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Comparison of Δ*relA* Strains of *Escherichia coli* and *Salmonella enterica* Serovar Typhimurium Suggests a Role for ppGpp in Attenuation Regulation of Branched-Chain Amino Acid Biosynthesis

K. TEDIN* AND F. NOREL

Unité de Génétique des Bactéries Intracellulaires, Institut Pasteur, F-75724 Paris Cedex 15, France

Received 19 December 2000/Accepted 16 August 2001

The growth recovery of *Escherichia coli* K-12 and *Salmonella enterica* serovar Typhimurium Δ*relA* mutants were compared after nutritional downshifts requiring derepression of the branched-chain amino acid pathways. Because wild-type *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 strains are defective in the expression of the genes encoding the branch point acetohydroxy acid synthetase II (*ilvGM*) and III (*ilvIH*) isozymes, respectively, Δ*relA* derivatives corrected for these mutations were also examined. Results indicate that reduced expression of the known global regulatory factors involved in branched-chain amino acid biosynthesis cannot completely explain the observed growth recovery defects of the Δ*relA* strains. In the *E. coli* K-12 MG1655 Δ*relA* background, correction of the preexisting *rph*-1 allele which causes pyrimidine limitations resulted in complete loss of growth recovery. *S. enterica* serovar Typhimurium LT2 Δ*relA* strains were fully complemented by elevated basal ppGpp levels in an *S. enterica* serovar Typhimurium LT2 Δ*relA spoT1* mutant or in a strain harboring an RNA polymerase mutation conferring a reduced RNA chain elongation rate. The results are best explained by a dependence on the basal levels of ppGpp, which are determined by *relA*-dependent changes in tRNA synthesis resulting from amino acid starvations. Expression of the branched-chain amino acid operons is suggested to require changes in the RNA chain elongation rate of the RNA polymerase, which can be achieved either by elevation of the basal ppGpp levels or, in the case of the *E. coli* K-12 MG1655 strain, through pyrimidine limitations which partially compensate for reduced ppGpp levels. Roles for ppGpp in branched-chain amino acid biosynthesis are discussed in terms of effects on the synthesis of known global regulatory proteins and current models for the control of global RNA synthesis by ppGpp.

Guanosine tetraphosphate (ppGpp) is a low-molecular-weight effector molecule which accumulates to high levels during amino acid starvation and correlates with a rapid inhibition of RNA synthesis, both phenotypes being associated with the stringent response (reviewed in reference 12). The enzyme responsible for the synthesis of ppGpp during amino acid starvation in wild-type *Escherichia coli* and *Salmonella enterica* serovar Typhimurium strains is a ribosome-associated protein encoded by the *relA* gene, also referred to as ppGpp-synthetase I (PSI). PSI-dependent synthesis of ppGpp occurs during an idling reaction of ribosomes stalled during translation of mRNA in the presence of uncharged tRNAs. Mutations in *relA* severely reduce or abolish the accumulation of this nucleotide during amino acid starvation, and, consistent with the inverse correlation of ppGpp levels and RNA synthesis, there is an increase in RNA synthesis, the bulk of which is stable rRNA and tRNA. In addition, during amino acid starvation of *relA* mutants, not only does ppGpp fail to accumulate but also the prestarvation basal levels of ppGpp effectively disappear (30, 31, 50). The basal levels of ppGpp during steady-state growth in different media are the same in both *relA*$^+$ and *relA* strains and are most probably synthesized by the product of the *spoT* gene, believed to encode a bifunctional enzyme with both ppGpp-synthetic (PSII) as well as ppGpp-degradative activities (24, 65). Suggestive of this, strains of *E. coli* K-12 and *S. enterica* serovar Typhimurium in which both the *relA* and *spoT* genes are deleted (Δ*relA*Δ*spoT* strains) contain no detectable ppGpp levels (65; K. Tedin and F. Norel, unpublished data). The PSII activity of SpoT is unstable, with a half-life of approximately 30 s, similar to that of ppGpp itself, whereas the ppGpp-degradative activity is stable (12, 29, 39). How the PSII and/or degradative activities are regulated remains unclear, although evidence suggests that the ratio of uncharged to charged tRNA in the cell in some way provides the regulatory signal (39, 48); discussed in reference (12).

A characteristic phenotype of Δ*relA*Δ*spoT* strains of *E. coli* K-12 is amino acid requirements, suggesting a role for ppGpp in amino acid biosynthesis (65). Likewise, defects in the biosynthesis of and sensitivities to amino acids are characteristic of *relA* mutants of *E. coli* K-12 strains, including a failed derepression of *ilvBN*, encoding the branched-chain amino acid acetohydroxy acid synthetase (AHAS) isozyme I (18) and sensitivities to leucine and serine (1, 55, 56).

Branched-chain amino acid biosynthesis is complex and regulated at a number of different levels. The first reaction in the biosynthetic pathways is the synthesis of either acetolactate from two molecules of pyruvate or the formation of acetohydroxybutyrate from pyruvate and ketobutyrate (KB), with the latter compound being derived from threonine by threonine deaminase, the *ilvA* gene product (Fig. 1). Both reactions are carried out by the branch point AHAS isozymes, responsible for the first committed step in the pathways (for a review, see reference 54). Three isoforms of the AHAS enzymes have

* Corresponding author. Mailing address: Institut Pasteur, Unité de Génétique des Bactéries Intracellulaires, 28 Rue du Docteur Roux, F-75724 Paris Cedex, France. Phone: (033)-01-4061-3164. Fax: (033)-01-4568-8228. E-mail: ktedin@pasteur.fr.

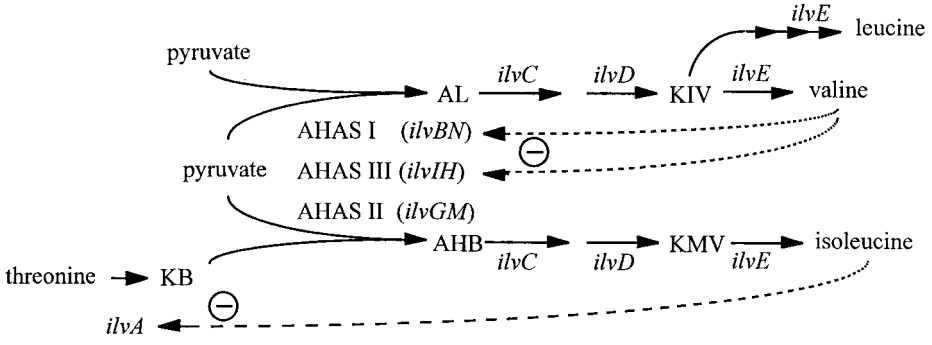

FIG. 1. Schematic representation of the branched-chain amino acid pathways. Solid lines with arrowheads represent the reaction pathways, and dotted lines with arrowheads represent the targets for end product inhibitions (denoted by a minus symbol). Abbreviations: AL, acetolactate; AHB, α-hydroxybutyrate; KIV, α-ketoisovalerate; KMV, α-keto-β-methylvalerate. Genes of the *ilvGMEDA* and *ilvYC* operons encoding enzymes in common for both pathways are indicated: *ilvA*, threonine deaminase; *ilvC*, acetohydroxy acid isomeroreductase; *ilvD*, dihydroxy acid dehyratase; *ilvE*, transaminase B.

been characterized, AHAS I, AHAS II, and AHAS III. While all three isozymes are capable of performing the same reactions, their substrate specificities differ significantly (5, 17). The AHAS I (*ilvBN*-encoded) isozyme uses pyruvate almost exclusively as the second substrate in this reaction to form AL, the precursor leading to valine synthesis. The immediate precursor of valine, ketoisovalerate, is itself a branch point reaction product used for leucine biosynthesis. Leucine can also be synthesized directly from valine through a deamination of valine to ketoisovalerate (54). AHAS II (*ilvGM* gene product) shows a marked preference for KB as a substrate for the condensation reaction with pyruvate to form AHB, the precursor leading to isoleucine biosynthesis, and while AHAS III (*ilvIH*–encoded) is somewhat intermediate in its second substrate preference, it also appears to favor isoleucine biosynthesis (17). The AHAS isozyme activities are also differentially regulated; AHAS I and AHAS III are end product repressible by valine, while AHAS II is valine insensitive.

In addition to the differences in substrate specificities and end product repression, the regulation of expression of the genes encoding the various isozymes differs. The *ilvBN* and *ilvGMEDA* operons are regulated by an attenuation mechanism involving the branched-chain amino acid codons present in the leader peptide coding regions upstream of the operons (54). The expression of AHAS I, encoded by the *ilvBN* genes, is bivalently repressed by leucine and valine; *i.e.*, derepression is observed when either amino acid is limiting. The *ilvBN* genes are also under catabolite control, requiring cyclic AMP (cAMP)-cAMP receptor protein (CRP) for activation of expression (18, 53). The expression of AHAS II, encoded by the *ilvGM* genes of the *ilvGMEDA* operon, is derepressed when any one of the three branched-chain amino acids is limiting, consistent with the requirement of both pathways for the common enzymes encoded in this operon. The expression of AHAS III (*ilvIH* gene products) is inhibited by leucine, apparently due to a requirement for the leucine-responsive regulatory protein (Lrp) for activation of *ilvIH* transcription. In the presence of leucine, binding of Lrp is reduced, resulting in reduced transcriptional activation of *ilvIH* expression (references 60 and 61 and references therein). In addition to these two global regulatory systems, integration host factor (IHF) is involved in the expression of the genes involved in branched-chain amino acid biosynthesis (for reviews, see references 11, 40, and 54).

In another study to be described elsewhere, Δ*relA* and Δ*relA* Δ*spoT* strains of *S. enterica* serovar Typhimurium were constructed and partially characterized (Tedin and Norel, unpublished). Like the *E. coli* derivatives, the *S. enterica* serovar Typhimurium Δ*relA* Δ*spoT* strains synthesized no detectable levels of ppGpp and showed the same lack of growth on minimal media in the absence of amino acids. However, the *S. enterica* serovar Typhimurium Δ*relA* derivatives acquired a number of additional amino acid requirements, particularly for the branched-chain amino acids, which were not observed in *E. coli* K-12 Δ*relA* strains. Because defects in branched-chain amino acid biosynthesis are characteristic phenotypes of *relA* mutants (1, 18), we chose to investigate the bases for these differences in the two bacterial species. Growth recoveries after nutritional downshifts of *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 Δ*relA* strains were compared under conditions requiring derepression of the branched-chain amino acid pathways. In addition, because *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 strains are naturally defective in the expression of the *ilvG* and *ilvI* genes, respectively, Δ*relA* derivatives of *E. coli* K-12 and *S. enterica* serovar Typhimurium strains which express the full complement of AHAS isozymes were also examined. The results indicate that the different *relA* dependencies for branched-chain amino acid biosynthesis between *relA* strains of *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 are due to preexisting mutations in the wild-type strains which, when corrected, result in essentially equivalent *relA*-dependent defects in branched-chain amino acid derepression patterns. The role of ppGpp in the expression of known global regulatory factors involved in branched-chain amino acid biosynthesis, as well as additional roles for ppGpp based on current models for the control of global RNA synthesis by this nucleotide, is discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Growth of cultures was performed in either L broth (36) or M9 minimal medium (38) supplemented with 0.2% glucose, 1 μg of thiamine per ml, and 1 μg of calcium pantothenate per ml. Additional supplements are indicated in the table footnotes. Growth of overnight cultures of Δ*relA*

TABLE 1. Bacterial strains and bacteriophage used in this study

Strain	Genotype[a]	Source or reference[b]
S. enterica serovar Typhimurium		
LT2	Wild-type prototroph *ilvI* (SGSC 1412)	SGSC
CDC 331-86	Wild-type prototroph *ilvI*$^+$	CDC (10)
CDC 1119-83	Wild-type prototroph *ilvI*$^+$	CDC (10)
CH1107	LT2 *cya*::Tn*10*	J. C. D. Hinton
CH1108	LT2 *crp*::Tn*10*	J. C. D. Hinton
GA446	LT2 *dhuA1 lrp-1*::Tn*5*	G. F.-L. Ames (23)
KP1469	LT2 *cdd-9 cod-8 deoD201 udp-11*	K. F. Jensen (26)
KP1475	LT2 *cdd-9 cod-8 deoD201 rpoBC udp-11*	K. F. Jensen (26)
TT66	LT2 *ilvG1007*::Tn*10*	SGSC
TT206	LT2 *leu-1151*::Tn*10*	J. R. Roth
TR6478[c]	LT2 *spoT*$^+$	49
TR6479[c]	LT2 *spoT1*	49
TT7262	LT2 *ara-9* Δ(*ilvB-uhp*)*650 ilvG236 pan-187*	J. R. Roth
KT2146	SL1344 *his* Δ*relA71*::*kan rpsL spoT*$^+$	Tedin and Norel, unpublished
KT2160	SL1344 *his* Δ*relA71*::*kan rpsL* Δ*spoT281*::*cat*	Tedin and Norel, unpublished
KT2184	LT2 Δ*relA71*::*kan*	This study
KT2192	LT2 Δ*relA71*::*kan* Δ*spoT281*::*cat*	This study
KT2222[c]	TR6478 Δ*relA71*::*kan spoT*$^+$	This study
KT2224[c]	TR6479 Δ*relA71*::*kan spoT1*	This study
KT2244	KP1469 Δ*relA71*::*kan*	This study
KT2246	KP1475 Δ*relA71*::*kan rpoBC*	This study
KT2274	LT2 *leu*-1151::Tn*10*	This study
KT2282	LT2 *ilvI*$^+$	This study
KT2286	LT2 *ilvI*$^+$ Δ*relA71*::*kan*	This study
KT2290	LT2 *ilvG1007*::Tn*10*	This study
KT2298	LT2 *ilvI*$^+$ Δ*relA71*::*kan* Δ*spoT281*::*cat*	This study
KT2314	CDC 331-86 Δ*relA71*::*kan*	Tedin and Norel, unpublished
KT2316	CDC 1119-83 Δ*relA71*::*kan*	Tedin and Norel, unpublished
KT2354	LT2 *ilvG236 ilvI*$^+$	This study
KT2358	LT2 *ilvG236 ilvI*$^+$ Δ*relA71*::*kan*	This study
KT2454	LT2 *ilvG236 ilvI*$^+$ Δ*relA71*::*kan* Δ*spoT281*::*cat*	This study
KT2402	LT2 *lrp-1*::Tn*5*	This study
KT2404	LT2 *ilvI*$^+$ *lrp-1*::Tn*5*	This study
KT2406	LT2 *ilvG236 ilvI*$^+$ *lrp-1*::Tn*5*	This study
E. coli K-12		
MG1655	Wild-type *E. coli* K-12 *ilvG rph-1*	CGSC (20)
CF1652	MG1655 Δ*relA251*::*kan*	65
CF1693	MG1655 Δ*relA251*::*kan* Δ*spoT207*::*cat*	65
CF7968	MG1655 Δ(*lacIZ*) *rph*$^+$	M. Cashel
CF7974	MG1655 Δ(*lacIZ*) Δ*relA251*::*kan rph*$^+$	M. Cashel
CF7976	MG1655 Δ(*lacIZ*) Δ*relA251*::*kan rph*$^+$ Δ*spoT207*::*cat*	M. Cashel
KT2268	MG1655 *ilvG*$^+$	This study
KT2270	MG1655 *ilvG*$^+$ Δ*relA251*::*kan*	This study
KT2302	MG1655 *ilvG*$^+$ Δ*relA251*::*kan* Δ*spoT207*::*cat*	This study
KT2448	MG1655 *ilvG*$^+$ Δ(*lacIZ*) *rph*$^+$	This study
KT2450	MG1655 *ilvG*$^+$ Δ(*lacIZ*) Δ*relA251*::*kan rph*$^+$	This study
KT2452	MG1655 *ilvG*$^+$ Δ(*lacIZ*) Δ*relA251*::*kan rph*$^+$ Δ*spoT207*::*cat*	This study
SK2226	Δ*argH1 galK2*(Oc) Δ(*gpt-proA*)*62 glnV44 hisG4*(Oc) *lacY1 mtl-1 trpE3*(Oc) *tsx-33 zif-290*::Tn*10* (100% linked to *ilv*)	CGSC
T31-4-590	*ilvG468* (*ilvG*$^+$) *thi-1 trpA9761 trpE9829*	G. W. Hatfield (35)
Bacteriophage		
P1D7H	P1*vir*	Laboratory stock
P22HT105/1	P22*int*	Laboratory stock

[a] Unless noted otherwise, all *E. coli* K-12 MG1655 derivatives are *ilvG* and *rph*, as is the wild type. Likewise, all *S. enterica* serovar typhimurium LT2 derivatives are *ilvI* unless noted otherwise.

[b] CDC, Centers for Disease Control and Prevention; CGSC, *E. coli* Genetic Stock Center; SGSC, *Salmonella* Genetic Stock Center.

[c] Strains TR6478 and TR6479 and their Δ*relA* derivatives KT2222 and KT2224 harbor an uncharacterized mutation preventing growth with pyruvate as a carbon source (unpublished observations).

Δ*spoT* strains was carried out at 32°C in the presence of antibiotics to avoid the accumulation of revertants capable of growth on minimal glucose medium (12). Experimental cultures were inoculated from −80°C stocks into 5-ml culture tubes and grown aerobically at 32°C, and when visibly turbid, these precultures were used to inoculate larger volumes (25- to 50-fold dilutions) of the same, prewarmed medium to ensure steady-state growth conditions. Downshift plate tests were performed using mid-log (optical density at 600 nm [OD_{600}] <0.5) cultures grown in L broth as above, followed by two centrifugations with resuspension in 0.15 M NaCl each time to wash the cells. Identical results were obtained in control experiments with either M9 salts or 1 mM $MgSO_4$ as the wash and resuspension medium. Washed cells were streaked for single colonies rather than plating dilutions since cell densities are known to affect some of the phenotypes of *relA* mutants (55). Downshift tests were performed at least twice for all strains. Green plates for screening of *S. enterica* serovar Typhimurium P22

transductants to eliminate lysogens and infected cells were prepared as previously described (52), except that the NaCl concentration was reduced to 5 g/liter. M9 salts were used as the basis for the SMG medium used for screening of *relA* mutants (49, 55). Kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), or tetracycline (20 μg/ml) was added where appropriate for the screening of genetic markers. Antibiotics were omitted for experimental cultures.

Strain constructions. Construction of the *S. enterica* serovar Typhimurium strain SL1344 Δ*relA71*::*kan* and Δ*relA71*::*kan* Δ*spoT281*::*cat* derivatives KT2146 and KT2160 (Table 1) will be described elsewhere (Tedin and Norel, unpublished). *S. enterica* serovar Typhimurium LT2 Δ*relA* and Δ*relA*Δ*spoT* strains were constructed by P22 transductions using lysates of strains KT2146 and KT2160 with selection for kanamycin or chloramphenicol resistance, respectively. The *S. enterica* serovar Typhimurium LT2 *ilvI*$^+$ strains KT2282 and KT2284 were constructed by introduction of the *leu-1151*::Tn*10* allele from strain TT206 into strain LT2 (SGSC 1412) followed by infection of the resulting strain (KT2274) with lysates of *ilvI*$^+$ strain CDC 331-86 or CDC 1119-83, respectively, with selection for growth on minimal glucose and screening for loss of tetracycline resistance (the *leuABCD* and *ilvIH* operons are very closely linked [7]). Control experiments without P22 phage infection of strain KT2274 showed no colonies after up to 3 days on minimal glucose plates. Since all phenotypes were identical for the Δ*relA* strains KT2282 and KT2284 and derivatives, results are shown only for KT2282 and related constructs.

Strain KT2290 was constructed by infection of *ilvI*$^+$ strain KT2282 with P22 lysates prepared on LT2 *ilvG1007*::Tn*10* strain TT66 with selection for tetracycline resistance and screening for lack of growth on minimal glucose. Strain KT2354 was constructed by infection of strain KT2290 with P22 lysates prepared on strain TT7262 (*ilvG236*) with selection for growth on minimal glucose and screening for loss of tetracycline resistance and valine sensitivity (IlvG$^-$ phenotype). Note that *S. enterica* serovar Typhimurium LT2 *ilvG ilvI* strains require isoleucine supplementation for growth on minimal glucose medium (51). Δ*relA* derivatives of strains KT2244 and KT2246 were constructed by transduction of strains KP1469 and KP1475 to kanamycin resistance as above. Four independent Δ*relA* isolates of KP1475 were screened on L broth plates to verify the absence of fast-growing revertants of the slow-growth phenotype on rich media conferred by the *rpoBC* allele (26). The *lrp-1*::Tn*5* mutants KT2402, KT2404, and KT2406 were constructed by P22 transduction of strains LT2, KT2282, and KT2354, respectively, to kanamycin resistance using lysates prepared on strain GA446 followed by screening for serine sensitivity at 42°C (Lrp$^-$ phenotype [40]). *S. enterica* serovar Typhimurium LT2 *cya*::Tn*10* and *crp*::Tn*10* mutants (CH1107 and CH1108, respectively) were screened for lack of growth on M9 minimal glycerol (0.4%) medium. Phage P22 transductions were performed as previously described (52), except that in all P22 transductions involving the Δ*spoT281*::*cat* allele, only the 20- to 30-min infection was at 37°C; subsequent incubations were performed at 32°C.

P1*vir* phage transductions for strain constructions in *E. coli* K-12 were performed by standard methods. Strains KT2268 and KT2448 were constructed by infection of the *E. coli* K-12 strains MG1655 and CF7968, respectively, with P1*vir* lysates of *ilvG468* (*ilvG*$^+$) strain T31-4-590 followed by selection for growth on minimal glucose plates containing 25 to 50 μg of valine per ml and screening on minimal glucose without amino acid supplements and minimal glucose containing leucine (25 μg/ml). Verification of acquisition the *ilvG468* (*ilvG*$^+$) allele was performed by transduction of valine-resistant isolates to tetracycline resistance using strain SK2226 as the donor (*zif-290*::Tn*10* 100% linked to *ilv*) and screening for loss of valine resistance. Sequencing verified the presence of the *ilvG468* mutation. Introduction of the Δ*relA251*::*kan* and Δ*spoT207*::*cat* alleles was performed by transductions using P1*vir* lysates of strains CF1652 and CF1693, respectively, with selection for kanamycin or chloramphenicol resistance. As with the *S. enterica* serovar Typhimurium P22 transductions to Δ*spoT*, only the infections using P1*vir* lysates of CF1693 were carried out at 37°C, and incubations were at 32°C.

RESULTS

Δ*relA* and Δ*relA*Δ*spoT* derivatives of *S. enterica* serovar Typhimurium LT2 have more extensive amino acid requirements than their *E. coli* K-12 counterparts. During the initial characterization of Δ*relA* and Δ*relA*Δ*spoT* mutants of *S. enterica* serovar Typhimurium strains (Tedin and Norel, unpublished), the *S. enterica* serovar Typhimurium Δ*relA* derivatives appeared to show more amino acid requirements than did previously reported *E. coli* K-12 Δ*relA* derivatives (65), particularly for the branched-chain amino acids. The full patterns of apparent amino acid requirements of the *S. enterica* serovar Typhimurium LT2 Δ*relA* strains were determined from cultures grown to mid-logarithmic phase in L broth and downshifted to M9 minimal glucose medium containing combinations of 19 amino acids (dropout plates). As shown in Table 2, histidine, phenylalanine, and the branched-chain amino acids isoleucine, leucine, and valine were consistently very strong if not absolute requirements for the *S. enterica* serovar Typhimurium LT2 Δ*relA* strains KT2184 and KT2222. In contrast, the *E. coli* K-12 Δ*relA* strain CF1652 showed requirements only for valine and isoleucine (65) (Table 2).

The Δ*relA* Δ*spoT* derivatives of both *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 showed similar patterns of amino acid requirements (compare strains CF1693 and KT2192 [Table 2]), suggesting that the differences in the Δ*relA* mutants regarding the amino acid requirements were related to differences in the basal (SpoT- or PSII-derived) levels of ppGpp. Because defects in branched-chain amino acid biosynthesis are characteristic phenotypes of *relA* mutants (1, 18), the basis for these apparent differences in the *relA*-dependencies between *E. coli* K-12 and *S. enterica* serovar Typhimurium was more closely investigated.

***relA*-dependent branched-chain amino acid requirements.** It should be stressed that *relA* mutants do not have amino acid requirements in the strict sense; the apparent *relA*-dependent amino acid requirements are due to sensitivities to certain amino acids or combinations and to defects in expression of amino acid biosynthetic operons, particularly after nutritional downshifts or amino acid starvations (1, 18, 55). Valine sensitivity is characteristic for wild-type *E. coli* K-12 strains which do not express the valine-insensitive AHAS II isozyme due to a frameshift in *ilvG*, encoding the large subunit of the enzyme (35). *E. coli* K-12 strains therefore starve for isoleucine in the presence of valine, since the AHAS isozymes are required for the first, committed reaction step in the valine-leucine and isoleucine biosynthetic pathways (54) (Fig. 1). In contrast, wild-type *S. enterica* serovar Typhimurium LT2 strains do not express the AHAS III isozyme due to a nonsense mutation in *ilvI* encoding the large subunit of this isozyme (47). Since wild-type *S. enterica* serovar Typhimurium LT2 strains express the *ilvGM*-encoded isozyme, AHAS II, excess valine does not lead to isoleucine starvation. Despite these differences in AHAS isozyme expression patterns, however, the regulation of branched-chain amino acid biosynthesis is considered to be essentially equivalent in the two microorganisms (21, 54).

Another reported characteristic of *E. coli* K-12 Δ*relA* derivatives is their lack of growth on minimal glucose containing all 17 non-branched-chain amino acids (65). To determine whether the *S. enterica* serovar Typhimurium LT2 Δ*relA* strain showed a similar growth defect, strain KT2184 and the *E. coli* K-12 Δ*relA* strain CF1652 were tested for growth recovery after a downshift to either M9 minimal glucose or M9 minimal glucose containing all 17 non-branched-chain amino acids (M9/17 medium). In addition, since the branched-chain amino acid-dependent attenuation regulation of the various AHAS isozymes differs (54) (see Introduction), growth on these media supplemented with combinations of the branched-chain amino acids was also determined.

Both the *E. coli* K-12 wild-type and Δ*relA* strains were un-

TABLE 2. Amino acid requirements of Δ*relA* and Δ*relA* Δ*spoT* mutants[a]

Strain	Genotype[b]	Requirement for amino acid omitted from full complement[c]																			
		A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
S. enterica serovar Typhimurium LT2																					
LT2	Wild type (*ilvI*)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2184	Δ*relA*	+	±	+	+	+	+	+	+	±	±	−	+	±	−	+	±	+	+	+	−
KT2192	Δ*relA* Δ*spoT*	+	±	±	±	+	+	+	±	−	−	−	±	±	−	±	−	±	−	+	−
KT2222[d]	Δ*relA*	+	+	+	+	+	+	+	+	±	±	−	+	+	±	+	+	+	+	+	−
KT2224[d]	Δ*relA spoT1*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2282	*ilvI*$^+$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2286	*ilvI*$^+$ Δ*relA*	+	+	+	+	+	+	+	+	−	±	−	+	±	±	+	+	+	+	+	−
KT2298	*ilvI*$^+$ Δ*relA* Δ*spoT*	+	±	+	+	±	+	+	±	−	−	−	+	−	−	±	−	−	−	+	−
KT2354	*ilvG ilvI*$^+$	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	±	+	+
KT2358	*ilvG ilvI*$^+$ Δ*relA*	+	+	+	+	+	+	+	+	−	−	−	+	±	±	+	+	±	±	+	−
KT2454	*ilvG ilvI*$^+$ Δ*relA* Δ*spotT*	+	±	±	+	+	+	±	±	−	−	−	±	−	−	±	−	−	−	±	−
KT2402	*ilvI lrp*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+
KT2404	*ilvI*$^+$ *lrp*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+
KT2406	*ilvG ilvI*$^+$ *lrp*	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	±	+	+	+
E. coli K-12																					
MG1655[e]	Wild type (*ilvG rph*)	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
CF1652[e]	Δ*relA*	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	−
CF1693[e]	Δ*relA* Δ*spoT*	+	−	+	+	+	+	+	−	−	−	−	+	±	−	+	−	−	+	+	−
KT2268	*ilvG*$^+$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2270	*ilvG*$^+$ Δ*relA*	+	+	+	+	+	+	+	+	±	+	±	+	+	±	+	+	+	+	+	−
KT2302	*ilvG*$^+$ Δ*relA* Δ*spoT*	+	±	±	+	+	+	+	−	−	−	−	±	−	−	+	±	−	±	−	−
CF7968	*rph*$^+$	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
CF7974	*rph*$^+$ Δ*relA*	+	+	+	+	+	+	+	+	−	−	−	+	±	−	+	+	−	±	+	−
CF7976	*rph*$^+$ Δ*relA* Δ*spoT*	+	±	±	±	±	+	+	−	−	−	−	±	−	−	+	−	−	−	−	−

[a] Amino acid requirements were scored after downshift a of a mid-log ($OD_{600} < 0.5$) L broth culture to M9 minimal glucose plates containing combinations of 19 amino acids at 25 μg/ml with 1 of the full set of 20 omitted. Control plates included one with the full complement of 20 amino acids and one without amino acid supplements (M9 minimal glucose). All strains grew overnight on plates containing the full complement of amino acids, and no growth was observed for any of the Δ*relA* Δ*spoT* strains without amino acid supplements.

[b] Abbreviated genotypes shown. For full genotypes, see Table 1.

[c] Symbols: +, normal growth; ±, poor growth (reduced CFU) or microcolonies; −, no visible growth after 24 to 48 h of incubation at 32°C relative to the isogenic wild-type controls on the same media. Results are from at least two independent determinations on different days.

[d] KT2222 and KT2224 are derived from the *S. enterica* serovar Typhimurium LT2 strains TR6748 and TR6749 (see Table 1).

[e] Results as reported for *E. coli* K-12 strains by Xiao et al. (65) included for comparison.

able to grow on minimal glucose plates containing valine without added isoleucine (MG1655 and CF1652) (Table 3). Plates containing leucine alone in minimal glucose also showed no growth recovery of CF1652, consistent with the leucine sensitivity characteristic for *E. coli* K-12 *relA* strains (1). Unlike valine, which inhibits AHAS isozyme I and III by end product inhibition, leucine is not known to interfere with the activities of any of the enzymes in the branched-chain pathways but is known to inhibit the expression of the *ilvIH* genes, encoding AHAS III (11, 40) (see Introduction). The *S. enterica* serovar Typhimurium LT2 Δ*relA* strain, KT2184, showed reduced growth in the presence of any one of the branched-chain amino acids and combinations without valine (Table 4).

Consistent with the results obtained from the dropout plates (Table 2), the M9/17 combinations corresponding to the full complement of amino acids omitting either valine or isoleucine also resulted in no growth of the *E. coli* K-12 Δ*relA* strain CF1652 (Table 3) while those omitting leucine resulted in reduced growth, with slow-growing microcolonies. The results for the *S. enterica* serovar Typhimurium LT2 Δ*relA* strain KT2184 were similar, except that rather than a requirement for isoleucine as in the *E. coli* K-12 derivative, strain KT2184 apparently required leucine and valine (Table 4).

A full complement of AHAS isozymes improves the growth recovery of *E. coli* K-12 Δ*relA* mutants. To allow a more direct comparison of the possible role of differences in AHAS isozyme patterns of expression and the *relA* dependencies of the *E. coli* K-12 and *S. enterica* serovar Typhimurium strains and to circumvent the valine-mediated growth inhibition of *E. coli* K-12 strains, *ilvG*$^+$ (AHAS II$^+$) strains of *E. coli* K-12 and Δ*relA* derivatives were constructed.

Introduction of the *ilvG*$^+$ allele reversed the valine sensitivity of the wild-type *E. coli* K-12 strain MG1655 (strain KT2268, Tables 2 and 3). For the *ilvG*$^+$ Δ*relA* derivative KT2270, the most obvious changes were the reversal of growth inhibition in the presence of valine or leucine (or both) (Table 3). In addition to loss of valine sensitivity, the growth recovery patterns of strain KT2270 became identical to those of the *S. enterica* serovar Typhimurium LT2 Δ*relA* strain KT2184 (compare Tables 3 and 4). Although strain KT2270 showed improved

TABLE 3. Branched-chain amino acid responses of *E. coli* K-12 Δ*relA* mutants[a]

Strain	Genotype[b]	Branched-chain amino acid(s) in medium[c]															
		M9								M9/17							
		None	I	L	V	IL	IV	LV	ILV	None	I	L	V	IL	IV	LV	ILV
MG1655	Wild type (*ilvG rph*)	+	+	+	−	+	+	−	+	+	+	+	−	+	+	−	+
CF1652	Δ*relA*	+	±	−	−	±	+	−	+	−	−	−	−	−	±	−	+
KT2268	*ilvG*$^+$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2270	*ilvG*$^+$ Δ*relA*	+	±	±	±	±	±	+	+	±	−	−	±	−	±	+	+
CF7968	*rph*$^+$	+	+	+	−	+	+	−	+	+	+	+	−	+	+	−	+
CF7974	*rph*$^+$ Δ*relA*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
KT2448	*ilvG*$^+$ *rph*$^+$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2450	*ilvG*$^+$ *rph*$^+$ Δ*relA*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	+

[a] Growth recovery was scored after a downshift of a mid-log ($OD_{600} < 0.5$) L broth culture to either M9 minimal glucose plates (M9) or M9 minimal glucose plates containing all amino acids except Ile (I) Leu (L), and Val (V) (M9/17) at 25 μg/ml each and supplemented in both cases with the indicated amino acids also at 25 μg/ml each.
[b] Abbreviated genotypes are indicated. For full genotypes, see Table 1.
[c] Symbols are as defined in Table 2. Results are from at least two independent determinations on different days.

growth in M9/17 medium relative to the *ilvG* Δ*relA* strain CF1652, in most cases the growth defects were only partially relieved. Since isoleucine addition alone did not restore growth, it appeared that the *ilvG*$^+$ Δ*relA* strain KT2270 remained compromised for the valine-leucine pathways, particularly in the presence of the non-branched-chain amino acids (M9/17, Tables 2 and 3).

Despite the apparent improvements in growth recovery for the *E. coli* K-12 Δ*relA* derivative KT2270 on M9 or M9/17 medium, the presence of the *ilvG*$^+$ allele in the Δ*relA*Δ*spoT* background in the *E. coli* K-12 strain KT2302 did not alleviate any of the amino acid requirements. Indeed, in the absence of both the *relA* and *spoT* genes, additional requirements appeared for methionine and tyrosine (Table 2). The *ilvG*$^+$ Δ*relA* Δ*spoT* strain KT2302 also acquired an apparent rich-medium growth defect, with extremely long culture doubling times on L broth plates and in liquid culture, a phenotype not as apparent with M9 minimal glucose medium supplemented with all amino acids (data not shown).

A full complement of AHAS isozymes worsens the growth recovery of *S. enterica* serovar Typhimurium LT2 Δ*relA* mutants. In *S. enterica* serovar Typhimurium LT2, derepression of the branched-chain amino acid pathways should occur when any one of the branched-chain amino acids is limiting, provid-

TABLE 4. Branched-chain amino acid responses of *S. enterica* serovar Typhimurium LT2 Δ*relA* mutants[a]

Strain	Genotype[b]	Branched-chain amino acid(s) in medium[c]:															
		M9								M9/17							
		None	I	L	V	IL	IV	LV	ILV	None	I	L	V	IL	IV	LV	ILV
LT2	Wild type (*ilvI*)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2184	Δ*relA*	+	±	±	±	±	±	+	+	±	−	−	±	−	±	+	+
KT2282	*ilvI*$^+$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2286	*ilvI*$^+$ Δ*relA*	+	±	−	±	±	±	±	+	±	−	−	−	−	−	+	+
KT2354	*ilvG ilvI*$^+$	+	+	+	−	+	+	−	+	+	+	+	−	+	+	−	+
KT2358	*ilvG ilvI*$^+$ Δ*relA*	+	±	−	−	±	±	−	±	±	−	−	−	−	−	−	+
TR6478	*relA*$^+$ *spoT*$^+$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TR6479	*relA*$^+$ *spoT1*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2222	Δ*relA spoT*$^+$	+	±	±	+	±	±	+	+	±	−	±	−	±	±	+	+
KT2224	Δ*relA spoT1*	+	+	+	+	+	+	+	+	+	±	±	+	+	+	+	+
KT2402	*ilvI lrp*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2404	*ilvI*$^+$ *lrp*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2406	*ilvG ilvI*$^+$ *lrp*	+	+	+	−	+	+	−	+	+	+	−	−	+	+	−	+
KP1469[d]		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2244[d]	Δ*relA*	+	±	−	−	±	±	+	+	±	−	−	−	±	±	+	+
KP1475[d]	*rpoBC*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KT2246[d]	Δ*relA rpoBC*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Growth conditions and composition of the downshift media are as described in Table 3, footnote *a*.
[b] Abbreviated genotypes are indicated. For full genotypes, see Table 1.
[c] Symbols are as defined in Table 2. Results are from at least two independent determinations on different days.
[d] Results for these strains are reported after 36 to 48 h owing to the slow growth of strain KP1475.

ing not only AHAS II but also the other enzymes encoded by the *ilvGMEDA* operon required for both the valine and isoleucine pathways (54) (Fig. 1). For comparison with the *E. coli* K-12 *ilvG*$^+$ strains, an *ilvI*$^+$ (AHAS III$^+$) strain of *S. enterica* serovar Typhimurium LT2 and a Δ*relA* derivative were constructed, along with Δ*relA* derivatives of two recent, clinical isolates of *S. enterica* serovar Typhimurium which have been found naturally to express AHAS III (10). In addition, an *ilvG ilvI*$^+$ derivative of *S. enterica* serovar Typhimurium LT2, i.e., a "pseudo"-*E. coli* K-12 strain with regard to the pattern of AHAS isozyme expression, was constructed and growth recoveries for all Δ*relA* derivatives were determined.

The patterns of *relA*-dependent growth defects in the three *ilvI*$^+$ Δ*relA* derivatives indicated that in *S. enterica* serovar Typhimurium, a full complement of AHAS isozymes conferred no apparent advantages for growth recovery from nutritional downshifts in the Δ*relA* derivatives. Indeed, in the presence of the *ilvI*$^+$ allele, more growth defects or inhibitions were observed, particularly in the presence of the non-branched-chain amino acids (Table 4). Since essentially identical results were obtained with Δ*relA* derivatives of the two *ilvI*$^+$ clinical isolates (data not shown), these results suggested that the worsening of growth recovery was directly related to the presence of the *ilvI*$^+$ allele in a Δ*relA* background.

The *S. enterica* serovar Typhimurium LT2 *ilvG ilvI*$^+$ *relA*$^+$ and *ilvG ilvI*$^+$ Δ*relA* derivatives, KT2354 and KT2358, were valine sensitive, as are *E. coli* K-12 strains. Likewise, essentially the same growth recovery defects appeared in the *S. enterica* serovar Typhimurium LT2 *ilvG ilvI*$^+$ Δ*relA* strain KT2358 as in *E. coli* K-12 Δ*relA* mutants on M9 minimal glucose medium (compare strains CF1652 and KT2358, Tables 3 and 4). However, in contrast to the *E. coli* K-12 *ilvG*$^+$ Δ*relA* strain KT2270, a strong leucine-sensitive phenotype appeared in both *ilvI*$^+$ Δ*relA* strains, independent of the status of the *ilvG* gene (compare strains KT2286 and KT2358, Table 4).

An *ilvI*$^+$ Δ*relA* Δ*spoT* derivative of *S. enterica* serovar Typhimurium LT2 (strain KT2298) was also constructed and scored for amino acid requirements. As seen in Table 2, the presence of the *ilvI*$^+$ allele alleviated none of the amino acid requirements, and two additional requirements appeared for methionine and threonine, which were also present in the *E. coli* K-12 *ilvG*$^+$ Δ*relA* Δ*spoT* derivative (compare strains KT2192, KT2298, and KT2302). For both the *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 strains, therefore, the presence of a full complement of AHAS isozymes conferred no growth improvements in the absence of a source of ppGpp. These results further suggested that the *relA*-dependent differences observed were related to differences in the SpoT- or PSII-derived basal ppGpp levels in these strains.

Elevated basal ppGpp levels compensate for the growth defects in *S. enterica* serovar Typhimurium Δ*relA* mutants. To more directly test the role of the basal ppGpp levels, the Δ*relA* allele was transduced into two isogenic *S. enterica* serovar Typhimurium LT2 strains, TR6478 and TR6479, the latter of which harbors the *spoT1* allele and shows a two- to three-fold-increased basal level of ppGpp (49). These strains and their Δ*relA* derivatives, KT2222 and KT2224, respectively, were subjected to the same nutritional downshifts and scored for growth recovery. As shown in Tables 2 and 4, all amino acid requirements or sensitivities resulting from deletion of the *relA* gene were compensated for by the *spoT1* mutation in the *S. enterica* serovar Typhimurium LT2 Δ*relA spoT1* strain KT2224. These results verified that the amino acid requirements could be alleviated by elevations in the basal ppGpp levels alone and further indicated that the growth defects were a direct consequence of the loss of the *relA* gene product (PSI).

cAMP does not compensate for the *relA*-dependent growth recovery defects, and neither adenylate cyclase nor CRP is required for growth recovery of *relA*$^+$ strains. Previously, the *relA* dependence for branched-chain amino acid biosynthesis was found to be compensated by the addition of exogenous cAMP to *E. coli* K-12 strains, attributed to a requirement for cAMP-CRP for *ilvBN* expression (18, 53). The *E. coli* K-12 Δ*relA* strains were compared with the *S. enterica* serovar Typhimurium LT2 Δ*relA* derivatives under the same conditions but in the presence of cAMP in the downshift medium. No significant differences for any of the Δ*relA* strains were observed, regardless of the pattern of AHAS isozyme expression (data not shown). Only M9/17 plates containing both isoleucine and valine showed partial recoveries for the *E. coli* K-12 Δ*relA* strains, but, as shown in Table 3, recovery was observed on these combinations without cAMP addition. These results indicated that exogenous cAMP was not capable of compensating for the growth recovery defects after nutritional downshifts from a rich medium such as L broth.

S. enterica serovar Typhimurium LT2 *relA*$^+$ *cya*::Tn*10* and *crp*::Tn*10* mutants were also subjected to the same downshifts. Although growth of the *cya* and *crp* mutants was somewhat slower, a characteristic phenotype observed as well on L broth plates, growth recovery was observed with all branched-chain amino acid combinations of M9 or M9/17 media (data not shown). Combined with the lack of improvement of growth recoveries of the Δ*relA* strains on addition of exogenous cAMP, it therefore appeared that a *relA*-dependent source of ppGpp was both necessary and sufficient for growth recovery requiring derepression of the branched-chain amino acid pathways in the absence of cAMP-CRP.

Lrp is not required for growth recovery in the presence of a *relA*$^+$ allele in *S. enterica* serovar Typhimurium. As mentioned in the Introduction, the expression of *ilvIH* (AHAS III) in *E. coli* K-12 is activated by Lrp (11, 40, 60) whose expression in turn is strongly dependent on ppGpp (32). To determine whether the loss of *lrp* gene expression would reproduce some of the *relA*-dependent growth recovery defects observed in the *S. enterica* serovar Typhimurium LT2 Δ*relA* strains, the *S. enterica* serovar Typhimurium LT2 *lrp relA*$^+$ derivatives KT2402, KT2404, and KT2406 were subjected to the same downshifts in either M9 or M9/17 medium. As shown in Table 4, none of the combinations showed significant growth recovery defects for any of the *lrp relA*$^+$ strains. In particular, the *S. enterica* serovar Typhimurium LT2 *ilvG ilvI*$^+$ *lrp* strain KT2406 should be dependent on AHAS III for isoleucine biosynthesis on glucose-containing media (17), yet this strain was found to grow on all combinations except those containing valine without added isoleucine. As shown in Table 4, however, both *ilvI*$^+$ Δ*relA* strains showed a strong leucine sensitivity on M9 medium (strains KT2286 and KT2358), irrespective of the status of *ilvG*. These observations indicated that in the presence of a functional *relA* gene, Lrp is dispensable for *ilvIH* (AHAS III) expression.

It was also determined whether introduction of the *lrp* allele would result in any other amino acid requirements after downshifts using the dropout plate combinations shown in Table 2. Consistent with the results in Table 4, the *lrp* gene was not essential for recovery after downshifts in a *relA*$^+$ background. While no absolute amino acid requirements were apparent, the presence of the *lrp* allele conferred a partial threonine requirement, seen as colony heterogeneity and reduced growth.

Loss of growth recovery of *E. coli* K-12 Δ*relA* strains in an *rph*$^+$ background. The growth recovery differences between *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 Δ*relA* strains expressing all three AHAS isozymes (KT2270 and KT2286, respectively, Tables 3 and 4) were unexpected and seemed unusual based on the known patterns of regulation in these microorganisms (21, 54). A possible explanation based on other aspects of the genetic backgrounds of these strains was therefore considered. One important genetic difference peculiar to the *E. coli* K-12 MG1655 strain used in this study is the presence of a frameshift mutation in the *rph* gene, encoding the tRNA processing enzyme RNase PH (28). While this mutation results in loss of function of RNase PH, polynucleotide phosphorylase (PNPase) or other RNases can apparently catalyze the same reactions in vivo (67). However, the *rph-1* mutation has polar effects on the downstream *pyrE* gene, resulting in pyrimidine limitations particularly at fast growth rates (28, 44). The growth rate of MG1655 is only slightly affected by this mutation but is stimulated by exogenous uracil (reference 28 and references therein). It was reasoned that perhaps the better recovery of the *E. coli* K-12 *ilvG*$^+$ Δ*relA* strain was somehow related to pyrimidine limitation after the downshifts, which might alter the RNA chain elongation rate of the RNA polymerase and affect the attenuation regulation and derepression of the genes involved in branched-chain amino acid biosynthesis (33, 54) (see Discussion).

To test this idea, the growth recoveries of *rph*$^+$ *relA*$^+$ and *rph*$^+$ Δ*relA* derivatives of *E. coli* K-12 were compared. As seen in Table 3, the presence of the *rph*$^+$ allele in a Δ*relA* background (strains CF7974 and KT2450) resulted in a complete loss of growth recovery with the exception of media containing the full complement of amino acids. In addition, the effect of the *rph*$^+$ allele in the Δ*relA* strains was independent of the status of *ilvG*, since growth recovery was not improved in the *ilvG*$^+$ *rph*$^+$ Δ*relA* derivative KT2450 relative to CF7974. Despite somewhat slower growth recovery after the downshifts, particularly on M9 medium containing isoleucine or leucine, none of the *relA*$^+$ *rph*$^+$ derivatives (CF7968 and KT2448) were otherwise adversely affected after the downshifts. Although less severe, exogenous uracil in the downshift media also reduced the growth recovery of the *E. coli* K-12 MG1655 Δ*relA* *rph*-1 derivatives in a similar manner to that observed in the presence of an *rph*$^+$ allele, suggesting that the observed effects were indeed related to the pyrimidine pools (data not shown).

The loss of growth recovery on minimal glucose medium containing all three branched-chain amino acids (M9, Table 3) suggested that the *rph*$^+$ allele resulted in more pleiotropic effects in the Δ*relA* strains in addition to defective derepression of the branched-chain amino acid pathways. Consistent with this, the *rph*$^+$ Δ*relA* strain CF7974 showed no growth on dropout plates lacking histidine, phenylalanine, or threonine and reduced growth on plates lacking methionine or tryptophan (Table 2). Likewise, the presence of the *rph*$^+$ allele in a Δ*relA* Δ*spoT* background (strain CF7976) resulted in additional amino acid requirements for tryptophan and tyrosine compared to the other *E. coli* K-12 or *S. enterica* serovar Typhimurium LT2 Δ*relA* Δ*spoT* strains (Table 2). The combined results of the branched-chain amino acid downshifts shown in Table 3 and the additional amino acid requirements which appeared suggested that the *rph-1* mutation conferred an advantage to the *E. coli* K-12 MG1655 strain in a *relA* background. Since the *rph*$^+$ allele did not alter any other obvious tested phenotypes of the *relA*$^+$ derivative, CF7968, the observed growth defects were probably a direct result of correction of the *rph-1* mutation in the wild-type *E. coli* K-12 strain combined with the loss of a *relA*-dependent source of ppGpp after nutritional downshifts.

An RNA polymerase with a reduced RNA chain elongation rate compensates for all branched-chain amino acid requirements in the *S. enterica* serovar Typhimurium LT2 Δ*relA* strain. The loss of growth recovery of the *rph*$^+$ Δ*relA* derivatives of *E. coli* K-12, CF7974 and KT2450 (Table 3), suggested that pyrimidine limitations were at least partially responsible for the ability of the MG1655 *E. coli* K-12 *relA* mutants to overcome the amino acid imbalances resulting from nutritional downshifts. Since attenuation regulation is sensitive to transcriptional-translational coupling (33), one interpretation of these results is that a reduced RNA chain elongation rate of the RNA polymerase was required for derepression under conditions of amino acid limitations and that a reduction in the RNA chain elongation rate could be achieved either by pyrimidine limitation or by the use of elevated ppGpp levels. ppGpp reduces the RNA chain elongation rate *in vivo* in *E. coli* K-12 (58, 59). It was reasoned that it should be possible to compensate for the reduced basal ppGpp levels after downshifts of a *relA* mutant in a strain harboring an RNA polymerase with a reduced RNA chain elongation rate without resorting to pyrimidine limitations. To test this idea, the Δ*relA* allele was introduced into a strain of *S. enterica* serovar Typhimurium LT2, KP1475, expressing an RNA polymerase with a reduced RNA chain elongation rate (26, 27). As seen in Table 4, the Δ*relA* derivative of the parent strain, KT2244, showed recovery defects similar to the other *S. enterica* serovar Typhimurium LT2 Δ*relA* derivatives, but in all cases the defects were reversed in the presence of both leucine and valine. In contrast, all branched-chain amino acid requirements and/or sensitivities were compensated after downshifts of the Δ*relA* derivative of the *rpoBC* mutant strain, KT2246. Strain KT2246 was also found to be resistant to the histidine analogue 3-amino-1,2,4-triazole (unpublished observations), a resistance also conferred by elevated basal ppGpp levels in *S. enterica* serovar Typhimurium LT2 *spoT1* mutants (49). These results were therefore consistent with the idea that a reduction in the RNA chain elongation rate could compensate for defects in derepression of the branched-chain amino acid pathways resulting from reduced basal ppGpp levels following nutritional downshifts of the *S. enterica* serovar Typhimurium LT2 Δ*relA* mutant.

DISCUSSION

The basis for the differences in some of the branched-chain amino acid requirements which appear after nutritional down-

shifts of *relA* strains of *E. coli* and *S. enterica* serovar Typhimurium was investigated. As noted above, a *relA* mutation does not introduce amino acid auxotrophies or requirements in the strict sense, and the *relA* strains in this study are all capable of growth on unsupplemented minimal glucose medium. With the exceptions of the preexisting *ilvG* and *ilvI* mutations in *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2, respectively, the genes necessary for the de novo synthesis of amino acids are present in these strains. The observed requirements and growth inhibitions are the result of defects in expression of the genes required for branched-chain amino acid biosynthesis resulting from nutritional downshifts or amino acid starvations in a *relA* background.

The major observations of this study are that the growth recovery defects of *relA* mutants are related in part to preexisting mutations and that the individual contributions of known global regulatory factors involved in branched-chain amino acid biosynthesis are insufficient to explain the growth defects. These growth defects are compensated by (i) elevation of the basal ppGpp levels, (ii) pyrimidine limitations in *E. coli* K-12 MG1655 (*rph*-1) strains, (iii) a reduction in the RNA chain elongation rate of the RNA polymerase. These results suggest a role for ppGpp in attenuation regulation of these genes and operons. A mechanism to explain these latter observations is discussed below.

The roles of preexisting *ilv* mutations in *relA*-dependent growth defects. Results from the *E. coli* K-12 *ilvG*$^+$ Δ*relA* derivative (KT2270) clarify both the isoleucine requirement and leucine sensitivity of *E. coli* K-12 *relA* mutants. Growth of the *ilvG*$^+$ Δ*relA* strain KT2270 after downshifts to leucine- or valine-containing glucose minimal medium shows that this strain is able to express the valine-insensitive AHAS II. These results suggest that the isoleucine requirement for both the Δ*relA* strains of *E. coli* K-12 in the presence of valine is solely the result of the preexisting *ilvG* mutation in the wild-type strain. Since all *ilvG*$^+$ *relA* derivatives of both *E. coli* K-12 and *S. enterica* serovar Typhimurium LT2 were rescued by leucine and valine additions to the downshift media, this might suggest that isoleucine biosynthesis and, by implication, *ilvGM* (AHAS II) gene expression were not severely affected under the downshift conditions examined (however, see below).

Lrp. Leucine sensitivity of *E. coli relA* mutants has been reported previously, and the growth inhibitions were reversed by addition of isoleucine and valine (1, 55). The leucine sensitivity of *E. coli* K-12 *relA* mutants can now be interpreted in terms of the Lrp-mediated activation of *ilvIH* (AHAS III) expression. Lrp activates *ilvIH* expression (60) and is thought to repress *ilvGM* expression (46). In addition to autogenous regulation, the expression of *lrp* is growth rate or growth medium regulated and is strongly dependent upon ppGpp (32, 61). In the otherwise wild-type (*ilvG*) *E. coli* K-12 Δ*relA* background, the presence of leucine in the downshift medium would prevent the Lrp-mediated activation of AHAS III expression, resulting in an isoleucine requirement, consistent with the improvement in growth on glucose minimal medium containing both isoleucine and leucine (Tables 3 and 4). The isoleucine requirement most probably results from the very-low-level contribution of AHAS I to isoleucine biosynthesis (17), since the *ilvG*$^+$ Δ*relA* strains were able to partially overcome the apparent isoleucine requirement in the presence of leucine. However, the *S. enterica* serovar Typhimurium *ilvI*$^+$ Δ*relA* strains acquired a strong leucine sensitivity, irrespective of the status of the *ilvG* locus (M9, Table 4), and isoleucine at least partially relieved the strong leucine sensitivity, indicating that in *S. enterica* serovar Typhimurium LT2 a *relA* (PSI)-dependent source of ppGpp is necessary for both AHAS II and III expression after such downshifts.

The general lack of isoleucine compensation for the leucine sensitivity in the presence of the non-branched-chain amino acids (M9/17 medium) might stem from the observations of Chen *et al.*, who found that the α-ketoglutarate or oxaloacetate families of amino acids also resulted in repression of Lrp expression (14). This would also lead to reduced AHAS III expression and an isoleucine requirement, as with leucine addition to the K-12 Δ*relA* strain. However, the absence of growth of the *ilvG*$^+$ Δ*relA* strains on M9/17 supplemented with isoleucine indicates that neither end product (valine) repression nor leucine sensitivity (Lrp inactivation) is sufficient to explain the growth requirements; under such conditions, all branched-chain pathways should have been derepressed. If the reason for the growth defects in M9/17 were related to a defect in AHAS III expression (e.g., reduced Lrp synthesis) and reduced isoleucine synthesis, AHAS II should have been able to provide isoleucine for the cell as it did in minimal glucose medium containing leucine. These observations indicate that the proposed Lrp-mediated repression of the *ilvGp2* promoter (46) is not responsible for the defects, since leucine in general did not allow growth recovery of the Δ*relA* strains on M9/17 medium (Tables 3 and 4).

While the lack of growth recovery of any of the *E. coli* K-12 or *S. enterica* serovar Typhimurium LT2 *relA* strains on M9/17 medium supplemented with isoleucine and the very good recoveries on plates containing both leucine and valine suggest a defect in valine-leucine biosynthesis, valine only partially rescued growth in the *ilvG*$^+$ *relA* strains and did so only in combination with preexisting mutations in either *ilvI* in *S. enterica* serovar Typhimurium LT2 or in the *rph-1* background in *E. coli* K-12. The levels of KB are expected to decrease due to feedback inhibition of threonine deaminase by isoleucine, favoring AHAS I activity and valine-leucine synthesis (17). These observations suggest that the rescue of growth inhibition by leucine and valine additions is not due to a reduced ppGpp requirement for *ilvGM(EDA)* expression but, rather, is due to inhibition of the enzymatic activities of AHAS I, permitting the common enzymes encoded by *ilvEDA* to devote their activities solely to isoleucine biosynthesis.

Since all *S. enterica* serovar Typhimurium LT2 *lrp relA*$^+$ strains, including the *ilvG ilvI*$^+$ *lrp* strain KT2406, were unaffected by the same downshifts (Table 4), this suggests that the previously observed (nonleucine) amino acid repression of Lrp synthesis is indirect occurring through a reduction in ppGpp synthesis or accumulation. Furthermore, this indicates that a *relA*-dependent source of ppGpp can compensate for any leucine-mediated inactivation of Lrp, either through elevated *lrp* expression or through other ppGpp-dependent mechanisms (see below).

Role of cAMP-CRP in *ilvBN* (AHAS I) expression. Freundlich and coworkers found that high concentrations of exogenous cAMP after a downshift of a *relA1* mutant under limiting leucine conditions (in the presence of all other amino acids)

could compensate for the resulting growth recovery defect, attributed to the failed derepression of *ilvBN* (AHAS I) under these conditions (18, 53). In this study however, exogenous cAMP was not capable of rescuing any of the growth recovery defects when strains were precultured in a medium supporting very low basal ppGpp levels. In addition, *S. enterica* Serovar Typhimurium LT2 *relA*$^+$ strains defective in *cya* or *crp* expression were found to completely recover from the same downshifts (data not shown). These observations indicate that rather than a cAMP compensation for the *relA* dependence for derepression of *ilvBN* in a *relA* mutant, cAMP additions in those prior studies represented an effect in addition to the elevated basal ppGpp levels resulting from the preculture conditions. Indeed, the preculture conditions strongly affect the responses of *relA* mutants on the SMG medium of Uzan and Danchin (55, 56), with essentially no recovery of *relA* strains possible if the mutants were precultured in L broth whereas cultures pregrown in M9 minimal glucose with or without all three branched-chain amino acids are rescued by isoleucine and valine and other combinations (unpublished observations).

IHF. Friden et al. (19) investigated the observation that *E. coli* K-12 strains containing mutations in the genes encoding the subunits of IHF required isoleucine and valine for growth on minimal medium and showed growth inhibition in minimal medium supplemented with leucine. The expression of AHAS I and enzymes encoded by the *ilvGMEDA* operon was reduced two- to sevenfold in the *ihfA* (previously known as *himA*) strains, correlating with an equivalent reduction in *ilvB*- or *ilvGMEDA*-specific mRNA, and added cAMP was not capable of compensating for the defect in expression. IHF activates the transcription of *ilvBN* and the *ilvGp2* promoter (41, 42). Like Lrp (14, 32), IHF accumulation and expression is growth phase dependent (3, 16) and expression of the genes encoding IHF (*ihfA* and *ihfB*) apparently requires ppGpp (2). The *relA* (or ppGpp)-dependent growth defects could therefore stem from a reduction in IHF expression due to the reduced levels of ppGpp during the relaxed response. However, the majority of growth recovery defects were at least partially corrected by valine additions in the *ilvG*$^+$ *relA* strains, suggesting that isoleucine synthesis was less affected after the downshifts. One would therefore have to postulate that IHF was present at sufficient levels for expression of *ilvGM* but not of *ilvBN*. It therefore appears that like cAMP-CRP and Lrp, reduced levels of IHF alone are insufficient to explain all the observed growth recovery defects of the *E. coli* and *S. enterica* serovar Typhimurium Δ*relA* strains.

Contribution of ketobutyrate toxicity. The *ilvG* mutation present in wild-type *E. coli* K-12 strains and the *ilvG236* mutation used in construction of the *S. enterica* serovar Typhimurium LT2 *ilvG* mutant differ in that the former shows polar effects on the downstream genes of the *ilvGMEDA* operon (43) while the latter does not (45). Since KB is derived from threonine, a possible explanation for the more severe effects of the Δ*relA* allele in *S. enterica* serovar Typhimurium is the accumulation of toxic levels of KB after the downshifts. Accumulation of KB in *ilvG* mutants of *S. enterica* serovar Typhimurium correlates with growth inhibitions and apparently contributes to the toxicity of sulfometuron methyl, an inhibitor of AHAS II (34, 45, 57). However, the toxic effects of KB should be reversed by isoleucine addition, rather than aggravated as observed (Tables 3 and 4). In addition, valine should exacerbate KB toxicity by inhibiting AHAS I and III (34), but valine was consistently one of the amino acids in whose presence at least partial recovery was observed for the *ilvG*$^+$ Δ*relA* strains. Therefore, KB toxicity does not appear to explain the growth inhibitions of the *E. coli* K-12 and *S. enterica* serovar Typhimurium Δ*relA* strains.

PSI and PSII are intimate partners in determining the basal ppGpp levels. Paradoxically, the results indicate that amino acids other than the branched-chain (i.e. regulatory) amino acids play a decisive role in the regulation of the branched-chain amino acid pathways: the more amino acids present in the downshift medium, the greater the inability for growth recovery. That all *relA* strains, regardless of the patterns of AHAS isozyme expression, showed a reduced capacity for growth recovery in M9/17 relative to minimal glucose suggests that the most likely cause for the growth defect(s) on M9/17 medium is related to a reduction in the basal ppGpp levels due to the presence of additional amino acids in the downshift medium.

As discussed by Murray and Bremer (39), multiple amino acid limitations are expected to reduce overall tRNA charging. The result would be elevation of the PSI-derived ppGpp levels as many ribosomes encountered uncharged tRNAs during translation. Although a Δ*relA* strain has no such mechanism for the synthesis of ppGpp, in the Δ*relA spoT*$^+$ strains the reduced tRNA charging would also result in a PSII-dependent increase in the basal levels of ppGpp, through elevated ppGpp synthesis and/or inhibition of the SpoT ppGpp-degradative activity. Deprivation of only one or a few amino acids in the presence of all others in a Δ*relA spoT*$^+$ mutant is expected to elevate overall tRNA charging, since high amino acid pools would permit charging of the respective tRNAs. Elevated tRNA charging would lead to increased ppGpp-degradative activity and the low, basal ppGpp levels would be expected to rapidly disappear. Limitation for a single amino acid would therefore have greater consequences for a *relA* strain than would limitation for multiple amino acids. While somewhat counterintuitive, this suggestion would be consistent with the proposed regulation of SpoT-dependent ppGpp basal level synthesis and degradation by the levels of tRNA charging in the cell (39, 48) and provides an explanation for the apparent paradox of decreased growth recovery capacity with improved nutritional quality of the downshift medium in the Δ*relA* strains.

Based on the known attenuation regulation patterns of the branched-chain pathways, it appeared unusual that isoleucine addition should result in the observed worsening of growth recovery seen in all the *relA* strains. This curious effect of isoleucine addition is most noticeable in the M9/17 medium and is independent of the patterns of AHAS isozyme expression. This suggests that the deleterious effect of isoleucine addition might be best explained in terms of tRNA synthesis and charging.

Characteristic for the relaxed response of *relA* mutants to amino acid starvation is the continued synthesis of stable rRNA and tRNA, correlating with the decreased ppGpp levels (reviewed in reference 12). In *E. coli* K-12, three of four $tRNA^{Ile}$ genes are cotranscribed with rRNA operons (7). The increase in stable RNA synthesis after amino acid starvation of a *relA* mutant would lead to a corresponding increase in the levels of isoleucyl-tRNA, which could lead to increased

ppGpp-hydrolase activity, preventing elevation of the basal levels of ppGpp after the downshift. That sufficient isoleucyl-tRNA charging is possible is suggested by downshift experiments of cultures in L-broth to M9/17 medium containing isoleucine. Despite no measurable growth after such a downshift, *S. enterica* serovar typhimurium LT2 Δ*relA* cultures recovered almost immediately after addition of leucine and valine, indicating that uptake of the branched-chain amino acids was also not affected (unpublished observations). Based on these observations, it therefore appears that increased tRNA charging is the most likely cause of the *relA*-dependent growth recovery defects observed on M9/17 medium. In addition, this implies that the PSI and PSII activities are not completely independent pathways in ppGpp metabolism but would be intimately connected through tRNA synthesis and charging levels.

Role of ppGpp in promoter activation. Prior work on the effects of ppGpp on transcription has focused on the inhibitory effects at promoters showing similarities to a consensus "stringent" promoter, containing a GC-rich "discriminator" sequence immediately downstream of the −10 region. The paradigm for these stringent promoters is the rRNA (*rrn*) P1 promoters, where point mutations within the GC-rich motif alter both the stringent and growth rate control of the promoters (12, 66). In contrast, Artz and coworkers have examined the role of ppGpp in the activation of the *his* promoter in great detail (reference 15 and references therein). The activation of *his* transcription is responsive to elevations of the basal ppGpp levels in *spoT* mutants defective in ppGpp-degradative activities, and the *his* promoter has a generally AT-rich discriminator region in which point mutations abolish the activation by ppGpp (50, 62, 63). A role for ppGpp in transcriptional activation of *ilvG* has previously been suggested based on the stimulation of transcription in vitro from the *ilvGp2* promoter and homology of the −10 and discriminator regions to those of ppGpp-activated promoters (discussed in reference (54).

A hypothesis to explain the ppGpp-dependent activation of promoter activities is based on consideration of the available pools of limiting RNA polymerase. Bremer and coworkers proposed a model to explain the effects of ppGpp on both stable RNA and mRNA gene expression (reference (4) and references therein). In this model, inhibition of stable RNA synthesis by ppGpp frees a fraction of limiting RNA polymerase for transcription of mRNA genes. Recently, Barker et al. (6) found no direct activation of amino acid gene promoters in vitro and concluded that the activation by ppGpp in vivo was indirect, consistent with a ppGpp-dependent partitioning of the pool of limiting RNA polymerase (4). In addition, Bremer and Ehrenberg (8) proposed a model which sought to explain the three- to fourfold-elevated levels of mRNA synthesis in Δ*relA* Δ*spoT* strains of *E. coli* K-12 (25). One premise of this model is that the low, basal levels of ppGpp divert a fraction of RNA polymerase from the stable RNA promoters to mRNA promoters, where the ppGpp-bound RNA polymerase is able to initiate transcription, but pauses during the elongation phase at sites at or near the mRNA promoters.

A role for ppGpp in modulation of the RNA chain elongation rate of the RNA polymerase in attenuation regulation. The majority of the amino acid requirements of the Δ*relA* *spoT*$^+$ and Δ*relA*Δ*spoT* strains fall into a class of genes and/or operons which are attenuation regulated, including the *his*, *phe*, *thr*, *trp*, and *ilv* genes and operons. One important feature of attenuation regulation is that of pausing by the transcribing RNA polymerase within the attenuator region (reviewed in reference 33). We suggest that the ppGpp-dependent effects on the mRNA chain elongation rate may also include or enhance pausing in attenuation-regulated genes and operons. In the absence of ppGpp, transcription might be subject to termination (hyper attenuation) or the mRNA would be more vulnerable to endonucleolytic cleavage as the average distance between the RNA polymerase and ribosome increases. The level of tRNA charging, dependent on amino acid availability, would form part of a regulatory loop involving translating ribosomes, which not only consume charged tRNAs but also signal PSI (RelA) as to the presence of uncharged tRNAs. If the amino acid starvation is severe, the PSI enzyme (associated with 1 to 2% of ribosomes) would respond by synthesizing ppGpp, which in turn binds the RNA polymerase. The ratio of charged to uncharged tRNAs would also affect the SpoT PSII-synthetic and/or ppGpp-degradative activities to modulate the basal ppGpp levels. Expression of amino acid biosynthetic operons not needed to keep up the charging level of the respective tRNAs would not be unnecessarily derepressed; the mechanism would require both events—a ppGpp-dependent pause by the RNA polymerase and ribosome stalling at the regulatory codons.

Vogel and Jensen found that ppGpp reduces the RNA chain elongation rate of the RNA polymerase in vivo and requires the termination-antitermination factor NusA for this effect (58, 59). Previously, Hauser et al. (22) found that RNA polymerase pausing in vitro at the *ilvB* attenuator region was increased in the presence of ppGpp and further increased on addition of L-factor (NusA). In the model described above, amino acid starvation of *relA* mutants would lead to decreased pausing (or increased RNA chain elongation or both) as the ppGpp levels declined, preventing derepression of these genes and operons. The requirement for a ppGpp-dependent pause in the *ilvBN* (AHAS I) attenuator region would explain the valine requirements observed. While ppGpp-dependent pausing has not been observed in the *his* or *trp* attenuator regions in vitro (13, 64), the activation of transcription by ppGpp at the *his* promoter may represent a different strategy adopted in regulation of the *his* operon (however, see reference 6). Hauser et al. (22) also found no increase in pausing in the presence of ppGpp in the *ilvG* attenuator region, whereas the addition of NusA increased the duration of the pause approximately twofold. The effects of both ppGpp and NusA on *ilvG* pausing were not examined, however (22).

The effects of the *rph*$^+$ allele in the *E. coli* K-12 Δ*relA* strains are related to this proposal. Partial pyrimidine starvation in the *rph-1* background would lead to a reduction in the RNA chain elongation rate and/or increased pausing, despite the reduction in ppGpp levels. The *rph*$^+$ Δ*relA* strain would no longer have this compensation for the reduced ppGpp levels. While it might be thought that an increased rate of RNA synthesis would lead to a concomitant increase in gene expression, Dreyfus and coworkers have shown that an increased RNA chain elongation rate can lead to reduced gene expression (reference 37 and references therein). The relatively improved growth recovery of the *ilvG*$^+$ *rph-1* Δ*relA* strain seen in Table 3 would be explained by relief of the polarity effects of the *ilvG* muta-

tion in the wild-type K-12 strain, affecting the expression of enzymes common to both pathways, combined with the reduced RNA chain elongation rate. That the *rph-1* allele provides the more important component seems clear since the same severe growth recovery defects were observed in both the *ilvG* and *ilvG*$^+$ Δ*relA rph*$^+$ strains (CF7974 and KT2450, respectively, Table 3).

The RNA polymerase mutation conferring a reduced RNA chain elongation rate (26, 27) compensated for all the branched-chain amino acid requirements in an *S. enterica* serovar Typhimurium LT2 Δ*relA* background (strain KT2246, Table 4). This mutant was identified as a suppressor conferring derepression of the attenuation-regulated *pyrBI* operon in pyrimidine biosynthesis, and the original studies also reported that it showed a two- to threefold-reduced basal ppGpp level (26). This suggests that a reduction of the RNA chain elongation rate by elevated ppGpp levels might to some extent be reciprocal; i.e., reduction of the RNA chain elongation rate by nucleotide limitations would be compensated by lowered ppGpp levels, resulting from reduced mRNA synthesis (discussed in reference 58). Further studies are required to determine whether the effects on expression are due to a specific role for ppGpp, i.e., ppGpp-dependent pausing of RNA polymerase at important regulatory sites during transcription, or a reflection of a more general effect on transcriptional-translational coupling affecting these genes and operons particularly severely.

In conclusion, at least three ppGpp-dependent means of regulation appear to contribute to branched-chain amino acid biosynthesis: (i) effects of ppGpp on transcriptional activation, as suggested for *ilvGp2* (55); (ii) indirect effects of ppGpp on expression of auxiliary factors such as Lrp or IHF; and (iii) a ppGpp-dependent reduction in the RNA chain elongation rate, enhancing the pausing required for derepression, as suggested here for *ilvBN* and possibly the other *ilv* genes and operons. The cAMP-CRP complex, Lrp, or IHF may act at different stages in the process of derepression, e.g. increasing promoter availability or RNA polymerase recruitment to increase the likelihood that a ppGpp-bound RNA polymerase will initiate transcription despite the low levels of ppGpp. Likewise, NusA could play a significant role in the regulation, where its function may differ depending on whether the RNA polymerase has bound ppGpp.

The absence or reduction of ppGpp levels has effects which are difficult for the cell to compensate for and underscores the importance of the basal ppGpp levels in the regulation of mRNA gene expression in addition to effects on stable RNA synthesis. Since RNA polymerase is limiting in the cell for transcription (reference (9) and references therein), it is not surprising that many genes and operons would utilize additional factors for the recruitment of RNA polymerase, factors whose own expression is coupled to the basal ppGpp levels, which change according to the growth conditions and nutrient availability. Considering the pleiotropic effects on gene expression characteristic of Δ*relA* Δ*spoT* strains and the severe growth recovery defects of the Δ*relA* strains observed in this study, one is inclined to agree with the suggestion of Lagosky and Chang (30) that the basal levels of ppGpp are an absolute requirement for normal bacterial growth.

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