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Polyamine Synthesis and Accumulation in *Escherichia coli* Infected with Bacteriophage R17

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We have studied the biosynthesis of polyamines during the multiplication of the RNA bacteriophage R17. R17-sensitive strains of *Escherichia coli* were derived from the stringent CP78 and the relaxed mutant derivative CP79. The cells were infected with R17 in the presence or absence of arginine, a required amino acid, and both the RNA and polyamine contents of the bacteria were determined before and after the infection. The uninfected CP79 *rel* derivative accumulated RNA and spermidine in the absence of arginine, unlike the stringent organism that accumulated neither under these conditions. After R17 infection, the stringent strain accumulated RNA and spermidine in the presence or absence of arginine. The data indicate a close correlation between the synthesis of RNA and spermidine, suggesting a significant role for this polyamine in the multiplication of phage R17.

During the past several years, considerable evidence has accumulated to suggest that polyamines play a role in regulating the synthesis of RNA and protein in bacteria, as well as in higher organisms (6). Some organisms are known that require these amines for growth (15), and in some strains of *Escherichia*, the inability to synthesize putrescine and, hence, spermidine leads to a marked decrease in growth rate (11, 17). In some strains of *E. coli*, the inability to synthesize adequate amounts of *S*-adenosylmethionine led to low levels of intracellular spermidine and a decrease in growth rate, which is partially increased by addition of exogenous spermidine (26). Bacterial cells contain large amounts of putrescine and spermidine, and the amount of the latter particularly relates to the amount of cellular RNA (6) that is present to a significant degree in ribosomes. Spermidine has been shown to be an obligate requirement for polypeptide synthesis on some ribosomes (5).

Numerous viruses, such as turnip yellow mosaic virus (4), T-even bacteriophages (3, 16), and even animal viruses, e.g., herpesvirus (14) and adeno virus (24), contain polyamines. According to Ames and Dubin (3), the amount of putrescine and spermidine present in phage T4 accounts for about 40% of the cations required to neutralize phage DNA-P. Studies by Dion and Cohen (11) showed that in a putrescine-depleted strain of *E. coli*, T4 infection resulted in a very slow rate of synthesis of virus DNA, which was markedly stimulated by addition of exogenous polyamine. Although polyamine availability controlled production of phage DNA, the reverse was not the case (10). Polyamine synthesis was essentially unchanged in cells infected by a T4 mutant incapable of DNA synthesis (10).

In view of the close correlation between the concentrations of RNA and spermidine in various biological systems (20, 21, 23), it seemed of interest to know whether polyamine biosynthesis and accumulation are affected during multiplication of RNA-containing viruses.

Bacteriophage R17 and MS2 each contains an RNA molecule of 1.05×10^6 daltons, and a large part of its nucleotide sequence has been established (1, 9, 25). In addition, physicochemical as well as biological studies have suggested the probable existence of loops, i.e., partial double-stranded regions of the RNA (1, 9, 25). Spermidine is known to stabilize double-stranded regions in various nucleic acids, e.g. tRNA (6, 8, 19, 22). Therefore it seemed possible that the phage RNA is also stabilized by spermidine. In fact, we have recently shown that R17 phage, after isolation from lysates, does contain large amounts of spermidine, enough to neutralize 60 to 80% of the RNA-P, as described by I. Fukuma and S. S. Cohen (Abst. Annu. Meet. Amer. Soc. Microbiol., 73rd, Miami Beach, Fla., p. 209, 1973).

In this investigation, we describe the effect of

phage R17 infection on the biosynthesis and accumulation of polyamines in host bacteria in the presence and absence of a required amino acid. Although significant differences between the members of a pair of stringent and relaxed strains (15 TAU) have been reported for polyamine synthesis and accumulation (7), these strains are not presently useful in studies of infection. The effects of R17 infection have been examined with an appropriate pair of stringent and relaxed strains of *E. coli*, i.e., CP78 *rel*$^+$ and CP79 *rel*, respectively, in which were introduced the F$^+$ factor essential for infection by an RNA phage. It was found that polyamine synthesis does continue during infection; indeed spermidine accumulation within the cells is markedly stimulated by infection of the stringent cell. Surprisingly, this occurred even in the absence of exogenous arginine, an amino acid essential for normal growth. The data presented in this paper suggest a significant role for spermidine may exist in the organization and multiplication of R17, an RNA-containing bacterial virus.

MATERIALS AND METHODS

Isolation of R17-sensitive derivatives of E. coli CP78 and CP79. The R17-sensitive derivatives (F$^+$) were obtained by conjugation of the original F$^-$ strains with a male strain of *E. coli*, W6. The strains CP78 F$^-$ *rel*$^+$ and CP79 F$^-$ *rel* are isogenic except in the *rel* locus and require arginine, histidine, leucine, threonine, and thiamine for growth (13). We have shown that the block in arginine synthesis occurs after formation of ornithine; these strains are therefore able to synthesize putrescine and spermidine in the absence of arginine. *E. coli* W6 F$^+$ requires methionine. These strains were grown at 37 C in nutrient broth to a density of about 5×10^8 cells/ml. The culture of strains CP78 F$^-$ or CP79 F$^-$ was mixed with an equal volume of the culture of W6 F$^+$ and incubated for 1 h at 37 C. After incubation, a sample was removed from the mixed culture, diluted 10^{-6}, and plated on nutrient agar. After incubation overnight at 37 C, more than 10 separate colonies were picked from each plate and examined for sensitivity to phage R17. Organisms sensitive to R17 were isolated from each strain and possessed the same nutritional requirements as the parent cultures; they were designated as CP78 F$^+$ *rel*$^+$ or CP79 F$^+$ *rel*. The plating efficiency of R17 on these strains was approximately 90% of that on *E. coli* W6 F$^+$.

Media, culture conditions, and phage infection. The minimal medium used throughout the present study contained 0.116 M Na_2HPO_4, 0.011 M KH_2PO_4, 0.015 M Na_2SO_4, 0.01 M NH_4Cl, 1.8×10^{-6} M $FeSO_4$, 9×10^{-5} M $CaCl_2$, and 8×10^{-5} M $MgSO_4$. The concentration of NH_4^+ ion was reduced to facilitate polyamine estimation. The medium was supplemented (per milliliter) with 2 mg of glucose, 40 μg each of arginine, histidine, leucine, and threonine, and 10 μg of thaimine. An overnight culture of *E. coli* CP78 F$^+$ *rel*$^+$ or CP79 F$^+$ *rel* in the complete medium was diluted with the same medium to a cell density of approximately 6×10^7/ml. The cells were grown at 37 C with aeration to a density of approximately 2×10^8/ml. The bacteria were collected by centrifugation, washed, and suspended in approximately one-eighth of the original volume of the minimal media. Wild-type R17 was added to a portion of this concentrated cell suspension at a multiplicity of infection of approximately 20. After 5 min of incubation at room temperature, the infected cells were centrifuged, suspended in prewarmed (37 C) complete media, and the culture was incubated at 37 C with aeration. In these experiments, more than 95% of the cells were infected, as estimated by plating for the ability to form colonies. The turbidities of cultures were monitored on a Klett colorimeter with a 420-nm filter. PFUs of R17 were assayed by the soft-agar overlay method (2).

Polyamine and RNA analyses. The polyamines in acid extracts of the bacteria or of the cultures were assayed as fluorescent dansyl derivatives (12) which were extractable into benzene. The compounds in the benzene extract were separated by thin-layer chromatography on activated silica gel plates (Analtech Inc., Newark, Del.), with ethyl acetate-cyclohexane (30:60, vol/vol). The fluorescent spots were scanned on a Farrand chromatogram-scanning fluorometer (Farrand Optical Co., Inc., New York).

In estimating the polyamine content of cells, small samples of the cultures were filtered on membrane filters (Millipore Corp., pore size 0.45 μm). The filters containing the cells were extracted in 1.0 ml of 3% perchloric acid (PCA). The polyamines were dansylated directly (free polyamines) or the acid extract was mixed with concentrated HCl to 6 N HCl and heated at 110 C for 18 h. After drying in vacuo over KOH pellets and concentrated H_2SO_4, the residue was dissolved in the original volume of water. Samples of the hydrolysates were used for dansylation of the polyamines (total polyamines).

The polyamine content (free and total) of the media was determined by difference, i.e., by subtracting the values for the cells from that of the entire culture. After acidification of the culture with 3% PCA and removal of the precipitate by centrifugation, the extract was adjusted to pH 13 with KOH, Na_2SO_4 was added, and the polyamines were extracted with *n*-butanol (20). HCl was added to the butanol, and the acid extract was taken to dryness in vacuo. The residue was dissolved in 3% PCA and analyzed for free and total polyamines, as given above. For the estimation of RNA from the culture, bacterial pellets derived from the acidified culture were suspended and extracted two times with cold 5% trichloroacetic acid. The washed pellets were then hydrolyzed in 5% trichloroacetic acid at 80 C for 15 min. The hot extract was assayed for RNA by the orcinol procedure (18).

RESULTS

One-step growth of bacteriophage R17 on E. coli CP78 F⁺ rel⁺ and CP79 F⁺ rel. As shown in Fig. 1A, the multiplication of bacteriophage R17 was similar in both the stringent and the relaxed strain of *E. coli*. In this experiment, the cells grown in the complete minimum medium were collected, infected at 1.6×10^9 bacteria/ml, and resuspended at 2×10^8 cells/ml in the same medium with or without arginine. The infected cultures were aerated at 37 C, and portions of the culture were removed at intervals for assay of plaque-forming units. No significant readsorption of liberated phage was detected at this bacterial concentration. Phage release began at about 40 min after infection in both the stringent and the relaxed strains, and the burst was maximal at about 70 min (Fig. 1A). The turbidity of the cultures increased after infection during this period and attained a maximum at 90 min after infection. Lysis of the infected cells, detected by turbidity measurements, took place thereafter and was complete at about 120 min after infection with both strains (Fig. 1B). From the final level of PFUs in the cultures, the burst size was estimated to be approximately 2,000 in both host strains.

When arginine, which is required for the growth of host bacteria, was omitted from the medium, the phage titer did not increase above the original level for 2 h after infection. There was no loss of infectious centers during this incubation. During this period, the turbidity increase was only 8% of the original value.

Synthesis of polyamines in uninfected and infected cultures. As shown in Fig. 2A, the initial production of spermidine by the relaxed (CP79) strain was slightly greater than that by the stringent (CP78) strain during exponential growth. On the other hand, the initial production of putrescine by the relaxed strain was somewhat less than that of the stringent strain. During the multiplication of R17 phage, however, the production of both putrescine and spermidine by the relaxed strain was much less than that produced by the infected stringent strain. Although the uninfected control cultures of these strains continued to produce polyamines during the experimental 2-h period, after R17 infection, the production ceased at about 90 min when the turbidity of the cultures began to fall. Nevertheless, in the initial 60-min period, the increment of spermidine in the infected stringent culture was about three times that in the infected relaxed culture.

It has been reported previously from this laboratory (7) that polyamine biosynthesis is markedly different in a relaxed strain of *E. coli*, 15 TAU, as compared with the stringent parent under conditions of arginine starvation. It was shown that the rate of spermidine production in the relaxed strain is three times greater than that in the stringent strain, whereas the rate of putrescine production in the relaxed strain was one-third of that in the stringent organism. It appears that polyamine syntheses in strains CP78 (stringent) and CP79 (relaxed) differ in the same directions, although the magnitude of the differences are less pronounced when compared with the one-step mutant pairs of strain 15 TAU. In the experiment presented in Fig. 2B, exponentially growing cells of the two strains were washed and suspended to the same turbidity in the medium lacking arginine. Total pu-

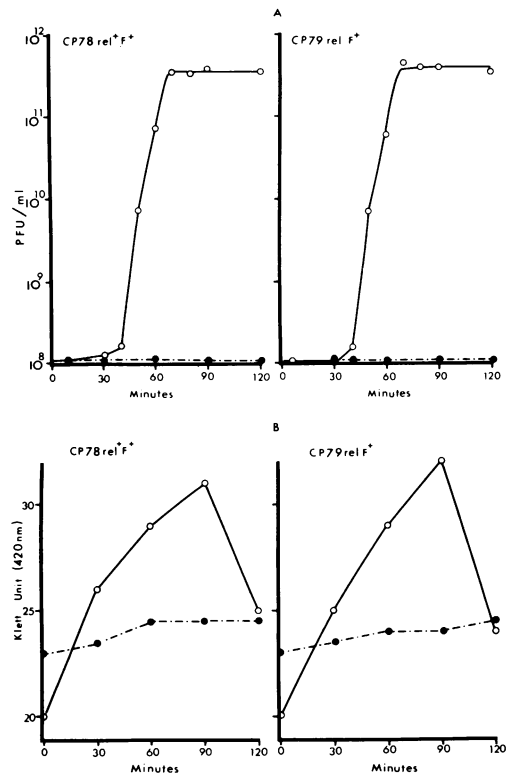

FIG. 1. *One-step growth of R17 and turbidity changes in infected E. coli. CP78 F⁺ rel⁺ and CP79 F⁺ rel were grown and infected as described in Materials and Methods. Samples for plaque-forming units were removed at the designated times, diluted in saline (0.15 M NaCl, 0.001 M $MgCl_2$, and 0.001 M $CaCl_2$), and assayed. A, PFU; B, turbidity. ○, In complete media; ●, without arginine.*

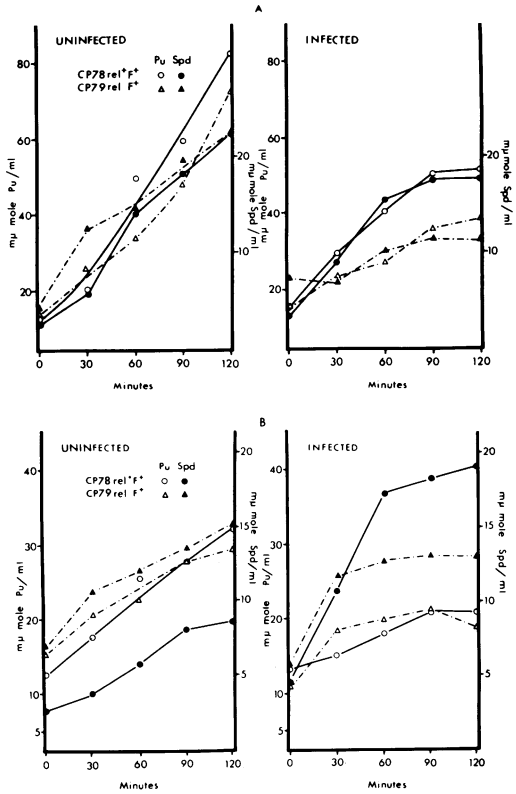

FIG. 2. *Total polyamine synthesis in cultures of E. coli CP78 and CP79. Assays were performed on the same samples described in Fig. 1A. Uninfected or infected cells were suspended in the complete (A), or in arginine-lacking (B), media, and the polyamine contents of both cells and media were determined at the intervals indicated as described in Materials and Methods.*

trescine synthesis by relaxed cells in a 2-h interval was about 30% less than that by stringent cells. Both strains behaved similarly in the rate of spermidine synthesis, but the total amount of spermidine in the relaxed culture was about two times higher than in the stringent culture.

When cells were infected with R17 in the absence of arginine, total polyamine synthesis continued, but the patterns of the increments were quite different from those in the uninfected control cultures. In the absence of arginine, the total production of putrescine was reduced to about one-half by infection in both strains, although the patterns in the two strains were slightly different. On the other hand, spermidine production sharply increased in both strains. Curiously, the final level of spermidine in the infected stringent culture significantly exceeded that produced by the infected relaxed strain. Therefore, the accumulation of RNA was examined next under these conditions, i.e., in the presence and absence of arginine.

Accumulation of RNA. The rate of RNA accumulation was very similar in the two strains during the exponential growth (Fig. 3A). When arginine was omitted from the culture media, the accumulation of RNA stopped almost completely in the stringent cells, and its increment in 2 h after the deprivation was only 4% of the amount accumulated in the control culture (with arginine) during the same amount of time (Fig. 3A). In the relaxed culture, on the other hand, the increment of RNA in the absence of arginine was about 60% of the amount made in the presence of the amino acid after 1 h of incubation. The RNA content leveled off around this time in the absence of arginine; the percentage of newly formed RNA was greater than 60% of the initial RNA content.

During normal infection (i.e., in the presence of arginine), the accumulation of RNA continued for up to 90 min in both strains, though its rate was somewhat higher in the stringent strain than in the relaxed bacteria (Fig. 3B).

When the cells were infected in the absence of arginine, on the other hand, the patterns of RNA accumulation were completely different from those observed in the uninfected control cultures under the same conditions. Although RNA had continued to increase without arginine only in the uninfected relaxed culture (Fig. 3A), even the stringent strain continued to accumulate RNA after infection in the absence of arginine (Fig. 3B). Thus, R17 infection ap-

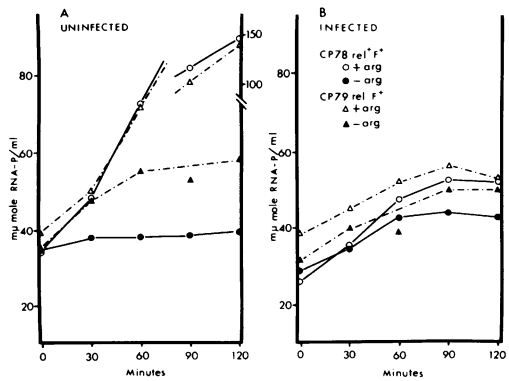

FIG. 3. *RNA synthesis in E. coli CP78 and CP79. Assays were performed on the samples described in Fig. 1A.*

peared to relax the stringent cells with respect to both RNA accumulation and spermidine synthesis.

Cellular polyamines. The intracellular polyamines would be more important than total intra- and extracellular levels in an analysis of the regulatory function of these compounds on RNA synthesis. From the cultures in which we estimated total polyamine production, portions were removed, cells were collected on membrane filters (Millipore Corp.), and the total polyamine contents of the cells were determined. The intracellular putrescine concentration in cells in a growing culture of the stringent strain remained fairly constant (Fig. 4). The level was not significantly influenced by the omission of arginine from the medium. On the other hand, spermidine in the cells increased for a period of 60 min to more than twice the original level. The removal of arginine caused a slight reduction in the initial rate of spermidine accumulation in the cells.

After infection, the intracellular polyamine contents were drastically changed. The cellular spermidine increased very sharply. In the absence of arginine, this increase was more than 300% in 2 h and indeed was even greater than in the presence of arginine. Although the putrescine level showed a transient increase, it was rapidly restored to the original level.

Figure 5 shows the results obtained with the relaxed strain. As in the stringent strain described above, the cellular putrescine level remained unchanged either in the uninfected control or in the R17-infected cells. The omission of arginine did not influence this putrescine level. The level in the relaxed cells was slightly (approximately 10%) higher than that in the stringent organism. Nevertheless, the behavior of these two strains was quite similar with respect to the intracellular level of putrescine in the presence or absence of arginine in the media.

On the other hand, the levels of cellular spermidine in the relaxed strain were different from those observed with the stringent cells. In the uninfected growing culture, the cellular spermidine level reached a maximum in 30 min after the onset of the incubation and decreased thereafter. At the end of the 2-h period, the cellular spermidine content was equivalent to the maximum level observed in the stringent organism. When the medium was deprived of arginine, the cellular levels of spermidine became significantly higher than those determined in the presence of the amino acid.

During the normal infection, the spermidine

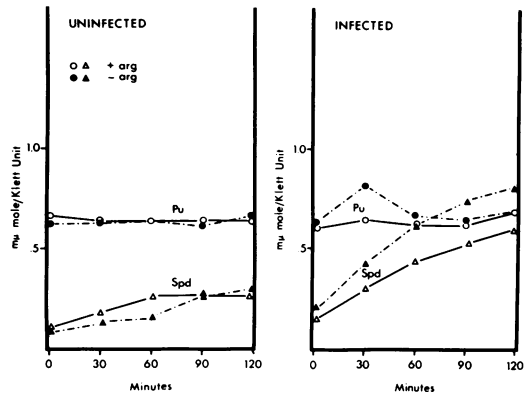

FIG. 4. *Total cellular polyamine levels in E. coli CP78. Assays were performed on the same samples described in Fig. 1A. The cellular polyamine contents are expressed as millimicromoles per Klett turbidity unit.*

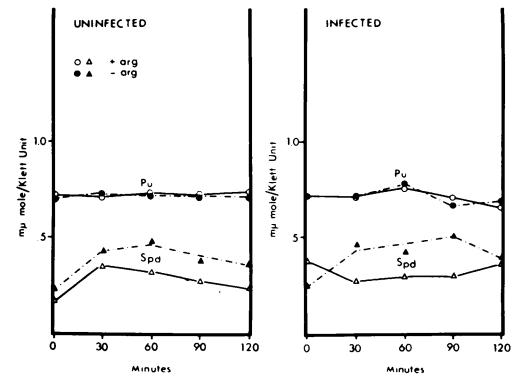

FIG. 5. *Total cellular polyamine levels in E. coli CP79. Assays were performed on the same samples described in Fig. 1A. The cellular polyamine contents are expressed as millimicromoles per Klett turbidity unit.*

content of turbidity unit did not increase, unlike that in the stringent strain under the same conditions. On the other hand, when the relaxed cells were infected in the absence of arginine, the cellular spermidine did increase sharply and remained about 50% higher than that observed during the normal infection.

DISCUSSION

We obtained R17-sensitive F^+ derivatives of both stringent and relaxed strains of *E. coli*. One-step growth curves for the phage in both strains were similar. Exogenous arginine was essential to a burst in each host. The burst of infectious centers, approximately 2,000 per cell, began at 40 min after infection and was completed in 30 min.

Although the requirement for polyamines in

phage T4 multiplication has been demonstrated (11), the role of these amines in the growth of RNA-containing bacteriophage is not known. The present study has shown that there is a close correlation between the RNA accumulation in the R17-infected cells and the biosynthesis and accumulation of spermidine. We have observed that (i) polyamine synthesis continued during infection by R17 of both the stringent (CP78) and the relaxed (CP79) strain; (ii) infection of the stringent strain resulted in a very considerable accumulation of newly synthesized spermidine; and (iii) this biosynthesis and accumulation was even augmented in the absence of arginine during infection. Thus, under conditions of amino acid deficiency, an R17-infected stringent bacterium showed a relaxed response with respect to both RNA and spermidine synthesis, although reproduction of virus was undetectable. It will be of interest to determine if the R17 replicase and viral RNA have been synthesized under these conditions. It is possible that some host arginine had been made available, perhaps by turnover, and that some protein synthesis had occurred. Preliminary results (data not shown) indicate that both cellular and viral RNA are synthesized in the absence of arginine.

The structural relations of the newly synthesized spermidine and of RNA will also be of interest, as well as the relations of these units in virus particles. From a calculation of the spermidine content of virus particles (700 to 1,000 spermidine molecules per phage) it can be estimated that the spermidine associated with virus (2,000 particles per cell) accounts for a high percentage of the newly synthesized polyamine. Whether any of the virus-associated spermidine is preformed in the uninfected host remains to be determined.

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