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Title: Guanosine Tetra- and Pentaphosphate Accumulation in Bacillus subtilis

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ABSTRACT

Stringent control of RNA synthesis has been fairly well established in Escherichia coli : (p)ppGpp is synthesized on the ribosomes under conditions which cause a block in translation. Associated with the rise in (p)ppGpp levels is a change in RNA polymerase transcriptional specificity. Although a similar mechanism has been found in Bacillus subtilis, there is evidence that the mechanisms involved in (p)ppGpp accumulation may differ. The series of experiments described in this text analyze conditions resulting in (p)ppGpp accumulation in B. subtilis. It has not yet been firmly established that (p)ppGpp has the same mode of action in B. subtilis as it does in E. coli.

A transient accumulation of ppGpp and pppGpp was found to occur in vegetatively growing Bacillus subtilis cultures after temperature increase, although net RNA accumulation was not interrupted sig-

nificantly. The addition of the RNA polymerase inhibitors rifampicin and lipiarmycin, which antagonize the initiation step in transcription, caused a large accumulation of MS nucleotides in vegetatively growing B. subtilis. Streptolydigin, however, caused no increase in MS nucleotide levels. The response to the addition of these drugs in a rif^r strain was markedly deficient, implicating an active role of RNA polymerase in (p)ppGpp accumulation in this instance. The rel^- strain, however, did accumulate noticeable amounts of (p)ppGpp after the addition of rifampicin or lipiarmycin. The results obtained here suggest a possible role for RNA polymerase in MS nucleotide synthesis in B. subtilis.

Fifteen to twenty minutes after the end of logarithmic growth (t_o), the addition of rifampicin or lipiarmycin to cultures did not cause an increase in (p)ppGpp levels over those present in untreated controls. Numerous changes occur in both the RNA polymerase and the ribosomes during the shift from vegetative growth to sporulation. Therefore, the loss of rifampicin/lipiarmycin-stimulated (p)ppGpp accumulation after vegetative growth could be attributed to alterations at either the transcription or translation level. It is not thought that rifampicin fails to enter the cell because: 1.) RNA synthesis is still inhibited by the drug in sporulating cells, and 2.) sporulation is blocked by drug addition even at late (t_5) times in the sporulating cells.

A temperature-sensitive Bacillus subtilis mutant, OSB 459, is described, which shows a rapid cessation of both stable and unstable RNA synthesis after shift to the restrictive temperature. This

mutant also accumulated (p)ppGpp transiently upon temperature upshift, but this accumulation appeared to be more temperature-sensitive than in wild-type. At the permissive temperature, rifampicin and lipiarmycin caused a large overaccumulation of (p)ppGpp relative to the wild-type strain. Since RNA polymerase had been implicated in (p)ppGpp accumulation, the possibility that the temperature sensitivity of OSB 459 is due to an altered RNA polymerase is presented.

Guanosine Tetra- and Pentaphosphate Accumulation in *Bacillus subtilis*

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Typed by Carlene Sousa for Virginia Lee Price

To my mother, Mona, whose examples of
strength and love have
always been a source of encouragement

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I. Preface

Several different modes of regulation of RNA synthesis have been deciphered: the heterogeneity of promotor regions, presumably each with differing affinities for RNA polymerase, has been described (3). Effector molecules such as inducers, repressors, or cAMP (and its receptor protein, crp) bind to DNA at specific sites to either promote or prevent the binding of RNA polymerase. Other effector molecules, like ppGpp, are thought to bind RNA polymerase directly to alter transcription (14-16). RNA polymerase itself is thought to exist in a number of different configurations ("conformers"). Travers (15) has described a "multiple forms model" for RNA polymerase specificity in which the enzyme exists in at least two functionally distinct forms, each of which preferentially initiates a certain class of promoters. It can be seen from this complexity that each method of control is not separate in and of itself, but overlaps, and may even depend on, the other forms of control.

The regulation of transcription must necessarily, at one point or another, involve the RNA polymerase. As Travers pointed out (15), mitochondrial RNA polymerase consists of just one polypeptide chain of about 6×10^4 daltons, which is adequate for the process of synthesizing RNA. The bacterial enzyme, on the other hand, is a large (ca. 4×10^5 daltons), extremely complex molecule, composed of four distinct subunits. This added complexity of the bacterial enzyme

perhaps reflects its multifunctional role in the regulation of transcription.

The coupling of transcription and translation has offered an intriguing hunt for a link between the two processes of RNA synthesis and the production of proteins. As it turns out, several such "links" may exist. One example appears to be the stringent control system in Escherichia coli, where a product of the translational apparatus, ppGpp, interacts with RNA polymerase to alter transcriptional specificity (14-16). Another candidate is elongation factor-G (EF-G) in Bacillus subtilis; a temperature-sensitive EF-G mutant has been described (6), which shows increased stable RNA synthesis at the restrictive temperature, although protein synthesis ceases and (p)ppGpp accumulates. This represents an uncoupling of RNA and protein synthesis, which is thought to be mediated, in part, by ppGpp. In another case, an EF-G mutant (fus^r) demonstrating temperature-sensitive sporulation has been isolated, which could be suppressed by introducing a rif^r mutation (containing an altered RNA polymerase) (7). Kobayashi et al. (7) suggested that EF-G functionally interacts with RNA polymerase and may be involved in the regulation of sporulation. A similar example is a rif^r, str A double mutant in E. coli described by Chakrabarti and Gorini (2). These two lesions, in combination, resulted in temperature-sensitive transcription, but individually, strains were not temperature-sensitive. Again, a functional interaction between RNA polymerase and the ribosomes was implicated. Also, formylmethionyl-tRNA$_f$ and EF-Tu are

two ribosome-associated factors which have been shown to alter E. coli RNA polymerase specificity in vitro (10,14).

Another major form of transcriptional control must also take place during sporulation of bacilli. It was, in fact, at one time thought that the sporulation process could provide a simple model for differentiation. However, it has been shown not to be just a matter of "turning off" vegetative genes and "turning on" sporulation-specific genes, since at least 80 percent of the transcripts present during vegetative growth continue to be expressed during sporulation (3,9,13). Many factors involved in sporulation have been described (3,13), but exact cause and effect relationships for the sequence of changes that occur has not been established. It is interesting to note that, although a cAMP-crp system has not been found in B. subtilis (13), some of the environmental "triggers" that result in stimulation of cAMP, (p)ppGpp, and sporulation are identical : a shortage of carbon, nitrogen, or phosphorous.

In B. subtilis then, the end of vegetative growth results in depletion of energy sources. This, in turn, stimulates both (p)ppGpp accumulation and the initiation of sporulation. Is the stringent response and sporulation merely coincidental in this case, or is there a cause and effect relationship? Evidence so far suggests that (p)ppGpp is not needed for sporulation (11) since a relaxed strain of B. subtilis sporulates without an increase in (p)ppGpp at the end of vegetative growth. However, Rhaese et al. (11,12) have described highly phosphorylated adenosine compounds (HPNs), especially pppAppp, which are produced at the onset of sporulation. Whether

these compounds are actually necessary for sporulation to occur, or only represent a change in the translational apparatus (and are thus a by-product of the process), has not yet been established (13).

A number of changes have been shown to occur at both the levels of transcription and translation during sporulation in B. subtilis. Specifically, RNA polymerase appears to undergo two important alterations: 1.) σ activity (σ promotes initiation of new RNA chains at specific sites, analogous to the initiation factors in the translational process (8)), and binding to core polymerase is greatly reduced (3,13) and 2.) at least two polypeptides not present during vegetative growth are found associated with the RNA polymerase isolated from sporulating cells (3,4). It is, at this point, still assumed that these sporulation-specific polypeptides play a role in altering the transcriptional specificity of RNA polymerase, as this has not yet been proven.

The vegetative RNA polymerase core, however, does not appear to change during the sporulation process. Sporulating cells remain sensitive to rifampicin throughout the entire process, and no difference has been observed in the in vitro RNA transcripts between the vegetative and sporulation RNA polymerase (3).

A number of lines of evidence have also alluded to a form of control involving the cell membrane. This could occur either by membrane-bound effector molecules, or enzymes with membrane-bound activity, or both. Gallant (5) has described an E. coli mutant designated shf (shiftless) which fails to produce either cAMP or ppGpp

during energy-source downshift. This mutant also seems to have a membrane defect. Adenyl cyclase, the enzyme responsible for cAMP formation, is membrane-bound, and the spo T reaction (ppGpp→ppG+X) in E. coli has been shows to be associated with membranes (1). The synthesis of pppAppp in B. subtilis, that Rhaese et al. reported to be part of the sporulation process, was shown by them to be a membrane-bound reaction (11). Membrane-associated control mechanisms would seem to be a logical place to monitor the external environment. The sporulation process itself obviously involves radical morphological changes in the outer structures of the cell, prespore septation being the first recognizable change.

It is becoming increasingly evident that there is an intricate relatedness and interaction of the many factors involved in the regulation of transcription. Because of this, the plieotropy resulting from the alteration of any one component makes it difficult to sort out cause and effect. With this in mind, an attempt has been made to reach conclusions concerning the role of RNA polymerase in promoting the accumulation of (p)ppGpp and the possible role of (p)ppGpp in the regulation of transcription in B. subtilis.

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II. The Effects of Temperature Upshift and RNA Polymerase Inhibitors on (p)ppGpp Accumulation in *Bacillus subtilis*

Introduction

The discovery of the regulatory nucleotides ppGpp and pppGpp[1] in *Escherichia coli* (6,7,9) and their role in the regulation of RNA synthesis during the stringent response has illustrated the importance of these compounds as controlling elements in the regulation of transcription. Several effects of these highly phosphorylated nucleotides have subsequently been demonstrated: their interaction with elongation factors and RNA polymerase, and both stimulation or repression of certain biosynthetic operons (1,4,21,41).

The possibility that ppGpp and pppGpp may play a regulatory role in other organisms was suggested when Swanton and Edlin isolated a relaxed strain of *Bacillus subtilis* and demonstrated the production of (p)ppGpp during the stringent response induced by amino acid starvation (43). The production of these compounds has also been demonstrated in yeast along with a stringent response to amino acid starvation, indicating that the regulatory role of (p)ppGpp may be a mechanism common to eukaryotes as well (34,46). However, differences have been shown to exist between *E. coli* and *B. subtilis* in the (p)ppGpp systems; for example, chloramphenicol prevents the accumulation of (p)ppGpp in *E. coli* and does not in *B. subtilis* (38).

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[1]Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) are also referred to as MS nucleotides or (p)ppGpp.

The physiological conditions that result in (p)ppGpp accumulation which have been defined in E. coli include energy source downshift and situations in which the amino acid charging of tRNA is prevented (2,3,5,6,8,18,19,21,28,42). Inhibitors of proteins synthesis such as chloramphenicol and tetracycline have been shown to antogonize the stringent response through their direct interaction with ribosomes. The production of (p)ppGpp in B. subtilis occurs under similar conditions of amino acid deprivation or energy source downshift (12,43), but the response to addition of inhibitors of protein synthesis differs. Drugs that inhibit protein synthesis do not relieve the stringent response in B. subtilis and instead, appear to result in an accumulation of MS nucleotides (38). In addition, there appear to be several highly phosphorylated compounds present in B. subtilis, including highly phosphorylated adenosine compounds, which have been implicated in the control of sporulation (37,38).

The effects of temperature upshift and the addition of various RNA polymerase inhibitors on the accumulation of (p)ppGpp and RNA synthesis in B. subtilis are described in this report. Both similarities and differences have been found with respect to E. coli under these conditions. A transient accumulation of (p)ppGpp occurs upon temperature upshift without cessation of stable RNA synthesis as Gallant et al. demonstrated in E. coli (14). We have obtained unexpected results upon addition of RNA polymerase inhibitors to B. subtilis; unlike the results of rifampicin addition to E. coli (10),

the addition of rifampicin and lipiarmycin, but not streptolydigin, caused a marked accumulation of (p)ppGpp.

Materials and Methods

Strains and Media

Bacillus subtilis OSB 158 has been described (20) and B. subtilis OSB 422 is a rifampicin-resistant strain obtained from OSB 158 by EMS mutagenesis. Strains BR 17 is a rel$^-$, lys$^-$ strain of Swanton and Edlen obtained through Dr. J. A. Gallant. Its parent strain BR 16, is a lys$^-$ derivative of OSB 158 which was EMS mutagenized to obtain the rel A$^-$ phenotype (43).

Media used for radioactive labeling of cells consisted of the tris-glucose media (TG) of Kaempfer and Magasanik (24) supplemented with 0.2% casamino acids and, in the case of BR16 and BR17, 20 µg/ml of each lysine and tryptophan (these strains are also "weak tryptophan auxotrophs" (43)). The final phosphate concentration was 0.5 millimolar.

Amino acid starvation for lysine of BR16 was achieved by centrifuging a 0.5 ml sample of cells in a Beckman microfuge then resuspending the pellet in an equal volume of TG media supplemented with an amino acid mixture containing 20 µg/ml of each of the following amino acids: glutamine, proline, arginine, valine, alanine, leucine, methionine, tryptophan, threonine, histidine, isoleucine, and arginine.

Extraction and Quantitation of Nucleotides

Cells were grown in the presence of 300 μCi/ml [^{32}P] orthophosphate (NEN, Carrier free) for one generation to allow equilibration with the phosphate pools. Growth was followed with a Klett-Summerson colorimeter (standardized to a Zeiss spectrophotometer at 650 nM for determining concentration of nucleotide per 0.D unit). Samples (0.1 ml) were removed at appropriate times for nucleotide extraction with formic acid by the method of Gallant et al. (15).

Highly phosphorylated nucleotides were resolved by two-dimensional thin layer chromatography on PEI-cellulose (Brinkman) which consisted of 4M HCOOH, 1 M LiCl development in the first dimension, and 1.5 M KH_2PO_4, pH 3.4 development in the second dimension. This is method 3 of Gallant et al. (15).

Radioactively labeled nucleotides were located by autoradiography and the corresponding spots cut out of the TLC plate. Identification of nucleotides was done by testing for norit-adsorption and co-migration with the authentic compound. Nucleotide triphosphates were obtained from Sigma, and ppGpp and pppGpp were obtained from PL labs. Radioactivity in each spot was determined in a toluene-based scintillant (containing 0.4% 2,5-diphenyl oxazole and 0.01%, 1,4-bis-[2-(5-phenyloxazolyl]-benzene) by liquid scintillation counting with a Beckman LS8000. A computer program was used to convert counts per minute to picomoles of nucleotide per 0.D. Adjustment for background radioactivity was made by cutting out an adjacent equal-sized blank spot from the PEI plate.

RNA and Protein Synthesis

RNA accumulation was measured by incorporation of [5-^{3}H]uridine (Amersham, 0.5 μCi/ml with 10 μg/ml non-radioactive uridine as carrier) into trichloracetic acid (TCA) precipitable material. Cultures were grown at 37°C in TG media to a cell density of 15-20 Klett units then labeled for 10 min. before shifting to a high temperature (49°C-51°C). Samples (0.5 ml) were removed at indicated times after addition of label and added to an equal volume of cold 10% TCA. Precipitation was allowed to occur at least 1 hour, then samples were filtered on a glass fiber prefilter (Millipore) and counted as described above. Pulse-labeling of RNA was done by exposing 0.5 ml samples of cells grown in TG containing 20 μg/ml non-radioactive uridine to [5-^{3}H]uridine (1 μCi/ml with 20 μg/ml unlabeled uridine) for two minutes. The labeling was stopped by adding 1 ml. of cold 10% TCA and chilling the samples on ice. Samples were then precipitated and counted as described above.

Protein synthesis was measured by growing cells in the minimal media described by Haworth and Brown (20) supplemented with .05% casamino acids and measuring the incorporation of [U-^{14}C]protein hydrolysate (Amersham) into TCA precipitable material as described above.

Results

Effect of Temperature Upshift on (p)ppGPP Accumulation and RNA Synthesis

Exponentially growing cultures of 158, BR16, and BR17 have generation times in TG at 37°C of 35 min, 35 min, and 30 min respectively. Upon shift to a high temperature of 49°C - 51°C an increased growth rate follows; these generation times are then 30 min, 28 min, and 26 min respectively indicating that growth is not restrictive at the high temperature. Net protein synthesis, as measured by [u-^{14}C]protein hydrolysate incorporation, also increased slightly upon temperature upshift (data not shown).

The accumulation of stable RNA of 158, BR16, and BR17 changes only slightly upon temperature upshift (Fig. 1A). Little difference in the net effect between *rel*$^+$ and *rel*$^-$ strains is observed in this instance, although a slight lag in the *rel*$^+$ strains immediately following temperature upshift is detected. [^{3}H]uridine uptake into RNA during a two-minute pulse does, however, undergo a brief change after shift to the high temperature. There is an immediate increase in the amount of [^{3}H]uridine incorporated in the 0-2 minute pulse after temperature upshift of the *rel*$^+$ strains (Fig. 1B). This increase in pulse-labeled RNA quickly returns to the pre-shift level. It should be noted that this measurement of pulse-labeled RNA cannot be taken as a determination of the rate of RNA synthesis since the specific activity of the precursor pools was not determined. The observed increase in pulse-labeled RNA may only represent an increased uptake of labeled precursor upon temperature upshift, thereby increasing the

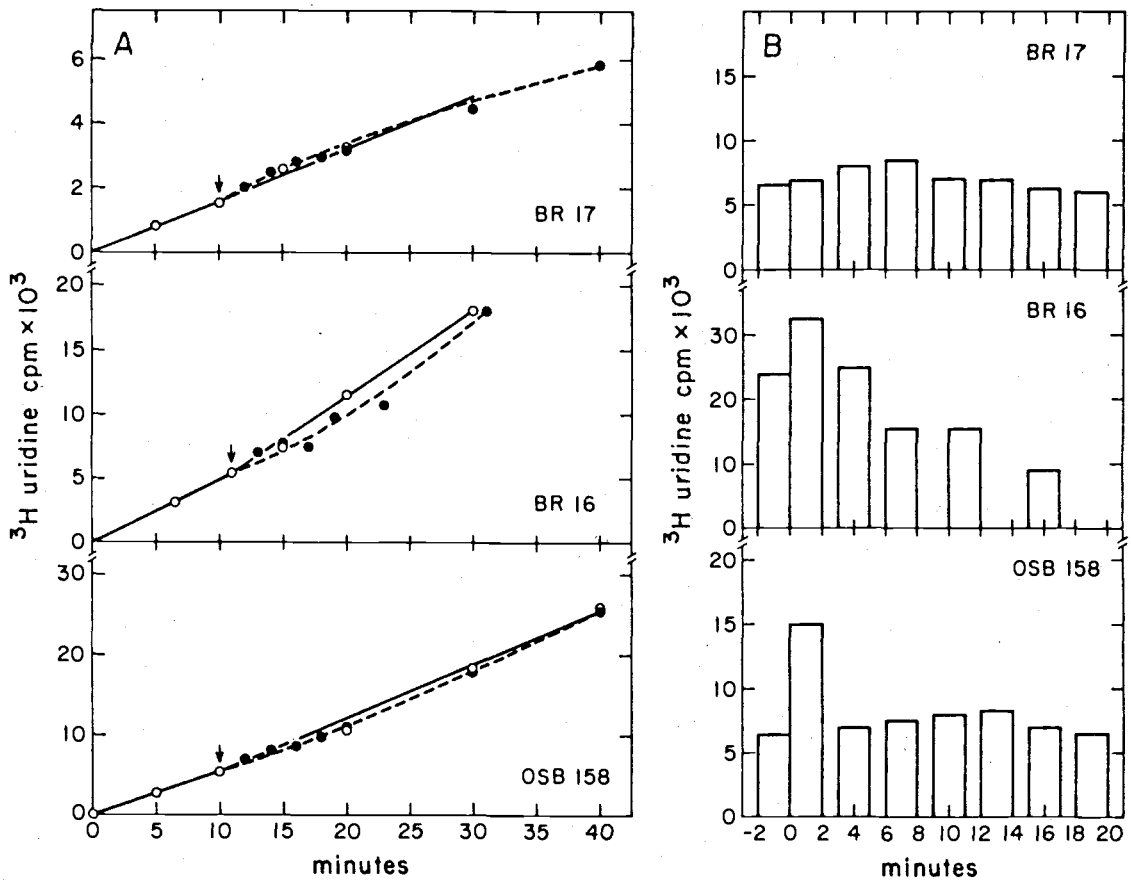

Fig. 1A. RNA accumulation after temperature upshift. The incorporation of [5^3]uridine was carried out as described in Materials and Methods. At the times indicated by arrows, a portion of each culture was shifted from 37°C (open symbols) to 49°C (closed symbols).

B. Pulse-labeled RNA after temperature upshift. Bars represent the amount of [5-^{3}H]uridine incorporated into a two minute pulse as described in Materials and Methods. At time zero, cultures were shifted from 37°C to 49°C.

specific activity of the internal pools. Whatever the mechanism for increased pulse-labeling of RNA upon temperature increase, the rel A^- strain does not show the same response. Rather, a slight increase is detectable over several minutes, but overall, there is little change in the profile of pulse-labeled RNA.

Also upon temperature upshift, a burst of (p)ppGpp is detected, reaching a maximum three to five minutes after temperature shift, then decreasing to basal levels by thirty minutes (Fig. 2). The levels detected in the rel A^+ strain are about ten to twenty-fold higher than basal levels, comparable to the levels attained during amino acid starvation. Fig. 2 shows the typical kinetics of MS nucleotide accumulation in strain OSB 158. Similar results were also obtained for the other rel^+ strain, BR16. The accumulation of MS nucleotides after temperature increase appears to be dependent on a functional rel gene product; when the rel^- strain was shifted from 37°C to 50°C, a change in (p)ppGpp levels was not detected. The rifampicin-resistant strain also accumulated significant amounts of (p)ppGpp at 50°C, in a manner similar to wild-type.

Effect of RNA Polymerase Inhibitors on (p)ppGpp Accumulation

The drugs rifampicin and lipiarmycin both inhibit the initiation of transcription, yet their mechanisms of action appear to be different. Rifampicin has been used as an inhibitor of the initiation step in transcription for quite some time, but recent evidence has shown that although it partially antagonizes dinucleotide synthesis, it appears to prevent elongation by blocking the release of σ and/or the first translocation (22,24). Rifampicin-resistance has also been

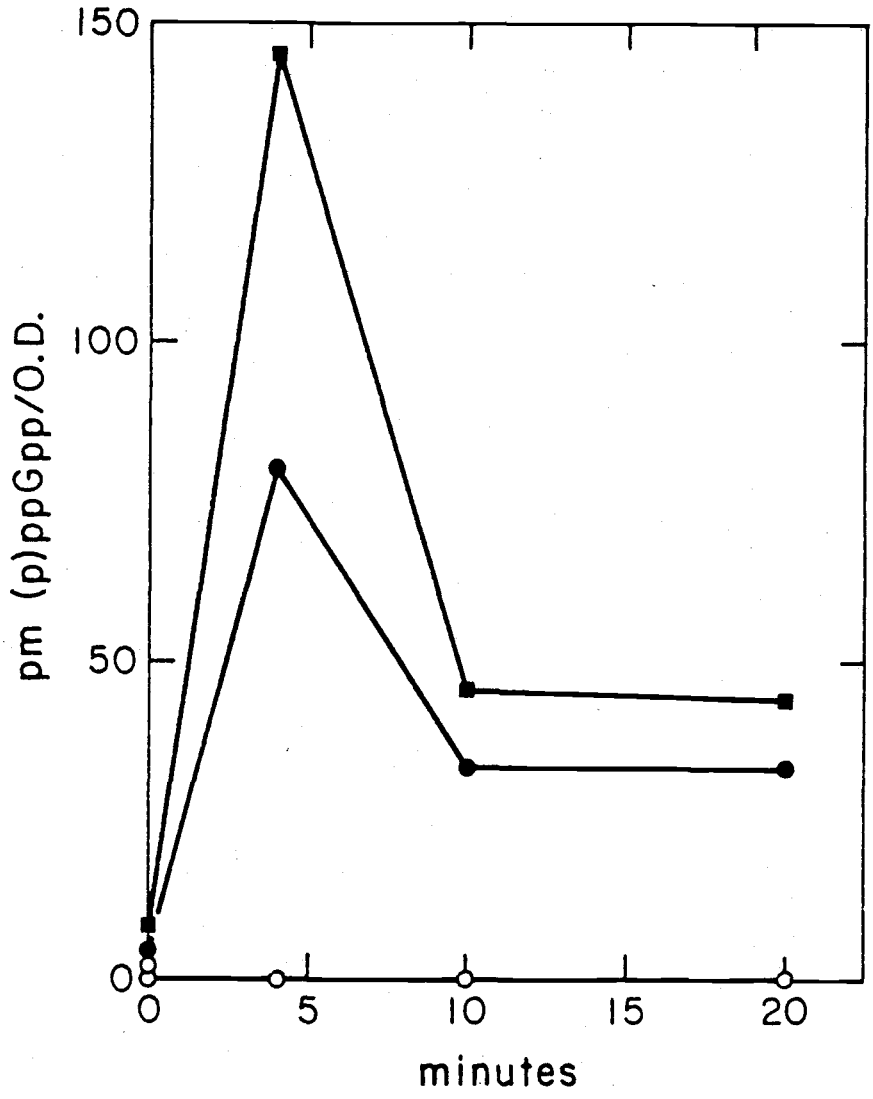

Fig. 2. Accumulation of MS nucleotides after temperature upshift. Cultures of OSB 158 (closed symbols) or BR17 (open symbols) were shifted to 50°C at time zero and assayed for ppGpp (circles) or pppGpp (squares) as described. The levels present at time zero represent basal amounts.

shown to lie in the β subunit of RNA polymerase (16). Lipiarmycin prevents the formation of the first internucleotide bond and is thought to bind to the β subunit. It does not seem to interfere with the formation of RNA polymerase - DNA complexes (40).

The addition of rifampicin (10 μg/ml) or lipiarmycin (2 μg/ml) to vegetatively growing cells resulted in a significant rise in (p)ppGpp levels in both *rel*$^+$ strains: wild-type *B. subtilis* OSB 158 and the lysine auxotroph BR16 (grown in the presence of lysine). Fig. 3 shows a typical accumulation of (p)ppGpp after addition of the drugs to OSB 158. Different kinetics of accumulation were observed however, with each of the two drugs. Upon addition of rifampicin, the levels of pppGpp rose significantly over the basal level, reaching a maximum six to eight minutes after exposure to the drug, then rapidly dropping to the levels of the controls within ten minutes. ppGpp also rose under these conditions but to a lesser degree, as was the case upon temperature upshift. Even larger amounts of both pppGpp and ppGpp accumulated in OSB 158 and BR16 after lipiarmycin exposure for up to ten minutes after the drug was added, then declined over the next 20 min. The levels of MS nucleotides reached in this instance were comparable to those present during the stringent response induced by lysine starvation of BR16 (see Table 1).

When rifampicin or lipiarmycin were added to the *rel*$^-$ strain BR17, a predominant increase in the levels of ppGpp took place (Fig. 4). The pentaphosphate also increased but to a much lesser extent, as though the ratios of the two MS compounds were reversed in the drug-treated *rel*$^-$ strain. Quantitatively, much less MS nucleotide

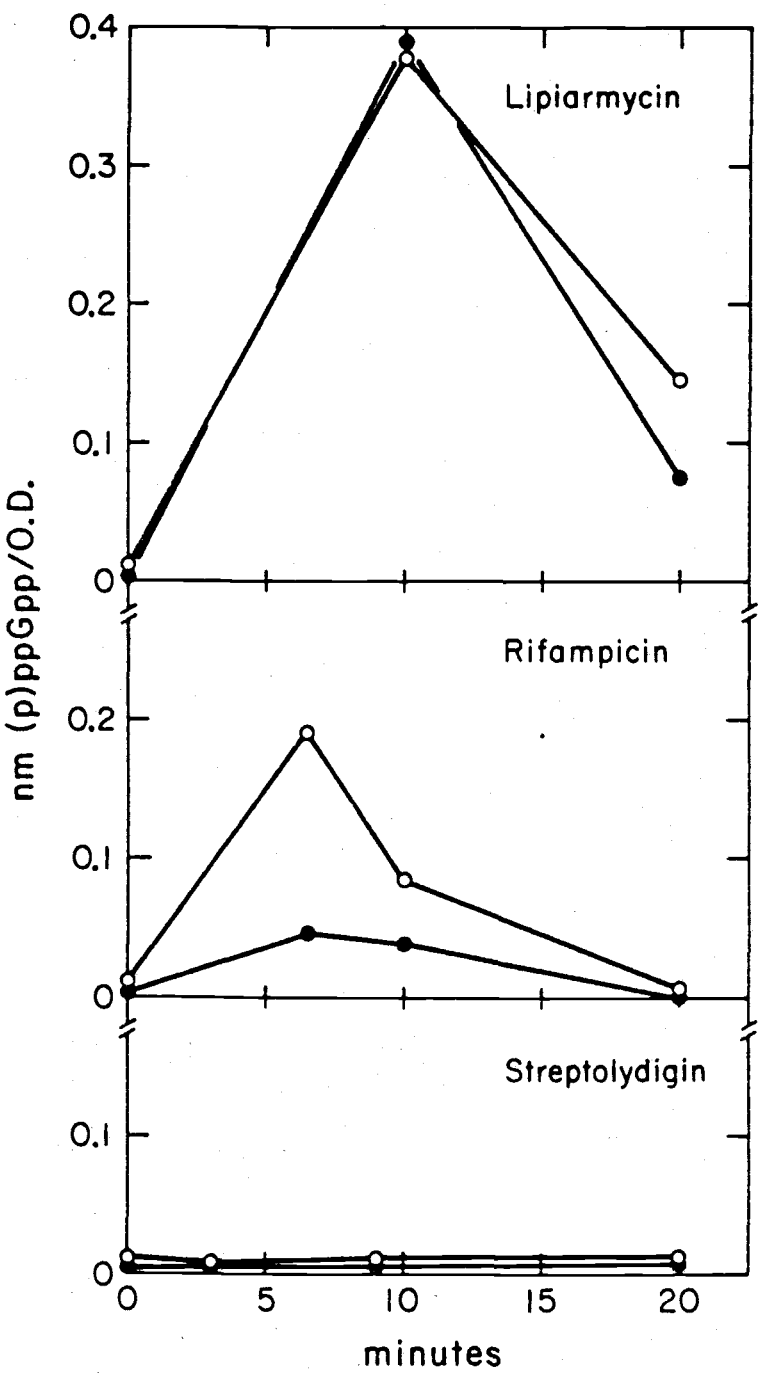

Fig. 3. Accumulation of MS nucleotides in OSB 158 after addition of RNA polymerase inhibitors. Various inhibitors were added at time zero as described in Materials and Methods and cultures were assayed for ppGpp (closed symbols) and pppGpp (open symbols) at the times indicated.

accumulated in the rel$^-$ strain. It should be noted that the basal levels of MS nucleotide detected in BR17 were extremely low, (1-2 picomoles for each compound), and in some extracts basal amounts were not detected by our methods.

The rif^r strain 422, when treated with rifampicin, showed a slight increase in the levels of pppGpp, but considerably less than either of the rel$^+$ or the rel$^-$ strains (compared to ppGpp in BR17) (Fig. 4). Lipiarmycin caused more of an accumulation of pppGpp than rifampicin, yet less than occurred in its parent rif^s strain 158 with the same treatment. Most noteably, the ppGpp accumulated is less than in all the other strains treated with lipiarmycin. To examine a different RNA polymerase mutant, a streptolydigin-resistant strain was used to determine the effects of temperature increase and RNA polymerase inhibitors on MS nucleotide accumulation. The std^r strain tested (OSB 406, isogenic to OSB 158) accumulated (p)ppGpp under conditions of both temperature increase and rifampicin and lipiarmycin addition (data not shown) to levels about one-third those of wild-type (Table 1).

Streptolydigin, which inhibits the elongation step of RNA synthesis, caused no accumulation of (p)ppGpp when added to cells at 100 µg/ml under the above conditions (Fig. 3). When streptolydigin was added prior to rifampicin or lipiarmycin, it prevented the accumulation above basal levels of MS nucleotides as seen when rifampicin or lipiarmycin was added alone.

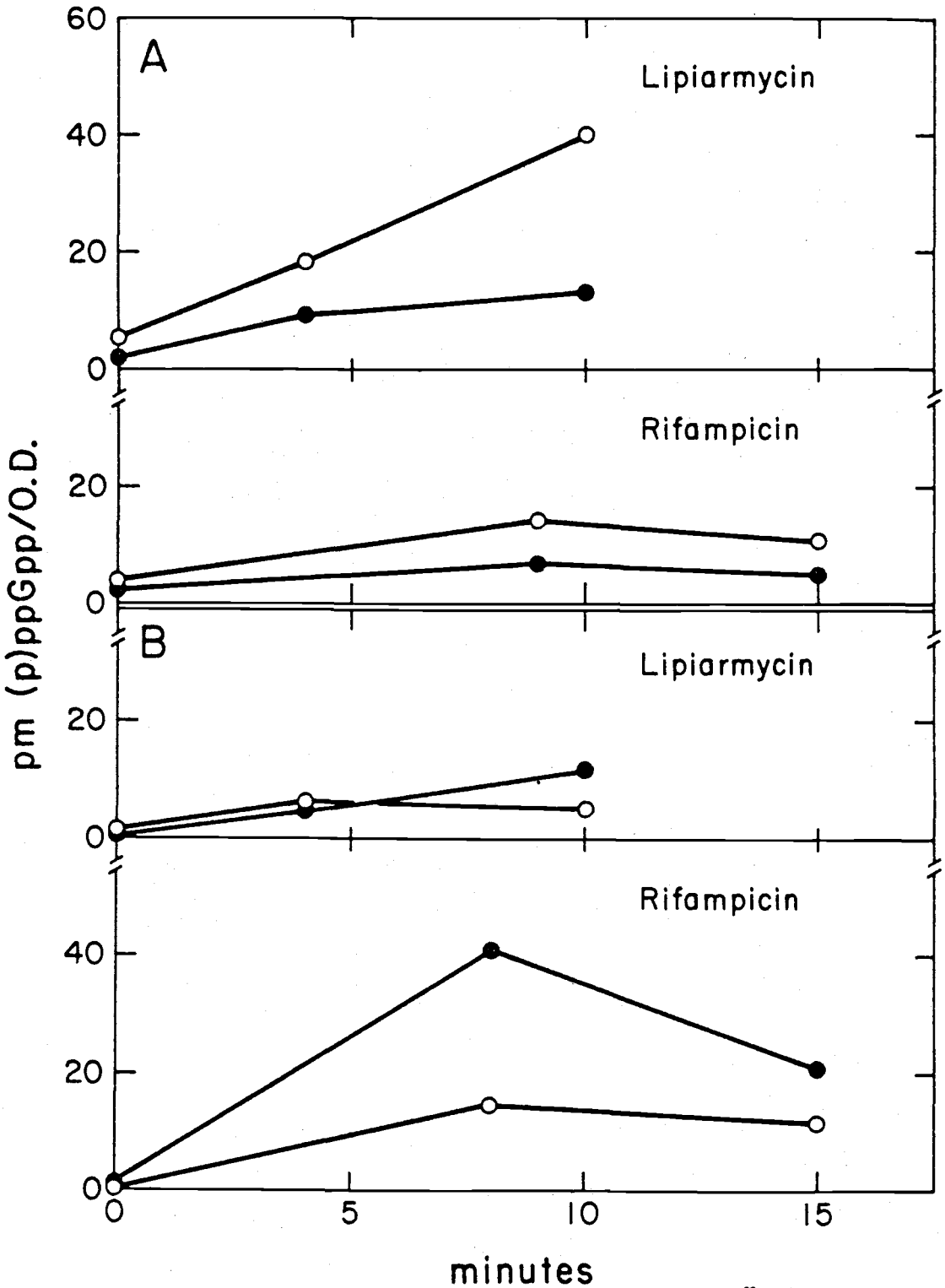

Fig. 4. Accumulation of MS nucleotides in A. the rif^r strain, OSB 422 and B. the rel$^-$ strain, BR17 after addition of RNA polymerase inhibitors. Rifampicin and lipiarmycin were added at time zero and cultures were assayed for ppGpp (closed symbols) and pppGpp (open symbols).

Table 1. Accumulation of (p)ppGpp, picomoles/O.D.

Treatment		OSB158	OSB422	OSB406	BR16	BR17
basal level	ppGpp	3	2	5	2	1
	pppGpp	8	5	5	4	2
Rifampicin	ppGpp	45	7	15	16	41
	pppGpp	190	14	38	65	15
Lipiarmycin	ppGpp	390	11	123	92	22
	pppGpp	375	40	174	194	10
temperature upshift to 50C	ppGpp	60	22	15		1
	pppGpp	100	55	42		2
amino acid starvation	ppGpp				95	
	pppGpp				120	

Values represent maximum levels of (p)ppGpp accumulated under various conditions, as described in the text.

It appears that in the cases of MS nucleotide accumulation described here, RNA synthesis is shut off prior to the build up of these compounds since both rifampicin and lipiarmycin cause cessation of RNA accumulation in less than two minutes (data not shown).

Effects of Trimethoprim on the stringent response

Trimethoprim has been shown to evoke the stringent response in E. coli (25) by restriction of the formylation of methionyl-$tRNA_f$ through inhibition of dihydrofolate reductase; ppGpp synthesis ensues with cessation of RNA accumulation. It was thought that this drug could be used in B. subtilis for the same purpose : to induce typical stringent response as a control.

When trimethoprim (10 μg/ml) was added to vegetatively growing B. subtilis, RNA accumulation ceased after five minutes, yet no accumulation of (p)ppGpp could be detected up to 20 minutes after addition of the drug (data not shown). The reason for this is not known.

Discussion

Following a nutritional upshift, bacterial cells undergo a transition resulting in a more rapid growth rate. In achieving a faster rate of growth, an immediate increase in the rate of stable RNA synthesis is observed in E. coli, mediated by a shift in transcription from mRNA genes to stable RNA, along with an increase in stable RNA chain growth rate (5). An increased rate of ribosomal protein synthesis also takes place. This represents a control of RNA synthesis distinct from the regulatory effects of ppGpp since relaxed strains exhibit these same changes upon enrichment (32).

When radioactive uracil is added to E. coli immediately following a nutritional upshift, an initial rapid uptake of isotope into RNA is observed. This has been attributed mainly to a higher specific activity of the intracellular precursor pools which occurs when the synthesis of stable RNA is increased along with a transient fall in the rate of mRNA synthesis (29-32).

Temperature upshift also results in a change to a faster growth rate. This is accounted for by an increase in the rates of reactions in general at a higher temperature, rather than qualitative changes in RNA and protein synthesis resulting from a redistribution of the RNA polymerase. An initial, brief increase in pulse-labeled RNA upon temperature upshift has been observed in rel^+ strains similar to the rapid uptake of labeled precursor observed immediately following a nutritional upshift. Whether or not this is due to actual increased RNA synthesis or to solely an increased uptake of precursor has not been established. The net accumulation of RNA upon temperature increase does not appear to change drastically however, unlike the effects of a nutritional upshift.

Upon temperature upshift, a transient period must necessarily exist before reaching the final temperature and attaining its commensurate rate of growth. During this period, the rate at which individual processes reach their new, faster rate may differ, causing a transient period of unbalance with regard to normally co-ordinated processes.

Whatever the mechanisms involved in the temperature effect are, the product of the B. subtilis rel gene appears to be involved in

the change in pulse-labeled RNA synthesis, besides the transient MS nucleotide accumulation, since rel A^- cells do not show, as rel^+ cells do, the magnitude of change in [^{3}H]uridine pulse-labeled RNA upon temperature shift. The brief accumulation of MS nucleotides after temperature upshift reaches a peak after three to four minutes at the high temperature, but is still at basal levels 1.5 minutes after upshift (data not shown). The two-fold increase in [^{3}H]uridine incorporated in a two-minute pulse occurs during the first two minutes after upshift, therefore before MS nucleotides accumulate significantly. Perhaps the transient increase in pulse-labeled RNA is due to transcription of additional genes upon temperature increase. One possible reason for the failure of (p)ppGpp to shut off stable RNA synthesis discussed by Gallant et al. (14) is that the increase in temperature could result in a transient change in promotor or RNA polymerase conformation, rendering the transcription apparatus insensitive to (p)ppGpp. Whether or not (p)ppGpp is involved in the regulation of any RNA synthesis during that time or is only coincidence with the transient increase in pulse-labeled RNA needs further study.

The temperature effect on MS accumulation is similar to results obtained by Gallant et al. (14) and Isaksson and Takata (22,44) in E. coli. This represents an anomolous situation where (p)ppGpp is present without the concomitant shutoff of stable RNA synthesis normally associated with this response. Kimura et al. (27) have also

described a temperature sensitive EF-G mutant of B. subtilis which shows increased stable RNA synthesis at the restrictive temperature along with a transient rise in MS nucleotides. The transient rise in (p)ppGpp levels after temperature upshift may be related to the above-mentioned hypothetical unbalance in the transcription and translation systems immediately following temperature increase.

The accumulation of (p)ppGpp in response to temperature shift described here takes place in defined media containing 0.2% casamino acids. The transient temperature effect on ppGpp accumulation reported by Gallant et al. (14), though, could be abolished by the simultaneous addition of certain amino acids. Also, unlike our results obtained with B. subtilis, the temperature effect in E. coli showed the rel A^- strain to accumulate ppGpp upon temperature upshift, albeit much less than the rel A^+ strain. It should be noted that the rel^- strain of B. subtilis, selected for its inability to synthesize RNA upon amino acid deprivation, has not been as well characterized as its E. coli rel A counterpart, eg. it has not been genetically mapped. It has recently been demonstrated that ribosomes from a strain bearing the BR17 rel^- mutation do not produce stringent factor, as ribosomes from B. subtilis rel^+ strains do (note added in proof, ref. 39).

Several researchers (11,29,36,41,45,47,48) have provided evidence that ppGpp in E. coli exerts its effect by preventing the initiation of RNA synthesis and that it interacts with RNA polymerase

to alter promotor specificity. Rifampicin is an antibiotic commonly employed as an antagonist of RNA polymerase in a number of bacteria. Our results show that both rifampicin and lipiarmycin, another RNA polymerase inhibitor, cause a significant accumulation of MS nucleotides when added to wild-type B. subtilis, a phenomenon not observed in E. coli (10).

This response to rifampicin is, for the most part, abolished in a rifampicin-resistant mutant (most likely containing an altered RNA polymerase since it maps in the ββ′ region of the B. subtilis chromosome). The MS nucleotide accumulation after lipiarmycin is added is drastically reduced in this strain also. A streptolydigin-resistant strain which contains an RNA polymerase altered at a different site (β′ subunit) (17) shows a response to rifampicin and lipiarmycin addition intermediate to the wild-type and rif^r strains (Table 1).

All this suggests a possible role of the transcription apparatus, specifically RNA polymerase, in the accumulation of MS nucleotides, at least under certain conditions. That pretreatment of cells with streptolydigin prevents this accumulation is further evidence that RNA polymerase is directly involved. (However, the possibility of secondary effects of streptolydigin, such as its interaction with stringent factor or the ribosomes and the prevention of MS nucleotide accumulation, has not been eliminated).

The relaxed strain of B. subtilis did not accumulate (p)ppGpp upon temperature upshift. However, there was a noticeable increase, especially in ppGpp, after exposure to rifampicin or lipiarmycin.

Whatever specific lesion produces the relaxed B. subtilis phenotype, it does not prevent the formation of MS nucleotides after drug addition. Therefore the two phenomena described here that cause MS nucleotide accumulation apparently occur via different processes or perhaps the same process with different sensitivities to external triggers. Since rel$^-$ strains are incapable of increasing their (p)ppGpp levels in response to amino acid starvation but can be made to accumulate these compounds under other conditions, it could be the specificity of the trigger that determines the rel$^-$ response. For example, Harshman and Yamazaki (19) have demonstrated NaCl-induced accumulation of ppGpp in E. coli which takes place in the presence of required amino acids and to an equal extent in both stringent and relaxed strains.

From the above results, it appears that there are several differences in the stringent control mechanisms between the two organisms. Chloramphenicol and most antibiotics that inhibit protein synthesis prevent ppGpp synthesis in E. coli by affecting the 50S ribosomal subunit on which it is made (13). However, Rhaese et al. (38) showed that chloramphenicol and several other antibiotics affecting the ribosome cause the accumulation of (p)ppGpp in B. subtilis. We also obtained a slight (3-4 fold) increase in MS nucleotides after 60 min of chloramphenicol treatment of OSB 158 (data not shown). Trimethoprim, when added to E. coli, elicits the stringent response by blocking formyl methionyl $tRNA_f$ formation (26). Yet, when we added this drug to B. subtilis, RNA accumulation

ceased after five minutes, but no increase in (p)ppGpp could be detected. Differences in the mechanism by which the MS nucleotides are generated or degraded may account for these varied responses.

Rel A^- strains of E. coli and B. subtilis are capable of maintaining basal levels of the MS nucleotides (28,43). In E. coli, rel A^- cells are able to increase these levels in response to carbon-source downshift as efficiently as rel^+ strains, apparently through lack of degradation rather than increased biosynthesis (13). It is not discernable from our studies whether the observed build-up of (p)ppGpp from either temperature increase or addition of RNA polymerase inhibitors is due to increased synthesis or decreased degradation. One possibility, therefore, would be that a temperature increase induces stringent factor-dependent synthesis of these compounds, and rifampicin/lipiarmycin or a drug-RNA polymerase complex prevents degradation of (p)ppGpp, thus the build-up of these nucleotides in both rel^+ and rel^- cells. This fails to explain the transience of the MS nucleotide build up after rifampicin-treatment, though.

A possible mode of rifampicin/lipiarmycin-induced synthesis of (p)ppGpp would be that an RNA polymerase-drug complex could bind a component on the ribosome, such as EF-G or formylmethionyl tRNA, and cause a type of "idling" reaction similar to that which occurs in the presence of an uncharged tRNA. It has been shown in E. coli that the formylmethionyl $tRNA^f$ can bind RNA polymerase (35), and there is evidence for interaction of EF-G with RNA polymerase in B. subtilis

(27). This mechanism is dependent on a functional stringent factor enzyme, however. Another possibility is that an essential step in protein synthesis, such as tRNA charging, is affected by the RNA polymerase-drug complex. If this were the case, one would not expect the *rel*$^-$ strain to accumulate MS nucleotides under these conditions, as it did.

Alternate mechanisms of MS nucleotide synthesis in *B. subtilis* must also be considered, as was mentioned above. Two hypothetical mechanisms for rifampicin/lipiarmycin-induced synthesis of (p)ppGpp, independent of stringent factor activity, will be presented. In the first case, a drug-RNA polymerase complex could interact with the ribosomes (or proteins associated with ribosome function) as described above, and the RNA polymerase itself catalyze the pyrophosphate transfer from ATP to GTP, synthesizing pppGpp. The second possibility would be that (p)ppGpp is formed by RNA polymerase during a block in the initiation of transcription on the DNA template. This would provide a second site for MS nucleotide production in *B. subtilis* involving the transcription apparatus. Evidence for or against these two alternate mechanisms has not yet been established. However, significant differences, not only in the stringent response, but in the RNA polymerase molecules between *E. coli* and *B. subtilis* have been noted (16) : the size of the RNA polymerase σ subunit is larger in *E. coli*, and it is the larger of the two largest *B. subtilis* polypeptides (β), which binds rifampicin, and the smaller (β') binds streptolidigin. (This inference is based on the finding that *rif*^{r}

mutants and *std*^{r} mutants contain altered β and β′ subunits, respectively (16,17)). The largest subunit of *E. coli* (β) binds both streptolidigin and rifampicin. These differences in RNA polymerase structure may make it possible for RNA polymerase to have an active role in (p)ppGpp synthesis in *B. subtilis*.

It is not known by what mechanism the transient accumulation of MS nucleotides occurs after temperature shift or after the addition or rifampicin or lipiarmycin to *B.* subtilis. The role of (p)ppGpp itself in *B. subtilis* as the direct effector of the stringent response comes under serious question, since a variety of conditions can be imposed to cause its accumulation both with and without concomitant cessation of RNA synthesis.

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III. The Effects of RNA polymerase Inhibitors on (p)ppGpp Accumulation During Sporulation of Bacillus subtilis

The putative regulatory nucleotides ppGpp and pppGpp have been shown to accumulate in vegetatively growing cultures of Bacillus subtilis soon after the addition of the RNA polymerase inhibitors rifampicin or lipiarmycin. Evidence has been presented for the direct involvement of RNA polymerase in the accumulation of MS nucleotides under these conditions (Price and Brown, manuscript submitted). Results obtained by several researchers (for example 17-19) suggest that ppGpp interacts directly with RNA polymerase to regulate transcription, but the possibility of RNA polymerase playing a role in the synthesis or induction of these compounds had not been established (prior to the above-mentioned report). RNA polymerase is also thought to be a key factor in the regulation of sporulation (10,15).

Modifications are known to occur in RNA polymerase during sporulation (4,8,12,14); therefore, it was of interest to examine the effects of rifampicin and lipiarmycin on the accumulation of MS nucleotides in sporulating cells. Also, we wished to search for the appearance of the highly phosphorylated nucleotides (HPNs) eg. pppAppp, reported to accumulate in sporulating cells by Rhaese et al. (11,12), under these conditions.

Formic acid extracts of B. subtilis wild-type strain OSB 158 were chromatographed at various times after t_o to examine the possibility of MS nucleotide or HPN accumulation at the onset of sporu-

lation. Fig. 1 shows that about 20 minutes after t_o there is an increase in the amount of ppGpp, which then decreases to basal levels over the next 60 min. Very little pppGpp accumulated at this time.

Cells were then also treated with rifampicin (10 μg/ml) for 6.5 min. or lipiarmycin (2 μg/ml) for 10 min. before the nucleotides were extracted, since these times showed maximum levels of (p)ppGpp to accumulate in vegetatively growing cells (Price and Brown, manuscript submitted). When rifampicin or lipiarmycin (both of which bind the β subunit of RNA polymerase and antagonize RNA chain initiation) were added to OSB at 158 at t_o, a time at which ppGpp had not yet accumulated significantly in the controls, a moderate increase in ppGpp levels was detected (Figs. 1 and 2). pppGpp did not accumulate to a significant degree in this instance, either. As Figs. 1 and 2 also show, the addition of rifampicin 40 min. after t_o, or lipiarmycin 15 min. after t_o, caused virtually no increase in the accumulation of MS nucleotides over the levels of the controls. Conversly, the addition of these RNA polymerase inhibitors did not prevent the rise in ppGpp observed shortly after t_o. Rifampicin was also added to cells at t_2 and $t_{2.5}$. No increase in the basal amounts of (p)ppGpp were observed, nor were any HPNs detected at those times (data not shown).

Although changes occur in the RNA polymerase holoenzyme during sporulation (reviewed in 10,15), sporulating cells do remain sensitive to rifampicin as far as its ability to prevent the initiation of transcription. Lipiarmycin, on the other hand, apparently binds holoenzyme preferentially (14), and its effect on RNA

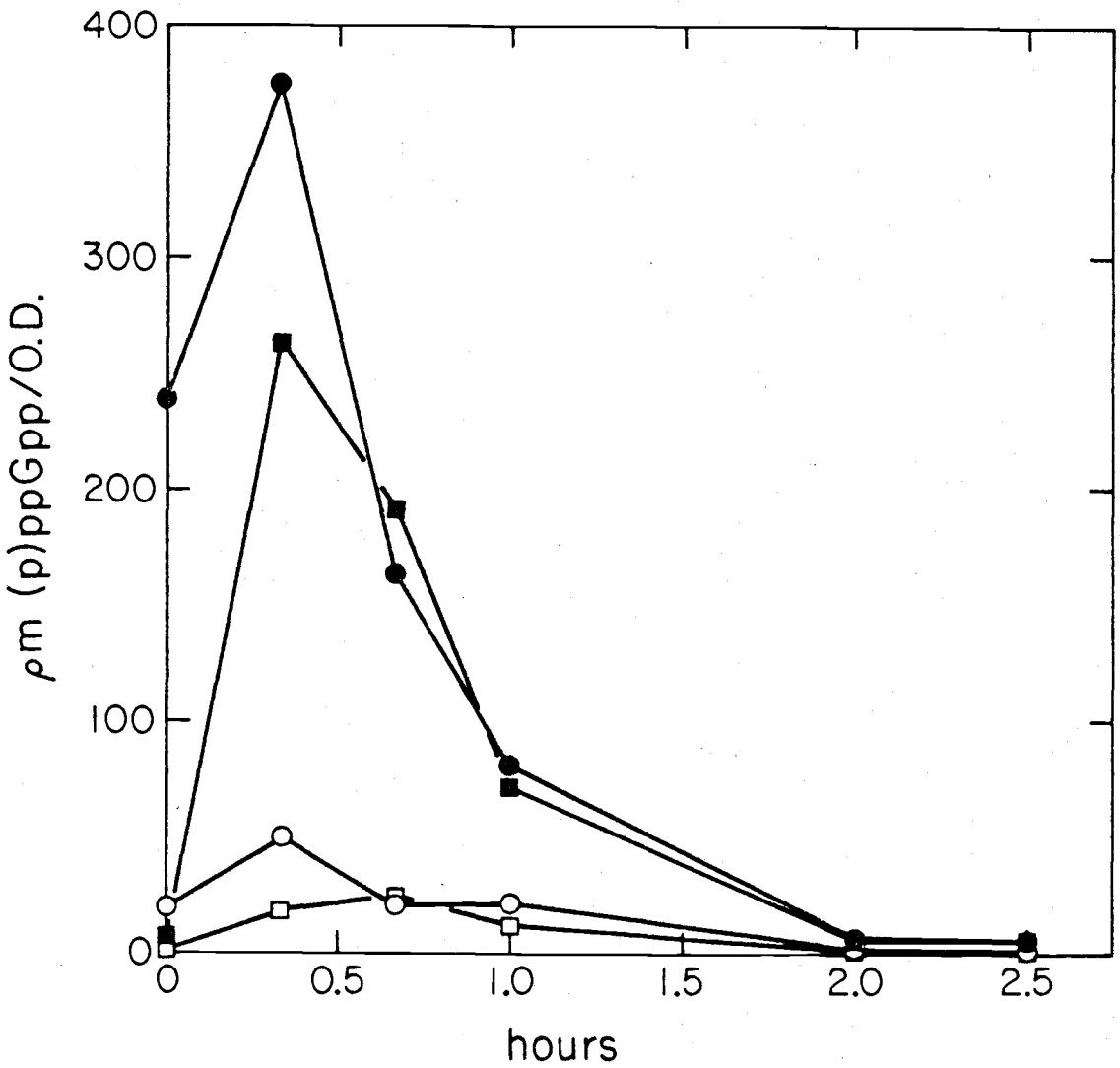

Fig. 1. Accumulation of (p)ppGpp in wild-type B. subtilis OSB 158 after the end of logarithmic growth (t_o) in control cultures (squares) and with the addition of rifampicin (circles). Cells were grown in a sporulation medium consisting of: .08 M NaCl, .02 M KCl, .02 M NH_4Cl, .12 M Tris-HCl pH 7.4, .35 mM $CaCl_2$, .02 mM $FeCl_2$, 2.5 mM Na_2SO_4, .5 mM K_2HPO_4, .025 mM $MnCl_2$, 1.5 mM $MgSO_4$, 5 mM glucose, and .2% casamino acids. About 80% of the culture sporulated in this media. At the various times indicated, 0.1 ml samples were removed for formic acid extraction and quantiation of MS nucleotides as described in (5). Closed symbols represent ppGpp, and open symbols, pppGpp.

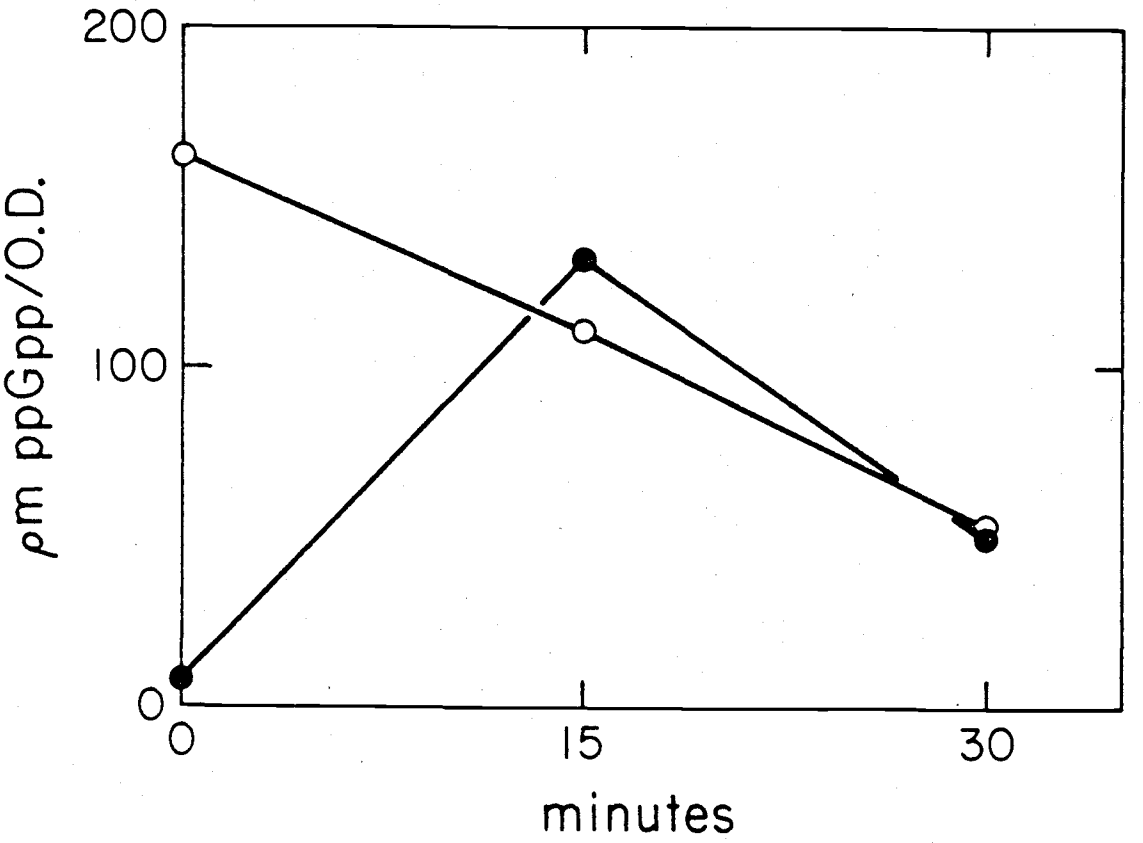

Fig. 2. Accumulation of ppGpp in OSB 158 during sporulation after the addition of lipiarmycin (closed symbols). Open symbols represent untreated control samples. The experiment was performed as described in Fig. 1. pppGpp levels did not significantly increase over basal amounts in either case, therefore are not included.

synthesis in sporulating cells has not been publicized. Sonenshein et al. found that neither core alone nor σ alone was inactivated by lipiarmycin to the same extent as holoenzyme, and postulated that a core subunit and σ could each provide a part of the lipiarmycin binding site. Like some rif^r mutants, lpm^r mutants have been isolated which concomitantly lose their ability to sporulate (14).

The accumulation of MS nucleotides after addition of rifampicin or lipiarmycin which was demonstrated in vegetatively growing B. subtilis (Price and Brown, manuscript submitted) did not take place when these drugs were added several minutes after t_o. This may be a further indication that the RNA polymerase molecule is directly involved in the anomolous MS nucleotide accumulation induced by rifampicin or lipiarmycin in vegetative cells. Furthermore, it suggests that σ may be an important factor in (p)ppGpp accumulation under these conditions for the following reasons: Sporulating cells have an inactive σ subunit, loosely associated with core enzyme (8,16). They, in a sense, become immune to the drug-induced MS nucleotide accumulation described above. Vegatative cells contain σ tightly bound to core in the holoenzyme (2) state, and accumulate large quantities of (p)ppGpp after drug addition. Vegetative cells also show a much greater accumulation of MS nucleotides in response to lipiarmycin than rifampicin. However, cells at t_o showed a slightly less response to lipiarmycin than they did to rifampicin. Fifteen minutes after t_o, the addition of lipiarmycin caused no increase in ppGpp levels above the control, wherease 20 min. after t_o, rifampicin still caused

in a 1.5 fold increase in the ppGpp levels induced in the untreated control (compare Figs. 1 and 2). In effect, it appears that at the end of vegetative growth, cells become less sensitive to lipiarmycin, and gain "immunity" to lipiarmycin-induced (p)ppGpp accumulation sooner than they do for rifampicin. Perhaps this is due to the loss of a σ binding site. Lipiarmycin and rifampicin are antibiotics that interfere with the initiation step in RNA synthesis; the only step, to date, in which σ subunit is known to be utilized (2,7). Streptolydigin, on the other hand, antagonizes elongation(3), and did not promote MS nucleotide accumulation in vegetative cells (Price and Brown, manuscript submitted).

The additional polypeptides found associated with RNA polymerase from sporulating cells (4,9), could also be a factor in the loss of rifampicin/lipiarmycin-induced MS nucleotide accumulation. Core enzyme from sporulating cells apparently remains unchanged (10), and RNA synthesis remains sensitive to rifampicin inhibition (S. Haworth, Ph.D. thesis, Oregon State Univ., Corvallis, Ore.). The addition of new polypeptides during sporulation, however, may change the conformation of a rifampicin (or lipiarmycin)-RNA polymerase complex in such a way so that MS nucleotide accumulation is not affected.

Since changes are also believed to occur in the ribosomes during sporulation (4,10,13,15), the possibility cannot be ignored that sporulation ribosomes are immune to the effects of a drug-RNA polymerase complex. (Assuming that the drug-induced MS nucleotide accumulation in vegetative cells takes place in the ribosomes). However, MS nucleotides accumulated soon after t_o in response to glucose

deprivation, so that at that point in the growth cycle, cells are perfectly capable of generating these compounds.

The appearance of ppGpp soon after t_o is similar to the results obtained by Rhaese et al. (11), and presumably occurs in response to glucose deprivation, a general signal for the onset of sporulation. A noticeable difference in MS nucleotide accumulation between vegetative and sporulating cells is that ppGpp is by far in excess of any pppGpp which accumulates at t_o or later (due to either drug addition or glucose deprivation). During vegetative growth, however, amino-acid starvation or the addition of rifampicin/lipiarmycin results in a greater accumulation of the pentaphosphate than the tetraphosphate. Perhaps this reflects a change in the polymerase, the ribosomes, or ribosome-associated proteins that occurs with sporulation. Another explanation for the difference in the relative levels of ppGpp and pppGpp in vegetative versus sporulating cells is that this may be due to a difference in membrane structure or membrane-bound enzymes present during the two stages of growth. It has been shown in E. coli the the spo T reaction (ppGpp→ppG+X) is membrane-bound (1) and that high ionic conditions promote ppGpp accumulation without pppGpp (6).

The HPNs described by Rhaese et al. (11,12) were not detected in the B. subtilis extracts prepared by the methods described here.

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IV. A *Bacillus subtilis* Mutant which Exhibits Temperature-sensitive RNA Synthesis

Introduction

The dual role of RNA polymerase in both the synthesis of RNA and the regulation of transcription has been well established. This regulation appears to be an intricate interaction between different classes of promoters, an RNA polymerase molecule that may itself have at least two conformations (20) and a number of effector molecules that seem to bind either the promotor or the RNA polymerase. There is also evidence suggesting further interrelationships between RNA polymerase and components of the translational system (reviewed in 20). Mutant strains which exhibit altered patterns of RNA synthesis, therefore, may have defects in one of a number of different regulatory components.

Mutants characterized so far that show defective RNA synthesis include those with an altered RNA polymerase; mutants in all four holoenzyme subunits have been identified in *E. coli* (7,8,10,13,15,19, 21) while only β or β′ mutations have been found in *B. subtilis* (5,6,17). Relaxed strains of both *E. coli* and *B. subtilis* have also been described (2,18). Thiostrepton-resistant mutants of *B. subtilis*, which appear to have a defect in the ribosomes, exhibit relaxed RNA synthesis (16), and a temperature-sensitive EF-G mutant increases RNA synthesis in the presence of (p)ppGpp at the restrictive temperature (11).

This report describes a B. subtilis mutant displaying temperature-sensitive RNA synthesis which has been genetically mapped at a locus distinct from either the β or β′ subunit of RNA polymerase. This strain, OSB 459, rapidly shuts off both stable and unstable RNA synthesis after shift to the restrictive temperature of 49°C. At 45°C, a semi-permissive temperature, this mutant undergoes changes in macromolecular synthesis resembling the stringent response. For this reason, the accumulation of MS nucleotides under restrictive conditions was analyzed. A transient burst of (p)ppGpp after temperature upshift was shown to occur, similar to wild-type (Price and Brown, manuscript submitted), although this accumulation appeared to be higher than the wild-type at 45° or 49°C and less at 51°C. It is not thought that the accumulation of MS nucleotides per se, is the primary cause of defective RNA synthesis, for reasons which will be discussed.

It has been shown that when rifampicin or lipiarmycin is added to wild-type B. subtilis a large, transient increase in MS nucleotide levels follows (Price and Brown, manuscript submitted). Due to this response to RNA polymerase inhibitors, the possibility was brought forth that RNA polymerase itself may be involved in the accumulation of these compounds. Since this mutant displayed both temperature-sensitive RNA synthesis and enhanced accumulation of MS nucleotides at semi-permissive temperatures, its ability to accumulate MS nucleotides in response to rifampicin and lipiarmycin was of interest. Results of this experiment showed a large overaccumulation of MS nucleotides in the mutant, at the permissive temperature, with either

rifampicin or lipiarmycin. For this reason, it is thought that the temperature sensitivity in OSB 459 may lie in either the σ or α subunit of RNA polymerase, although other possibilities cannot yet be ruled out.

Materials and Methods

Bacterial strains and bacteriophage

Bacillus subtilis OSB 158 has been described (9) and B. subtilis OSB 459 is a temperature-sensitive strain obtained from OSB 158 by mutagenesis, as described below. Bacteriophage Tsp-1 was obtained from La Montagne and McDonald and was propagated by their procedures (12). Spore preparation was similar to that described by Haworth and Brown (9). It was shown that Mn^{++} concentrations higher than those previously described should be avoided since the ability to mutagenize spores grown in high Mn^{++} concentrations is greatly reduced.

Mutagenesis and Phage Selection

Purified Bacillus subtilis spores, 1 x 10^8/ml from OSB 158, were diluted into sterile 0.2M Tris (hydroxymethyl) aminomethane (Tris) buffer pH 8.3 and equilibrated at 37°C in a water bath shaker. Ethylmethanesulfonate (EMS) was added at 0.2% and allowed to incubate with the spores for 30 minutes. The spores were centrifuged 332xg for 15 min, washed twice with 4 volumes of cold Tris buffer and resuspended in penassay broth (PAB) (Difco) + 0.1% glucose at a concentration of 2 x 10^7 spores/ml. Aliquots (30-50 in different experiments) were placed in small sterile tubes, incubated in a reciprocal shaker at 37°C for 3.5 h and 1 ml of TSP-1 phage (M.O.I. of 3) with

1 ml of fresh PAB added. The cultures were shifted to a 50°C incubator and shaken for 6-12 hr. until the culture lysed. The tubes were then placed at 37°C and allowed to incubate for an additional 2 hr. Each of the tubes was sampled and plated at 37°C on PAB agar (PAB + 1.5% agar). After overnight incubation at 37°C, the surviving clones were picked and tested for the ability to grow on PAB plates at 59°C. Temperature-sensitive mutants were then screened. Only one mutant clone from each original tube was kept to insure mutant of independent origin.

Incorporation of label into macromolecules

RNA, DNA, and protein synthesis were monitored by the incorporation of [5-^{3}H]uridine, [6-^{3}H]uridine, and [U-^{14}C]protein hydrolysate respectively, into acid precipitable material. Radioactive precursors were obtained from Amersham. Experiments determining net RNA and protein synthesis, and pulse-labeling of RNA were done as described in (Price and Brown, manuscript submitted) except the media used was that of Anagnostopoulos and Spizizen (1) supplemented with 0.05% casamino acids, 0.5% glucose, and 0.8% glutamic acid. Ribonuclease-resistant, TCA precipitable counts obtained from [6-^{3}H]uridine incorporation were assumed to be an indication of DNA synthesis. Incorporation experiments were done as described above using a final concentration of 1 μCi/ml [6-^{3}H]uridine and 40 μg/ml cold carrier uridine. Duplicate samples were removed for TCA precipitation one of which was treated with RNase as follows: neutralization was achieved by adding 0.3 mls of 1 M NaOH, RNase was added to a final

concentration of 45 μg/ml, and the samples were then made 10% in TCA by the addition of 50% TCA before filtering.

Extraction and quantiation of (p)ppGpp

Nucleotides were labeled by growing cultures in [^{32}P] orthophosphate (NEN)-containing media as described in (Price and Brown, manuscript submitted). Nucleotides were then formic acid extracted and separated by thin-layer chromatography on Brinkman polyethyleneimine TLC plates as described in (4).

Results

Growth

The wild-type strain, OSB 158, grew exponentially in PAB with generation times of 27 min at 37°C, 25 min. at 45°C, and 21 min. at 49°C. (Fig. 1 shows growth of OSB 158 at 37°C with a shift to 45°C as an example). The mutant strain, OSB 459 grew in the same medium at 37°C with a 33 min. generation time. When a log-phase culture of OSB 459 at 37°C was shifted to 45°C, turbidity increased at the pre-shift rate for approximately 20 min. then slowed to a 50 min. generation time (Fig. 1). The viable count, as determined by colony-forming ability, also continued to increase at 45°C, indicating that the cells were dividing (data not shown). The maximum optical density reached by cultures grown at 45°C was also less than that of cultures grown at 37°C. When OSB 459 was shifted to 49°C or 51°C, no growth occurred (data not shown). The optimum temperature for growth of OSB 459 lies within a narrow range of 35°-37°C, with growth ceasing at 49°C.

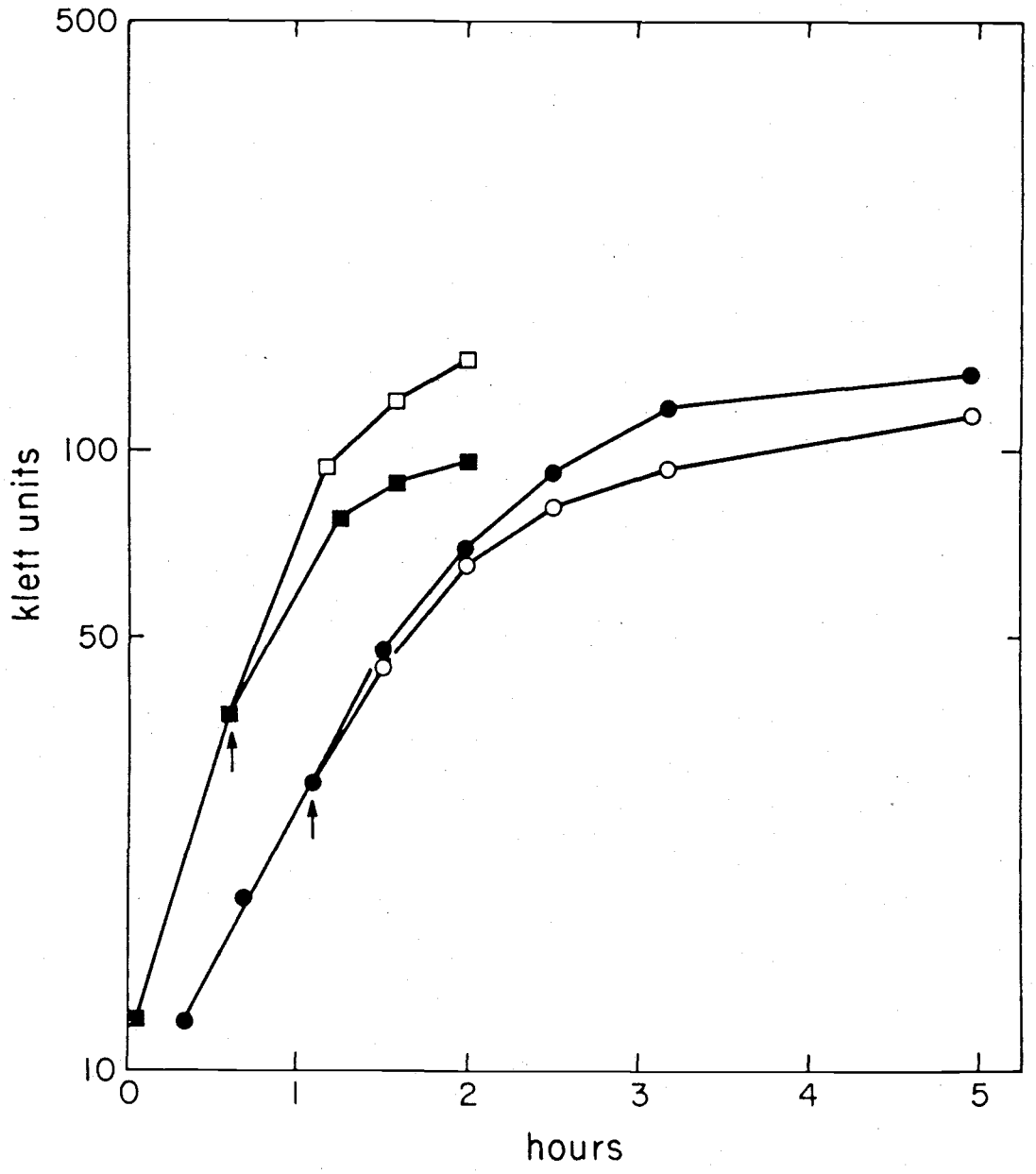

Fig. 1. Growth of OSB 158 (squares) and mutant OSB 459 (circles) in PAB. At times indicated by arrows, a portion of each culture was shifted from 37°C (closed symbols) to 45°C (open symbols).

Wild-type cells grew optimally over a much broader temperature range : 37°-49°C, and growth did not cease until 54°C was reached (data not shown).

The ability of OSB 459 to form spores was determined by counting spores under a phase-contrast microscope. At the permissive temperature, about 40% of the mutant culture formed spores after 48 hrs. By comparison, 80% of the parental, wild-type strain sporulated under the same conditions. After incubation at 45°C, sporulation did not occur in OSB 459, as it did in OSB 158.

RNA synthesis

The incorporation of [5-^{3}H]uridine into acid insoluble material was used to measure RNA accumulation. Cultures of OSB 158 showed little, if any, change in net RNA accumulation after shift from 37°C to 45°C (Fig. 2B). When a culture of OSB 459 was shifted from 37°C to a higher temperature, [5-^{3}H]uridine incorporation decreased to 76% at 41°C, 6-8% at 45°C, and ceased at 49°C (Fig. 2A). A culture of OSB 459 which was shifted back to the permissive temperature after 3 min. at 45°C resumed RNA accumulation 6-8 min. later. After 30 min. at 45°C, then downshift to 37°C, recovery was much slower (ca. 20 min.) (data not shown).

Pulse-labeling of RNA in OSB 459 after shift to 45°C showed that the amount of labeled precursor incorporated in a 2 min. pulse was greatly reduced at the restrictive temperature. (The limitations of pulse-labeling as done in these experiments have been discussed (Price and Brown, manuscript submitted)). During 2 min. pulses after

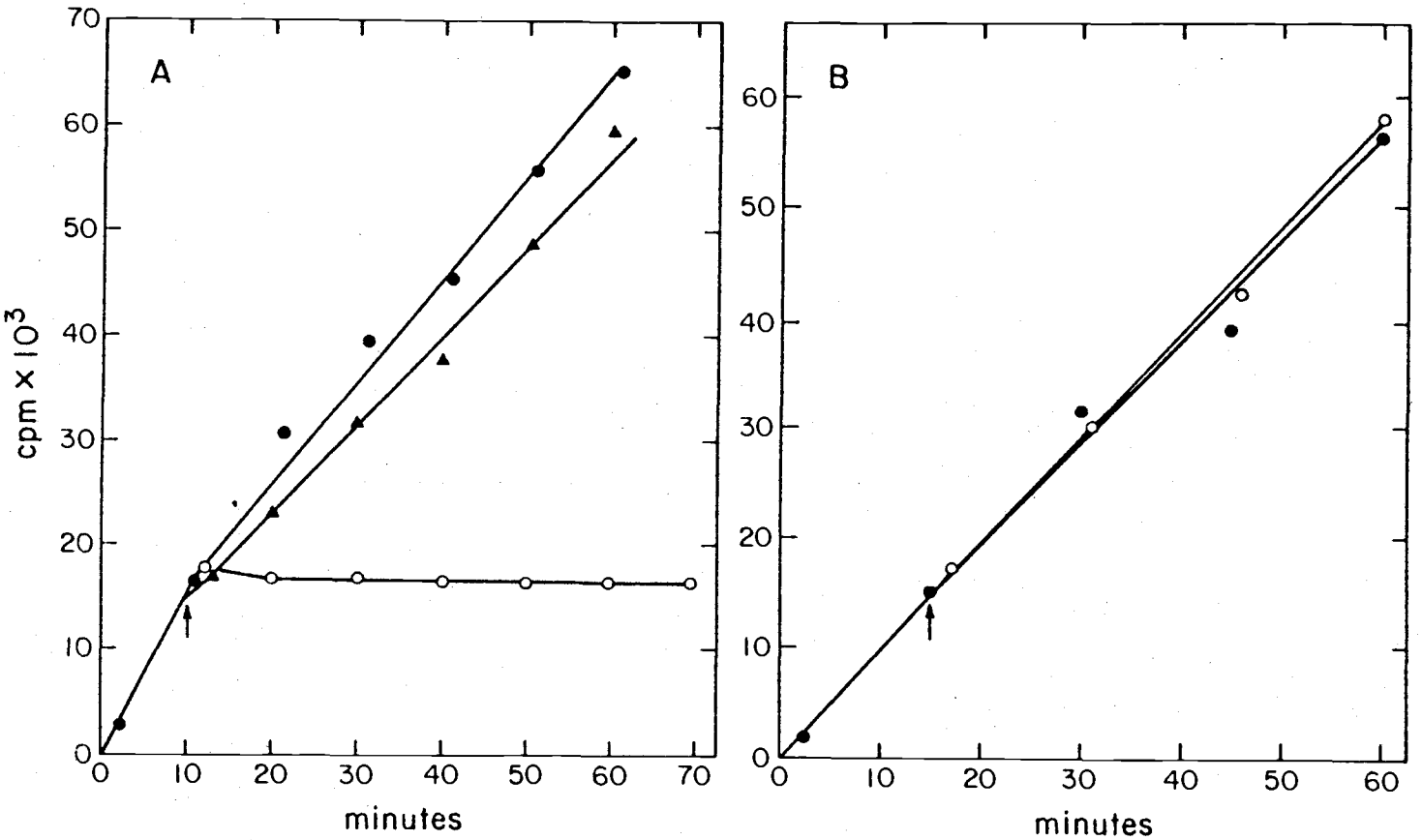

Fig. 2A. RNA accumulation after temperature upshift in OSB 158. The incorporation of [5-^{3}H]uridine was done as described in Materials and Methods. At the time indicated by the arrow, a portion of the culture was shifted from 37°C (closed circles) to 45°C (open circles).

B. RNA accumulation in OSB 459. Portions of the culture were shifted from 37°C (closed circles) to 41°C (triangles) or 45°C (open circles).

shifting a culture from 37°C to 45°C, there was an initial drop in the amount of pulse-labeled RNA accumulated, followed by partial recovery after about 20 min. (Fig. 3B). The partial recovery in pulse-labeled RNA amounted to 10-30% of the pre-shift level; a value which varied somewhat from one experiment to another. It was thought that since 45°C is close to the fully restrictive temperature for this strain, any slight variation in temperature from one experiment to another may have accounted for this. At 50°C, pulse-labeling under the same conditions showed no incorporation of label.

Wild-type B. subtilis undergoes a change in the incorporation of labeled RNA precursor in a 2 min. pulse upon temperature upshift as described in (Price and Brown, manuscript submitted). Fig. 3A lustrates this initial rapid increase followed by decrease to pre-shift levels.

Riva et al. (14) have described a B. subtilis temperature-sensitive RNA synthesis mutant in which stable and unstable RNA synthesis is rapidly shut off at 45°C. This temperature sensitivity was reversed by the addition of chloramphenicol. When a culture of OSB 459 was shifted to 45°C with the simultaneous addition of 150 µg/ml of chloramphenicol, a slight increase in net RNA synthesis was observed, but this could not be considered a reversal of the temperature-sensitivity (data not shown).

DNA and Protein Synthesis

The incorporation of [6-^{3}H]uridine into TCA precipitable counts followed by treatment with RNase was used to measure DNA synthesis.

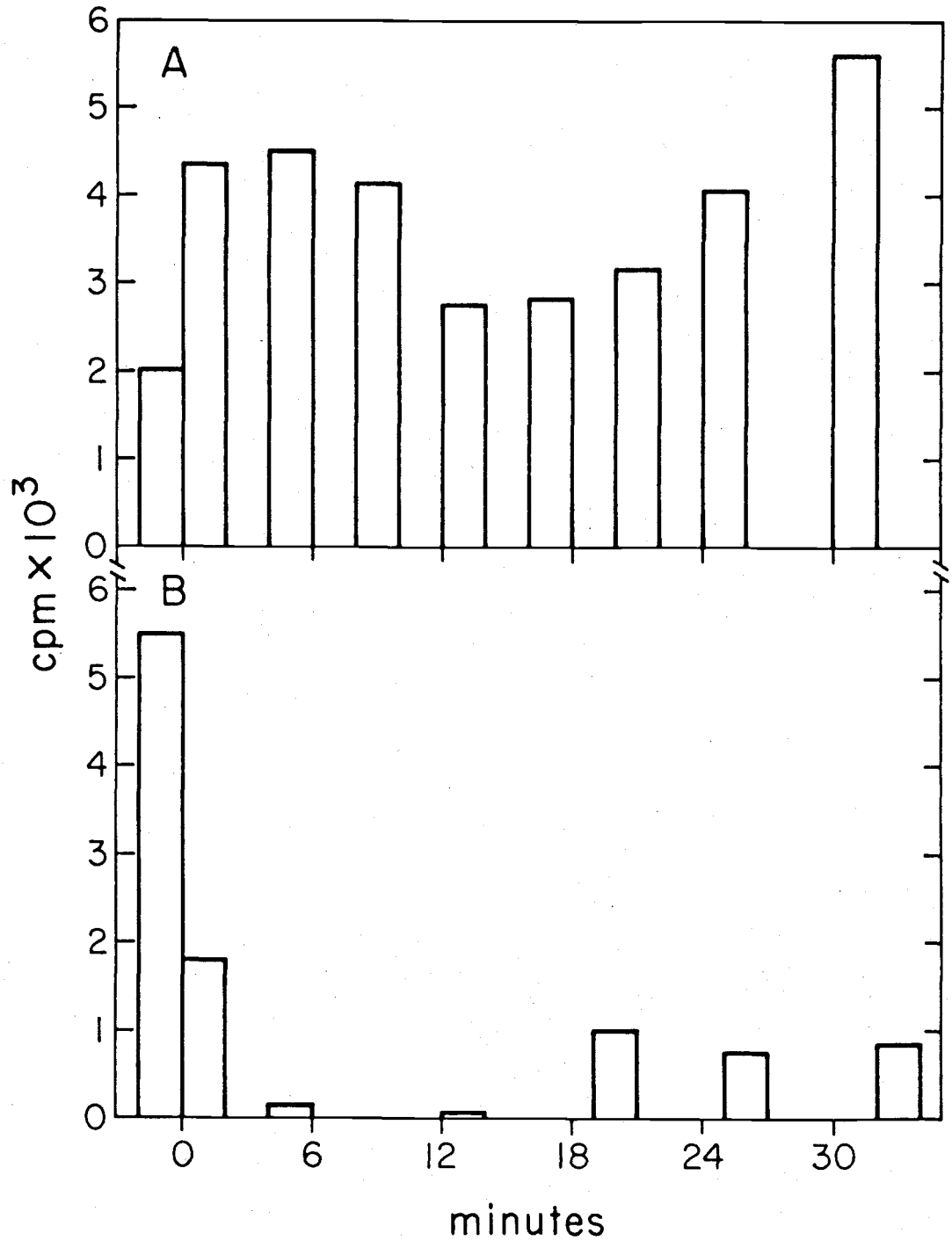

Fig. 3. Pulse-labeled RNA after temperature upshift was determined by incorporation of [5-^{3}H]uridine in a two minute pulse as described in Materials and Methods. At time zero, cultures were shifted from 37°C to 45°C. Squares represent OSB 158 and circles, OSB 459.

After shift to 45°C, OSB 459 continued to incorporate label for 15 min., then reduced uptake to about 55% of that at 37°C. Since [6-^{3}H]uridine continued to be incorporated to a greater extent than [5-^{3}H]uridine at 45°C, the large decrease in RNA synthesis observed was not likely due to the failure of exogenous uridine to enter the cell, (data not shown).

Incorporation of [6-^{3}H]uridine into OSB 158 DNA was 1 1/2 times more at 45°C than at 37°C.

Protein synthesis in the temperature-sensitive strain, monitored by the incorporation of [U-^{14}C]protein hydrolysate into acid insoluble material, continued for about 10 min. after shift from 35°C to 45°C, then slowed considerably (data not shown). Cultures of OSB 158 incorporated greater amounts of [U-^{14}C]protein hydrolysate at 45°C than at 37°C

Genetic Analysis

Genetic analysis was performed by Dr. James Hoch. Transformations with OSB 459 as donor and CU 479 (*ctr* A1, *Crp* c2) as recipient resulted in 69 *ctr*$^{+}$ transformants, 51 of which also gained temperature sensitivity. In the reciprocal cross, with OSB 459 as recipient and CU 479 as donor, 138 out of 180 temperature-resistant transformants were *ctr*$^{-}$. These results show that the temperature-sensitive locus is closely linked to *ctr* A1.

Effect of temperature increase on (p)ppGpp accumulation

Since the response of OSB 459 to temperature upshift to 45°C showed phenotypic changes similar to those which occur during the

stringent response, it was of interest to analyze the MS nucleotides from this mutant after temperature upshift. Wild-type B. subtilis demonstrates a transient rise in MS nucleotide levels upon temperature upshift which has been described (Price and Brown, manuscript submitted). During this accumulation, there is a rapid rise in (p)ppGpp which reaches a maximum 3-4 min after temperature increase then decreases to basal levels by 30 min. OSB 459 shows a similar rise in MS nucleotide levels after temperature upshift, with identical kinetics of accumulation. Fig. 4 shows a plot of temperature versus (p)ppGpp accumulated 3-4 min. after temperature upshift for the two strains. The levels of (p)ppGpp reached in wild-type cultures increases with increasing temperature, as it does in OSB 459, until the fully restrictive temperatures over 49°C are reached. The accumulation of (p)ppGpp in OSB 459 at elevated temperatures of 45°C (semi-permissive) and 49°C are higher than in OSB 158 at these temperatures, as though it accumulates these compounds more readily upon temperature increase. At 51°C, though, OSB 459 does not accumulate much (p)ppGpp and the wild-type strain accumulates levels comparable to those reached in the mutant at 49°C.

Pretreatment of the wild-type strain with streptolydigin (100 µg/ml) for 5 min. before temperature upshift greatly reduced the temperature effect on (p)ppGpp accumulation. When OSB 459 was treated similarly, MS nucleotide accumulation after temperature upshift was not reduced, but was slightly enhanced (data not shown).

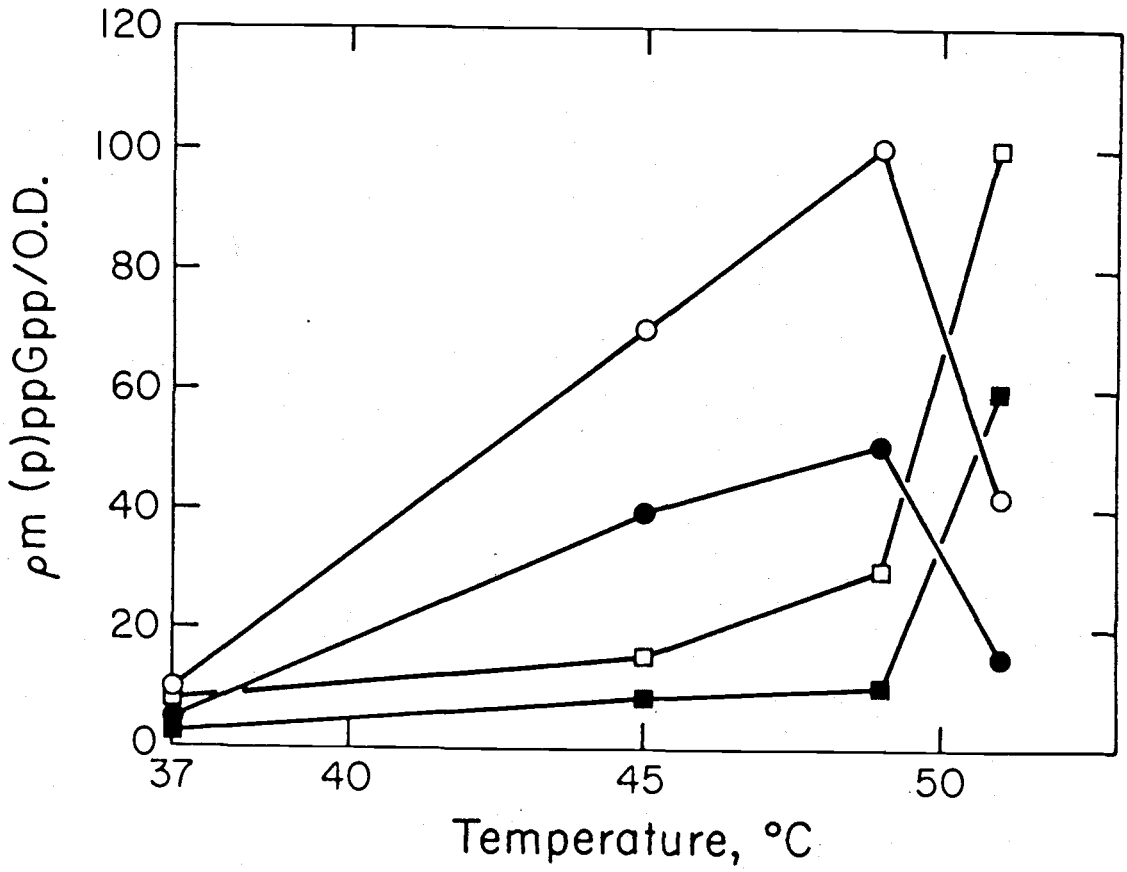

Fig. 4. Accumulation of (p)ppGpp after upshift to various temperatures. Levels represent maximum amounts of pppGpp (open symbols) and ppGpp (closed symbols) reached after shift from 37°C to the higher temperature for 3 min. Squares represent OSB 158 and circles, OSB 459.

Accumulation of (p)ppGpp after the addition of rifampicin or lipiarmycin

When rifampicin or lipiarmycin was added to OSB 459 at the permissive temperature, very high levels of (p)ppGpp accumulated, severalfold higher than occurred in the parental wild-type strain under these conditions. Fig. 5A shows also that the kinetics of accumulation of (p)ppGpp after the addition of rifampicin is slightly different than in OSB 158. MS nucleotides continue to build up for at least 12 min. after rifampicin is added to the mutant, while levels increase for only about 7 min. in OSB 158. Rifampicin-induced (p)ppGpp accumulation did not take place in the mutant at 49°C (data not shown). The response to lipiarmycin addition is also greatly enhanced over wild-type levels but with similar kinetics (Fig. 5B).

Pretreatment of both wild-type and mutant strain with streptolydigin, as described in the previous section, eliminated the build up of MS nucleotides in response to both rifampicin and lipiarmycin at 37°C (data not shown).

Revertant Analysis

The mutant demonstrated a reversion frequency of 10^{-6}, consistent with it being a point mutation.

A spontaneous revertant of OSB 459, OSB 459r, was analyzed for its ability to incorporate [5-^{3}H]uridine into TCA precipitable material at 49°C and to accumulate MS nucleotides in response to temperature upshift and rifampicin or lipiarmycin addition. The revertant strain continued to accumulate RNA at 49°C and showed a profile of pulse-labeled RNA similar to wild-type after temperature

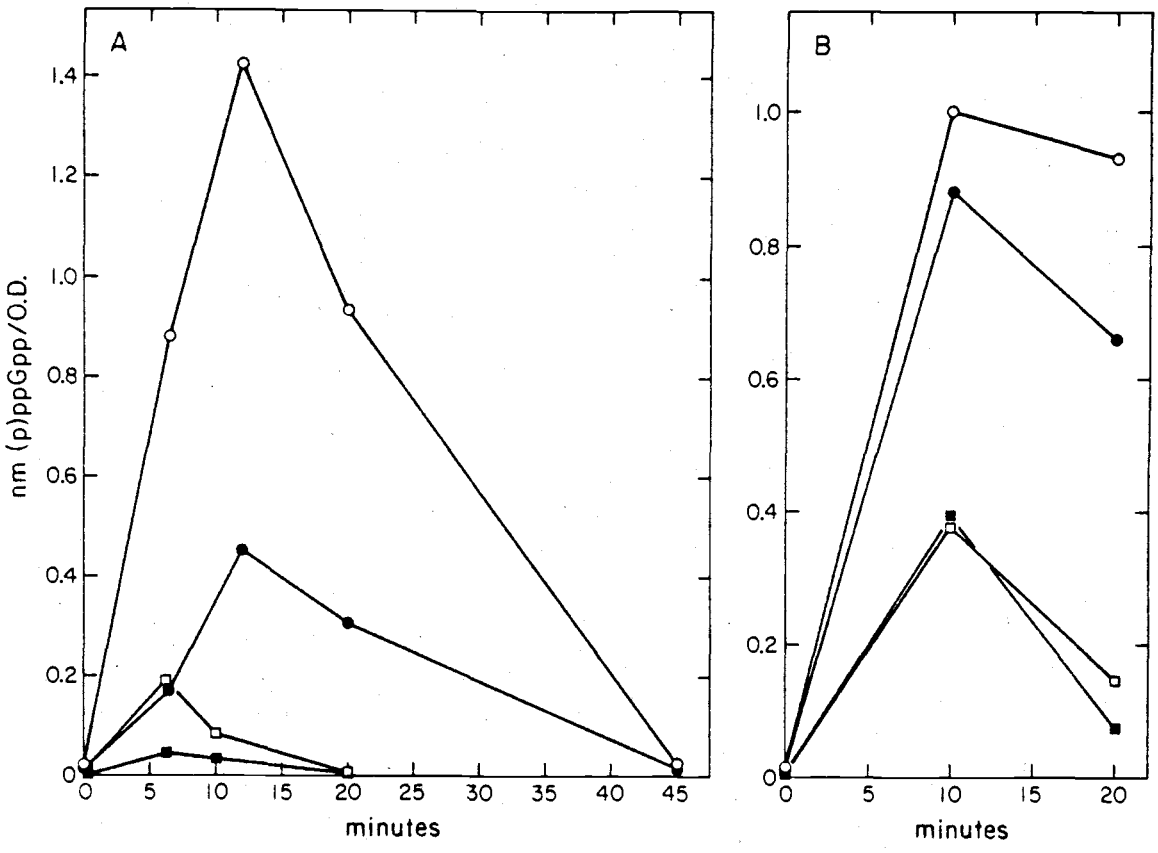

Fig. 5. Accumulation of (p)ppGpp in OSB 158 (squares) and OSB 459 (circles) after the addition of RNA polymerase inhibitors. A., rifampicin or B., lipiarmycin were added at time zero and cultures were assayed for pppGpp (open symbols) and ppGpp (closed symbols) at the times indicated.

upshift. The accumulation of MS nucleotides in response to both temperature increase and rifampicin or lipiarmycin was also similar to that of the wild-type strain (data not shown).

Discussion

The temperature-sensitive mutant, OSB 459, contains a defect which seems primarily to affect both stable and unstable RNA synthesis, as this aspect of macromolecular synthesis is most rapidly affected by temperature increase. At the semi-permissive temperature of 45°C, the mutant undergoes changes in RNA, DNA, and protein synthesis that appear to mimic the stringent response; e.g. RNA accumulation is immediately curtailed followed by a slowing of DNA and protein synthesis after several minutes. There is difficulty in sorting out the response to temperature upshift in this situation, however. The effects of temperature upshift involve changes in RNA synthesis and MS nucleotide levels in wild-type cells; this becomes superimposed on the altered responses of a mutant that demonstrates temperature-sensitive RNA synthesis.

The addition of rifampicin or lipiarmycin to vegetative B. subtilis is followed by an accumulation of (p)ppGpp (Price and Brown, manuscript submitted). At the permissive temperature, OSB 459 exhibits a greatly exaggerated response in the accumulation of (p)ppGpp under these conditions. The lesion causing temperature-sensitivity also appears to be responsible for the increased accumulation of MS nucleotides at the permissive temperature : A revertant of OSB 459, 459r, simultaneously regains the ability to synthesize RNA at 49°C, and shows accumulation of MS nucleotides after the addition of rifampicin or

lipiarmycin, or temperature upshift similar to wild-type. Another interesting aspect of the accumulation of (p)ppGpp in the mutant, which may be related to its temperature-sensitvity, is that pre-treatment with streptolydigin for 5 min. at the permissive temperature prevents the rifampicin/lipiarmycin-induced (p)ppGpp accumulation, as it does in wild-type cells. However, when OSB 459 is pretreated with streptolydigin at 37°C then shifted to 49°C, the accumulation of (p)ppGpp which follows temperature upshift was not prevented, as it was in the wild-type strain under similar conditions.

The results obtained with this mutant suggest that the temperature-sensitive lesion most probably lies in either the RNA polymerase itself, (other than in the β or β´ subunits, in view of the genetic mapping data) or a component of the translation system which interacts with the transcription apparatus. Ribosome-associated factors or ribosomal proteins altered in such a way as to overproduce MS nucleotides under certain conditions is one possibly for the exaggerated response to RNA polymerase inhibitors. This assumes that the rifampicin/lipiarmycin-induced accumulation of (p)ppGpp is due to increased synthesis of these compounds rather than a lack of degradation, though. Also, since the levels of (p)ppGpp obtained after temperature upshift do not seem to be sufficient to curtail RNA synthesis per se (for reasons to be discussed), a temperature-sensitive component of the translation system would have to some way interfere with RNA synthesis directly.

The other possibility mentioned, that the σ or α subunit of RNA polymerase (or perhaps another, unidentified polypeptide associated with RNA polymerase) is temperature-sensitive, will be considered in more detail.

Both the wild-type strain and the temperature-sensitive mutant exhibit increased levels of (p)ppGpp shortly after temperature upshift. From the graph of temperature versus (p)ppGpp accumulation for both strains, (Fig. 4) two aspects of this temperature-effect can be seen that distinguish mutant from wild-type: 1.) the temperature sensitive strain attains higher levels of (p)ppGpp when shifted to 45°C or 49°C, as though this response is also more temperature sensitive, and 2.) at the restrictive temperature of 51°C, the mutant begins to loose its ability to accumulate MS nucleotides in response to temperature upshift. The wild type strain, on the other hand, accumulates levels of (p)ppGpp at 51°C comparable to those reached in OSB 459 at 49°C, without adverse effect on RNA synthesis. These responses are not surprising if one takes the point of view that the temperature sensitive component in OSB 459 is also involved in the accumulation of MS nucleotides, as was discussed above. An important piece of information obtained from these results is that the levels of MS nucleotides attained in the mutant at 49°C are not significantly higher than those reached in the wild-type strain at 51°C. Therefore, it is not likely that the increased accumulation of (p)ppGpp in the mutant at semi-permissive temperatures is alone responsible for the curtailment of RNA synthesis. If the presence of (p)ppGpp in the mutant after temperature upshift contributes to the shutoff of RNA synthesis, a possible

reason could be increased sensitivity to these compounds. In turn, the most likely reason for increased sensitivity to (p)ppGpp would be an altered RNA polymerase. (Which, of course, would alter RNA synthesis itself). A possible role of RNA polymerase in the accumulation of MS nucleotides has already been presented (Price and Brown, manuscript submitted), in light of the accumulation of these compounds after rifampicin or lipiarmycin addition. If the temperature-sensitive component in OSB 459 was RNA polymerase, we suggest that the σ subunit may contain the defect, based on the following pieces of information: the σ subunit has been implicated in the accumulation of (p)ppGpp after addition of rifampicin or lipiarmycin, since this response is lost in sporulating cells (discussed in Price and Brown, manuscript submitted). The alt 1 mutation in E. coli (14), shown to affect the σ subunit by Travers et al. (21), shows altered patterns of RNA synthesis at the restrictive temperature (although sRNA is only slowly affected). Additionally, it shows an altered response of rRNA synthesis to low concentrations of ppGpp : at low (< 10mM) concentrations of ppGpp, wild-type E. coli RNA polymerase is stimulated to synthesize rRNA. The alt-1 mutant does not show this response. This illustrates that, at least in E. coli, a functional relationship between σ and ppGpp takes place. A temperature-sensitive σ mutant in E. coli described by Harris et al. (7) showed an abrupt decrease in pulse-labeled RNA synthesis upon temperature increase with DNA synthesis being affected more slowly. In addition, Riva et al. (14) have isolated a ts B subtilis mutant which they suggest produces an inhibitor of σ (such as is postulated to be

active during sporulation) with nearly identical responses to restrictive conditions with regards to RNA, DNA, and protein synthesis as OSB 459. Their mutant maps in the ctr A region, although distant from OSB 459, and the block in RNA synthesis is released by addition of chloramphenicol, which does not occur in OSB 459. If the mutation were in σ itself rather than a protein inhibitor, however, repression by chloramphenicol would not be expected.

The rapid shutoff of RNA synthesis in a σ mutant could be due either to the altered σ subunit entirely or perhaps to the combined efforts of altered σ subunit along with an increased sensitivity to (p)ppGpp, which accumulates transiently after temperature upshift. The levels of MS nucleotides reached in response to temperature increase, although not affecting wild-type RNA synthesis significantly, may be capable of interfering with a hypersensitive polymerase. This would provide an explanation for OSB 459 mimicking a stringent response upon temperature upshift.

Chakrabarti and Gorini(3) hypothesized a similar mechanism as a possible explanation for the temperature-sensitivity in a combined rif and str A E. coli mutant which, individually rif or str A, is temperature-insensitive. However, their mutant did not display rapid shutoff of stable RNA, expected if a stringent response was "activated" by an altered ribosome producing ppGpp and a hypersensitive RNA polymerase. They felt that the rif-str A interaction might mean a mechanical coupling of ribosomes and RNA polymerase for optimum transcription, and that altered configurations resulting from the rif and str A lesions at the restrictive temperature prevented this coupling.

Evidence that the temperature-sensitive lesion is OSB 459 lies in the RNA polymerase σ subunit, although suggestive, is by no means conclusive; if the RNA polymerase enzyme is temperature-sensitive, the α subunit must also be considered. If σ or α were the temperature-sensitive component, and therefore most likely involved in the generation of MS nucleotides, a situation would exist where the RNA polymerase molecule is involved in the synthesis (or induction) of a compound that is thought to be one of its effectors. It must be considered, however, that several differences have been shown to exist between the RNA polymerases and the so-called stringent control systems between E. coli and B. subtilis. Although (p)ppGpp has been shown to interact with E. coli RNA polymerase to alter template specificity, this same function has, so far, only been extrapolated to B. subtilis. The possibility that (p)ppGpp interacts differently in B. subtilis cannot be eliminated. This possibility does not necessarily detract from the model for a temperature-sensitive RNA polymerase, though.

A large part of the argument for a temperature-sensitive RNA polymerase rests on the previously described results that it is the RNA polymerase molecule itself that causes MS nucleotide accumulation in the presence of rifampicin or lipiarmycin (Price and Brown, manuscript submitted). Perhaps it would be more appropriate to conclude that, whatever mechanism elicits MS nucleotide accumulation in response to rifampicin/lipiarmycin inhibition, also appears to be the source of temperature-sensitivity in OSB 459.

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