5}
VC6158	*rpoB3449*	Δ532A	0.91
VC6160	*rpoB3401*	R529C	6.2×10^{-5}
VC6166	*rpoB3445*	Δ(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

[a] Ratio of colony formation at 42°C to colony formation at 30°C.

TABLE 3. Suppression of *relA1*-mediated temperature sensitivity by *spoT* mutant alleles

Strain	Relevant genotype	Plating efficiency[a]
VC7237	*relA1 spoT*[+]	2.3×10^{-3}
VC7239	*relA1 spoT202*	0.12
VC7240	*relA1 spoT203*	0.34
VC7241	*relA1 spoT204*	0.46

[a] Ratio of colony formation at 42°C to colony formation at 30°C.

dia, 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 (3). 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 (3). This is an unlikely explanation for the *relA*-mediated temperature sensitivity. For example, strains carrying the Δ*relA251::kan* mutation did not produce RelA but still exhibited an osmoremedial temperature-sensitive phenotype. Therefore, as an alternative, we are investigating the possibility that the osmoremedial characteristic may instead be related to some aspect of osmoregulated gene expression.

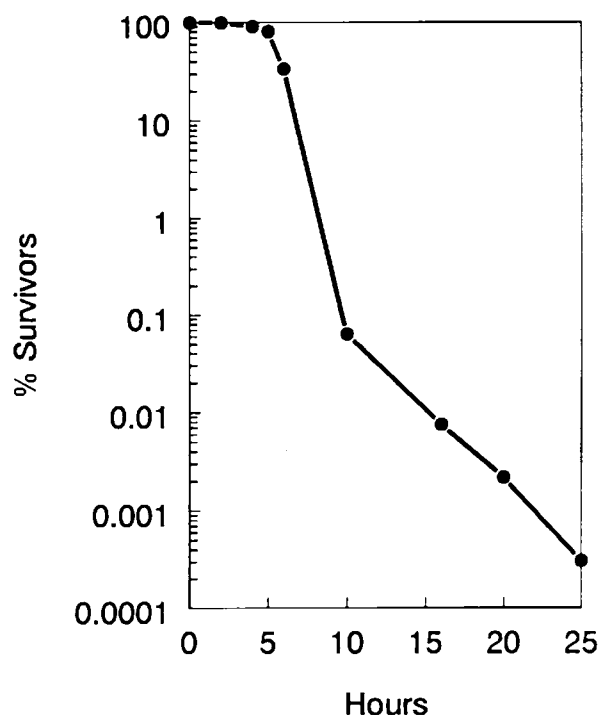The *relA*-mediated temperature sensitivity was suppressed

FIG. 4. Killing of strain VC6129 (Δ*relA251::kan*) at 42°C. Identical sets of plates inoculated with serial dilutions of strain VC6129 were incubated at 42°C beginning at 0 h. At the indicated times, sets of plates were removed and incubated further at 30°C to determine the number of survivors of the 42°C treatment.
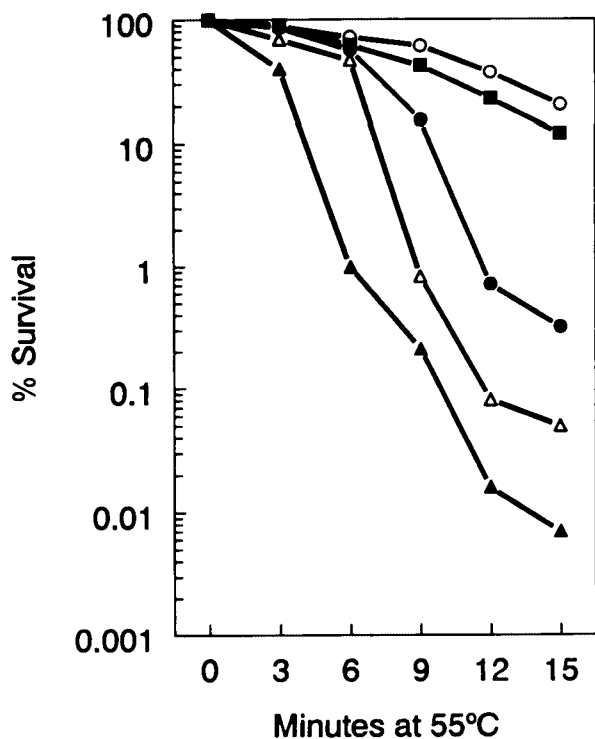
FIG. 5. Decreased thermotolerance conferred by *relA* mutation. Suspensions of bacteria in saline were incubated at 55°C. At the indicated times, samples were removed and the numbers of survivors were determined. Symbols: ○, strain VC6132 (wild type); △, strain VC6129 (Δ*relA251::kan*); ■, strain VC6129 carrying plasmid pALS10 (*relA*⁺); ▲, strain VC6130 (Δ*relA251::kan* Δ*spoT207::cat*); ●, strain VC6158 (Δ*relA251::kan rpoB3449*).

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 (11, 12). The connection between mutations in *rpoB* and ppGpp-dependent phenomena is well documented. The four *rpoB* alleles that suppress *relA*-mediated temperature sensitivity were previously shown to suppress the multiauxotrophic phenotypes of ppGpp-deficient mutants (1; M. Cashel, personal communication). These same alleles also enhanced the survival of ppGpp-deficient strains during prolonged stationary phase (1). Mutations in *rpoB* have also been reported to suppress the sensitivity of *relA* mutants to serine, methionine, and glycine (25, 26) and have been shown relieve the growth-inhibitory effects of high levels of ppGpp (24). The multiauxotrophic phenotype of ppGpp-deficient mutants is corrected not only by the aforementioned *rpoB* alleles but also by certain mutations in *rpoC* and *rpoD*, the genes encoding the β′ and σ⁷⁰ subunits of RNA polymerase, respectively (1, 9). 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 have the same underlying basis.

Evidence for the direct and specific binding of ppGpp to the β subunit of RNA polymerase has been presented (2, 19). The in vitro activities of RNA polymerase, to which azido-ppGpp

had been cross-linked, were compared on stringent and nonstringent promoters (2). The transcription of ribosomal genes was inhibited by azido-ppGpp, whereas transcription from the *lacUV5* promoter was unaffected. It will therefore be of interest to determine whether there is a relationship between these findings and ppGpp-dependent phenomena such as multiauxotrophy and thermotolerance.

We have considered the possibility that ppGpp is required for the expression of genes that are necessary for thermotolerance, and an obvious possibility was the involvement of those encoding heat shock proteins. The heat shock response is dependent on the concentration of σ³², and heat shock protein synthesis can even be induced in the absence of heat shock when σ³² is overproduced (5). We have shown that the overproduction of σ³² from plasmid pDS1 did not relieve the *relA*-mediated temperature sensitivity (data not shown). However, in retrospect, this is perhaps not surprising since VanBogelen et al. (27) have shown that induction of the heat shock regulon by induction of σ³² overproduction at 28°C is, for unknown reasons, insufficient to confer thermotolerance. More importantly, we did not detect any differences in gene expression patterns by DNA microarray technology in *relA*⁺ and *relA* mutant bacteria during heat shock induction, suggesting that the heat shock response is functional in *relA* mutant strains (X. Yang and E. E. Ishiguro, unpublished data). This confirms the unpublished data of A. D. Grossman (cited in reference 6). Moreover, VanBogelen and Neidhardt (29) have demonstrated that strain CF1946, a W3110 derivative carrying Δ*relA251::kan* Δ*spoT207::cat*, exhibited a heat shock response, albeit an altered one. They observed 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 occurred 10 min later than that observed in wild-type strain W3110. It is interesting that VanBogelen and Neidhardt (29) also observed in this experiment that CF1946 was temperature sensitive and failed to grow at 42°C, but no attempt was made to correlate the temperature sensitivity to the Δ*relA251::kan* and Δ*spoT207::cat* mutations. It has been previously noted that temperature upshifts result in the accumulation of ppGpp (18, 28), but VanBogelen and Neidhardt (29) concluded from their results that ppGpp was neither sufficient nor necessary for induction of the heat shock regulon. In an earlier study, Grossman et al. (6) 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 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. In contrast, subsequent studies by VanBogelen et al. (28) indicated that the heat shock response was not induced when the stringent response was invoked by isoleucine deprivation. Therefore, it is not clear what role, if any, ppGpp plays in induction of the heat shock response. Nevertheless, we conclude from our studies that the

expression of heat shock proteins is not sufficient to relieve the temperature sensitivity exhibited by *relA* mutant strains.

The *rpoS* gene encodes the σ^s transcription factor that is required for the expression of stationary phase-induced and osmoregulated genes in *E. coli* (7, 15). Gentry et al. (4) have shown that ppGpp is required for expression of the *rpoS* gene. Moreover, Kvint et al. (13, 14) have demonstrated that ppGpp is required for expression of RpoS-dependent genes in addition to σ^s. Because of the connection to ppGpp and, possibly, to osmoregulated gene expression, it was possible that the temperature sensitivity of the *relA* mutants could be attributable to their inability to express a key RpoS-dependent gene required for thermotolerance. However, strain UM122, which carries an insertion-inactivated *rpoS* gene (17), is not temperature sensitive (E. E. Ishiguro, unpublished data). Therefore, it seems unlikely that RpoS is involved in the *relA*-mediated temperature-sensitive phenotype.

In summary, the basis for *relA*-dependent thermotolerance appears to be complex. The suppression of temperature sensitivity in *relA* mutants by certain *rpoB* alleles suggests the involvement of an unknown aspect of gene expression. This characteristic is common to other *relA*-dependent phenotypes, and the relationship between *relA* and RNA polymerase must be elucidated in order to gain a better understanding of *relA*-dependent thermotolerance.

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