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RNA Synthesis in *Yersinia pestis* During Growth Restriction in Calcium-Deficient Medium

WILLARD T. CHARNETZKY[1] AND ROBERT R. BRUBAKER[2]

Department of Bacteriology and Public Health, Washington State University, Pullman, Washington 99164[1] *and Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824*[2]

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Yersinia pestis requires 2.5 mM Ca^{2+} for growth at 37°C but not at 26°C. After a shift from 26 to 37°C in a Ca^{2+}-deficient medium, an ordered series of metabolic alterations occur which result in transition from a growing cell to a viable but non-proliferating cell. The earliest known alteration in normal metabolism associated with this transition is a termination of net RNA synthesis. Competitive RNA/DNA hybridizations with uniformly labeled RNA and stable RNA competitor indicated identical mRNA to stable RNA ratios in growing cells and non-proliferating Ca^{2+}-deprived cells. Similar hybridizations with pulse-labeled RNA demonstrated that growing cells synthesized 57% mRNA, 37% rRNA, and 5% tRNA, whereas Ca^{2+}-deprived cells synthesized 95% mRNA, 4.7% rRNA, and 0.7% tRNA. After addition of radioactive uracil and rifampin to growing and Ca^{2+}-deprived cells, decay of approximately 40 and 90% of the newly synthesized RNA was found for growing and Ca^{2+}-deprived cells, respectively. The half-life of the mRNA was found to be 1.5 min for growing cells and 4.5 min for Ca^{2+}-deprived cells. *Y. pestis* elicited increases in the levels of guanosine tetraphosphate and guanosine pentaphosphate in response to amino acid deprivation and yielded transient increases in the levels of these phosphorylated nucleotides after a shift from 26 to 37°C. These increases were independent of Ca^{2+} availability and preceded the alteration in RNA synthesis by more than 1 h. The levels of these phosphorylated nucleotides then stabilized at about 80 and 40 pmol for Ca^{2+}-deprived and Ca^{2+}-supplemented cultures, respectively, and did not increase further in the Ca^{2+}-deprived culture at the time corresponding to the reduction in stable RNA synthesis. These findings indicate that the early lesion in RNA synthesis associated with the growth restriction of Ca^{2+}-deprived *Y. pestis* reflects a block in stable RNA synthesis and that this effect is not mediated by guanosine tetraphosphate or guanosine pentaphosphate.

Wild-type cells of *Yersinia pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*, the human pathogens of the genus *Yersinia*, require approximately 2.5 mM Ca^{2+} for growth at 37°C but not at 26°C. This temperature-dependent nutritional requirement for Ca^{2+}, which is unique among procaryotes, is dependent upon the presence of a 45-megadalton plasmid (10) and is correlated with the production of the V and W antigens (4). Little correlation with the function of Ca^{2+} in other bacterial systems is evident, since Ca^{2+} appears to play only nonessential roles in most bacteria, except during morphogenic change (9, 18, 25, 29). Unlike other divalent cations, Ca^{2+} is actively exported from growing bacteria (28) and therefore is found normally in only trace levels, presumably associated with the cell envelope (1, 5, 11, 27).

In *Y. pestis*, the Ca^{2+}-dependent cells, shifted from permissive (26°C) to restrictive (37°C) temperature in the absence of Ca^{2+}, undergo an ordered series of physiological alterations which result in termination of cell growth (15), conversion of compact nucleoids to axial filaments (14), increase in cell size (14), release of V and W antigens (21), and reductions of ribonucleotide pool size, adenylate energy charge (32), and the rates of DNA (30), RNA, and protein synthesis (32). In the sequence of events involved in the transition from a growing cell to a viable but non-proliferating cell, the earliest alteration observed is a reduction in the rate of accumulation of RNA (2 h postshift), which precedes the next established alterations, the reductions in protein and cell mass accumulation, by approximately 1 h (32). The molecular interactions with Ca^{2+} which permit continued cell growth, and the regulatory events which permit the ordered termination of cell growth in the absence of Ca^{2+} are unknown.

The purpose of this report is to show that the decrease in the net accumulation of RNA by Ca^{2+}-deprived cells results from alteration of the classes of RNA synthesized from the normal distribution of 57% mRNA, 37% rRNA, and 5% tRNA to predominantly mRNA (95%). We also show that the reduction in transcription of stable RNA is not accompanied by an accumulation of high levels of guanosine tetraphosphate (ppGpp) or guanosine pentaphosphate (pppGpp), even though *Y. pestis* is capable of accumulating high levels of these phosphorylated nucleotides during starvation for a required amino acid.

MATERIALS AND METHODS

Bacterium. *Y. pestis* strain EV76 used in this study, possesses the plasmid associated with Ca^{2+} dependence at 37°C (10) but is avirulent because of an unrelated mutational loss of the ability to bind hemin and related planar compounds (4).

Medium and cultural conditions. The defined synthetic medium of Higuchi et al. (15) as modified by Brubaker (3) was used routinely. For isotopic labeling with $H_3{}^{32}PO_4$, cells were grown in the defined synthetic medium modified by the omission of K_2HPO_4 and the addition of 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. Potassium phosphate was then added to give a final phosphate concentration of 0.21 mM. When cells were grown for extraction of unlabeled RNA or DNA, a medium containing 3% (Z)-(N)-amine, 0.01 M D-xylose, 0.01 M lactic acid, 2.5 mM Na_2SO_3, and 2.5 mM Mg^{2+} (pH 7.0) was used. The growth conditions and the rationale for the temperature shifts have been described previously (32).

Purification of DNA and preparation of filters. DNA from cells grown at 26°C was prepared by the procedure of Marmur (22) with the additional RNase and pronase steps of Gillespie and Spiegelman (12). After purification, the DNA was denatured by heating (23), applied to nitrocellulose filters (Schleicher & Schuell, Inc., B-6 coarse), and fixed by heat treatment (12). The amount of DNA bound to the filter was determined by monitoring the absorbance of the DNA solution at 260 nm (23) before and after passage through the filters.

Preparation of stable RNA competitors. Cells were grown at 37°C in the presence of Ca^{2+} to an optical density of 1.0, and 200 μg of rifampin per ml was added to inhibit further RNA synthesis. Incubation was continued at 37°C for 30 min to allow degradation of mRNA. The cells were poured over iced 0.85 M phosphate buffer (pH 7.2), collected by centrifugation, suspended in 0.01 M Tris-acetate buffer (pH 7.2), containing 1 mM magnesium acetate, and disrupted by passage through a French pressure cell at 15,000 lb/in^2. After treatment with 50 μg of DNase per ml for 30 min at 37°C, the extracts were centrifuged at 30,000 × *g* for 30 min, and the supernatant was used for the extraction of total stable RNA. When rRNA was desired, the ribosomes were collected from the supernatant fluid by centrifugation (140,000 × *g* for 3 h), resuspended, recentrifuged to remove residual tRNA, and resuspended in the Tris-magnesium-acetate buffer described above. Purity was verified by sucrose density sedimentation. An equal volume of chloroform containing 3% isoamyl alcohol was added to either the initial supernatant or to the purified ribosomes, and the mixture was shaken for 5 min. After centrifugation at 20,000 × *g* for 5 min, the aqueous phase was carefully removed and repeatedly extracted until no material was evident at the interface. A total of 2.5 volumes of cold ethanol was added to the final aqueous phase, and the suspension was incubated overnight at −20°C. The resulting flocculent material was sedimented by centrifugation at 10,000 × *g* for 10 min, washed in cold ethanol, and resuspended in 0.1 M Tris-hydrochloride (pH 7.2) containing 0.01 M $MgCl_2$ and 0.01 M KCl. After treatment with DNase (10 μg/ml) for 30 min at 30°C and pronase (100 μg/ml) for 30 min at 37°C, the preparation was chilled to 4°C. An equal volume of phenol saturated with the Tris-$MgCl_2$-KCl buffer just described, but adjusted to pH 8.0, was then added. The mixture was agitated for 5 min at 4°C and centrifuged at 20,000 × *g* for 5 min, and the aqueous phase was removed and extracted two additional times. RNA was removed from the final aqueous phase by the addition of 2.5 volumes of cold ethanol, then redissolved in the same buffer (pH 7.2), precipitated with cold ethanol two additional times to remove residual phenol, and stored in a freezer under 95% ethanol until used. The amount of RNA recovered was determined by absorbance at 260 nm.

Preparation of radioactively labeled RNA. For uniformly labeled RNA, cells were grown for six generations in a medium containing $H_3{}^{32}PO_4$ (10 μCi/ml). Transfers were made when cells were in the exponential growth phase. For pulse-labeling, cells were grown in a low-phosphate medium for four generations at 26°C in the presence or absence of Ca^{2+} and shifted to 37°C. After 3 h, $H^{32}PO_4$ (200 μCi/ml) was added 1 min before collection. In neither case was phosphate growth limiting. In both cases, the cells were poured over crushed ice made from 0.85 M phosphate buffer (pH 7.2) and collected by centrifugation. The RNA was extracted and purified as described above except that the initial DNase treatment was omitted.

RNA/DNA hybridizations. Hybridizations were performed in a manner similar to that described by Kennell and Bicknell (17). Nitrocellulose filters (Schleicher & Schuell, Inc., B-6 coarse) with 100 μg of *Y. pestis* DNA and 0.1 μg of $H_3{}^{32}PO_4$-labeled whole-cell RNA were placed in scintillation vials containing 50% formamide and 3× standard saline citrate (SSC) where 1× SSC is 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0) (13). When specified, 20 μg of purified rRNA or total stable RNA was added as competitor. After incubation at 44°C for 72 h, the samples were transferred to an ice bath, washed by filtration for 30 s with 2× SSC, treated with pancreatic RNase (5 μg/ml) for 1 h at 37°C, and washed twice by filtration for 30 s with 2× SSC. Nonspecific binding was determined by using the same procedures except that the filters had 100 μg of yeast DNA rather than *Y. pestis* DNA.

Rifampin. Cells were grown for four generations at 26°C in the presence or absence of Ca^{2+} and shifted to 37°C 3 h before use. The procedures used for rifampin treatment and for determination of [5-^{3}H]uracil incorporation into trichloroacetic acid (TCA)-precipitable alkaline-resistant material were similar to those of Bremer et al. (2) and Dennis and Bremer (8), except that a different synthetic medium (3) was used and

rifampin (100 μg/ml) was added 3 min, rather than 4 min, after the termination of EDTA treatment. Rifampin and [5-^{3}H]uracil were added at zero time (T_0; see Fig. 1 and 2), or [5-^{3}H]uracil was added at T_0 or 30 s later (T_{30}), and rifampin was added at T_{30} (see Table 3). In the latter case, each culture was sampled at T_{40} and $T_{1,500}$. The incorporation in a 30-s period, corrected for the delay in the onset of rifampin, equilibration of precursor pools, and the differences in the pool sizes were determined by subtracting the [5-^{3}H]uracil incorporation into TCA-precipitable alkaline-resistant material in the culture to which isotope had been added at T_{30} from the incorporation into the same material in the culture to which isotope had been added at T_0. For incorporation into total RNA, samples taken at T_{40} were used. For incorporation into stable RNA, samples taken at $T_{1,500}$ were used. (For theoretical considerations, see reference 8.) For determination of incorporation of [^{14}C]histidine into protein, 0.2-ml samples were added to 2 ml of 5% TCA. After 30 min at 4°C, precipitated material was collected on 0.45-μm nitrocellulose filters, washed with 5% TCA, and dried. All samples were counted in 3a70b liquid scintillation fluid (Research Products, Inc.). The EDTA treatment had no apparent effect on the accumulation of cellular mass (measured by absorbance at 620 nm), protein, DNA, or RNA. The addition of rifampin to EDTA-treated cultures resulted in essentially complete shutoff of RNA chain initiations within 10 s.

ppGpp and pppGpp. Cells were grown at 26°C for two generations in a medium containing 375 μCi/mol of ^{32}P before being sampled. Nucleotides were extracted with cold 1 M formic acid as described by Cashel (6) except that the cells were sedimented in a clinical centrifuge. The supernatants were frozen until used. Storage time never exceeded 4 days. Other phosphorylated compounds were separated from ppGpp and pppGpp on presoaked, dried, polyethyleneimine cellulose plates by using a 1.5 M KH_2PO_4 (pH 3.4) solvent system (6). The positions of the phosphorylated compounds were determined by autoradiography. The portions of the chromatogram containing the ppGpp and pppGpp (4 and 2 cm above the origin, respectively) were carefully cut out and counted in 3a70b liquid scintillation fluid. To minimize the contribution of phosphorylated compounds in the chromatographic trail, corrections were then made for counts in the sections immediately above and below those of interest. In samples containing low ppGpp or pppGpp levels, the fraction of the total counts in the sample sections originating from the trail was significant.

RESULTS

Hybridizations with uniformly labeled RNA. The extent of hybridization occurring at selected RNA/DNA ratios from 1:10 to 1:2,000 was similar to that reported for *Escherichia coli* (16). The results (not presented) indicated that at a ratio of 1:1,000, all species of RNA transcripts were limiting, whereas at a ratio of 1:20 only mRNA transcripts were limiting and that rRNA and tRNA transcripts were present in excess of available DNA sites and contributed only a small fraction of the RNA hybridized. To determine the relative concentrations of mRNA and stable RNA present within growing and Ca^{2+}-deprived cells, uniformly labeled RNA was hybridized to DNA at a ratio of 1:1,000 in the presence and absence of total stable RNA competitor. The results showed that the ratios of mRNA to stable RNA present under both culture conditions were similar (Table 1).

Hybridizations with pulse-labeled RNA. To determine the distribution of newly synthesized RNA in growing and Ca^{2+}-deprived cells, the fraction of pulse-labeled RNA binding at an RNA/DNA ratio of 1:1,000 was compared with the fraction binding at an RNA/DNA ratio of 1:20. The values obtained by this procedure were in close agreement with those obtained by competitive hybridizations at an RNA/DNA ratio of 1:1,000 with pulse-labeled RNA and total stable RNA and rRNA competitors (Table 2). These results indicate that cells growing at 26°C, or at 37°C with added Ca^{2+}, synthesized about 57% mRNA, 38% rRNA, and 5% tRNA. In contrast, the Ca^{2+}-deprived cells synthesized significantly more mRNA (95%) relative to rRNA (4.7%) and tRNA (0.5%). These results indicate that the termination of the growth of Ca^{2+}-deprived cultures was associated with a block in the synthesis of stable RNA.

Inhibition of transcription and decay of unstable RNA. To confirm the values determined by RNA/DNA hybridizations and to obtain information regarding translation-dependent decay of mRNA, rifampin, which inhibits transcription initiations, was used. The addition of rifampin to cells of *Y. pestis* grown at 37°C with Ca^{2+} and pretreated with EDTA yielded results essentially the same as those found in *E. coli* (26). Net accumulation of TCA-precipitable [^{3}H]uracil continued for about 2 min after the addition of rifampin, followed by a decrease of approximately 60% by 8 min (Fig. 1A). This decrease reflects the decay of unstable RNA, primarily mRNA (26), with some loss from processing of stable RNAs and perhaps some loss of ribosomal RNA (31). The accumulation of protein, as determined by the incorporation of [^{14}C]histidine into TCA-precipitable material, was evident up to 8 min after the addition of rifampin (Fig. 1A). When non-proliferating Ca^{2+}-deprived cells were similarly treated, net accumulation of TCA-precipitable [^{3}H]uracil continued for 3 min, and over 90% of this material was subsequently lost in the ensuing 15 min. Net accumulation of [^{14}C]histidine into TCA-precipitable material also continued for 18 min (Fig. 1B). To correct for precursor pool sizes, pool equilibration, and the lag in the onset of rifampin-induced inhibition of transcription, the method of Dennis and Bremer (8) was used to determine the relative amounts of stable and unstable

TABLE 1. RNA/DNA hybridizations with uniformly labeled $H_3{}^{32}PO_4$ RNA

Culture condition[a]	Time	Hybrids (cpm)[b]		% Unstable RNA
		No competitor	Stable RNA competitor[c]	
37°C, 2.5 mM Ca^{2+}	6 h	48,320	2,040	4.2
	6 h	34,100	1,250	3.7
37°C, no Ca^{2+}	6 h	45,190	1,710	3.8
	3 h	33,890	1,340	4.0
	3 h	46,270	1,610	3.5

[a] Cells were grown for six generations at 26°C, transferred to the indicated culture condition, and incubated for the time indicated before harvest.

[b] Hybridizations were prepared in triplicate. Each value is the average of the counts per minute of at least two filters. Individual filters generally gave values within 10% of the average. Occasional samples varied by more than 50% of the average; these were disregarded. All samples were counted for 5 or 10 min. Background (10 cpm) and nonspecific binding (0.4% of input) were subtracted before calculations. The specific activities ranged from 5.2×10^5 to 7.4×10^5 cpm/μg of RNA. In the absence of competitor RNA, 63 to 65% of the RNA remained on the filter after treatment with pancreatic RNase and washings.

[c] Unlabeled total cell stable RNA added to 200-fold excess of labeled RNA.

RNA synthesized under these conditions. The values obtained by this procedure were consistent with those obtained by RNA/DNA hybridizations and indicated that growing cells and restricted cells produced 43 and 4% stable RNA, respectively (Table 3).

Degradation of the unstable RNA fraction was slower in Ca^{2+}-deprived cells than in actively proliferating cells (Fig. 1). To determine the decay rates of mRNA, stable RNA present 20 min after the addition of rifampin was subtracted from values obtained in earlier samples. The resulting exponential decrease in the unstable RNA fraction (Fig. 2) permitted calculation of half-lives of 1.5 and 4.5 min for the mRNA of growing and Ca^{2+}-deprived cells respectively.

Biosynthesis of ppGpp and pppGpp. To determine whether *Y. pestis* was capable of producing

TABLE 2. RNA/DNA hybridizations with pulse-labeled $H_3{}^{32}PO_4$ RNA[a]

Culture condition[b]	RNA/DNA, 1:1,000		RNA/DNA, 1:20
	rRNA competitor[c]	Total stable RNA competitor	No competitor
26°C, no Ca^{2+}	62.3	57.2	58.1
37°C, 2.5 mM Ca^{2+}	62.9	57.6	56.9
37°C, no Ca^{2+}	95.3	94.1	94.6

[a] Expressed as the percentage bound relative to that bound at an RNA/DNA ratio of 1:1,000. Each value is the average of two experiments; hybridizations were done in triplicate. The specific activity of the RNA ranged from 1.8×10^5 to 2.5×10^5 cpm/μg for Ca^{2+}-deprived cultures at 37°C and 4.6×10^5 to 6.0×10^5 cpm/μg for Ca^{2+}-supplemented cultures at 37°C and 26°C. In the absence of competitor RNA, 63 to 65% of the radiolabeled RNA remained on the filters. Samples were counted for 5 or 10 min. Background (10 cpm) and nonspecific binding (0.4% of input) were subtracted before calculations.

[b] Cells in exponential growth at 26°C were shifted to the indicated condition and incubated for 3 h before the addition of $^{32}P_i$.

[c] Competitor RNA was added to 200 times the concentration of ^{32}P-RNA.

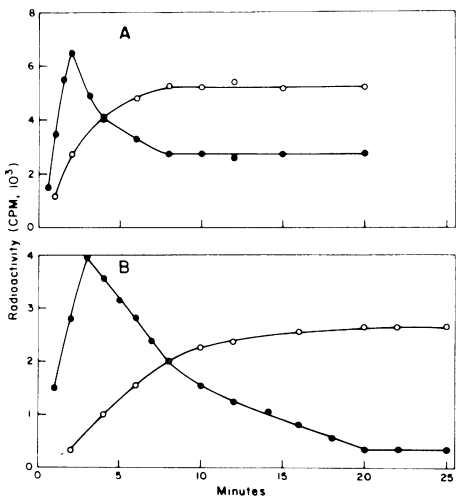

FIG. 1. Incorporation of [5-^{3}H]uracil (●) and [^{14}C]histidine (○) by cells incubated at 37°C in medium to which 2.5 mM Ca^{2+} was added (A) or omitted (B). Cells in exponential growth at 26°C were transferred to 37°C and incubated 4 h before treatment with EDTA. Values are expressed as counts per minute per 0.2 ml at an optical density at 620 nm of 1.0.

TABLE 3. Relative rates of incorporation of [5-^{3}H]uracil[a] into stable RNA

Culture condition[b]	T_0 (40)[c]	T_{30} (40)	T_0 (1,500)	T_{30} (1,500)	i_s/i_{tot}
37°C, 2.5 mM Ca^{2+}	7.85	2.57	15.07	12.79	0.43
37°C, no Ca^{2+}	2.90	1.52	0.49	0.44	0.04

[a] Rate of incorporation is expressed as 10^3 cpm/0.5 ml at an optical density of 1.0.

[b] Cells in log phase at 26°C were shifted to the indicated culture condition and incubated 4 h before treatment.

[c] After termination of the EDTA treatment, the cells were separated into two cultures, and isotope was added to one culture at zero time (T_0) and to the other 30 s later (T_{30}) as indicated by the subscripts. Samples were taken at 40 and 1,500 s as indicated in the parentheses. The incorporation into total RNA (i_{tot}) and stable RNA (i_s) in a 30-s period was determined by subtracting the incorporation in the culture to which isotope had been added at T_{30} from that in which isotope had been added at T_0, with i_{tot} and i_s determined from 40- and 1,500-s samples, respectively. The portion of the total incorporation which was into stable RNA (i_s/i_{tot}) was then determined as T_0 (1,500) − T_{30} (1,500)/T_0 (40) − T_{30} (40).

ppGpp and pppGpp during starvation for an essential amino acid, the organisms were cultivated at 37°C with reduced L-phenylalanine (2 μg/ml). As commonly observed in *E. coli* (6), the levels of ppGpp (Fig. 3C) and pppGpp (not illustrated) increased before growth became limited by phenylalanine depletion. These levels, however, did not rise as rapidly as reported for *rel*$^+$ *E. coli*, but rather increased gradually reaching plateau levels of 400 and 350 pmol/ml per optical density unit of culture for ppGpp and pppGpp, respectively. Although this response was not completely typical of stringent *E. coli*, the results showed that *Y. pestis* is capable of significant synthesis of ppGpp and pppGpp in response to amino acid starvation.

To determine whether a similar response occurred upon starvation for Ca^{2+}, cells grown at 26°C in the presence or absence of Ca^{2+} were shifted to 37°C. In both cases, a transient increase was observed for both nucleotides. Levels of approximately one-third of those detected during amino acid starvation were reached (Fig. 3A and B). The levels then fell to values slightly higher than those present at 26°C in the Ca^{2+}-supplemented cultures and about twofold-higher levels were maintained in the Ca^{2+}-deprived cultures. These (p)ppGpp levels were maintained for over 1 h before and 1 h after the time alterations in RNA synthesis were evident in the Ca^{2+}-deprived cells. These findings indicate that the growth restriction caused by Ca^{2+} deficiency was not mediated by (p)ppGpp.

DISCUSSION

Under the conditions used in these experiments, net synthesis of RNA ceases about 2 h after shift from 26 to 37°C in a Ca^{2+}-deficient medium (32). This shutoff is the first metabolic change known to occur during the onset of the growth restriction and undoubtedly accounts, either directly or indirectly, for the subsequent reductions in the rates of accumulation of other macromolecules. Results obtained by RNA/DNA hybridizations with pulse-labeled RNA showed that accumulation of RNA in restricted cells was reduced as a result of a block in synthesis of stable RNA. This result, of course, is not unique as a variety of other environmental changes resulting in nutritional stepdown are known to promote shutoff of stable RNA synthesis in procaryotes (24). However, the proportion of total RNA present as mRNA remained constant throughout the period of Ca^{2+} deprivation examined. Although this finding indicated that the steady-state level of mRNA in restricted cells was identical to that of growing organisms, it provided no information regarding rates of transcription- or translation-dependent decay. The results of experiments with rifampin con-

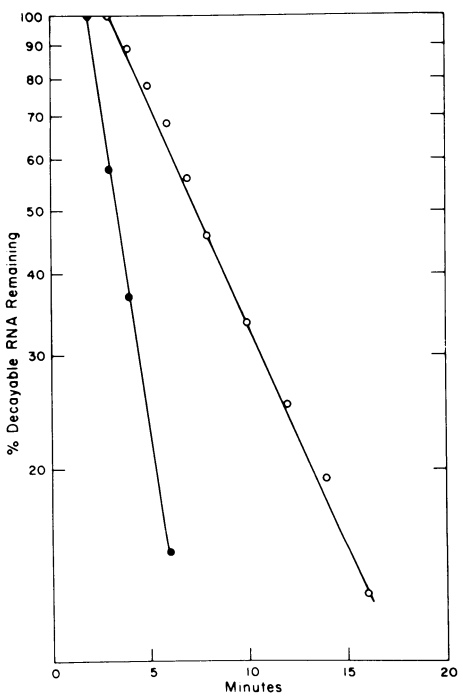

FIG. 2. Decay of unstable RNA in cells incubated at 37°C in medium to which 2.5 mM Ca^{2+} was added (●) or omitted (○). Values were determined from points in Fig. 1.

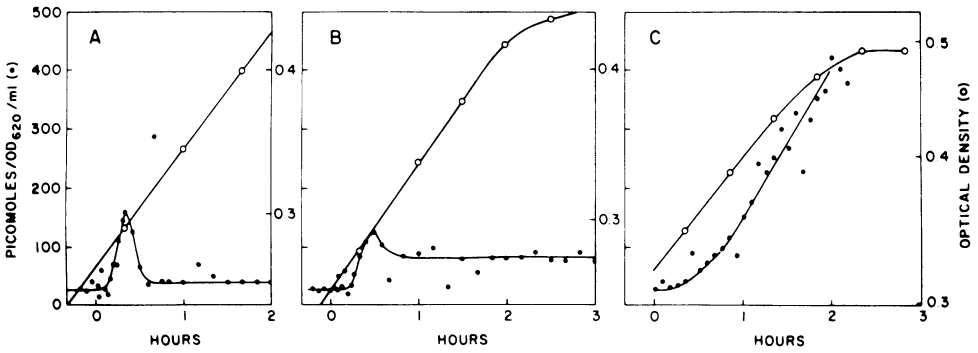

FIG. 3. ppGpp accumulation. After a shift from 26 to 37°C in the presence (A) or absence (B) of 2.5 mM Ca^{2+} or during the onset of phenylalanine starvation (C), samples were processed and analyzed by the method of Cashel (6) for ppGpp. The total available phosphate permitted log-phase growth to an optical density at 620 nm of 0.7.

firmed the block in stable RNA synthesis and, in addition, indicated that the rate of mRNA decay in Ca^{2+}-deprived cells was significantly slower than that in growing cells. Accordingly, to maintain a steady-state level consistent with that of growing cells, the rate of mRNA transcription during the period of growth restriction must be correspondingly reduced to balance that of its degradation. We conclude that Ca^{2+} starvation at 37°C results in a reduction in synthesis of stable RNA transcripts and also promotes a possible secondary decrease in rate of mRNA transcription even though this species then constitutes about 95% of the RNA synthesized. It is understood, however, that although a primary lesion in stable RNA synthesis could account for the observed growth restriction, unknown prior events may be responsible for the alteration in the pattern of RNA synthesis. Further study will be required to determine whether this change in distribution reflects some direct modification of the RNA polymerase or stable RNA promoter regions (reviewed in reference 7), or involves a novel mechanism peculiar to *Yersinia*.

To determine whether shutoff of RNA synthesis was associated with production of (p)ppGpp, we compared the ability of the organisms to synthesize and accumulate these regulatory nucleotides during starvation for Ca^{2+} and during starvation for an essential amino acid. Although the cells produced significant (p)ppGpp as a consequence of phenylalanine deficiency, the accumulation of (p)ppGpp was not entirely typical of stringent *E. coli*, and the levels of (p)ppGpp remained elevated after depletion of the amino acid. Nevertheless, this determination illustrated that *Y. pestis* is indeed capable of (p)ppGpp biosynthesis. A transient increase in the level of ppGpp was seen in both Ca^{2+}-deprived and Ca^{2+}-supplemented cultures which appeared to reflect a consequence of the temperature shift rather than the cation deficiency. In the Ca^{2+}-supplemented culture, the ppGpp level returned to near preshift levels within 40 min. In the Ca^{2+}-deprived culture, a new ppGpp level which was approximately twofold higher than this was maintained. Higher basal levels of ppGpp have been reported in *relA spoT* than *relA spoT*$^+$ strains of *E. coli* under conditions of balanced growth which relate to the rates of RNA accumulation (20). The differences in basal levels of ppGpp were similar to those differences reported here, but the difference in the rates of RNA accumulation were minor compared to the rather dramatic alterations in RNA synthesis reported here. It is possible, but unlikely, that the increase in ppGpp basal levels seen under conditions of Ca^{2+} deprivation relate directly to the reduction in stable RNA synthesis, since (i) the ppGpp levels were still low compared with the transient peak level and the level seen in amino acid-starved cells, and (ii) this level was maintained from this time until past the time (2 h postshift) when stable RNA synthesis was reduced. We conclude that *Y. pestis*, although capable of (p)ppGpp biosynthesis, does not utilize these regulatory nucleotides to mediate its temperature-dependent response to Ca^{2+}-deficient environments. It has been reported that the procedure we used for extracting ppGpp results in considerable degradation of this compound (19). It is likely that the absolute concentrations of ppGpp are, therefore, considerably higher than those reported here. However, since all samples were treated identically with the same batches of reagents, it is unlikely that the degradation altered the pattern of results presented here.

The described anabolic changes which result in the Ca^{2+} deprivation-imposed growth restriction are not unique. The unusual feature of these findings is that Ca^{2+} is not an essential nutrient of procaryotes other than *Yersinia* cultivated at the temperature of their mammalian hosts. Since

this requirement is plasmid mediated (10) and essential for expression of virulence (4), it seems reasonable to expect that its elucidation will provide an interesting, possibly important, and (based on the results presented here) novel example of metabolic regulation.

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