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Functional Analysis of a *relA*/*spoT* Gene Homolog from *Streptococcus equisimilis*

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We examined the functional attributes of a gene encountered by sequencing the streptokinase gene region of *Streptococcus equisimilis* H46A. This gene, originally called *rel*, here termed $rel_{S.\ equisimilis}$, is homologous to two related *Escherichia coli* genes, *spoT* and *relA*, that function in the metabolism of guanosine 5′,3′-polyphosphates [(p)ppGpp]. Studies with a variety of *E. coli* mutants led us to deduce that the highly expressed $rel_{S.\ equisimilis}$ gene encodes a strong (p)ppGppase and a weaker (p)ppGpp synthetic activity, much like the *spoT* gene, with a net effect favoring degradation and no complementation of the absence of the *relA* gene. We verified that the $Rel_{S.\ equisimilis}$ protein, purified from an *E. coli relA spoT* double mutant, catalyzed a manganese-activated (p)ppGpp 3′-pyrophosphohydrolase reaction similar to that of the SpoT enzyme. This $Rel_{S.\ equisimilis}$ protein preparation also weakly catalyzed a ribosome-independent synthesis of (p)ppGpp by an ATP to GTP 3′-pyrophosphoryltransferase reaction when degradation was restricted by the absence of manganese ions. An analogous activity has been deduced for the SpoT protein from genetic evidence. In addition, the $Rel_{S.\ equisimilis}$ protein displays immunological cross-reactivity with polyclonal antibodies specific for SpoT but not for RelA. Despite assignment of $rel_{S.\ equisimilis}$ gene function in *E. coli* as being similar to that of the native *spoT* gene, disruptions of $rel_{S.\ equisimilis}$ in *S. equisimilis* abolish the parental (p)ppGpp accumulation response to amino acid starvation in a manner expected for *relA* mutants rather than *spoT* mutants.

As typified by extensive studies with members of the family *Enterobacteriacae*, bacterial cells subjected to nutrient exhaustion respond with rapid and complex adjustments that involve the metabolism of GTP and GDP analogs bearing pyrophosphate derivatives at the ribose 3′-hydroxyl position, collectively abbreviated (p)ppGpp (8). Restriction of accumulation of most stable RNA species, stimulation of certain anabolic activities, and induction of stationary-phase-specific gene expression generally accompany these responses, presumably to facilitate adaptation to the nutritional stress or to ensure cell viability if adaptation is not possible (21).

In *Escherichia coli*, the products of the *relA* and *spoT* genes can regulate the accumulation of (p)ppGpp by two apparently independent mechanisms. Synthesis is regulated by the RelA protein, which catalyzes the pyrophosphorylation of GTP (or GDP) using an ATP donor and is activated by codon-specific uncharged tRNA binding to ribosomes engaged in protein synthesis elongation (13, 25, 26, 49). Increased ratios of uncharged to charged tRNA seem to comprise the sole signal for *relA*-dependent induction of (p)ppGpp synthesis (22). Degradation of (p)ppGpp is inhibited when a primary energy source becomes limiting (17) and leads to (p)ppGpp accumulation without necessarily increasing synthesis; this occurs by inhibiting the activity of a manganese-dependent (p)ppGpp 3′-pyrophosphohydrolase encoded by the *spoT* gene (27–29, 61). The mechanism of inhibition of this hydrolytic activity is poorly understood. In addition to energy source exhaustion, a variety of other stress conditions also provoke (p)ppGpp accumulation in a *spoT*-dependent fashion (8).

The presence of (p)ppGpp in *relA*-deleted strains indicates that a second source of (p)ppGpp synthesis exists; abolition of all detectable (p)ppGpp by an additional deletion or mutation in *spoT* has led to the proposal that *spoT* encodes the second source of this synthetic activity (30, 66). The existence of homology between the SpoT and RelA proteins (44) has been taken as supporting the notion that SpoT can function as a bifunctional pyrophosphohydrolase and transferase.

In addition to the *Enterobacteriaceae*, most eubacteria so far examined are capable of forming (p)ppGpp (56), but there are exceptions or examples of natural isolates of *rel*-like mutants (1, 40). Archaebacteria also do not apparently form (p)ppGpp (2, 12). Mutations that abolish (p)ppGpp accumulation during a stringent response to amino acid starvation have been noted for a marine *Vibrio* sp. (16), *Bacillus subtilis* (59), and a *Streptomyces* sp. (48). Synthetic and degradation activities for (p)ppGpp have been identified in extracts of *Bacillus stearothermophilus*, *Bacillus brevis*, and *B. subtilis* (14, 51, 60) and *Streptomyces antibioticus* (32) as well as with *E. coli* extracts. Sequence homologs for comparison to *relA* (45) and *spoT* (54) are available: a *relA* homolog from a *Vibrio* sp. (16), a *relA*/*spoT* homolog from *Mycobacterium leprae* (GenBank accession number U00011), *relA* and *spoT* homologs from *Haemophilus influenzae* (U32718 and U32847), and a *relA*/*spoT* homolog from *Mycoplasma genitalium* (U39707) as well as $rel_{S.\ equisimilis}$ (42).

Given the complexity of the responses to nutrient limitation in general and our limited knowledge of these responses in other bacteria, particularly pathogenic ones, we thought it worthwhile to explore the functional attributes of a *relA*/*spoT* gene homolog from *Streptococcus equisimilis* found in the streptokinase gene (*skc*) region of the chromosome (42). This gene was originally called *rel* but is designated $rel_{S.\ equisimilis}$ here, reserving the terms *relA* and *spoT* exclusively for the *E. coli* genes.

On the chromosome of *S. equisimilis*, the coding sequence of $rel_{S.\ equisimilis}$ is followed by an unknown reading frame, ORF1, and the two genes are cotranscribed convergently to *skc*. Incidentally, the ORF1 product shows a high degree of sequence

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference
E. coli		
CF1648	Wild-type MG1655	66
CF1652	As CF1648 but Δ*relA251*	66
CF1693	As CF1652 but Δ*spoT207*	66
CF4941	As CF1648 but *galK2 zib563*::Tn*10* Δ*relA251*	This study
CF4943	As CF4941 but *spoT203*	
S. equisimilis		
H46A	Wild type; human serogroup C strain	11
H46A $rel_{S.\ equisimilis}$::pVH45	$rel_{S.\ equisimilis}$ insertion; orientation I, Em^r	This study
H46A $rel_{S.\ equisimilis}$::pVH46	$rel_{S.\ equisimilis}$ insertion; orientation II, Em^r	This study
Plasmids		
pHX41	*spoT* plasmid; ColE1 replicon, Ap^r	66
pUC19	Vector; *lacZpo*; ColE1 replicon, Ap^r	
pURS24	As pUC19 but *Pst*I $rel_{S.\ equisimilis}$ fragment in *lacZpo* sense orientation	This study
pURS1	As pUC19 but *Pst*I $rel_{S.\ equisimilis}$ fragment in *lacZpo* antisense orientation	This study
pVA891	Vector; p15A replicon, Em^r Tc^r Cm^r	39
pVA8912	As pVA891 but lacking 3,133-bp Tc^r Cm^r fragment and containing pUC19 MCS	This study
pVRL1224	As pVA8912 but containing $rel_{S.\ equisimilis}$ under its natural and *lacpo* promoter, Em^r	This study
pVR3	As pVA8912 but containing $rel_{S.\ equisimilis}$ under its natural promoter, Em^r	This study
pALS10	P_{tac}::*relA lacI*^{q}, ColE1 replicon, Ap^r, inducible full-length (743-aa) RelA	58
pALS13	As pALS10 but *relA′* 455-aa RelA fragment	58
pALS14	As pALS10 but *relA′* 331-aa RelA fragment; catalytically inactive control RelA	58
pVH45	As pVA891 but containing internal *Hin*dIII $rel_{S.\ equisimilis}$ fragment (555 bp), orientation I	This study
pVH46	As pVH45, $rel_{S.\ equisimilis}$ fragment orientation II	This study

[a] MCS, multiple cloning site; aa, amino acid.

similarity (66%) to a deduced protein encoded by *E. coli* ORF *o145* (accession number L19201), located at about 87 min, whose function is also unknown.

Our approach for characterizing potential functions encoded by the $rel_{S.\ equisimilis}$ gene is to take advantage of well-defined *relA* and *spoT* mutants and measure the in vivo complementation activities of $rel_{S.\ equisimilis}$ expressed in *E. coli*. These tests revealed that while the streptococcal gene does not functionally replace a deleted *relA* gene, it does specify two phenotypes normally associated with the *spoT* gene, namely, a strong (p)ppGppase as well as a weak (p)ppGpp synthetic activity. The net effect of $rel_{S.\ equisimilis}$ expression in *E. coli* is to facilitate (p)ppGpp degradation. These findings led us to search for the corresponding enzymatic activities of the purified $Rel_{S.\ equisimilis}$ protein expressed in an *E. coli* strain otherwise devoid of endogenous sources of (p)ppGpp degradation and synthesis as well as to examine the effects of insertional inactivation of $rel_{S.\ equisimilis}$ on (p)ppGpp accumulation behavior in its native host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are described in Table 1. For the construction of pURS24 and pURS1, a 2,741-bp *Pst*I DNA fragment containing $rel_{S.\ equisimilis}$, including its promoter (42), was inserted into pUC19 in the sense and antisense orientations relative to the *lac* gene promoter-operator (*lacpo*). Plasmid pVA8912 was formed by removal of the 412-bp *Pvu*II fragment of pVA8911 (42). The 2,973-bp *Pvu*II-*Eco*RI fragment of pURS24 carrying $rel_{S.\ equisimilis}$ and *lacpo* was inserted into pVA8912 digested with *Eco*RI and *Sma*I to give pVRL1224. Deletion of the *lacpo*-containing 216-bp *Sph*I fragment of pVRL1224 resulted in pVR3.

E. coli strains were grown with agitation at 32°C in LB or MOPS (morpholinepropanesulfonic acid) glucose minimal medium containing 0.3 mM phosphate (for uniform ^{32}P labeling), 0.4% glucose, amino acids (40 μg/ml each), and five nucleosides (A, G, C, U, and T; 20 μg/ml each). Ampicillin (100 μg/ml) and erythromycin (200 μg/ml) were added as needed for plasmid maintenance. *S. equisimilis* H46A was grown at 37°C without shaking in chemically defined medium (65), brain heart infusion broth (Difco), or LB plates containing, if needed, 2.5 μg of erythromycin per ml for screening nucleotide patterns after $^{32}P_i$ labeling. Strains CF4941 and CF4943 were constructed from CF3700, a CF1648 derivative containing the *galK2* allele introduced for another purpose, with a *spoT*-linked Tn*10* element *zib563*::Tn*10. The relA251* allele was introduced by P1 transduction with lysates grown on CF1693, selecting for Km^r to yield strain CF3725, which was then transduced with a P1 stock grown on CF940, selecting for Tc^r (*zib-563*::Tn*10*) transductants. These were screened as either large, aminotriazole-sensitive *spoT*$^+$ recombinants (CF4941) or small, aminotriazole-resistant *spoT203* recombinants (CF4943) as described by Sarubbi et al. (53).

Plasmid insertion mutagenesis of $rel_{S.\ equisimilis}$. The internal 555-bp *Hin*dIII fragment of $rel_{S.\ equisimilis}$ was cloned into pVA891 to yield pVH45 (orientation I) and pVH46 (orientation II). Electrotransformation of H46A with the two plasmids, which cannot replicate in streptococci, and selection for erythromycin-resistant transformants as described before (39, 42) yielded H46A $rel_{S.\ equisimilis}$::pVH45 and H46A $rel_{S.\ equisimilis}$::pVH46. Insertions in both strains were confirmed by Southern hybridization of their chromosomal DNA with a $rel_{S.\ equisimilis}$ plasmid probe (data not shown).

(p)ppGpp measurements. Strains were screened for patterns of (p)ppGpp accumulation by labeling cells grown on LB plates in carrier-free $^{32}P_i$ minimal medium (7, 66). Changes in (p)ppGpp abundance were quantitated with cells grown in liquid cultures uniformly labeled with $^{32}P_i$ in MOPS medium with nucleosides and amino acids present as above, but omitting serine and using DL-serine hydroxamate (1 mg/ml) or isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) to provoke the amino acid starvation response (63) and to induce P_{tac}::*relA* plasmid-borne fusions, respectively (55, 58). Nucleotide abundance was quantitated with a radiologic imaging system; amounts of pppGpp and ppGpp were expressed as fractions of the total activity of blank-corrected GTP, ppGpp, and pppGpp after resolution by one-dimensional polyethyleneimine (PEI)-thin layer chromatography (TLC) developed with 1.5 M KP_i (pH 3.4) (8). Nucleotide identities were verified by cochromatography with standards after two-dimensional PEI-TLC in 3.3 M ammonium formate–4.2% ammonium borate (pH 7) in the first dimension and 1.5 M KP_i (pH 3.4) in the second dimension (10). The conditions for alkaline hydrolysis of labeled (p)ppGpp were 1-h exposures to 0.5 M NaOH–10 mM $MgCl_2$ at 37°C. The products of alkali digestion and enzymatic hydrolysis were resolved by PEI-TLC in 0.75 or 1.5 M KP_i.

(p)ppGpp preparation. Preparative amounts of pppGpp and ppGpp were synthesized from 500-ml reactions containing low-salt washed ribosomes from an *E. coli* strain bearing pALS10 with IPTG-induced overexpression of *relA* but otherwise purified as described previously (6). Preparations of (p)ppGpp ^{32}P labeled in specific positions were obtained from small reaction mixtures containing either low-salt washed ribosomes as above or purified $Rel_{S.\ equisimilis}$ protein (see below) and [α-^{32}P]GTP to obtain (p)pp*Gpp or [γ-^{32}P]ATP to obtain (p)ppGpp*, purified by elution with LiCl on small QAE-Sephadex columns, and ethanol precipitated with unlabeled pppGpp or ppGpp as the carrier.

Purification of $Rel_{S.\ equisimilis}$ and SpoT proteins. The proteins were from cells overexpressing $rel_{S.\ equisimilis}$ or *spoT*. In the case of $rel_{S.\ equisimilis}$, strain CF1693(pURS1), deleted for *relA* and *spoT* to eliminate endogenous sources of SpoT and RelA proteins, was found to express appreciable $Rel_{S.\ equisimilis}$ protein

without IPTG induction. SpoT was purified from an overproducing strain (20). Cells were resuspended in 3 volumes of lysis buffer (50 mM Tris acetate [pH 8.0], 5 mM EDTA, 0.23 M NaCl, 100 μg of phenylmethylsulfonyl fluoride per ml, 1 mM dithiothreitol [DTT]) and lysed by a combination of treatments with lysozyme (100 μg/ml) and a (nitrogen) pressure disruption bomb. After removal of debris by centrifugation (16,000 rpm for 30 min, Sorval SS34 rotor), protein aggregates were precipitated with 25% ammonium sulfate (0.134 g/ml) and dissolved in TGED (10 mM Tris-HCl [pH 8.0], 5% glycerol, 0.1 mM EDTA, 0.1 mM DTT). This suspension was again cleared by centrifugation and precipitated with 50% ammonium sulfate. The precipitate was redissolved in TGED containing 1 M NaCl and purified on a Sephacryl 300 sizing column in the same buffer, and the major SpoT protein band recovered was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as reacting with anti-SpoT antibody and verified by the (p)ppGppase assay (see below). Purification of both $Rel_{S.\ equisimilis}$ and SpoT proteins to near homogeneity could be achieved by this procedure; SDS-PAGE analysis showed that each consisted of a major band by Coomassie blue staining, with only trace amounts of other proteins. The SpoT protein used in the assays described below was purified further by chromatography on a 5.0-ml heparin column (19).

Western immunoblot analysis. Western analyses were performed as previously described (21) on whole-cell extracts electrophoresed on 9% polyacrylamide–SDS gels, transferred to polyvinylidene difluoride membranes, exposed to antibodies to RelA or SpoT proteins, developed with a chemiluminescent reagent (Dupont) for detection of anti-rabbit immunoglobulin G (IgG)/IgM-horseradish peroxidase conjugate, and detected by chemiluminescent exposures of X-ray films. Rabbit polyclonal antibodies were purified by preadsorption to extracts from strains deleted for *relA* or *spoT*; it was necessary to affinity purify the anti-SpoT to reduce residual background reactivity (18).

Assay for (p)ppGppase. (p)ppGpp hydrolysis was measured by adding enzyme (1 to 12.5 μg/ml, final concentration) to tubes warmed to 37°C containing [^{32}P](p)ppGpp (0.5 to 2 mM; about 0.1 μCi/μmol), 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, and 0.1 mM EDTA. Enzyme preparations, stored in TGED plus 1 M NaCl, were diluted in the same buffer so that the complete reaction mixture contained 100 mM NaCl. At 2-min intervals, 4-μl aliquots were spotted on PEI-cellulose presoaked in 0.15 M KP_i (pH 3.4), air dried (stopping the reaction), and subjected to PEI-TLC resolution of reaction products (GTP) or GDP and PP_i from (p)ppGpp substrates, allowing quantitation of (p)ppGpp remaining and labeled product accumulated. Hydrolysis rates were calculated from substrate/product ratios in a given sample. Specific activities observed were 52.0 ± 5.0 μmol/min/mg for $Rel_{S.\ equisimilis}$ protein and 17.3 ± 0.8 μmol/min/mg for SpoT protein under conditions of linearity of activities (2-min incubation times) over a 10-fold protein concentration range.

Assay for pppGpp synthetase. Reactions (20 μl) contained 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 mM NaCl, [γ-^{32}P]ATP (4 mM, 1 μCi/μmol), 13 mM GTP, and 20 μg of purified $Rel_{S.\ equisimilis}$ protein per ml. These were incubated at 32°C, sampled, and subjected to PEI-TLC as for hydrolysis but with 30-min sampling intervals. Accumulation of pppGpp could be visualized by UV absorption and quantitated after subtraction of activities present in a blank lacking enzyme. (The commercial labeled ATP preparation was contaminated with activity migrating near the pppGpp region of the chromatogram.) Specific activities observed averaged 0.17 ± 0.03 μmol/min/mg for $Rel_{S.\ equisimilis}$ protein.

RESULTS

The $rel_{S.\ equisimilis}$ gene in *E. coli* is not equivalent to *relA*. The *E. coli* wild-type strain CF1648 could be readily transformed with plasmids bearing the $rel_{S.\ equisimilis}$ gene in high-copy pUC19 derivatives (pURS1 or pURS24) or low-copy pACYC184 derivatives (pVRL1224 or pVR3). The presence of $rel_{S.\ equisimilis}$ causes expression of an 84-kDa protein at high copy number and phenotypic alterations at low copy number (see below). Therefore, the ability of $rel_{S.\ equisimilis}$ to complement the phenotypes of various *relA* and *spoT* mutants was explored to assess possible effects on (p)ppGpp metabolism.

The double deletion of *relA* and *spoT* in strain CF1693 eliminates all detectable (p)ppGpp, a phenotype abbreviated as (p)ppGpp^0, resulting in an inability to grow on minimal medium unless supplemented with multiple amino acids (66). Introduction of a wild-type *relA* gene, in single or multiple copy, is lethal in such strains because of excessive levels of (p)ppGpp arising from the absence of (p)ppGppase due to Δ*spoT* (66). Viable pURS1 transformants of strain CF1693 can be obtained; viability itself indicates that $rel_{S.\ equisimilis}$ in multicopy is unlikely to give ppGpp accumulation behavior equivalent to that of even single-copy *relA*. Strain CF1693 transformed with $rel_{S.\ equisimilis}$ plasmids grows, although slowly, on minimal medium, and therefore multiple amino acid requirements are suppressed. Since similar behavior is shown by the defective *relA1* insertion allele with low levels of residual (p)ppGpp synthetic activity (43), suppression by $rel_{S.\ equisimilis}$ suggests that $rel_{S.\ equisimilis}$ also provides a source of (p)ppGpp synthesis (66).

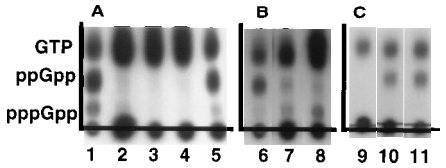

FIG. 1. Effects of multicopy $rel_{S.\ equisimilis}$ and *spoT* genes on (p)ppGpp accumulation responses. (A and B) (p)ppGpp responses to serine hydroxamate (1 mg/ml). Lanes 2 to 5, Δ*relA* hosts; lanes 1 and 6 to 8, wild-type hosts. (C) Responses of (p)pGpp^0 hosts after a 30-min exposure to 30 mM picolinic acid. Specific strains tested: 1, CF1648; 2, CF1652; 3, CF1652(pUC19); 4, CF1652 (pURS1); 5, CF1652(pALS10); 6, CF1648(pUC19); 7, CF1648(pURS1); 8, CF1648 (pHX41); 9, CF1693(pUC19); 10, CF1693(pURS1); 11, CF1693(pHX41). Cells were nonuniformly labeled with $^{32}P_i$ in the absence of amino acids by the screening procedure and grown on LB plates (A and B) or MOPS glucose amino acid plates lacking manganese (C). The positions of standards after PEI-TLC development in 1.5 M KP_i are marked above the origin (solid line).

The addition of picolinic acid gives a *spoT*-dependent accumulation of ppGpp by apparently selectively inhibiting the manganese-dependent (p)ppGppase (31, 66). Figure 1C shows that when cells are assayed for (p)ppGpp in the presence of picolinic acid, detectable (p)ppGpp is indeed observed in pURS1 $rel_{S.\ equisimilis}$ plasmid transformants of strain CF1693, much the same as transformants with plasmid pHX41 carrying a minimal *spoT* gene, but not in the pUC19 vector control.

Figure 1A shows that neither $rel_{S.\ equisimilis}$ in multicopy nor *spoT* restores the ability to accumulate (p)ppGpp during amino acid starvation of a *relA*-deleted host. This property differs from that of the wild-type *relA* in single or multicopy (55, 58) as well as that of the mutant *relA1* in multicopy (45). This failure to reverse the relaxed response was shown by all the $rel_{S.\ equisimilis}$ plasmids listed in Table 1 and was verified by 3-aminotriazole (52) and serine-methionine-glycine (64) plate growth tests, which discern between wild-type and *relA* mutant hosts (data not shown). The inability of $rel_{S.\ equisimilis}$ to complement a *relA* deletion in *E. coli* is notable, considering evidence just cited that it mediates synthetic activity.

Figure 1B shows that pURS1 transformants of a wild-type strain apparently behave like those transformed with the pHX41 *spoT* plasmid and markedly limit the accumulation of ppGpp during amino acid starvation (see below), as detected by the rapid nucleotide screening procedure (see Materials and Methods). Behavior under these conditions implies that the net effect of the synthetic and degradative activities of $rel_{S.\ equisimilis}$ expression, like those of *spoT*, favors (p)ppGpp degradation.

$rel_{S.\ equisimilis}$, like *spoT*, reverses the growth defects caused by (p)ppGpp. The *spoT203* allele is known (53) to limit (p)ppGpp degradation activity during growth without abolishing *spoT*-mediated (p)ppGpp synthetic activity and thereby confers a slow-growth phenotype accompanied by an 8- to 10-fold elevation in (p)ppGpp levels. This growth defect is reversed and ppGpp basal levels are lowered by *spoT* gene expression (53, 66). When the slow-growing Δ*relA spoT203* strain CF4943 is transformed with high-copy pURS1 or low-copy pVR3 (but not with vector control pUC19 or pVA8912), the growth defects after overnight growth on LB plates are largely reversed

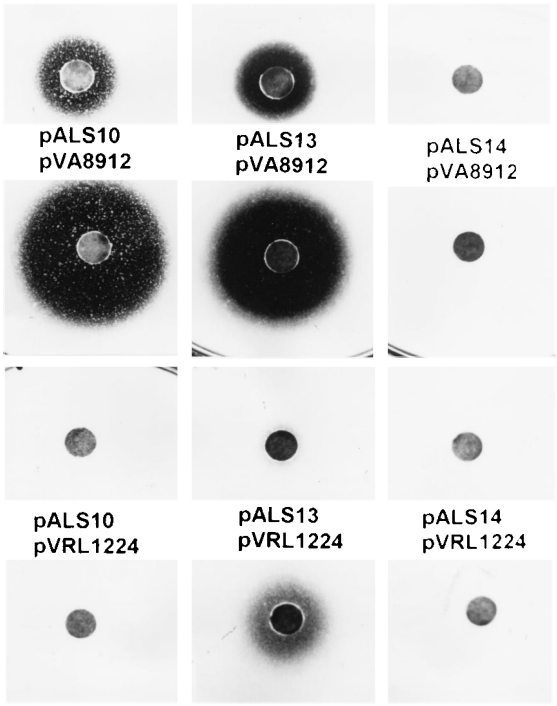

FIG. 2. Reversal of growth inhibition due to IPTG induction of *relA* by the $rel_{S.\ equisimilis}$ gene. The indicated plasmids were transformed into wild-type *E. coli* CF1648, and LB plates seeded with a lawn of cells were exposed to filter disks soaked in 10 mM IPTG (first and third rows) or 100 mM IPTG (second and fourth rows) and grown overnight to visualize zones of growth inhibition surrounding the disks.

(data not shown), implying that the modest ppGpp elevations due to *spoT*203 can be lowered by the presence of $rel_{S.\ equisimilis}$.

We devised a double-plasmid assay system to determine if plasmids bearing $rel_{S.\ equisimilis}$ can reverse cessation of growth due to gratuitous induction of even higher levels of (p)ppGpp generated from a second, compatible, plasmid (Fig. 2). The (p)ppGpp-generating set of plasmids are derived from pALS10, which is a P_{tac} promoter fusion to a full-length *relA* gene (55, 58). After IPTG exposure, pALS10 transformants are known to induce large amounts of RelA protein, which results in higher levels of (p)ppGpp than can be achieved with *spoT203* mutants, giving the 20- to 50-fold elevations seen during the stringent response to amino acid starvation and complete growth inhibition (55, 58). pALS13 contains a portion of the *relA* gene that shows IPTG inducibility, but the truncated RelA′ protein is apparently active without ribosome participation. The control pALS14 plasmid encodes an even larger C-terminal deletion that does not give IPTG-inducible (p)ppGpp levels (55, 58). Figure 2 shows the effects of disks impregnated with 10 or 100 mM IPTG on overnight growth of a lawn of strain CF1648 containing each member of the (p)ppGpp-generating set of plasmids paired with the $rel_{S.\ equisimilis}$ plasmid pVRL1224 or its vector control (pVA8912). It can be seen that the presence of pVRL1224 either reduces (with pALS13) or abolishes (with pALS10) growth inhibition surrounding the IPTG disks to mimic the behavior of the *relA* induction control (pALS14). The effects shown in Fig. 2 were not specific for growth on LB plates but could also be obtained on minimal glucose medium with and without amino acid supplementation. Small differences in sizes of single colonies of these strains grown on LB or M9 glucose-casamino acid plates were also consistent with $rel_{S.\ equisimilis}$ suppression of growth inhibition when pALS10 was present but not with pALS13 without IPTG present (data not shown).

$rel_{S.\ equisimilis}$ prevents accumulation of ppGpp but not pppGpp after IPTG induction of pALS10. It seemed likely that the ability of pVRL1224 to reverse growth inhibition due to (p)ppGpp accumulation was because $rel_{S.\ equisimilis}$ specified a (p)ppGppase. Alternative explanations judged less likely were that $rel_{S.\ equisimilis}$ expression might inhibit IPTG induction of active RelA protein or reverse growth-inhibitory effects despite normal induction of (p)ppGpp. Therefore, the kinetics of uniformly $^{32}P_i$-labeled (p)ppGpp accumulation after induction with 1 mM IPTG was measured in liquid cultures. The strain bearing pALS10 paired with pVRL1224 was chosen for study (along with its vector control) because it appears (Fig. 2) to be the most resistant to growth inhibition by IPTG.

Figure 3 shows that the presence of the pVRL1224 plasmid did greatly reduce the extent of pALS10-mediated induction of ppGpp accumulation compared with the pVA8912 vector control, indicating that $rel_{S.\ equisimilis}$ provides high levels of ppGppase activity.

Figure 3 also surprisingly shows that pppGpp is induced to accumulate not only in excess of ppGpp but with nearly equivalent kinetics in the pALS10 strains containing either pVRL1224 or pVA8912. As will be discussed, this result is interesting because it seems to conflict with a previous observation in another strain background of growth inhibition associated with excessively high levels of pppGpp (57). Growth inhibition under these labeling conditions is similar to growth inhibition on LB (Fig. 2). Measurements of growth effects during an hour after IPTG addition, shown in Fig. 3, confirmed that growth of induced CF1648(pALS10, pVRL1224) is only mildly inhibited compared with the strongly inhibited CF1648

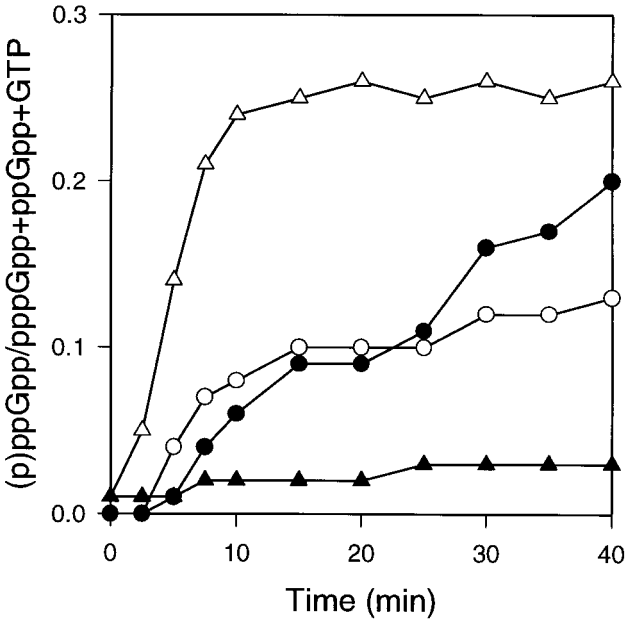

FIG. 3. Accumulation of ppGpp and pppGpp after IPTG induction of strain CF1648(pALS10) containing either the $rel_{S.\ equisimilis}$ plasmid pVRL1224 or a pVA8912 vector control. Cultures were grown and uniformly labeled with $^{32}P_i$ in MOPS glucose medium supplemented with all 20 amino acids. After isotope equilibration for two generations of growth, 1 mM IPTG was added (time zero), and aliquots were withdrawn and assayed for their content of ppGpp (triangles) and pppGpp (circles). Open symbols, pALS10 plus pVA8912; solid symbols, pALS10 plus $rel_{S.\ equisimilis}$ plasmid pVRL1224.

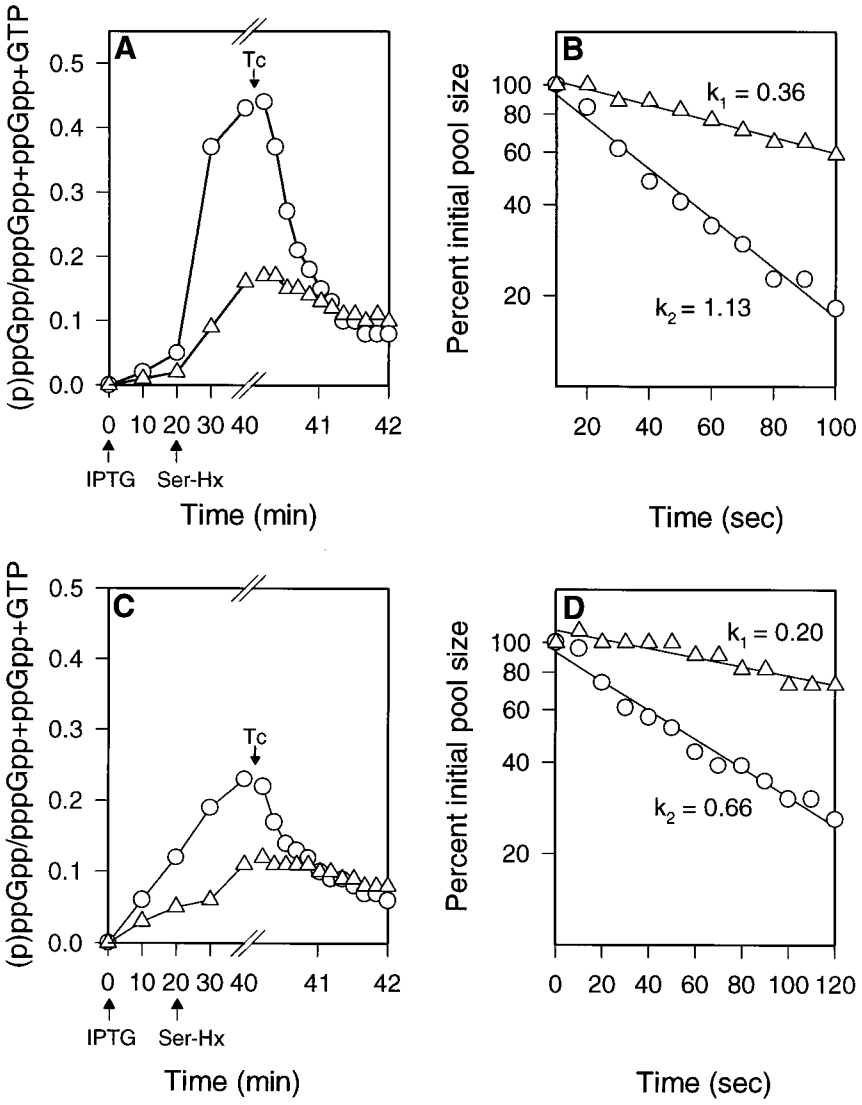

FIG. 4. Accumulation and decay of ppGpp and pppGpp in strains with $rel_{S.\ equisimilis}$ as the sole source of (p)ppGppase containing *relA* on pALS10 or *relA′* on pALS13. Cultures were grown and uniformly $^{32}P_i$ labeled in MOPS glucose medium supplemented with 19 amino acids, each at 40 μg/ml (serine omitted). At time zero, 1 mM IPTG was added; after 20 min of induction, DL-serine hydroxamate (Ser-Hx) (1 mg/ml) was added for an additional 20 min, after which tetracycline (Tc, 200 μg/ml) was added with serine (1 mg/ml), and aliquots were sampled rapidly thereafter. (A and B) Data obtained with CF1693(pALS10, pVRL1224); (C and D) data obtained with CF1693(pALS13, pVRL1224). Circles, pppGpp; triangles, ppGpp.

(pALS10, pVA8912) strain (data not shown). The preferential accumulation of pppGpp in excess of ppGpp could also be taken to suggest that the $Rel_{S.\ equisimilis}$ protein might more efficiently degrade ppGpp than pppGpp (see below). The presence of pppGpp in induced CF1648(pALS10, pVA8912) suggests the RelA protein is induced. In order to verify that induction was equivalent, we subjected protein extracts of the two strains induced as in Fig. 3 to SDS-PAGE followed by Western analysis with anti-RelA antibody (data not shown).

Quantitation of effects of $rel_{S.\ equisimilis}$ on degradation of (p)ppGpp. The effects of $rel_{S.\ equisimilis}$ on in vivo degradation of (p)ppGpp were estimated by using strain CF1693(pALS10, pVRL1224) in order to eliminate the endogenous source of ppGppase by deletion of the *spoT* gene. It is noteworthy that strain CF1693 is gpp^+ and therefore is expected to possess normal pppGpp γ-phosphohydrolase activity (24). The presence of pALS10 is necessary to allow an inducible source of (p)ppGpp that can be further amplified by the stringent response to serine hydroxamate addition but can be blocked to allow measurements of (p)ppGpp turnover. Because the strains tested are Cm^r, Em^r, and Km^r, tetracycline at 0.2 mg/ml was chosen to inhibit synthetic activity of the full-length RelA protein by blocking uncharged tRNA binding to ribosome acceptor sites (34). However, a note of caution comes from a report that tetracycline inhibits SpoT ppGppase in vitro (29; but see reference 61) and gives inhibition in vivo at 1 mg/ml (4).

It is notable that viable pALS10 (or pALS13) transformants of strain CF1693 cannot be obtained unless CF1693 is first transformed with the $rel_{S.\ equisimilis}$ plasmid pVRL1224; thus, pVRL1224 but not the pVA8912 vector control suppresses the nonviability of pALS10 (or pALS13) in the $ppGpp^0$ strain.

Figure 4 shows that when CF1693(pALS10, pVRL1224) was induced with 1 mM IPTG for 20 min, it accumulated about half

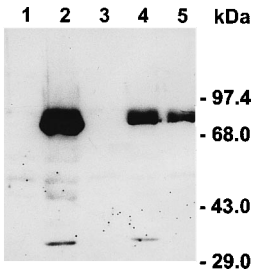

FIG. 5. Western blot analysis of the *rel*$_{S.\ equisimilis}$ gene product with *E. coli* anti-SpoT polyclonal antibody. Protein extracts were made from CF1693 strains carrying pUC19 (control, lane 1), pURS1 (*rel*$_{S.\ equisimilis}$, lane 2), pVA8912 (control, lane 3), pVR3 (*rel*$_{S.\ equisimilis}$, lane 4), and pVRL1224 (*rel*$_{S.\ equisimilis}$, lane 5).

as much (p)ppGpp as obtained with the wild-type CF1648 (pALS10, pVRL1224) (Fig. 3). Induction of CF1693(pALS13, pVRL1224) (Fig. 4C) gave more pppGpp than in Fig. 4A. In both strains, IPTG-induced pppGpp levels were in excess of ppGpp levels (Fig. 4). When the induced cells were then exposed to serine hydroxamate for 20 min, an additional stimulation of (p)ppGpp accumulation occurred, as expected, for the full-length RelA (pALS10) but not the RelA′ truncation (Fig. 4). After the addition of tetracycline, quantitative (p)ppGpp measurements revealed that the disappearance of (p)ppGpp (Fig. 4A and C) approximates first-order decay rates (Fig. 4B and D). Estimates of the observed first-order decay constants, from the equation $k = \ln 2/\text{half-life}$, were higher for pppGpp (1.13/min and 0.66/min) than for ppGpp (0.36/min and 0.20/min) and could well reflect a contribution from *gpp*-mediated conversion of pppGpp to ppGpp (see Discussion). Nevertheless, the metabolic labilities of (p)ppGpp observed are within the range found among various *spoT*$^+$ strains, with half-lives ranging from 20 s to a few minutes, compared with about 30 min for a *spoT* deletion mutant of the CF1648 strain used here (20). Thus, the presence of *rel*$_{S.\ equisimilis}$ evidently is responsible for ppGpp degradation at nearly normal rates; these may be underestimates, given the possibility of weak inhibition of (p)ppGppase due to the use of tetracycline to block (p)ppGpp synthesis.

Rel$_{S.\ equisimilis}$ protein cross-reacts with anti-SpoT but not with anti-RelA antibodies. Polyclonal antibodies that are specific for RelA or SpoT (18) were used to determine their reactivity with Rel$_{S.\ equisimilis}$ protein produced in the Δ*relA* Δ*spoT* strain CF1693 transformed with high-copy (pURS1) or low-copy (pVR3 or pVRL1224) *rel*$_{S.\ equisimilis}$ plasmids. Western blots of whole-cell extracts subjected to SDS-PAGE showed a prominent anti-SpoT-reactive band at a position consistent with the calculated molecular mass of Rel$_{S.\ equisimilis}$, i.e., $M_r \sim 84{,}000$ (Fig. 5). The differences of intensity for anti-SpoT reactivity of this band are consistent with expected differences in *rel*$_{S.\ equisimilis}$ plasmid copy number. Reactivity with anti-RelA antibody was not observed under conditions that could readily detect RelA in wild-type strain CF1648 (data not shown).

Purified Rel$_{S.\ equisimilis}$ protein shows manganese-dependent (p)ppGpp 3′-pyrophosphohydrolase activity in vitro. Staining of proteins resolved from extracts of CF1693(pURS1) revealed a plasmid-specified protein apparently comigrating with anti-SpoT antibody reactivity on Western blots. This protein was purified to near homogeneity by a method devised for the purification of overexpressed SpoT from extracts. The hydrolytic activity of Rel$_{S.\ equisimilis}$ towards (p)ppGpp and the ensuing reaction products are shown in Fig. 6 to be comparable to those of SpoT. The SpoT enzyme is a known manganese-dependent (p)ppGpp 3′-pyrophosphohydrolase (27–29, 50, 61). Preliminary exploration of optimal conditions for Rel$_{S.\ equisimilis}$ hydrolytic activity revealed parallels with SpoT that include (i) stimulation of activity by Mn^{2+} ions (not replaced by Ca^{2+}, Co^{2+}, Zn^{2+}, or Fe^{2+}); (ii) broad optimal salt concentrations (50 to 150 mM) with only minor effects of substituting KCl, LiCl, or NH_4Cl for NaCl; and (iii) a broad pH optimum around pH 8.

For analysis of (p)ppGpp products obtained after hydrolysis with Rel$_{S.\ equisimilis}$, the RelA ribosome-dependent reaction was used to synthesize ppGpp* with the 3′ β-phosphate specifically labeled with ^{32}P by using GTP and [γ-^{32}P]ATP as substrates (38, 62). The 3′-pyrophosphohydrolase of SpoT releases *PPi rather than *Pi from (p)ppGpp*. Furthermore, the absence of hydrolytic activity towards guanosine 5′-polyphosphates will be revealed if ppp*Gpp and pp*Gpp (labeled in the 5′-α-phosphate position by synthesis with [α-^{32}P]GTP) yield labeled GTP and GDP, respectively, after enzyme treatment. Figure 6 shows the Rel$_{S.\ equisimilis}$ and SpoT hydrolysis products with ppp*Gpp, pp*Gpp, or ppGpp* as the substrate together with nonhydrolyzed control reactions lacking enzyme. It is apparent that the two enzymes behave similarly. It is notable from lanes 7 to 9 that radioactive PP_i is indeed recovered as chromatographically separable from the UV-absorbing GDP product as well as P_i after both Rel$_{S.\ equisimilis}$ and SpoT hydrolyses. The radioactivity ascribed to PP_i can be further verified as not adsorbed to charcoal (data not shown). Hydrolysis of ppp*Gpp and pp*Gpp with both enzymes results in radioactivity stably associated with GTP and GDP as end products, respectively. Thus, the lack of Rel$_{S.\ equisimilis}$ enzymatic cleavage of 5′-polyphosphate residues is also verified.

The results in Fig. 6 show that both pppGpp and ppGpp can be substrates for Rel$_{S.\ equisimilis}$ hydrolysis. The in vivo observations of unexpected pppGpp hyperabundance (Fig. 3 and 4) raise the need to ask whether Rel$_{S.\ equisimilis}$, unlike SpoT, shows a marked preference for ppGpp over pppGpp as a substrate for hydrolysis. In Fig. 7, the enzyme concentration used in each set of six reactions gave nearly complete digestion of each single substrate at 1 mM. We asked whether the rates of hydrolysis of each substrate are slowed by the presence of the other upon admixture (total substrate concentration of 2 mM). Inspection of the data in Fig. 7 reveals that this occurs for Rel$_{S.\ equisimilis}$ as well as for the SpoT enzyme, suggesting no strong substrate preference. The reason for the slight differ-

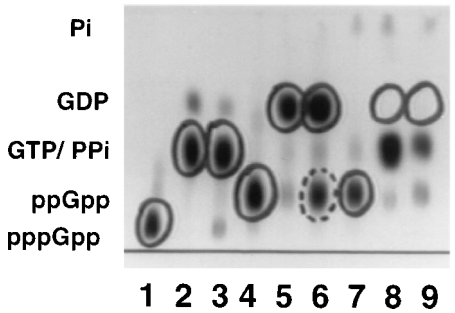

FIG. 6. Comparison of Rel$_{S.\ equisimilis}$- and SpoT-catalyzed hydrolysis of ppp*Gpp, pp*Gpp, and ppGpp*. Reaction mixtures containing 0.5 mM ppp*Gpp (lanes 1 to 3), pp*Gpp (lanes 4 to 6), or ppGpp* (lanes 7 to 9) were incubated for 5 min in the absence of enzyme (lanes 1, 4, and 7) or with SpoT (lanes 2, 5, and 8) or Rel$_{S.\ equisimilis}$ (lanes 3, 6, and 9) for 5 min at 37°C, resolved by PEI-TLC, developed with 1.5 M KP_i, autoradiographed, and viewed under UV. Regions showing UV absorption are circled. A spot weakly absorbing UV is circled with a dashed line in lane 6. The positions of standards are shown on the left.

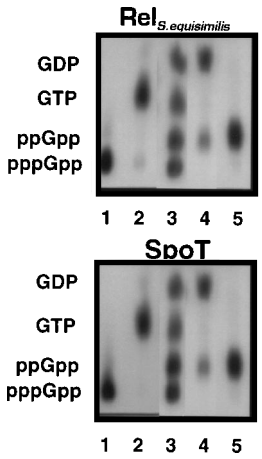

FIG. 7. Lack of preferential hydrolysis of ppGpp in mixtures of pppGpp and ppGpp by Rel$_{S.\ equisimilis}$ and SpoT. Reaction mixtures containing 1 mM ppp*Gpp (lanes 1 and 2), 1 mM pp*Gpp (lanes 4 and 5), or a mixture of 1 mM ppp*Gpp and 1 mM pp*Gpp (lane 3) were incubated for 10 min at 37°C in the absence of enzyme (lanes 1 and 5) or in the presence of enzyme (lanes 2, 3, and 4). (Top panel) Rel$_{S.\ equisimilis}$ protein; (bottom panel) SpoT protein. PEI-TLC development in 1.5 M KP_i was followed by autoradiography. The mobilities of nucleotide standards are indicated at the left.

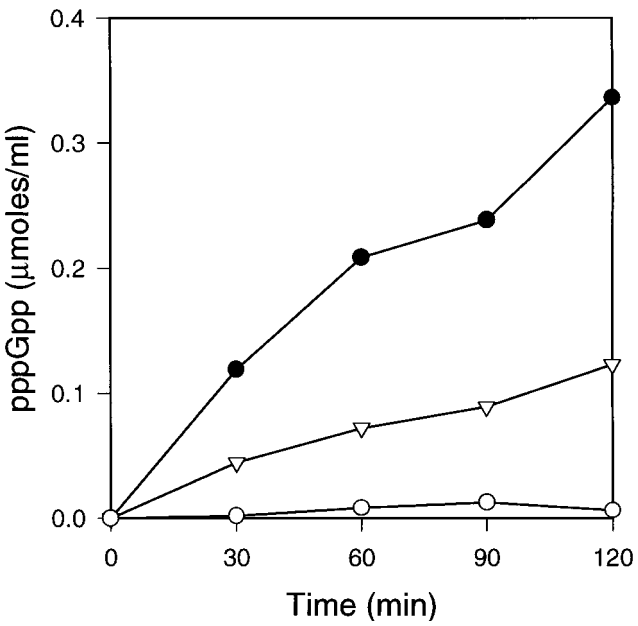

FIG. 8. Synthesis of pppGpp by Rel$_{S.\ equisimilis}$. All reactions contained 20 μg of Rel$_{S.\ equisimilis}$ protein per ml, 10 mM $MgCl_2$, and 4 mM [γ-^{32}P]ATP and, in addition, 13 mM GTP (solid circles), 4 mM GTP (open circles), or 13 mM GTP plus 2 mM ppGpp (triangles). After sampling at the times indicated and PEI-TLC development, activities accumulating in the pppGpp region of the chromatogram were quantitated.

ences in the degree of hydrolysis of single substrates in Fig. 7 is unknown.

Purified Rel$_{S.\ equisimilis}$ protein catalyzes pppGpp synthesis by an ATP:GTP 3′-pyrophosphoryltransferase activity. The results shown in Fig. 1C led us to ask whether Rel$_{S.\ equisimilis}$ possesses (p)ppGpp synthetic activity in vitro. Preliminary experiments revealed that this highly purified protein preparation catalyzed the appearance of pppGpp by UV absorbance in synthetic reactions after one- or two-dimensional TLC with 10 to 20 times more enzyme than in the hydrolysis assays, using incubation times of hours instead of minutes, omitting manganese from reaction mixtures, and increasing the total nucleotide concentrations to levels exceeding the $MgCl_2$ concentrations. Equivalent reactions with the SpoT protein did not yield detectable (p)ppGpp synthesis, nor did extracts of strains deleted for *relA* and *spoT* in the absence of *rel*$_{S.\ equisimilis}$ (data not shown). Various ratios of ATP to GTP, with the total nucleotide concentration held constant at about 13 mM (with $MgCl_2$ at 10 mM), revealed that optimal pppGpp formation with the Rel$_{S.\ equisimilis}$ protein was obtained at ratios of at least 1.5. When the 1.5 ratio is fixed but the total nucleotide concentration is varied at 4 mM $MgCl_2$, optimal pppGpp synthesis occurred at about 5 mM ATP plus GTP (data not shown). Low but significant rates of pppGpp synthesis at 10 mM $MgCl_2$ over extended time periods were seen at 4 mM ATP and 13 mM GTP (ATP/GTP ratio of about 0.3) that were decreased by the presence of 2 mM ppGpp, the homolog of the pppGpp product, and markedly decreased by lowering GTP to 4 mM (increasing the ATP/GTP ratio to 1 but giving a total nucleotide concentration below that of $MgCl_2$) (Fig. 8). Despite the inefficiency of synthesis, this condition allows us to determine if the 3′-β-phosphate residue of the pppGpp product is selectively labeled with [γ-^{32}P]ATP as it is with ATP:GTP 3′-pyrophosphoryltransferase catalyzed by the RelA ribosome-dependent (p)ppGpp synthetic reaction. If this occurs, the labeled 3′-β-phosphate should be uniquely labile to alkaline hydrolysis and released as $^*P_i$ (9, 62).

The pppGpp was prepared with the Rel$_{S.\ equisimilis}$ synthetic reaction with the higher-specific-activity [γ-^{32}P]ATP as the substrate. Table 2 shows the results of an analysis of alkali hydrolysis of the purified product compared with authentic ppGpp* (and pp*Gpp and ppp*Gpp controls) synthesized in the RelA reaction. It appears that pppGpp labeled with [γ-^{32}P]ATP during synthesis by Rel$_{S.\ equisimilis}$ does show alkali lability of the 3′-β-phosphate residue in much the same manner as the analogous RelA reaction product. This, in turn, suggests that the synthetic activity of Rel$_{S.\ equisimilis}$ also consists of a pyrophosphate transfer reaction, as summarized for reactions I and II in Fig. 9. Control hydrolyses with α-labeled (p)ppGpp did not release labeled P_i and revealed that (p)pp*Gp is a stable hydrolysis product.

In *S. equisimilis* H46A, accumulation of ppGpp during the stringent response depends on a functional *rel*$_{S.\ equisimilis}$ gene. When wild-type strain H46A was subjected to the stringent

TABLE 2. Analysis of alkali hydrolysis products of (p)ppGpp[a]

Substrate	Source of substrate	Radioactivity (cpm)			
		Unhydrolyzed control		0.5 M NaOH hydrolysis	
		(p)ppGpp	P_i	(p)ppGpp	P_i
pppGpp*	Rel$_{S.\ equisimilis}$ [γ-^{32}P]ATP	17,985	198	184	19,885
ppGpp*	RelA [γ-^{32}P]ATP	1,823	14	48	1,845
ppp*Gpp	RelA [α-^{32}P]GTP	1,903	18	2,029	19
pp*Gpp	RelA [α-^{32}P]GTP	1,986	15	2,089	34

[a] Preparations of (p)ppGpp were labeled with [γ-^{32}P]ATP or [α-^{32}P]GTP by synthesis with Rel$_{S.\ equisimilis}$ or RelA enzymes as indicated. The purified pppGpp or ppGpp products were treated for 1 h at 37°C in water (nonhydrolyzed control) or hydrolyzed in alkali as described in Materials and Methods. Samples were neutralized and resolved by PEI-TLC, and radioactivity was quantitated in regions of nucleotide and P_i products. Samples before hydrolysis were at least 85% pure; activities found in the major hydrolysis products account for at least 95% of expected yields.

```
I    (p)ppG + p*ppA  -----> (p)ppGpp* -----> (p)ppGp + *Pi
      γ βα     γ βα           γ βα αβ          γ βα α

II   (p)pp*G + pppA  -----> (p)pp*Gpp -----> (p)pp*Gp + Pi
      γ βα     γβα           γ βα  αβ          γ βα  α
```

FIG. 9. Alkali hydrolysis products of (p)ppGpp. The deduced labeled phosphate positions are marked with asterisks for [γ-^{32}P]ATP (reaction I) and [α-^{32}P]GTP (reaction II).

response by the amino acid starvation screening protocol involving labeling with carrier-free $^{32}P_i$ as described in Materials and Methods, one-dimensional PEI-TLC of the nucleotide extracts revealed the presence of (p)ppGpp comigrating with (p)ppGpp synthesized by *E. coli* under similar conditions (Fig. 10). Two-dimensional chromatography of samples from *S. equisimilis* verified the comigration of putative labeled ppGpp with unlabeled marker ppGpp visualized by UV absorbance (Fig. 11). The radioactive spot comigrating with pppGpp is also present but in relatively minor amounts, indicating that ppGpp is the major accumulation product in the streptococcal strain, as in *E. coli*. The (p)ppGpp accumulation response following amino acid starvation of the wild type was not observed in either of the $rel_{S.\ equisimilis}$ insertion mutants assayed under comparable conditions (Fig. 10). This suggests that the accumulation of (p)ppGpp in response to amino acid starvation depends on a functional $rel_{S.\ equisimilis}$ gene, a phenotype characteristic of *relA* but not of *spoT* mutants of *E. coli.*

DISCUSSION

Studies of *E. coli* and closely related organisms have established that (p)ppGpp is a major but perhaps not invariant regulatory signal during the stringent response to amino acid starvation and other nutritional stress conditions (see the introduction). Global regulatory mechanisms have rarely been studied in host-associated streptococci; we are aware of only one report that explores ppGpp accumulation upon amino acid starvation of three streptococcal species (40). These authors showed that while all of them display some attributes of a stringent response, only in *Enterococcus hirae* was this response coupled with high ppGpp levels. The characterization of the $rel_{S.\ equisimilis}$ gene and effects of insertion alleles will help to characterize the stringent response in this metabolically and ecologically diverse group of organisms at the molecular level.

Despite several sequences now available for genes encoding proteins homologous to RelA and SpoT (see the introduction), motifs diagnostic of RelA or SpoT function have not yet been identified. Indeed, synthesis of 3′-polyphosphorylated nucleotides can be catalyzed by a protein with no apparent homology to either RelA or SpoT (47). While the deduced $Rel_{S.\ equisimilis}$ protein sequence has 59.4% overall sequence similarity with RelA and SpoT (41), the N-terminal half of $Rel_{S.\ equisimilis}$ is more closely related to that of SpoT than that of RelA, with optimized homology scores of 864 versus 699, respectively. However, the activities of the N-terminal halves of both SpoT and RelA proteins seem to be responsible for opposing activities, since it is the region for mutations affecting the degradation activity of SpoT (53) as well as the (p)ppGpp synthetic activity of RelA, as shown by pALS13 (55, 58).

We find that when $rel_{S.\ equisimilis}$ is expressed in *E. coli*, it provides a function similar to that of the *E. coli spoT* gene: $rel_{S.\ equisimilis}$ specifies a strong (p)ppGpp 3′-pyrophosphohydrolase as well as a weaker source of (p)ppGpp synthetic activity that is unresponsive to a serine hydroxamate-imposed stringent response. This conclusion is consistent with effects of

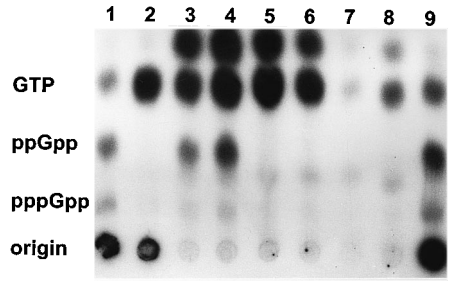

FIG. 10. Effects of chromosomal $rel_{S.\ equisimilis}$ insertions in *S. equisimilis* H46A on radioactivity comigrating with ppGpp and pppGpp after resolution by PEI-TLC. Cultures were nonuniformly labeled with $^{32}P_i$ and amino acid starved after growth on LB plates as described in Materials and Methods. Lanes 1 and 9, CF1648, wild type; lane 2, CF1652, Δ*relA*; lanes 3 and 4, independent cultures of H46A, wild type; lanes 5 and 6, independent cultures of H46A $rel_{S.\ equisimilis}$::pVH45; lanes 7 and 8, independent cultures of H46A $rel_{S.\ equisimilis}$::pVH46.

$rel_{S.\ equisimilis}$ expression on the growth of *E. coli* strains in which (p)ppGpp levels were elevated by mutation or induction of genes involved in (p)ppGpp metabolism and verified by measurements of in vivo (p)ppGpp degradation and synthesis in a mutant strain lacking endogenous sources of these activities (Fig. 1 and 4). Western analysis of extracts of cells containing the $Rel_{S.\ equisimilis}$ protein show specific immunological reactivity with anti-SpoT but not anti-RelA antibody (Fig. 5); this reactivity was used to monitor the protein during its purification (see Materials and Methods). The protein purified from a host deleted for *relA* and *spoT* possessed a manganese-activated pyrophosphohydrolase activity towards (p)ppGpp, with specific activities at least as high as those observed with purified SpoT protein (Fig. 6). The highly purified $Rel_{S.\ equisimilis}$ protein catalyzed the formation of pppGpp in a manner deduced to occur by an ATP:GTP 3′-pyrophosphoryltransferase activity (Fig. 8 and Table 2). This synthetic activity, which has not been demonstrated for the SpoT enzyme in vitro, is not likely to be due to a protein other than $Rel_{S.\ equisimilis}$ because the cellular host is devoid of this activity. The high ATP and GTP substrate concentrations required for demonstrating the $Rel_{S.\ equisimilis}$ pppGpp synthetic activity suggest that synthesis might occur by a mechanism closely related to reversal of the

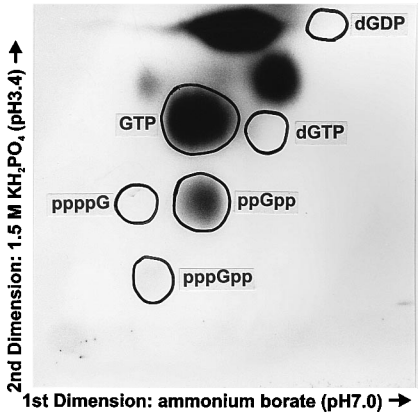

FIG. 11. Identification of ppGpp and tentative identification of pppGpp among $^{32}P_i$-labeled nucleotides of *S. equisimilis* H46A by two-dimensional PEI-TLC. Wild-type cells were labeled as for lanes 3 and 4 in Fig. 8, and formic acid cell extracts were resolved by two-dimensional chromatography and autoradiographed. UV-absorbing spots corresponding to added nucleotide standards are circled.

degradation reaction, for example, pyrophosphate group transfer from (p)ppGpp to water for hydrolysis and from ATP to GTP for synthesis. Attempts to measure simultaneous synthesis and degradation by searching for labeled AMP or pyrophosphate with [^{32}P]ATP labeled in the α or γ position, respectively, have not yet been revealing (data not shown). Systematic studies of substrate specificities and product inhibition measurements for the hydrolytic and synthetic reactions with purified enzyme are clearly warranted.

These studies raise interesting questions with respect to both *S. equisimilis* and *E. coli*. First, although $rel_{S.\ equisimilis}$ expressed in *E. coli* functions like *spoT*, insertional inactivation of the native gene in *S. equisimilis* suggests that it functions like *relA* rather than like *spoT*. The wild-type *S. equisimilis* was found to accumulate (p)ppGpp in response to amino acid starvation; this response apparently was abolished in strains isolated with an insertion in the $rel_{S.\ equisimilis}$ gene (Fig. 10 and 11). However, judging from the residual activities of *relA* and *spoT* gene insertions and partial deletions (20, 55), uncertainty exists as to whether an insertion in $rel_{S.\ equisimilis}$ comprises a null allele, whether residual levels of (p)ppGpp exist for the insertion mutant, and whether a second *relA/spoT* homolog might exist. It is noteworthy that the introduction of an insertionally inactive *spoT* gene in *E. coli* is predicted to cause slow growth, if not lethality, unless there is an accompanying simultaneous inactivation of *relA* (66). Both $rel_{S.\ equisimilis}$ insertion mutants of *S. equisimilis* isolated do grow slowly on both a chemically defined medium containing, inter alia, all 20 amino acids (65), and on rich media, but the presence of an extragenic suppressor is unlikely because a minimal $rel_{S.\ equisimilis}$ plasmid complements the defects in (p)ppGpp accumulation and growth during the stringent response (41). Thus, the highly qualified speculation can be made that a single $rel_{S.\ equisimilis}$ gene in a fully sequenced region might substitute for both *relA* and *spoT* functions encoded by separate genes in *E. coli.*

A potentially analogous situation has been reported for *B. subtilis*, in which a mutation in a gene originally presumed to be like *relA* (59) has been shown to inactivate the (p)ppGpp accumulation responses to both amino acid starvation and glucose deprivation. A 3-aminotriazole-resistant mutation isolated in this host and genetically mapped to the region of the original mutation restores the carbon starvation response but not the stringent response (23). However, the carbon source starvation responses in *S. equisimilis* are unknown, as are the sequences of the *rel* region of *B. subtilis.*

The observations presented in Fig. 2 and 3 present two challenges to what is currently understood of (p)ppGpp metabolism and regulation in *E. coli*. The first is that the presence of the $rel_{S.\ equisimilis}$ plasmid results in pppGpp accumulating in excess of ppGpp during IPTG induction of either the wild-type CF1648(pALS10) (Fig. 3) or CF1963(pALS10), especially evident after serine hydroxamate addition (Fig. 4). One explanation for this behavior is that $Rel_{S.\ equisimilis}$ could preferentially hydrolyze ppGpp over pppGpp; however, a strong preference is not apparent in vitro (Fig. 7). Preferential accumulation of pppGpp during the stringent response is also seen with *gpp* mutants; the *gpp* gene encodes the major source of pppGpp γ-phosphohydrolase as well as a source for polyphosphate degradation (5, 24, 36, 57). Thus, $rel_{S.\ equisimilis}$ might somehow interact with one or another of these functions to give a *gpp* mutant phenocopy.

Another explanation can be proposed that relates to the mechanism (4) explaining how a *spoT* mutant deficiency in (p)ppGppase gives a phenotype termed spotless, i.e., pppGpp << ppGpp (37), during the stringent response. When high rates of (p)ppGpp synthesis are activated early in the stringent response, both pppGpp and ppGpp are measurable (15, 35). At later times in the response, RelA-dependent synthesis of (p)ppGpp is thought to be progressively feedback inhibited by ppGpp, allowing more complete Gpp enzyme-dependent conversion of existing pppGpp to ppGpp and thereby yielding the normally relatively low ratios of pppGpp to ppGpp. A *spoT* mutant accumulates higher levels of ppGpp as a result of the ppGppase deficiency, which leads to even stronger feedback inhibition of (p)ppGpp synthesis and, in turn, vanishingly small amounts of pppGpp accumulation (3, 4).

Conversely, the excessively high pppGpp/ppGpp ratios seen in Fig. 3 and 4 might well simply reflect a consequence of excessive (p)ppGppase activity encoded by $rel_{S.\ equisimilis}$. It can be expected that the heightened (p)ppGppase activity would lower (p)ppGpp accumulation rates during IPTG induction or during the stringent response and thereby facilitate high rates of continued synthesis of (p)ppGpp by full-length RelA encoded by pALS10. This, in turn, would provide a constant influx of pppGpp and high pppGpp/ppGpp ratios despite rapid degradation, as shown in Fig. 4. A strong suggestion of this effect on pppGpp abundance can even be seen during the stringent response in strain CF1648 (with a single-copy $relA^+$ gene) when ppGppase is amplified by either $rel_{S.\ equisimilis}$ or *spoT* in multicopy, although (p)ppGpp abundance is low (Fig. 1B).

The faster decay rates for pppGpp than for ppGpp (Fig. 4B and D) presumably reflect the ability of both $Rel_{S.\ equisimilis}$ and Gpp to degrade pppGpp, whereas slower loss of ppGpp reflects the balance of ppGpp hydrolysis by $Rel_{S.\ equisimilis}$ together with the gain of ppGpp from Gpp attack on pppGpp.

The differences in (p)ppGpp accumulation after IPTG induction of pALS10 and pALS13 in the presence of multicopy $rel_{S.\ equisimilis}$ might be explained as being due to the relative resistance of RelA′ to ppGpp inhibition compared with the sensitivity of full-length RelA (Fig. 4A and C). Measurements have also been made under the conditions in Fig. 3, with pALS13 replacing pALS10. In this case, *relA′* induction also resulted in substantially enhanced rates of (p)ppGpp synthesis, and in the presence of the $rel_{S.\ equisimilis}$ plasmid, high levels of (p)ppGpp persisted (data not shown). The previously known differences in ppGpp synthetic responses to amino acid deprivation of strains bearing these two plasmids are thought to reflect a defect in ribosome-dependent activation (55, 58). Thus, we propose that RelA′ is insensitive to ppGpp feedback inhibition compared with full-length RelA; this insensitivity could well be closely related to the known RelA′ activation defect. This conjecture also accounts for differences in the IPTG sensitivity of growth caused by the pALS13 and pALS10 plasmids shown in Fig. 2.

The second result that is difficult to explain is the lack of strong growth inhibition during an hour after IPTG induction of pppGpp in the presence of pVRL1224 despite induction of pppGpp to levels comprising 10 to 20% of GTP activity (Fig. 3), implying that growth inhibition might be specific for ppGpp and not for pppGpp. This result is unexpected because *gpp-1* mutations were originally reported to cause increased pppGpp/ppGpp ratios during the stringent response as well as increased total (p)ppGpp basal levels, slow growth, and even lethality in *spoT1* mutant backgrounds (57). However, these reported growth defects apparently show a high degree of strain specificity in *E. coli* strains; a Δ*gpp* derivative of strain CF1648, used here, that neither grows slowly on rich or minimal medium nor contributes specific growth-limiting effects when combined with *spoT203* mutations has been constructed (33, 46). If ppGpp but not pppGpp is growth inhibitory in the strain CF1648 background, then Δ*gpp* mutant hosts would be expected to show reduced growth sensitivity to IPTG when trans-

formed with pALS10 in the absence of multicopy genes encoding a (p)ppGppase; instead, growth sensitivities to IPTG are equivalent with pALS10 transformants of otherwise isogenic strains differing only by the presence or deletion of the *gpp* gene (data not shown). The reason that strain CF1648 grows despite containing pppGpp at levels of about 10% of GTP pools (Fig. 3) may be related to our finding that CF1648 is rather insensitive to (p)ppGpp concentrations compared with N99 or NF161 derivatives and has correspondingly higher basal levels of ppGpp during normal growth (20). We therefore suspect that the reversal of growth inhibition despite persistence of pppGpp that accompanies expression of $rel_{S.\ equisimilis}$ may be related to the tolerance of strain CF1648 to elevations of (p)ppGpp.

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