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Characterization of the Stringent and Relaxed Responses of *Streptococcus equisimilis*

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The 739-codon rel_{Seq} gene of *Streptococcus equisimilis* H46A is bifunctional, encoding a strong guanosine 3′,5′-bis(diphosphate) 3′-pyrophosphohydrolase (ppGppase) and a weaker ribosome-independent ATP:GTP 3′-pyrophosphoryltransferase [(p)ppGpp synthetase]. To analyze the function of this gene, (p)ppGpp accumulation patterns as well as protein and RNA synthesis were compared during amino acid deprivation and glucose exhaustion between the wild type and an insertion mutant carrying a rel_{Seq} gene disrupted at codon 216. We found that under normal conditions, both strains contained basal levels of (p)ppGpp. Amino acid deprivation imposed by pseudomonic acid or isoleucine hydroxamate triggered a rel_{Seq}-dependent stringent response characterized by rapid (p)ppGpp accumulation at the expense of GTP and abrupt cessation of net RNA accumulation in the wild type but not in the mutant. Tetracycline added to block (p)ppGpp synthesis caused the accumulated (p)ppGpp to degrade rapidly, with a concomitant increase of the GTP pool (decay constant of ppGpp, $\approx$0.7 min^{-1}). Simultaneous addition of pseudomonic acid and tetracycline to mimic a relaxed response caused wild-type RNA synthesis to proceed at rates approximating those seen under either condition in the mutant. Glucose exhaustion provoked the (p)ppGpp accumulation response in both the wild type and the rel_{Seq} insertion mutant, consistent with the block of net RNA accumulation in both strains. Although the source of (p)ppGpp synthesis during glucose exhaustion remains to be determined, these findings reinforce the idea entertained previously that rel_{Seq} fulfils functions that reside separately in the paralogous *relA* and *spoT* genes of *Escherichia coli*. Analysis of (p)ppGpp accumulation patterns was complicated by finding an unknown phosphorylated compound that comigrated with ppGpp under two standard thin-layer chromatography conditions. Unlike ppGpp, this compound did not adsorb to charcoal and did not accumulate appreciably during isoleucine deprivation. Like ppGpp, the unknown compound did accumulate during energy source starvation.

Bacteria have evolved elaborate strategies that enable them to adjust their macromolecular syntheses when their nutrient supply becomes limiting or other environmental stresses are imposed. Among the responses involving the modulation of gene expression is the well-known stringent response, the hallmarks of which include the restriction of the synthesis of stable RNAs, the stimulation of certain anabolic reaction pathways, and the induction of stationary-phase-specific genes as results of amino acid limitation or inhibition of tRNA amino acylation. This pleiotropic response is mediated by the accumulation of GDP and GTP derivatives that carry a pyrophosphate group on the 3′-hydroxyl position of ribose, i.e., ppGpp and pppGpp, respectively, hereafter together designated (p)ppGpp (see references 3 and 9 for recent reviews). Depending on the nature of the nutritional stress imposed, there are two different sources for (p)ppGpp accumulation in *Escherichia coli*. During amino acid deprivation, (p)ppGpp accumulates above low basal steady-state levels in a reaction catalyzed by the *relA* gene product, also known as (p)ppGpp synthetase I (PSI). This enzyme pyrophosphorylates GDP and GTP in a ribosome-dependent reaction with ATP as the donor and is activated by a decreased ratio of charged to uncharged cellular tRNAs on the aminoacyl acceptor site of the ribosome (8, 17–19, 31). During energy source exhaustion, *relA* null mutants continue to accumulate (p)ppGpp in a *spoT* gene-dependent fashion which is characterized by the poorly understood inhibition of the (p)ppGpp 3′-pyrophosphohydrolase activity of the SpoT protein (15). The important observation that *relA-spoT* double null mutants lack all detectable (p)ppGpp under any conditions [the $(p)ppGpp^0$ phenotype] led to the proposal that SpoT is bifunctional and also acts as (p)ppGpp synthetase, thereby representing the second source of this synthetic activity (PSII) (20, 44). Although the PSII activity of SpoT has not yet been demonstrated in vitro, a recent deletion analysis of plasmid-borne *spoT* with chromosomal mutants defective in each activity for complementation tests has revealed that the two competing enzymatic activities reside in independent but overlapping regions of the N-terminal portion of the SpoT protein (16).

Pleiotropic physiological responses mediated by (p)ppGpp in organisms other than *E. coli* are less well understood, and there is only one report that approaches this problem in the pathogenic streptococci (26). While all three species explored in this study exhibited some features of the stringent response upon amino acid starvation, only in *Enterococcus hirae* was this response accompanied by increased ppGpp levels. More recently, a cloned and sequenced gene from *Streptococcus equisimilis* H46A has become available that is homologous to *relA* and *spoT* from *E. coli* and has been functionally analyzed in some detail. This gene, previously called *rel* (28) or $rel_{S.\ equisimilis}$ (27) and designated rel_{Seq} here, encodes a strong (p)ppGpp 3′-pyrophosphohydrolase activity [(p)ppGppase] and a weaker ribosome-independent (p)ppGpp synthetic activity resulting from its ATP:GTP 3′-pyrophosphoryltransferase capacity. Both activities are detectable in vivo by complementation experiments with appropriate *E. coli* mutants and could be verified in vitro by activity assays of the purified Rel_{Seq} protein (27). In vivo, the net effect of the dual Rel_{Seq} activities favors (p)ppGpp degradation, resulting in the failure of rel_{Seq} to com-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
E. coli		
CF1648	Wild-type MG1655	44
CF1652	As CF1648 but Δ*relA256*	44
S. equisimilis		
H46A	Wild type; human serogroup C	7
H46A*rel*$_{Seq}$::pVH46	*rel*$_{Seq}$ insertion; Em^r	27
H46A*rel*$_{Seq}$::pVRH550	*rel*$_{Seq}$ insertion; Em^r	This study
Plasmids		
pVA8912	Vector; p15A origin; Em^r	39
pMG36	Vector; pWV01 origin; Km^r	41
pMR9	As pMG36 but containing *rel*$_{Seq}$ under its natural promoter and *lacZpo*; Km^r	This study
pUC19	Vector; *lacZpo*; ColE1 replicon; Ap^r	45
pURS1	As pUC19 but containing *Pst*I *rel*$_{Seq}$ fragment in *lacZpo* antisense orientation	27

plement the absence of *relA* and, hence, in its functional likening to *spoT*. However, disruptions of chromosomal *rel*$_{Seq}$ by targeted insertion mutagenesis abolish the (p)ppGpp accumulation response following amino acid starvation of wild-type H46A, a phenotype characteristic of *E. coli relA* but not *spoT* mutants. Thus, the possibility exists that one gene in *S. equisimilis* fulfils the functions that reside in separate *E. coli* genes.

We attempt here to characterize the role of *rel*$_{Seq}$ in streptococcal physiology, asking if regulatory responses to amino acid starvation and energy source exhaustion exist in parallel to those of *E. coli* along with patterns of ppGpp accumulation and phenotypic effects of the *rel*$_{Seq}$ mutation. The possibility of a unique *rel*$_{Seq}$ gene in *S. equisimilis*, unaccompanied by a sister *relA*/*spoT* gene ortholog, leads us to ask if the *rel*$_{Seq}$ disruption allele confers a complete absence of (p)ppGpp rather than simply a block in ppGpp accumulation during amino acid starvation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are described in Table 1. The two plasmid insertion mutations of the H46A chromosomal *rel*$_{Seq}$ gene, *rel*$_{Seq}$::pVH46 and *rel*$_{Seq}$::pVRH550, disrupt *rel*$_{Seq}$ at codon 216 but differ in their vector components, the former carrying pVA891 (27) and the latter pVA8912 (39). *E. coli* strains were grown with agitation at 32°C in Luria broth or, for [^{32}P]orthophosphate ($^{32}P_i$) labeling, in MOPS (morpholinepropanesulfonic acid) glucose minimal medium as described previously (2). *S. equisimilis* was grown at 37°C without shaking in brain heart infusion broth (Difco) or low-phosphate medium derived from the chemically defined medium described by van de Rijn and Kessler (42). Low-phosphate medium differed from the chemically defined medium by containing, unless indicated otherwise, 2% glucose, 26 mM MOPS, 4 mM Tricine, 6 g of sodium acetate liter^{-1}, and 0.2 g each of cystine and tyrosine liter^{-1}. Furthermore, the basal low-phosphate medium was prepared free of phosphate and, before use, supplemented with P_i at the desired concentrations from a stock solution containing 1.54 g of KH_2PO_4 liter^{-1} and 1.04 g of K_2HPO_4 liter^{-1} (159 mM P_i).

(p)ppGpp measurements. Screening for patterns of (p)ppGpp accumulation in *E. coli* was performed as described by Cashel (2). For the same purpose, nonuniformly $^{32}P_i$-labeled *S. equisimilis* cells were obtained by growing strain H46A overnight on plates with low-phosphate medium (0.4 mM P_i), followed by incubation of the cells with 150 μCi of $^{32}P_i$ per ml under conditions otherwise identical to those used for *E. coli*. For uniform $^{32}P_i$ labeling of H46A cells, cultures grown overnight in liquid low-phosphate medium containing 0.7 to 1.0 mM P_i were diluted 1:100 in the same medium. After incubation to an optical density at 600 nm (OD_{600}) of 0.05, 150 μCi of carrier-free $^{32}P_i$ per ml was added, and the cultures were further incubated for two doubling times under normal conditions before being amino acid starved or glucose depleted.

For amino acid starvation experiments, the isoleucine content of the low-phosphate medium was reduced to 1/10 the normal concentration (i.e., 10 μg/ml), and pseudomonic acid (Beecham Pharmaceuticals), an inhibitor of isoleucyl-tRNA synthetase (21), was added at 200 to 300 ng/ml when the cultures had reached an OD_{600} of 0.25. To achieve energy source exhaustion, the glucose concentration of the low-phosphate medium was reduced to 0.07%, causing the cessation of exponential growth at OD_{600} values ranging from 0.25 to 0.32. To determine nucleotide patterns, at appropriate times during incubation 50- to 100-μl culture aliquots were dispensed into the wells of microtiter plates containing equal volumes of ice-cold 13 M formic acid. After three freeze-thaw cycles, the samples were centrifuged in a microcentrifuge at 4°C, and the supernatant fluids were subjected to one-dimensional polyethyleneimine (PEI) thin-layer chromatography (TLC) developed with 1.5 M potassium P_i (pH 3.4). Nucleotide spots were quantitated by PhosphorImager analysis with the BAS1000 Fuji bioimaging system and TINA 2.07c software from Raytest (Straubenhardt, Germany). Amounts of pppGpp and ppGpp were expressed as fractions of the total nucleotide pool of blank-corrected GTP, ppGpp, and pppGpp. The (p)ppGpp abundance reported under conditions of amino acid starvation represents nucleotide accumulation above the basal level of radioactivity which prevailed before the addition of pseudomonic acid. For ppGpp, this level, ranging from 11 to 16% of the total radioactivity in GTP, ppGpp, plus pppGpp, represented the activity of ppGpp itself plus that of an unknown phosphate-labeled compound which comigrated with ppGpp but, in contrast to the latter, did not adsorb to charcoal (see below). Including the unknown phosphate into basal-level deduction appeared legitimate since its abundance did not markedly respond to amino acid starvation.

Identification of ppGpp and its separation from the unknown phosphorylated compound. The methods for ppGpp identification and separation were adapted from procedures described by Cashel and Kalbacher (4) for the preparation of ppGpp or communicated personally by M. Cashel. Cultures of strain H46A or H46A *rel*$_{Seq}$::pVRH550 (750 μl) were uniformly labeled with $^{32}P_i$ at 400 μCi/ml during growth in low-phosphate medium for two doublings. As source of authentic ppGpp, *E. coli* CF1648 was nonuniformly $^{32}P_i$ labeled in MOPS minimal medium containing serine hydroxamate (1 mg/ml) and valine (300 μg/ml). Samples (250 μl) of the uniformly labeled streptococcal cultures were withdrawn before and after imposition of amino acid starvation for 25 min by the addition of pseudomonic acid at 200 ng/ml and added to the same volume of 13 M ice-cold formic acid. The cells were extracted and centrifuged as described above, and 350- to 400-μl volumes of the supernatant fluids were streaked along the origin of a PEI cellulose sheet (10 by 10 cm), using intermittent fan drying at room temperature. The sheet was chromatographed in 1.5 M potassium P_i (pH 3.4), dried, and briefly autoradiographed. The region corresponding to the mixture of ppGpp and the unknown phosphate was cut out, immersed in water to remove residual $^{32}P_i$, and then transferred to 90% ethanol before being air dried. The radioactivity was eluted from the PEI with a 0.5-ml solution of 4 M LiCl–0.5 M sodium formate (pH 3.4), and ppGpp was separated from the unknown phosphate by adsorption to and elution from charcoal (Norit A; Serva) in the presence of 20 mM GTP as the carrier. To this end, a small amount of charcoal (5 to 10 mg) was added to the eluate suspension, which, after thorough mixing, was kept on ice for 10 min before being centrifuged for 10 min at 4°C to obtain the charcoal pellet and supernatant fraction. The entire adsorption procedure was repeated five times, and the pooled charcoal fractions were washed with distilled water. The ppGpp adsorbed to charcoal was eluted with 150 μl of a 50% methanol–50% 0.5 mM EDTA–2% ammonium hydroxide solution. The suspension was vortexed and kept on ice for 10 min before being centrifuged to save the supernatant fraction and repeat the elution procedure six more times. To get rid of tiny bits of residual charcoal in the supernatant fluids, the fluids were passed through cotton plugs contained in pipet tips. The pooled supernatant fluids were evaporated to dryness in a Speedvac, and the pellets were dissolved in 100 to 200 μl of a 20 mM Tris (pH 8.0)–0.5 mM EDTA solution and frozen for later use.

To recover the radioactivity not adsorbing to charcoal, the pH of the supernatant fluids was adjusted to 2.0 with concentrated formic acid and the radioactivity was precipitated stepwise as the lithium salt by the addition of absolute ethanol in 1-volume increments, allowing 10 min on ice for precipitation. The suspensions were centrifuged, and the pelleted radioactivity was monitored in a scintillation counter. A 3- to 5-volume portion of ethanol was able to precipitate 62 to 94% of the original activity. The pellets were washed with ethanol, dried in

a Speedvac, and dissolved at ~100 cpm/μl in a 100 mM sodium formate solution (pH 3.4). After recentrifugation, a small insoluble residue was discarded and the supernatant fluid was stored in the freezer after being pH adjusted to 6.0 to 7.0 with 1 M Tris (pH 8.0).

The two phosphate preparations obtained as described above were partially characterized by testing their susceptibility to alkaline hydrolysis, acid hydrolysis, and calf intestinal phosphatase (Boehringer Mannheim), monitoring radioactivity by one-dimensional PEI TLC. Alkali hydrolysis was performed overnight at 37°C in 2.4 N NaOH containing 10 mM magnesium acetate and 0.5 mM EDTA. Nonhydrolyzed samples kept in water served as controls. The time course of alkali hydrolysis was determined in 0.3 N NaOH. Acid hydrolysis was performed in 1 M HCl for 20 min at room temperature, a treatment that did not affect authentic GTP but resulted in complete hydrolysis of ppGpp prepared in parallel from *E. coli*. For treatment with calf intestinal phosphatase, the substrates were exposed to 2 U of the enzyme under conditions recommended by the supplier.

Protein and RNA synthesis assays. The rates of net protein and RNA synthesis were measured by determining the incorporation of, respectively, [^{35}S]methionine and [^{3}H]uridine into trichloroacetic acid (TCA)-precipitable material. Streptococcal cultures growing in low-phosphate medium with decreased concentrations of methionine and isoleucine (10 μg/ml each) and with uridine at 200 μM were labeled at an OD_{600} of 0.05 with 1 μCi of [^{35}S]methionine per ml or 10 μCi of [5,6-^{3}H]uridine per ml. At an OD_{600} of 0.1, 100-μl samples were withdrawn at the indicated intervals and applied to filter discs previously soaked with a 10% TCA solution. The filters were washed in bulk three times in at least 1 liter of ice-cold 5% TCA solution and then in ethanol and acetone before being dried for scintillation counting of radioactivity in a toluene-based fluor. To impose amino acid starvation in these experiments, pseudomonic acid (300 ng/ml) was added to the cultures at an OD_{600} of 0.25, and, to imitate a relaxed phenocopy, tetracycline (125 μg/ml) was added at the same time. Cultures subjected to glucose exhaustion (0.07% glucose in low-phosphate medium) were divided at an OD_{600} of 0.2 into equal portions, one of which (the control) was replenished with 2% glucose.

Determination of the specific activities of the ribonucleoside triphosphate pool. ppGpp is known to exert inhibitory effects on labeled RNA precursor uptake in *E. coli* giving overestimates of inhibition of RNA synthesis if this is measured by isotope labeling (13, 24, 30, 43). To test whether the UTP-plus-CTP pool in wild-type H46A and its *rel$_{Seq}$*::pVRH550 mutant exhibited lower specific activities during amino acid starvation imposed by pseudomonic acid (300 ng/ml), ^{3}H and ^{32}P activities for these nucleotides were estimated in starved and control cultures under conditions of uniform $^{32}P_i$ labeling (20 μCi/ml) and pulse-labeling with [5,6-^{3}H]uridine (20 μCi/ml). Sampling (80 μl) into formic acid was started 20 min after pseudomonic acid addition and continued for 25 min at 5-min intervals. The extracted nucleotides were resolved by two-dimensional PEI TLC and identified with standards as described previously (27). UTP-plus-CTP spots visualized by autoradiography were cut out, and their activity was determined by scintillation counting. For the wild-type and mutant strains, the ^{3}H counts determined under amino acid starvation were, respectively, about 2 and 1.2 times as high as those determined under control conditions, and approximately the same numbers were obtained from unstarved cultures uniformly labeled with $^{32}P_i$. We therefore conclude that uridine labeling of *Streptococcus*, unlike *E. coli*, yields reasonable estimates of the rates of stable RNA synthesis during amino acid deprivation of both the wild-type and the *rel$_{Seq}$* insertion mutant strains studied here.

RESULTS

***rel$_{Seq}$* is the source of (p)ppGpp synthetic activity during amino acid starvation.** As shown previously (27), wild-type H46A cells accumulate (p)ppGpp during amino acid deprivation whereas *rel$_{Seq}$* disruption mutants do not. The introduction of a plasmid-borne *rel$_{Seq}$* gene into a strain carrying insertionally inactivated *rel$_{Seq}$* restored the (p)ppGpp accumulation response following amino acid starvation (Fig. 1) as well as the growth defect of *rel$_{Seq}$* insertion mutants in low-phosphate medium (doubling time, 48 ± 6 min for the wild type and 87 ± 9 min for the mutants). This shows that *rel$_{Seq}$* rather than an extragenic suppressor is responsible for the phenotype of the *rel$_{Seq}$*::pVRH550 mutant carrying pMR9. The autoradiograms in Fig. 1 also show the *rel$_{Seq}$*-independent occurrence in the ppGpp region of ^{32}P activity that did not vary substantially in normal and amino acid-starved H46A cells when samples from identical strains in neighboring lanes were compared and, in addition, GTP/ppGpp ratios were estimated. In contrast to ppGpp (and GTP), this streptococcal material did not adsorb to charcoal (Fig. 1, bottom panel), indicating that it is not a nucleotide. In numerous TLC experiments performed under

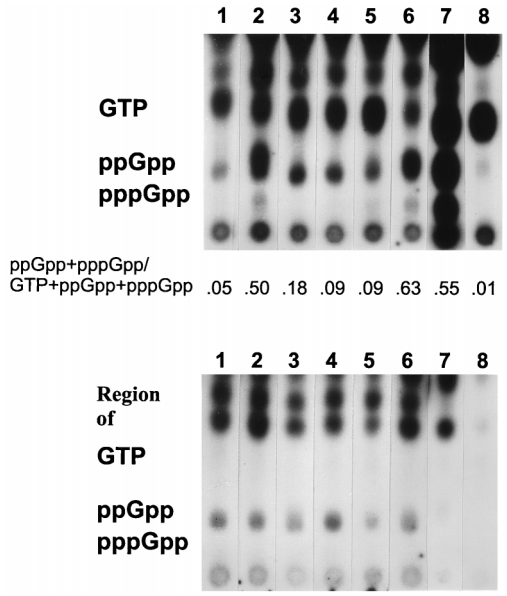

FIG. 1. Effects of the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of pseudomonic acid at 200 ng/ml on the accumulation responses of radioactivity that migrates in one-dimensional PEI TLC with pppGpp or ppGpp in *S. equisimilis* wild-type (lanes 1 and 2) or *rel$_{Seq}$*::pVHR550 (lanes 3 to 6) cultures. Cells were grown in low-phosphate medium (0.7 mM P_i, 10 μg of isoleucine per ml) and uniformly $^{32}P_i$ labeled (150 μCi/ml) before being treated with pseudomonic acid for 20 min at 37°C and then sampled in formic acid. Nonuniformly $^{32}P_i$ labeled (10 μCi/ml) *E. coli* cultures subjected to amino acid deprivation in MOPS minimal medium (no amino acids except valine at 0.5 mg/ml and serine hydroxamate at 1 mg/ml) served to provide standards for (p)ppGpp accumulation patterns (lanes 7 and 8). The autoradiograms were obtained from acid extracts before (above) and after (below) charcoal adsorption. (p)ppGpp pool sizes (given below the lanes of the unadsorbed samples) were determined after deduction of both basal-level radioactivity and activity present in the unknown phosphorus compound comigrating with ppGpp (below). The specific strains tested were H46A (lanes 1 and 2) H46A *rel$_{Seq}$*::pVHR550 (pMG36) (vector control) (lanes 3 and 4), H46A *rel$_{Seq}$*::pVHR550 (pMR9 *rel$_{Seq}$*) (lanes 5 and 6), CF1648 (wild type) (lane 7), and CF1652 (Δ*relA251*) (lane 8).

standard conditions, the unknown compound showed a somewhat variable migration behavior that depended on the particular lot of PEI sheets used. With most sheets, it comigrated with ppGpp or slightly slower (Fig. 1), and only rarely were sheets encountered on which it was well resolved from ppGpp, migrating between the latter and pppGpp. ^{32}P activity not attributable to ppGpp was not seen in *E. coli* examined in parallel (Fig. 1, lanes 7 and 8).

Kinetics of (p)ppGpp accumulation and decay. Optimal concentrations of pseudomonic acid needed to induce isoleucine starvation were determined by measuring ppGpp levels after exposing uniformly $^{32}P_i$-labeled wild-type cells to the antibiotic for 25 min. The amount of ppGpp as a fraction of the total radioactivity in GTP, ppGpp, plus pppGpp increased in a pseudomonic acid concentration-dependent manner to reach a plateau of 50 to 55% at antibiotic concentrations of ≥150 ng/ml (Fig. 2). Under the same conditions, pppGpp accumulated to approximately 1/10 the amount of ppGpp. Determination of the kinetics of (p)ppGpp accumulation at optimal concentrations of pseudomonic acid (200 ng/ml) revealed an extremely fast increase of the tetra- and pentaphosphate concentrations (Fig. 3A), associated with a fast decrease of the GTP level (data not shown). However, GTP decreased more rapidly than could be accounted for by the simultaneous increase of the (p)ppGpp level, suggesting that GTP was additionally converted to something different from (p)ppGpp (data not shown). Concentrations of 0.5 to 1 mg of isoleucine hy-

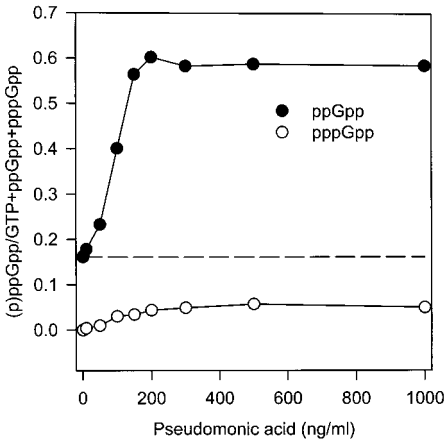

FIG. 2. (p)ppGpp pool size in *S. equisimilis* H46A as a function of the concentration of pseudomonic acid. Cells were grown and uniformly $^{32}P_i$ labeled (150 μCi/ml) in low-phosphate medium containing 0.7 mM P_i and all amino acids at standard concentrations except isoleucine, whose concentration was lowered to 1/10 the regular concentration (10 μg/ml). Exposure to pseudomonic acid was carried out for 25 min at 37°C. The amount of radioactivity migrating with ppGpp in samples taken before the addition of pseudomonic acid (the basal level) is marked by a dashed line.

droxamate per ml were also found to increase the (p)ppGpp pool with kinetics similar to those shown in Fig. 3A for pseudomonic acid. As expected from the results of Fig. 1, the *rel*$_{Seq}$::pVRH550 insertion mutant subjected to amino acid starvation by treatment with pseudomonic acid as described above did not exhibit the (p)ppGpp accumulation response (data not shown).

To monitor the kinetics of (p)ppGpp decay, tetracycline at 250 μg/ml was added to the starved cells and (p)ppGpp was quantitated. In *E. coli*, tetracycline inhibits the synthetic activity of RelA by blocking the binding of uncharged tRNA to the ribosome acceptor sites (22). Figure 3 shows that tetracycline caused the accumulated (p)ppGpp to disappear, following first-order kinetics. The turnover of (p)ppGpp was accompanied by a simultaneous increase of the GTP level. Estimates of the rate constants from the equation k = ln 2/half-life amounted to 0.7/min and 2.3/min for the decay of ppGpp and pppGpp, respectively, values that compared well with decay constants of 0.20/min to 2.1/min for these nucleotides in *spoT*$^+$ strains of *E. coli* (reviewed in reference 3). However, the scant accumulation of pppGpp makes the estimates of its decay constant inaccurate. Another inhibitor of protein synthesis, chloramphenicol (250 μg/ml), weakly shared with tetracycline the ability to reverse (p)ppGpp accumulation provoked by pseudomonic acid addition (data not shown).

Pseudomonic acid-induced (p)ppGpp accumulation triggers the stringent response in strain H46A. Addition of pseudomonic acid (300 ng/ml) to wild-type H46A or its *rel*$_{Seq}$::pVH46 insertion mutant growing exponentially in low-phosphate medium at reduced methionine and isoleucine concentrations (10 μg/ml) caused the immediate cessation of growth and protein synthesis (Fig. 4A and B). In the wild type, inhibition of protein synthesis was coupled with an immediate block of net RNA accumulation (Fig. 4C), whereas in the *rel*$_{Seq}$::pVH46 mutant, RNA continued to accumulate, albeit at reduced rates (Fig. 4D). Simultaneous addition of pseudomonic acid and tetracycline (125 μg/ml) did not alter the protein synthesis blockade for either the wild type (Fig. 4A) or the mutant (Fig. 4B). However, the stringent control of RNA accumulation displayed by the pseudomonic acid-treated wild-type strain was significantly relaxed by tetracycline addition. This effect of tetracycline reflects relaxed control rather than a generalized effect of tetracycline because the *rel*$_{Seq}$ mutant continued to accumulate RNA at equal rates whether or not tetracycline was present in addition to pseudomonic acid (Fig. 4D). The relaxed pattern of RNA accumulation in the mutant (Fig. 4D) could be restored to the wild-type stringent control by the presence of the plasmid pMR9 bearing the minimal *rel*$_{Seq}$ gene (Fig. 4E). These data, taken together with the (p)ppGpp accumulation behavior presented above, indicate that pseudomonic acid-mediated impairment of isoleucine amino acylation provokes a stringent response involving both (p)ppGpp and the *rel*$_{Seq}$ gene.

Effects of glucose exhaustion. The observation that ppGpp accumulation in response to carbon deprivation in *E. coli* occurs despite a *relA* deletion (29) prompted us to analyze the impact of *rel*$_{Seq}$ on ppGpp accumulation and macromolecular

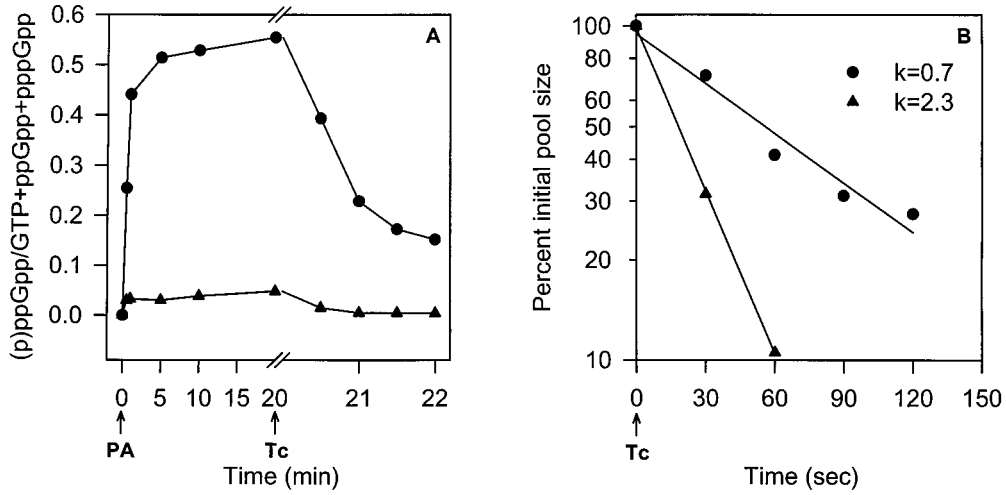

FIG. 3. Accumulation (A) and decay (B) of ppGpp (circles) and pppGpp (triangles) in *S. equisimilis* H46A. Cultures were grown and uniformly $^{32}P_i$ labeled as described in the legend to Fig. 2. At zero time, pseudomonic acid (PA, 200 ng/ml) was added to provoke amino acid starvation for 20 min before tetracycline (Tc, 250 μg/ml) was added. The basal level of radioactivity (12.5%; see the legend to Fig. 2) migrating in the region of ppGpp was deducted from all samples.

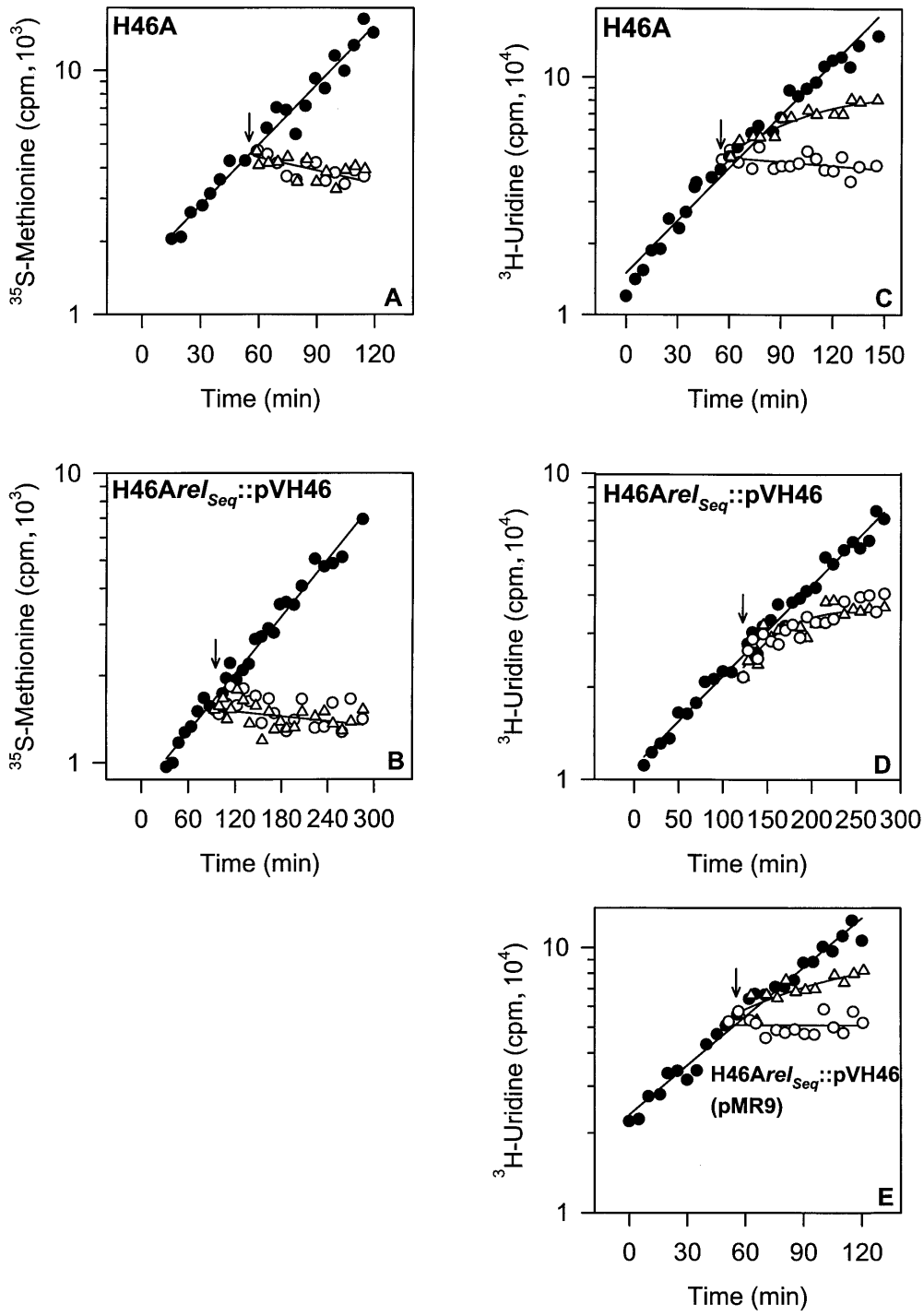

FIG. 4. The stringent response in *S. equisimilis* H46A. The response was measured by the incorporation of [^{35}S]methionine and [^{3}H]uridine in the TCA-precipitable material during growth of the indicated strains in low-phosphate medium containing uridine at 200 μM and all amino acids at standard concentrations except methionine and isoleucine, whose concentration was lowered to 1/10 the regular concentration (10 μg/ml each). At an OD_{600} of about 0.05, [^{35}S]methionine (1 μCi/ml) or [^{3}H]uridine (10 μCi/ml) was added to the cells and samples were taken as indicated, starting at an OD_{600} of about 0.1. The arrows mark the time at which the cultures were split and further incubated in the absence (solid circles; controls) or presence of either pseudomonic acid (300 ng/ml) alone (open circles) or pseudomonic acid plus tetracycline (125 μg/ml) (open triangles).

synthesis after exhaustion of glucose. As expected, exponential growth stopped abruptly for both mutant and wild-type cells when 0.07% glucose was exhausted at an OD_{600} of 0.25 to 0.32 (see Fig. 6); growth was restored by resupplementation with 2% glucose (data not shown). Monitoring the nucleotide pools by TLC showed that at the point of growth arrest in both the wild-type and mutant strains, the radioactivity migrating in the region of ppGpp increased and the GTP level decreased (Fig. 5). Charcoal adsorption experiments revealed that 40 to 60% of the radioactivity that accumulated at any time during the

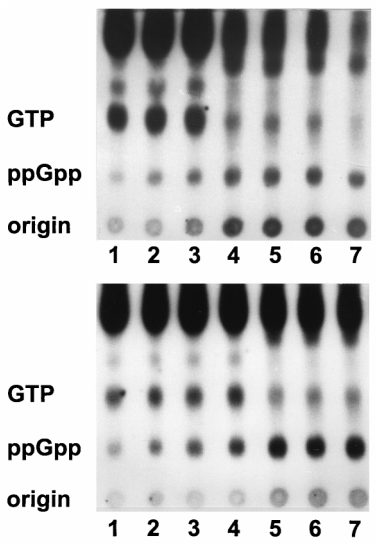

FIG. 5. Amount of radioactivity migrating with ppGpp in *S. equisimilis* H46A wild type (top) and its rel_{Seq}::pVH46 insertion mutant (bottom) before (top, lanes 1 to 3; bottom, lanes 1 to 4) and after (top, lanes 4 to 7; bottom, lanes 5 to 7) the break of the growth curves as a result of glucose depletion. Note that no pppGpp is seen. Cultures were grown and uniformly $^{32}P_i$ labeled in low-phosphate medium containing 0.07% glucose, 1 mM P_i, and all amino acids at standard concentrations. Sampling intervals were 20 min for wild-type cultures and 30 min for mutant cultures.

stationary phase in the ppGpp region of either the wild type or mutant failed to absorb to charcoal; i.e., it did not represent ppGpp. Attempts to separate the two phosphates by two-dimensional TLC (development in 4.2% borate–3.3 M ammonium formate [pH 7.0] in the first dimension and in 1.5 M KH_2PO_4 [pH 3.4] in the second dimension) also failed, as shown by the presence of the entire amount of radioactivity in a spot corresponding to nonradioactive authentic ppGpp included in the same chromatogram and visualized by UV absorption. Thus, during glucose exhaustion, both ppGpp and the unknown phosphate accumulated in the wild type and in the mutant, indicating that a strain carrying the rel_{Seq}::pVH46 allele does not exhibit a $ppGpp^0$ phenotype.

Evidence in support of this conclusion was provided by experiments designed to measure protein and RNA synthesis under conditions of glucose deprivation. As shown in Fig. 6, in both the wild type and the mutant, protein synthesis as well as net RNA accumulation immediately stopped at the point of growth arrest; i.e., both strains responded similarly to carbon source exhaustion by sharply limiting RNA and protein accumulation.

Evidence for the identity of ppGpp and characterization of the unknown phosphate. The activities comigrating with ppGpp on TLC from phosphate-labeled acid extracts were purified from the chromatogram and then processed by charcoal adsorption to yield adsorbed and unadsorbed fractions (see Materials and Methods). Each fraction was then exposed to 2.4 N NaOH in the presence of 10 mM magnesium acetate and 0.5 mM EDTA. Table 2 shows that under these hydrolysis conditions, the ppGpp standard purified from extracts of *E. coli* cells uniformly labeled with phosphate had the expected single labile phosphate residue, with ppGp and P_i as products (4, 27). Furthermore, the distribution of radioactivity in ppGp and P_i occurred as a 3:1 ratio when corrected for activity distributions in unhydrolyzed controls (Table 2).

In contrast, all *S. equisimilis* fractions not adsorbing to charcoal were alkali stable. The presence of completely alkali-stable material clearly indicates the occurrence of a compound present in *S. equisimilis* but not in *E. coli* and eliminates the possibility that ppGpp is present in this fraction. The distinction between this phosphorylated compound and ppGpp is also clearly seen by TLC resolution on some but not all batches of PEI-cellulose plates. The reason for this different behavior is unknown.

Also unlike *E. coli*, all charcoal adsorbed fractions from *S. equisimilis* contained a significant portion of activity resistant to hydrolysis in alkali, ranging from about 20 to 40% (Table 2). We are uncertain of the identity of this alkali-stable material, but we suspect that it represents contamination with the same material that is not adsorbed to charcoal. To estimate the presence of ppGpp in the charcoal-adsorbed fractions, we must therefore focus on the activity ratios of the ppGp and P_i alkali hydrolysis products. These ratios are indistinguishable from the ppGpp standard for amino acid-starved strain H46A and the unstarved mutant strain, a clear indication of ppGpp. Apparently, activity ratios higher than 3:1 are found from material from growing strain H46A and the starved mutant, suggesting the presence of putative ppGpp together with significant amounts of an alkali hydrolysis product in addition to, and comigrating with, ppGp. Alternatively, ppGp/P_i ratios deviating from 3 may simply reflect methodological inaccuracies.

To be certain of the presence of ppGpp, we have therefore attempted to further verify the identity of ppGpp in these fractions by measuring the kinetics of disappearance of ppGpp and appearance of hydrolysis products detected after exposures to mild alkali, mild acid, and calf intestine phosphomonoesterase. Figure 7 shows that disappearance of ppGpp in 0.3 N NaOH was quantitatively accounted for by the appearance of ppGp and P_i in activity ratios of 3:1 during the hydrolysis.

Although the data are not shown, authentic ppGpp from *E. coli* CF1648 was completely degraded to ppGp and P_i within 20 min at 37°C in the presence of 1 M HCl (4), and the same was true of the charcoal-adsorbable material from strains H46A and H46A rel_{Seq}::pVRH550. Their alkali-stable material not adsorbed to charcoal was also acid stable, like authentic GTP included as a control.

It is known that authentic ppGpp is completely sensitive to phosphomonoesterases (4), and this behavior also is found for putative ppGpp isolated from *S. equisimilis*. In addition, the kinetics of complete enzymatic digestion of the charcoal-adsorbed activity and appearance of the P_i hydrolysis product is shown in Fig. 8A, with a transient appearance of hydrolysis intermediates that comigrated with guanine nucleotides. The material not adsorbed to charcoal was also analyzed (Fig. 8B) and was also found to be completely hydrolyzed by the enzyme but with slower kinetics and with a less noticeable accumulation of transient hydrolytic intermediates.

We interpret this analysis as demonstrating the existence of ppGpp in extracts of the wild type as well as of the rel_{Seq} insertion mutant, whether growing or amino acid starved. This reinforces the notion already mentioned that the insertion mutants are not completely devoid of ppGpp and therefore are not the equivalent of *relA-spoT* double mutants of *E. coli* with respect to ppGpp.

DISCUSSION

Previous functional analysis of rel_{Seq} has shown that this gene suppresses the multiple amino acid requirements of an *E. coli* $(p)ppGpp^0$ strain and permits ppGpp to be detected when such cells are examined in the presence of picolinic acid to inhibit Mn-dependent (p)ppGppase activity. Direct evidence

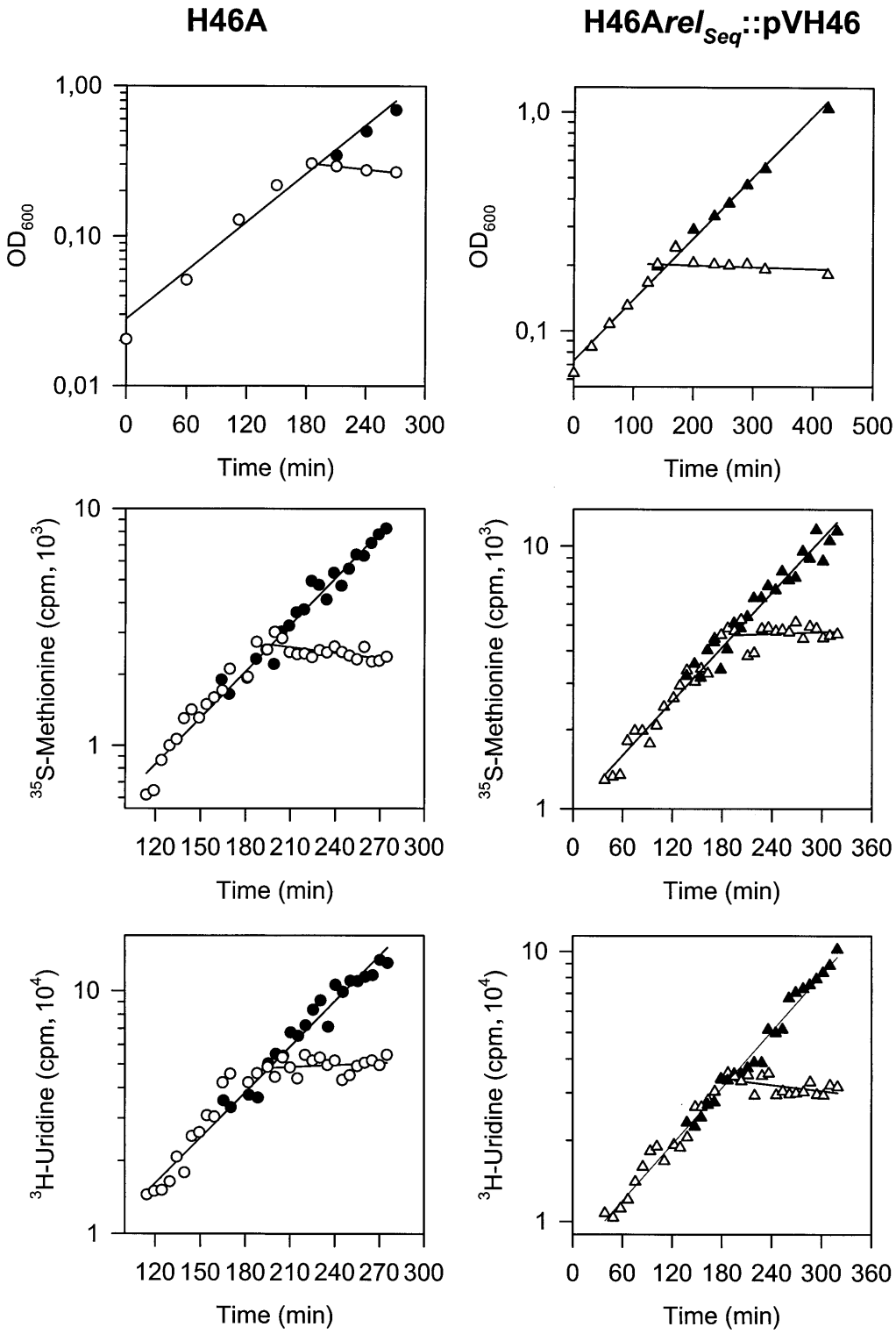

FIG. 6. Incorporation of [^{35}S]methionine (1 μCi/ml) or [^{3}H]uridine (10 μCi/ml) into TCA-precipitable material during the growth of *S. equisimilis* H46A and its *rel*$_{Seq}$::pVH46 insertion mutant during glucose exhaustion. Cultures were grown in low-phosphate medium containing 200 μM uridine and all amino acids at standard concentrations except methionine, which was present at 10 μg/ml. Both strains were exhausted for glucose by limiting its concentration to 0.07%. Doubling times during exponential growth were 45 and 87 min for the wild type and insertion mutant, respectively. The cultures were labeled at an OD$_{600}$ of about 0.05 and sampled for ^{35}S or ^{3}H counting at short time intervals as indicated. Control cells supplied with normal glucose concentrations were obtained by dividing the cultures at an OD$_{600}$ of 0.2 to 0.25 and adding glucose to a final concentration of 2% to the controls (solid symbols).

TABLE 2. Alkali hydrolysis of ppGpp or "unknown phosphate" after overnight exposure to 2.4 N NaOH

Source of substrate	Culture conditions		Charcoal behavior		% Radioactivity, in unhydrolyzed control, in region of:			% Radioactivity, in NaOH hydrolysis, in region of:		
	Amino acid starved	Not starved	Adsorbed	Not adsorbed	ppGpp	ppGp	P_i	ppGpp	ppGp	P_i
H46A		+	+		95.7	2.3	2.0	41.4	42.7	15.9
		+		+	98.6	0.2	1.2	97.7	0.9	1.4
	+		+		96.0	0.7	3.3	20.2	63.7	16.1
	+			+	98.9	0.0	1.1	97.2	0.8	2.0
H46A*rel*$_{Seq}$::pVRH550		+	+		92.8	1.4	5.8	20.2	62.7	17.1
		+		+	98.1	0.1	1.8	97.4	0.5	2.1
	+		+		96.0	1.5	2.5	40.6	43.3	16.1
	+			+	97.6	0.1	2.3	95.4	1.5	3.1
CF1648	+		+		94.6	1.2	4.2	2.1	70.6	27.3

for *rel*$_{Seq}$-determined (p)ppGpp synthetic activity has been provided by the demonstration in vitro of Rel$_{Seq}$-catalyzed ATP:GTP 3′-pyrophosphoryltransferase activity and by the observation that the (p)ppGpp accumulation response in amino acid-starved *S. equisimilis* strains depends on a functional *rel*$_{Seq}$ gene. Nevertheless, *rel*$_{Seq}$ is not equivalent to *relA* from *E. coli*, as it fails to restore the (p)ppGpp accumulation response during amino acid starvation of a *relA*-deleted *E. coli* host. Moreover, additional genetic, enzymatic, and serological evidence has shown that *rel*$_{Seq}$ functions like *spoT* in encoding a strong (p)ppGpp 3′-pyrophosphorylase, the activity of which favors (p)ppGpp degradation over synthesis in the heterologous host (27). To unravel further the function of *rel*$_{Seq}$, this work concentrated on its action in the homologous host, including its effect on RNA and protein synthesis during amino acid starvation and carbon source exhaustion. Regarding the measurement of RNA synthesis by uracil labeling, it should be pointed out that uridine uptake barriers arising during the stringent response in *E. coli* (13, 30, 43) do not appear to accompany the accumulation of (p)ppGpp in the streptococcal strains studied here.

Pseudomonic acid at low concentrations (150 to 200 ng/ml) proved to be an efficient agent to induce amino acid starvation in *S. equisimilis* and to provoke the *rel*$_{Seq}$-dependent accumulation of (p)ppGpp (Fig. 1). Evidence provided for the identity of (p)ppGpp includes (i) its migration behavior in one-dimensional and two-dimensional TLC, (ii) its nucleotide-like ability to adsorb to charcoal, (iii) the sensitivity of its 3′ β-phosphate group to acid and alkali hydrolysis, and (iv) the release of P_i as the only phosphorus end product following digestion with alkaline phosphatase. Additionally, in the uniformly $^{32}P_i$-labeled wild-type H46A strain exposed to pseudomonic acid, (p)ppGpp accumulates at the expense of GTP as fast as it does in amino acid-starved *E. coli* wild-type strains, reaching a level at maximum induction approximately equivalent to the level of GTP (Fig. 1). Conversely, the kinetics of (p)ppGpp decay when (p)ppGpp synthesis is inhibited shows that degradation is accompanied by an increase of the GTP concentration. In amino acid-starved *E. coli* cells, the pppGpp/ppGpp ratio is generally smaller than 1 but varies widely depending on the nature of the *spoT* or *gpp* alleles (reviewed in reference 3). In similarly starved *S. equisimilis* cells, we find a pppGpp/ppGpp ratio of ≤0.1, in contrast to the relative quantities of the two nucleotides in *Bacillus subtilis* (14) and *Staphylococcus aureus* (6), where pppGpp has been reported to be the more abundant nucleotide during amino acid starvation. Tetracycline efficiently blocks the net accumulation of (p)ppGpp (Fig. 3), and the ppGpp decay rate (k = 0.7/min, corresponding to a half-life of ≈1 min) determined in its presence (250 μg/ml) is similar to the *spoT*-determined ppGpp decay rate (k = 1.5/min, corresponding to a half-life of 20 to 30 s) determined in *E. coli* during the relief of valine-induced isoleucine starvation by the addition of isoleucine (10). It should be noted that in *S. equisimilis*, tetracycline was found to be similarly effective at lower concentrations (50 μg/ml). The fast breakdown of ppGpp even at high concentrations of this drug suggests that its reported inhibition of the ppGppase action of SpoT by the chelation of Mn ions (1) is not a factor that leads to a serious overestimation of the half-life of ppGpp in *S. equisimilis*. On the other hand, chloramphenicol, which is widely used in *E. coli* to relieve amino acid starvation (3, 15, 33, 34) and was used up to a concentration of 500 μg/ml in this work to estimate (p)ppGpp decay rates, led to unrealistically low k constants (data not shown). Since this antibiotic, like tetracycline, inhibits streptococcal growth at very low concentrations, the reason for the different behaviors of the two drugs remains unclear but seems worth noting since their utility for studying the kinetics of (p)ppGpp breakdown depends on the species examined.

Amino acid starvation-induced (p)ppGpp accumulation cor-

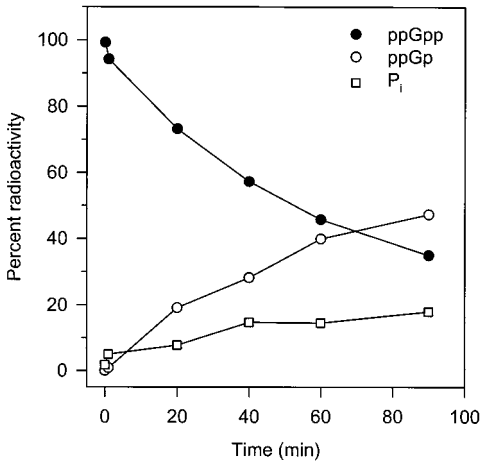

FIG. 7. Susceptibility to alkali hydrolysis (0.3 N NaOH containing 10 mM magnesium acetate and 0.5 mM EDTA) at 37°C of ppGpp purified from amino acid-starved *S. equisimilis* H46A cells.

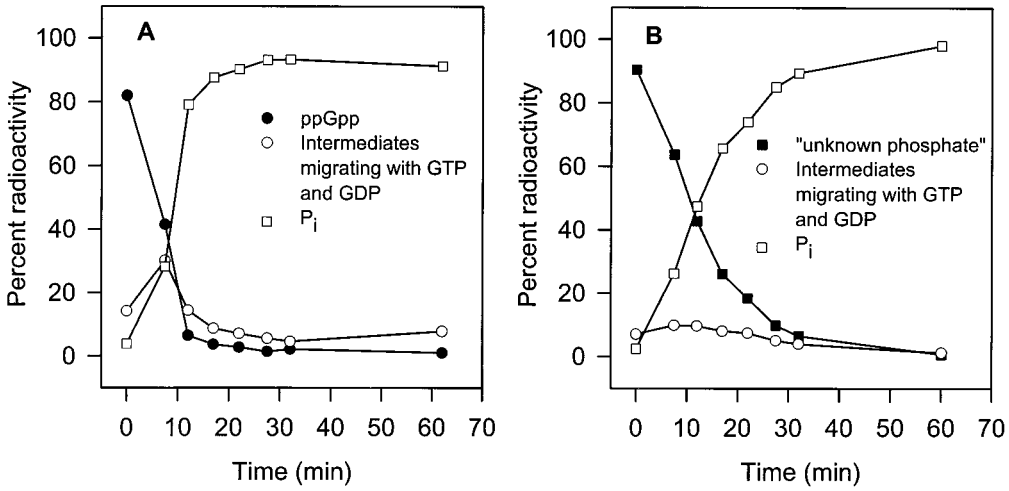

FIG. 8. Susceptibility of ppGpp (A) and the unknown phosphate (B) purified from amino acid-starved *S. equisimilis* H46A cells to digestion with calf intestinal phosphatase (2 U in a total volume of 40 μl).

relates with a stringent response in *S. equisimilis* as judged by the coupled cessation of net protein and RNA synthesis and the partial reversal of inhibition of RNA accumulation in the presence of tetracycline together with equivalent effects, apparently a relaxed response, conferred by an insertion mutation in rel_{Seq}. The relaxed response of the mutant operationally defines the rel_{Seq} gene as encoding the stringent factor of *S. equisimilis*. This, together with the fact that rel_{Seq}, unlike *relA* from *E. coli*, also determines a strong (p)ppGpp hydrolytic activity, reinforces the possibility raised previously (27) that the distinct functions of the paralogous *relA* and *spoT* genes from *E. coli* are fulfilled by a single *S. equisimilis* ortholog, rel_{Seq}, the competing activities of which are environmentally controlled. However, the insertionally inactivated rel_{Seq} gene determines a phenotype that is different from an *E. coli* (p)ppGpp^0 strain in that it maintains a low basal level of (p)ppGpp and responds, like wild-type *S. equisimilis*, to glucose exhaustion with ppGpp accumulation (Fig. 5). Consistent with these observations is the cessation of net RNA accumulation in response to glucose exhaustion in both the wild-type and mutant strains (Fig. 6). Although we did not differentiate between stable RNA synthesis and mRNA synthesis, there is evidence, reviewed by Cashel and Rudd (5), that not only the former but also part of the unstable RNA synthesis is subject to the stringent control. However, a cautionary note comes from observations in *E. coli* indicating that RNA degradation occurs during glucose starvation (reviewed in reference 3). We did not investigate this problem in *S. equisimilis*; thus, the contribution of possible RNA degradation to the arrest of RNA accumulation during glucose exhaustion remains an unresolved question. Possible differences in the kinetics of (p)ppGpp accumulation in response to glucose exhaustion between the wild-type and rel_{Seq}::pVH46 mutant strains, as reported for stringent compared to relaxed *E. coli* (24) or *Vibrio* (11) strains, also remain to be investigated, but such studies are presently hampered by the accumulation of an unknown phosphate during glucose deprivation. This phosphate compound, which cannot be readily separated from ppGpp by TLC, also interferes with the precise determination of the (p)ppGpp basal level in *S. equisimilis*, an important factor that, if known, might provide a clue to the pronounced difference in the growth rates of wild-type and rel_{Seq} insertion mutants.

We do not know the identity of the phosphorylated compound from *S. equisimilis* that does not adsorb to charcoal but have characterized it as migrating in the ppGpp region of chromatograms, as stable to exposures of both mild acid and alkali, and as completely degraded by phosphomonoesterase. The first property indicates that the compound is not a nucleotide; the last property suggests that it lacks cyclic monoesters and other blocked diesters. It remains uncertain whether the compound contains carbon atoms such as phosphorylated sugars. Instead, it could contain short-chain polyphosphates, which have been shown to accumulate in, for example, *Halobacterium volcanii* when entering the stationary phase (37). Similar phosphate compounds have been characterized in *Saccharomyces cerevisiae* (25, 38) and are apparently ubiquitous in nature (23). In particular, in *E. coli*, inorganic polyphosphates synthesized by polyphosphate kinase mediate the resistance of the bacteria to stressful agents and prolong the survival of the organism in the stationary phase (32). The occurrence in *S. equisimilis* of an unknown phosphate that comigrates with ppGpp in TLC justifies our caution and additional effort to identify a correctly positioned spot on a chromatogram with ppGpp.

The question of the source of the (p)ppGpp synthetic activity in *S. equisimilis* carrying the rel_{Seq} insertion alleles remains unresolved but deserves consideration. In view of what is known about the residual activity of a number of *relA* or *spoT* mutant alleles, it is quite possible that the 216-amino acid N-terminal segment preserved in the truncated proteins retains some residual activity. Of interest in this connection is a truncated 455-amino-acid RelA′ protein, which continues to specify (p)ppGpp synthesis but which is constitutively activated due to the C-terminal deletion (36, 40). There are also SpoT mutant proteins, like SpoT203, that lack hydrolytic activity but retain PSII activity (35). In an attempt to narrow the regions involved in the dual activities of the 702-amino-acid SpoT protein, Gentry and Cashel (16) have shown that the first 203 amino acids are sufficient for ppGppase activity and that the overlapping region from amino acids 67 to 374 is required for PSII activity. If the truncated Rel_{Seq} protein had lost its putative ribosome-binding capacity and retained, like *relA′* (40), unregulated (p)ppGpp synthetic as well as regulated (p)ppGpp hydrolytic activity, it would be expected to determine the mutant phenotype described in this paper, provided that there is no second gene for (p)ppGpp synthesis. The answer to this

question awaits the isolation of a rel_{Seq} null mutant or the determination of the complete genomic sequence of a pathogenic streptococcus. Our current attempts to isolate a viable rel_{Seq} deletion mutant have been unsuccessful so far, and complete *rel/spo* deletions have hitherto not been described in gram-positive species. However, it should be noted that there already exists a bacterial species, namely, *Mycoplasma genitalium*, which harbors only one *relA/spoT* ortholog (12). Furthermore, as yet, the genome sequence of *Streptococcus pyogenes*, which is 93% complete (URL, http://dna1.chem.uoknor.edu), shows only one gene that is homologous to rel_{Seq}.

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