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Lack of Production of (p)ppGpp in *Halobacterium volcanii* under Conditions That Are Effective in the Eubacteria

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The stringent halobacterial strain *Haloferax volcanii* was subjected to a set of physiological conditions different from amino acid starvation that are known to cause production of guanosine polyphosphates [(p)ppGpp] in eubacteria via the *relA*-independent (*spoT*) pathway. The conditions used were temperature upshift, treatment with cyanide, and total starvation. Under none of these conditions were detectable levels of (p)ppGpp observed. This result, in conjunction with our previous finding that (p)ppGpp synthesis does not occur under amino acid starvation, leads to the conclusion that in halobacteria both growth rate control and stringency are probably governed by mechanisms that operate in the absence of ppGpp. During exponential growth, a low level of phosphorylated compounds with electrophoretic mobilities similar, but not identical, to that of (p)ppGpp were observed. The intracellular concentration of these compounds increased considerably during the stationary phase of growth and with all of the treatments used. The compounds were identified as short-chain polyphosphates identical to those found under similar conditions in *Saccharomyces cerevisiae*.

In 1969, Cashel and Gallant reported that wild-type *Escherichia coli* is capable of producing two unusual compounds originally named magic spots I and II and later identified as guanosine tetra- and pentaphosphates, respectively [collectively referred to as (p)ppGpp] (3). These guanosine nucleotides are generated in a *relA*-dependent manner by the *relA* gene product, (p)ppGpp synthetase I, and in a *relA*-independent manner by the *spoT* gene product, the SpoT enzyme, which is also implicated in (p)ppGpp degradation. Kinetic studies have shown that pppGpp is formed from GTP, that ppGpp is derived from pppGpp, and that pppGpp inhibits its own synthesis (12).

In eubacteria, the rate of stable RNA synthesis at different growth rates is subject to a control mechanism that is distinct from that of other cellular molecules (growth rate control of RNA synthesis). An inverse correlation between the ppGpp concentration, produced in this case by the *relA*-independent pathway, and the rate of stable RNA synthesis was observed at a wide range of different growth rates (5), which suggested that ppGpp could act as an effector molecule negatively regulating stable RNA synthesis in growth rate control. The isolation by Xiao et al. (21) of *relA spoT* mutants unable to produce ppGpp permitted Gaal and Gourse (8) to show that ppGpp either is not the mediator or does not act alone, since growth rate control is normal in the double mutants. Hernandez and Bremer have recently shown that in wild-type *E. coli*, three factors increase with the growth rate: RNA polymerase concentration, RNA polymerase activity, and the distribution of active RNA polymerase between stable and mRNA genes. In an *E. coli*Δ*relA*Δ*spoT* mutant that does not produce ppGpp, RNA polymerase synthesis and activity increase with the growth rate but the distribution of active RNA polymerase between stable and mRNA genes does not. This is due to the absence of a mechanism that causes a slowing down of mRNA, but not rRNA, synthesis as the growth rate of the cells increases (11).

It has been shown that in eubacteria, ppGpp is involved in stringent control, a pleiotropic cellular response to amino acid starvation (5, 17). In a previous analysis (6), we showed that in halobacteria, a subdivision of the archaea, the level of guanosine tetraphosphate does not rise to detectable levels as a result of amino acid starvation, thus strongly suggesting that in these organisms the *relA*-dependent pathway for ppGpp production is absent. In the wild, these organisms can have either the stringent or the relaxed phenotype, and therefore when the stringent reaction is present, it is not mediated by ppGpp. However, low basal levels of compounds with chromatographic mobilities very similar to that of (p)ppGpp were always observed (6). If these compounds are (p)ppGpp, they must be produced by an alternate pathway, analogous to the *relA*-independent pathway described in *E. coli*. If ppGpp were indeed produced by such a mechanism, it could be involved in other aspects of halobacterial cell physiology, such as growth rate control.

In the present study, we attempted to cause (p)ppGpp production in the stringent halobacterial strain *Haloferax volcanii* by means of changes in the physiological conditions that are known to provoke an increase in the levels of guanosine polyphosphates in eubacteria. First, we examined (p)ppGpp production of exponentially growing cultures of *H. volcanii* after a temperature upshift: it is known that temperature upshift in *E. coli* results in changes in the intracellular concentration of (p)ppGpp levels in the absence of restriction of stable RNA synthesis (9). In a second series of experiments, we added potassium cyanide to the halobacterial cultures: in eubacteria, treatment with KCN results in levels of ppGpp that are even higher than those obtained with classical amino acid starvation. Since cyanide interferes with all energy-dependent reactions in the cell, it is likely that this compound activates the *relA*-independent (p)ppGpp pathway via carbon or energy source starvation (7). Finally, we examined guanosine polyphosphate production in *H. volcanii* after elimination of all nutrients from the growth medium (total starvation).

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *H. volcanii* 3757 was obtained from the Deutsche Sammlung von Mikroorganismen (Göttingen, Germany). *Salmonella typhimurium* TA997 (*aroC5 purF145 hisD2655*) was obtained from R. Cortese (IRBM, Pomezia, Italy). *Saccharomyces cerevisiae* SC was obtained from M. Polsinelli. *H. volcanii* was grown in salts medium (SM2) consisting of 19.5%

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NaCl, 6.5% $MgCl_2$, 4.1% $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2 \cdot 2H_2O$, 0.05% KCl, 0.02% $NaHCO_3$, 0.06% Nabromide, and 0.25% yeast extract. *S. typhimurium* was grown in low-salt medium (0.25% yeast extract, 1% NaCl) at 37°C. *H. volcanii* was grown with shaking and illumination at various temperatures ranging from 28 to 56°C.

Sources of reagents. Nutrients and agar were furnished by Difco (Detroit, Mich.), and pseudomonic acid was furnished by SmithKline Beecham Pharmaceuticals (Worthing, United Kingdom). GTP, PP_i, tripolyphosphate ($3P_i$), and tetrapolyphosphate ($4P_i$), used for colorimetric determinations, were from Sigma, St. Louis, Mo. All other chemicals were obtained from Merck (Darmstadt, Germany). [^{3}H]threonine was from New England Nuclear (Du Pont de Nemours, Firenze, Italy). [^{14}C]uridine and $^{32}P_i$ were from Amersham (Amersham, United Kingdom).

Synthesis of (p)ppGpp under a variety of physiological conditions. (i) Temperature upshifts. The two temperatures used were a low temperature of 31°C, which permits sufficiently rapid exponential growth, and 48°C, which is 3°C above the optimal growth temperature of 45°C.

(ii) Inhibition with cyanide. Inhibition with cyanide was achieved by adding KCN to the cells to a final concentration of 4 mM.

(iii) Total starvation. Total starvation of the cells was obtained by centrifugation and resuspension of the cells in nutrient-free SM2. Both the culture and resuspension medium contained $^{32}P_i$ at the same specific activity (100 μCi/ml).

Assay for guanosine polyphosphates. Guanosine polyphosphate production was analyzed by one-dimensional chromatography by the method described by Cimmino et al. (6). This technique permits the detection, in a single spot, of about 5 ng of P per 100 mg (dry weight) of cells.

Identification of short-chain polyphosphates. Short-chain polyphosphates were analyzed either by one-dimensional chromatography as described in the preceding paragraph or by the two-dimensional chromatographic method described by Guerrini et al. (10), with some minor modifications. PP_i, $3P_i$, and $4P_i$ (0.5 μmol of each dissolved in 1 N formic acid) were applied to a chromatographic plate prepared as described above, together with 25 to 200 μl of the same ^{32}P-labeled samples as those employed for one-dimensional ascending chromatography and with 0.2 μmol of GTP. A sheet of filter paper 15 cm long was stapled to the upper edge of the chromatographic plates. The samples were eluted in the first dimension with 1.5 M NaH_2PO_4 (pH 3.5). During this time period, the migration of GTP was monitored with a UV lamp and elution was terminated when the GTP reached the upper edge of the plate. The time required for this was 7 to 8 h. Subsequently, the filter paper was removed and the plates were thoroughly washed in methanol for at least 15 min. Without drying, the plates were briefly washed in distilled water and then dried under a flow of cold air. After rotation for the second dimension, a second sheet of filter paper was applied to the top of the plates, chromatography was performed in 2.7 M ammonium formate containing 4.2% boric acid, and the pH was adjusted to 7 with NH_4OH. This chromatographic step was arrested when the GTP spot had migrated more than half of the distance to the top of the plate (9 to 12 h). By this procedure, the ammonium formate solution removes from the plates essentially all of the P_i used as the first solvent, which would interfere with the subsequent coloration step.

Coloration of polyphosphates. For coloration of polyphosphates, each plate obtained by two-dimensional chromatography was dipped into a solution consisting of ammonium molybdate (1 g) dissolved in water (8 ml) and perchloric acid (3 ml; about 70%) and diluted with acetone to 100 ml. After 1 to 2 min, the plates were allowed to dry for 7 to 8 min at 85°C. With this hydrolytic method (2) the P_is react with the ammonium molybdate, giving rise to coloration. The positions of some of the standards were visible at this stage, but to develop color fully, the plates were allowed to regain moisture from the air and then exposed to ammonia gas. At this stage, the positions of the polyphosphates appeared as blue cyan spots against a straw-yellow background. By comparing the two-dimensional chromatograms colored as described above with their companion autoradiograms and looking for coincident spots, it was possible to identify the polyphosphates synthesized by *H. volcanii.*

RESULTS

Synthesis of (p)ppGpp by *H. volcanii*. The phenotypically stringent halobacterial species *H. volcanii* (6) was examined for production of guanosine polyphosphates under physiological conditions known to provoke an increase in the levels of guanosine polyphosphates in eubacteria (7, 9). In a first experiment, the cultures were temperature upshifted as described in Materials and Methods and the search for (p)ppGpp was carried out over an extended time period (Fig. 1). Before the upshift, two weak, barely visible autoradiographic signals were observed whose positions appeared to be similar to those of pppGpp and ppGpp. The intensity of these signals increased 6 h after the upshift and became quite marked after 12 h, when the cells reached the end of the exponential phase. The signals

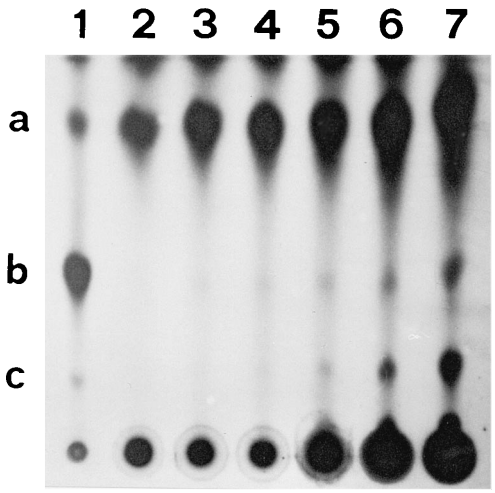

FIG. 1. Appearance of phosphorylated compounds in *H. volcanii* after a temperature upshift. Bacteria were labeled with $^{32}P_i$, and a formic extract was prepared and analyzed by ascending chromatography (8 h) as described in Materials and Methods. The locations of the nucleotides GTP (a), ppGpp (b), and pppGpp (c) were detected by autoradiography. Lanes: 1, control (*S. typhimurium* TA997) 5 min after addition of pseudomonic acid; 2, *H. volcanii* before the upshift; 3 to 7, *H. volcanii* 0.25, 1, 6, 12, and 24 h, respectively, after the upshift (48°C).

become manifest at times that are considerably later than what one would expect from studies on eubacterial production of (p)ppGpp, even if one takes into account the extremely long doubling times exhibited by *H. volcanii*. Furthermore, at these later times a number of additional spots appeared, at least one of which had barely moved away from the origin. Such signals could not be an effect of the temperature upshift for two reasons: their intensity did not increase appreciably immediately after the upshift, and control cultures maintained continuously at 31 and 48°C showed a similar set of spots that also appeared when the cells became nearly stationary.

In a subsequent experiment, KCN was added to an exponentially growing culture. Strong autoradiographic signals analogous to those seen in the previous experiment appeared, arising soon after KCN addition and persisting for the whole duration of the experiment. Subsequently (p)ppGpp production by *H. volcanii* was examined during total starvation by centrifuging the cells and resuspending them in a nutrient-free medium as described in Materials and Methods. The results were very similar to those obtained after the temperature shift: immediately after resuspension, there was no increase in the weak autoradiographic signals whereas strong signals in the same position were visible after 4 h and became stronger still after 14 h. Since this particular experimental procedure had never been used to cause (p)ppGpp production in eubacteria, a control experiment was performed with the stringent strain *S. typhimurium* TA997. As expected, large amounts of ppGpp were produced immediately after resuspension in the nutrient-free low-salt medium (data not shown).

To compare the migration of ppGpp and pppGpp with that of the spots obtained with *H. volcanii*, we carried out a careful chromatographic comparison of two samples: an *S. typhimurium* control sample and an *H. volcanii* sample containing high levels of the autoradiographic signals. The chromatographic run was prolonged from 8 to 33 h; at the end of the latter time period, the GTP had completely run onto the attached filter paper (see Materials and Methods). It became clear from the results of this experiment (shown in Fig. 2) that

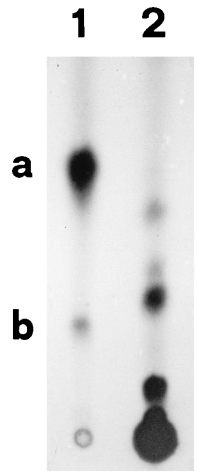

FIG. 2. Phosphorylated compounds in stationary-phase *H. volcanii*. Bacteria were labeled with $^{32}P_i$, and a formic extract was prepared and analyzed by ascending chromatography as described in Materials and Methods. Lanes: 1, control (*S. typhimurium*) 10 min after addition of pseudomonic acid (the locations of the nucleotides ppGpp (a) and pppGpp (b) were detected by autoradiography); 2, stationary-phase *H. volcanii*. The chromatographic run was prolonged to 33 h.

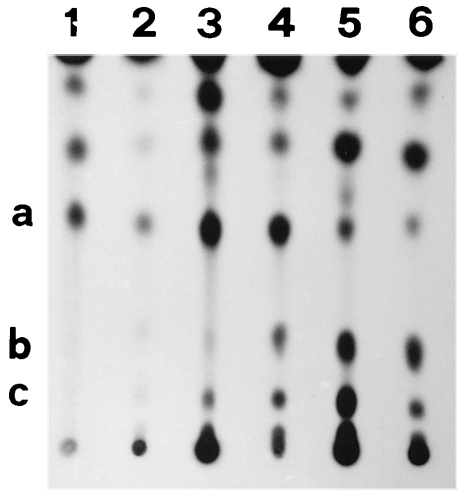

FIG. 3. Polyphosphates in late-exponential-phase *H. volcanii* and *S. cerevisiae*. Cells were labeled with $^{32}P_i$, and a formic extract was prepared and analyzed by ascending chromatography (10 h) as described in Materials and Methods. The locations of polyphosphates were detected by autoradiography. a, GTP; b, $3P_i$; c, $4P_i$ (10). Lanes: 1, *H. volcanii* one generation after addition of $^{32}P_i$ (time zero); 2, *S. cerevisiae* one generation after addition of $^{32}P_i$ (time zero); 3 and 5, *H. volcanii* 9.5 and 25 h, respectively, from time zero; 4 and 6, *S. cerevisiae* 13 and 35 h, respectively, from time zero.

the spots produced in late-exponential-phase *H. volcanii* had chromatographic mobilities that were different from those of ppGpp and pppGpp. When chromatography with NaH_2PO_4 (2.5 M, pH 3.5) was carried out for a long time (about 25 h), the appearance of a number of equally spaced spots was observed (data not shown). From these experiments, we can reasonably infer that these spots are low-molecular-weight members of a larger family of phosphorylated molecules.

Thus, a number of phosphorylated compounds are produced by *H. volcanii* at the inception of the late exponential phase of growth or after prolonged nutrient or energy starvation. One hypothesis for this phenomenon is that the molecules produced are short-chain polyphosphates, which have been shown to be produced by other microbial organisms (13, 18, 20). To verify this hypothesis, formic acid extracts were prepared from *S. cerevisiae*, an organism with well-characterized polyphosphate expression (13, 18), and the spots obtained were compared with those produced by *H. volcanii*. Samples obtained from both organisms, from the late exponential and stationary phases of growth, were analyzed by polyethylenimine thin-layer chromatography (Fig. 3). The spots obtained with *H. volcanii* and the spots corresponding to short-chain polyphosphates in *S. cerevisiae* had the same chromatographic mobility.

Identification of short-chain polyphosphates in *H. volcanii*. If the phosphorylated molecules produced by *H. volcanii* are indeed polyphosphates, the three members that are lowest in molecular weight should be PP_i, $3P_i$, and $4P_i$. To determine the nature of the radioactive spots produced by *H. volcanii*, the radioactive material was allowed to comigrate in two-dimensional chromatography with commericial samples of PP_i, $3P_i$, $4P_i$, and GTP that could be subsequently identified by coloration. Two polyethylenimine-cellulose plates were developed in the same chromatographic jar, one with samples of amino acid-starved *S. typhimurium* and one with samples of late-exponential-phase *H. volcanii*. Both plates also contained PP_i, $3P_i$, $4P_i$, and GTP. After autoradiography, the plates were colored as described in Materials and Methods. By comparing the colored plates with their companion autoradiograms and looking for coincident spots, we identified the upper of the two autoradiographic signals as $3P_i$ and the lower one as $4P_i$; moreover, there was an overproduction of PP_i, which by this method, as distinct from monodimensional polyethylenimine thin-layer chromatography, was quite distinct from GTP (Fig. 4). By comparing autoradiograms relative to *H. volcanii* and *S. typhimurium*, the distinction between (p)ppGpp and polyphosphates present in the halobacterial strain was confirmed.

DISCUSSION

In a previous study carried out with four different halobacteria (6), we reported the presence, during exponential growth, of low levels of phosphorylated compounds with chromato-

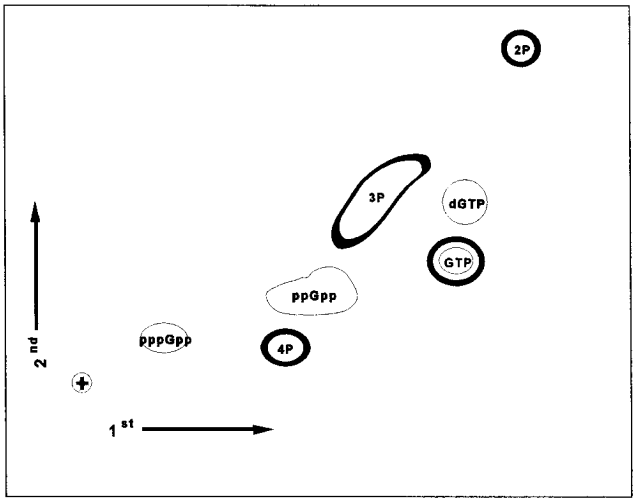

FIG. 4. Schematic representation of the identification by two-dimensional chromatography and coloration of phosphorylated compounds extracted from *H. volcanii* and *S. typhimurium* as described in Materials and Methods. ▨, phosphorylated compounds in *S. typhimurium* extract visualized by autoradiography; ■, phosphorylated compounds in *H. volcanii* extract visualized by autoradiography; ▭, colored spots obtained with polyphosphate standards.

graphic mobilities similar to those of guanosine penta- and tetraphosphates. The levels of these compounds, however, did not increase during the stringent response but were shown to rise, in both amino acid-starved and control samples, only in the late exponential phase of growth.

In eubacteria, including mutants exhibiting the relaxed phenotype, a small amount of (p)ppGpp is always present during exponential growth and the concentration of this nucleotide shows an inverse correlation with the growth rate. It has been shown that in this instance such compounds are produced by the *spoT* gene product. The possibility could not be ruled out, therefore, that the phosphorylated compounds observed during exponential growth in halobacteria are produced by a *relA*-independent (p)ppGpp-generating mechanism, analogous to the *spoT* function. In the present study, we caused a strain of *H. volcanii* to experience a series of physiological conditions that provoke a marked increase in intracellular levels of (p)ppGpp in eubacteria by virtue of the *relA*-independent pathway. None of the conditions used provoked a change in the intracellular concentration of the compounds having chromatographic mobilities similar to that of (p)ppGpp or an increase to detectable levels in other compounds that could be detected by this technique.

A more careful chromatographic comparison of the phosphorylated compounds present at low levels in *H. volcanii* cells with (p)ppGpp from *S. typhimurium* indicated a slight difference in their respective migrations. Furthermore, longer chromatographic runs showed that the compounds were members of a large family of phosphorylated molecules that accumulated in the late exponential phase of growth. This suggested, by analogy to other systems, that the compounds could be short-chain polyphosphates. In fact, a chromatographic comparison between the halobacterial compounds and short-chain polyphosphates produced by late-exponential-phase *S. cerevisiae* revealed a clear analogy with respect to both migration and the time course of expression. Bidimensional chromatography, followed by coloration with standards, confirmed that three of the compounds are PP_i, $3P_i$ and $4P_i$. In our monodimensional chromatographic system, PP_i comigrates with GTP (4) whereas $3P_i$ and $4P_i$ migrate with mobilities very similar to those of ppGpp and pppGpp, respectively. Further analysis strongly suggested that PP_i, $3P_i$, and $4P_i$ are low-molecular-weight members of a larger family of phosphorylated molecules. Polyphosphates are produced by a variety of organisms, including various eubacteria, fungi, algae, and even some higher eucaryotes. They have also been shown to be produced by some methanogenic archaeal strains (15, 16, 19). It is not particularly surprising, therefore, that they are also produced by halobacteria. By analogy to other systems, these compounds could have both an osmotic function and a phosphate storage function.

The main conclusion that can be drawn from this study is that ppGpp and pppGpp are not produced by *H. volcanii* under conditions that induce their production by eubacteria by means of a *relA*-independent pathway. If one keeps in mind our previous result strongly indicating that (p)ppGpp is not produced during the stringent response, one can conclude that all eubacterial (p)ppGpp-generating mechanisms appear to be absent in *H. volcanii*. Since the spots described here were shown to be present in four halobacterial strains (6), we can infer that all of the halobacteria examined produce short-chain polyphosphates and that they probably also all lack a mechanism for the synthesis of (p)ppGpp. It is likely, therefore, that the incapacity to produce (p)ppGpp is a general feature of halobacteria. This may be a general property of archaea, since two methanogens have been shown not to produce ppGpp (1). (p)ppGpp production and the ensuing control mechanisms could therefore be totally absent in archaea. The presence in eubacteria of a very important and sophisticated control mechanism having many effects on processes such as transcription, gene expression, and growth control may well constitute an enormous selective advantage for these organisms over archaea. This could be an important event that would help explain the preferential presence of eubacteria over archaea in all but extremophilic ecological niches.

It has been shown by others that in *Halobacterium saccharovorum* the growth rate varies in media with different compositions (14), which indicates that control of RNA synthesis is present. If this is the case, this control system probably acts without the participation of ppGpp. Whether such a putative mechanism is identical to the ppGpp-independent control system that is present in *E. coli* and that persists as the mechanism governing growth rate control in *relA spoT* mutants (8) remains an open question.

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