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Interrelation Between Guanosine Tetraphosphate Accumulation, Ribonucleic Acid Synthesis, and Streptomycin Lethality in *Escherichia coli* CP78

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The effects of streptomycin on the synthesis of ribonucleic acid (RNA) and guanosine-3′-diphosphate-5′-diphosphate have been examined in the isogenic strains CP78 (*rel*$^+$) and CP79 (*rel*−). In the absence of the required amino acids arginine, leucine, or threonine, streptomycin stimulates RNA synthesis in CP78 and this stimulation coincides with cell death. However, in the absence of histidine, also a required amino acid, streptomycin kills the cells without stimulation of RNA synthesis above that which occurs in the absence of streptomycin. In all of these instances, guanosine-3′-diphosphate-5′-diphosphate levels vary inversely with RNA synthesis, decreasing when a stimulation of RNA synthesis occurs. Streptomycin has little effect on guanosine-3′-diphosphate-5′-diphosphate levels in CP78 in the absence of histidine. Another histidine auxotroph, 15 T$^-$H$^-$U$^-$ (*rel*$^+$), does show streptomycin-stimulated synthesis of RNA which is coincident with cell death. CP79 (*rel*$^-$) is 10 times more susceptible to streptomycin than CP78 and streptomycin causes an inhibition of the relaxed synthesis of RNA. The greater susceptibility of CP79 may be due to a greater initial uptake of streptomycin by CP79.

Previous work from this laboratory (13, 14) on streptomycin-induced lethality in *Escherichia coli* 15 T$^-$A$^-$U$^-$ (*rel*$^+$) has shown that, in the absence of the required amino acid arginine, there is a marked stimulation of ribonucleic acid (RNA) synthesis which is coincident with the onset of cell death. Later studies by Freda and Cohen (7) showed that the synthesis of 16*S* ribosomal RNA is specifically stimulated by streptomycin and it was suggested that the synthesis of this class of RNA is in some way related to cell death. Consistent with this hypothesis was the observation that the isogenic relaxed mutant, 15 T$^-$A$^-$U$^-$ (*rel*$^-$), which synthesizes ribosomal RNA actively in the absence of arginine, was killed much more rapidly by streptomycin (3).

Whereas much is known about the inhibitory activity of streptomycin on ribosomes engaged in protein synthesis, the nature of the lethality of the antibiotic is far from clear. Although it has been suggested that the inactivation of ribosomes may produce the irreversible phenomenon known as killing, it is not clear how such inactivation would necessarily lead to cell death.

We have extended the studies on the isogenic strains of T$^-$A$^-$U$^-$ to the stringent and relaxed strains *E. coli* CP78 (*rel*$^+$) and CP79 (*rel*$^-$), K-12 derivatives which require thiamine and the amino acids arginine, histidine, leucine, and threonine. We have investigated the effect of streptomycin on cell viability, RNA synthesis, and guanosine-3′-diphosphate-5′-diphosphate (ppGpp) synthesis when each of the required amino acids is withheld singly. We have found that in CP78 streptomycin treatment in the absence of arginine, leucine, or threonine provides results consistent with those obtained in the stringent strain of 15 TAU. However, when histidine is withheld from CP78, streptomycin treatment, while still lethal to the cells, does not cause the stimulation of RNA synthesis. The accumulation of ppGpp, which is depressed when RNA synthesis is stimulated by streptomycin in the absence of the other required amino acids, is slightly stimulated in the absence of histidine. Another histidine-requiring strain, *E. coli* 15 T$^-$H$^-$U$^-$ (*rel*$^+$), does not show these effects in the absence of histidine. The result obtained with CP78 in the absence of histidine is the first indication of streptomycin-induced lethality in a strain of *E. coli* under stringent conditions which is not accompanied by a marked stimulation of RNA synthesis. However, this apparent exception may be due

to leakiness in the histidine mutation and the observations made under other conditions may still hold generally.

As found with 15 T⁻A⁻U⁻ (*rel*⁻), CP79 is much more susceptible to streptomycin than the isogenic stringent strain. Uptake experiments with ^{14}C-labeled streptomycin suggest that the greater susceptibility may be due to a higher initial uptake of streptomycin by the relaxed strain.

MATERIALS AND METHODS

Chemicals. [2-^{14}C]uracil (specific activity, 45.5 μCi/μmol) was obtained from Calbiochem. Carrier-free ^{32}P-inorganic phosphate was obtained from International Chemical and Nuclear Corp., Irvine, Calif. Uniformly labeled ^{14}C-labeled streptomycin (specific activity, 0.076 μCi/μmol) was the generous gift of Frank Wolf of Merck and Company. Streptomycin sulfate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Polyethyleneimine cellulose thin-layer chromatography plates were from Merck and Company. An authentic sample of ppGpp was the gift of M. Cashel. All other reagents used were the purest grades obtainable.

Strains and media. All strains used were from stocks maintained in our laboratory. Strains CP78 and CP79 require thiamine (used at 5 μg/ml) and arginine, histidine, leucine, and threonine (used at 40 μg/ml of each). Strain 15 T⁻H⁻U⁻ *rel*⁺ requires thymine (2 μg/ml), histidine (20 μg/ml), and uracil (10 μg/ml). For routine viability and uracil incorporation experiments, a standard high phosphate medium was used (8). Strains CP78 and CP79 with the above supplements and 2 mg of glucose per ml have a doubling time of 55 min in this medium. Strain 15 T⁻H⁻U⁻, in the presence of 1 mg of glucose, has a doubling time of 115 min by using 2 μg of thymine per ml; the doubling time is reduced to 85 min by using 10 μg of thymine per ml, although experimental cultures contained 2 μg of thymine per ml. Cell viability was determined by plating appropriate dilutions on nutrient agar plates.

For [^{14}C]uracil incorporation experiments, uracil was used at a concentration of 10 μg/ml with a specific activity of 100 counts/min per nmol. Incorporation of radioactivity into trichloroacetic acid-insoluble material was measured as described previously (13, 14). In ^{32}P-labeling experiments for the determination of ppGpp levels, a low phosphate medium buffered by *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) was used, consisting of (per liter) 11.9 g of HEPES, 1.0 g of $(NH_4)_2SO_4$, 0.15 g of KCl, 0.5 g of Na_3 citrate·$3H_2O$, 0.0016 g of $FeCl_3$, and 2×10^{-4} M KH_2PO_4, final pH 7.4. [^{32}P]inorganic phosphate was added to a final activity of 25 to 50 μCi/ml. Levels of ppGpp were determined by extraction of cells with 2 N formic acid followed by chromatography of the extracts on polyethyleneimine-cellulose thin-layer chromatography plates as described by Cashel (2). Regions of the chromatograms corresponding to authentic ppGpp were visualized under ultraviolet light, scraped into scintillation vials containing 10 ml of 3a70 scintillation fluid (Research Products International Corp.), and counted in a Packard model 3003 scintillation spectrometer.

Uptake of streptomycin was measured by collecting samples of bacteria treated with labeled streptomycin (80 μg/ml as free base, 5.8×10^4 counts/min per μmol) on membrane filters (Millipore Corp.) (0.45 μm), washing with 5 ml of water and counting the dried filters in 6 ml of Liquifluor (New England Nuclear).

RESULTS

Viability and RNA synthesis. In Fig. 1A are data on the viability of CP78 in the absence of arginine and in the presence of varying amounts of streptomycin (given as micrograms of free base per milliliter). Little loss of viability is seen with 40 μg of streptomycin per ml and viability decreases with increasing streptomycin concentrations. RNA synthesis (Fig. 1B) at the same time is initially at or below control levels but increases with the onset of cell death. Similar results are obtained if threonine or leucine (not shown) are withheld. If histidine is withheld, loss of viability occurs as previously (Fig. 2A); however, RNA synthesis is noticeably depressed with the addition of streptomycin (Fig. 2B). As the streptomycin concentration is increased, some stimulation of RNA synthesis occurs, although the levels remain below those obtained in the absence of streptomycin. As a comparison, if strain 15 T⁻H⁻U⁻, a histidine-requiring strain, is treated with streptomycin in the absence of histidine, loss of viability occurs which is coincident with the stimulation of RNA synthesis (6).

As found with 15 T⁻A⁻U⁻ (*rel*⁻), the relaxed strain CP79, in the presence or absence of required amino acids, is much more susceptible to streptomycin than CP78 (Fig. 3A), requiring approximately one-tenth the concentration to

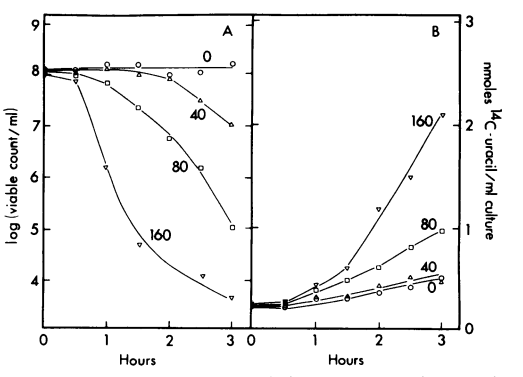

FIG. 1. *Viability (A) and RNA synthesis (B) in CP78 in the absence of arginine and the presence of varying concentrations of streptomycin (given as free base [μg/ml]).*

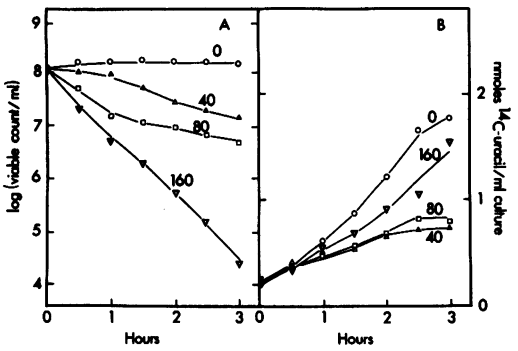

FIG. 2. *Viability (A) and RNA synthesis (B) as in Fig. 1, except histidine was withheld instead of arginine.*

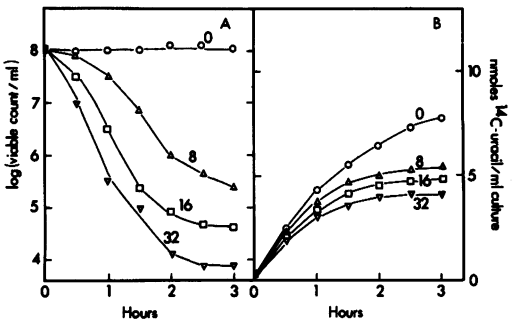

FIG. 3. *Viability (A) and RNA synthesis (B) in CP79 in the absence of arginine and the presence of varying concentrations of streptomycin (given as free base [μg/ml]).*

achieve the same degree of killing. In CP79, the synthesis of some stable RNA is occurring in the absence of the required amino acids, and the addition of increasing amounts of streptomycin results in a corresponding decrease in RNA synthesis (Fig. 3B).

Accumulation of ppGpp in CP78. In Fig. 4 is shown the synthesis of RNA in CP78 in the absence of arginine and in the presence and absence of streptomycin in the HEPES-buffered low phosphate medium. As found in high phosphate, there is a stimulation of RNA synthesis coincident with the onset of lethality. In this case, RNA stimulation and cell death both occur without a lag, and RNA accumulation is stimulated threefold after 3 h. Under these conditions, accumulation of ppGpp is stimulated threefold within 30 min of amino acid depletion, after which time accumulation proceeds more slowly (Fig. 5A). If streptomycin (80 μg/ml) is added, there is no net accumulation of ppGpp. Qualitatively similar results are found if threonine (Fig. 5B) or leucine (Fig. 6) are withheld. Leucine withdrawal leads to a fairly constant rate of accumulation of ppGpp; threonine withdrawal causes a rapid accumulation of ppGpp within 30 min, after which the level of ppGpp is maintained. When streptomycin is added to cultures under these conditions, there is little or no net accumulation of ppGpp. If histidine is withheld, there is no stimulation of RNA synthesis when streptomycin is added

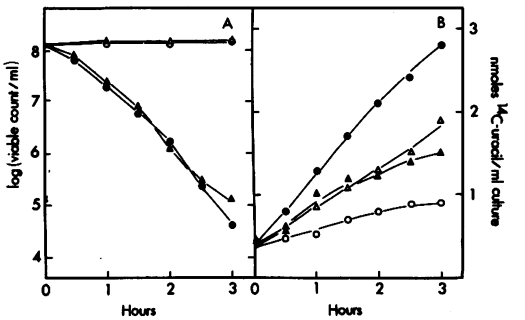

FIG. 4. *Viability (A) and RNA synthesis (B) in CP78 in the HEPES-buffered low phosphate medium in the absence of arginine (○, ●) and histidine (△, ▲), in the absence (○,△) and presence (●,▲) of 80 μg of streptomycin per ml.*

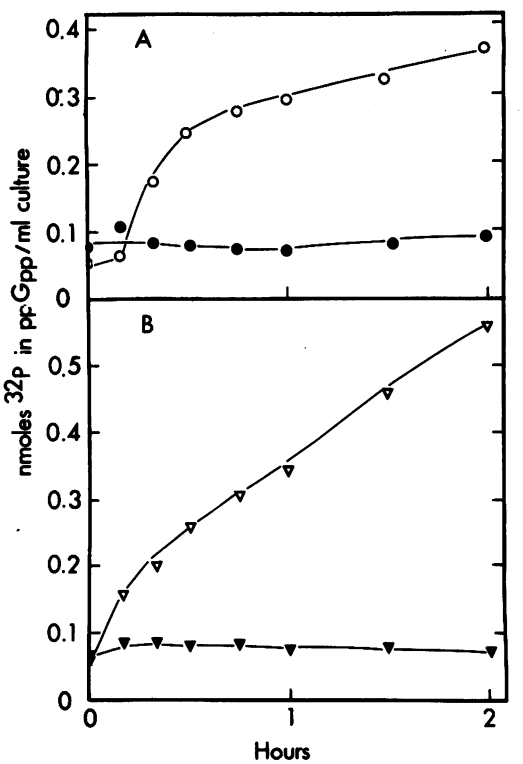

FIG. 5. *Accumulation of ppGpp in CP78 in the absence of arginine (A) and threonine (B) and in the absence (○,△) and presence (●,▲) of 80 μg of streptomycin per ml.*

(Fig. 4B). Under these conditions, cells lose viability at the same rate as cells depleted of arginine (Fig. 4A); however, accumulation of ppGpp is not inhibited by streptomycin, but is slightly stimulated (Fig. 6). 15 $T^-H^-U^-$, in the absence of histidine, shows the normal stimulation of RNA synthesis coincident with lethality (6). Accumulation of ppGpp is shut off in the presence of streptomycin as found with CP78 in the absence of arginine, leucine, or threonine.

It is noted that the greater susceptibility of cells to streptomycin in a tris(hydroxymethyl)aminomethane-buffered, low phosphate medium reported previously (13) is not seen here. This is attributed to the presence of the sulfonate group of HEPES which provides a counter ion as does phosphate ion.

Effect of adenosine and guanosine on CP78. To determine if histidine deficiency in CP78 is affecting RNA synthesis and streptomycin lethality by lowering purine levels, adenosine and guanosine were added separately to cultures of CP78 in the absence of histidine. Both nucleosides, when added to the cultures at a concentration of 0.1 mM, stimulate RNA synthesis slightly in the absence of streptomycin. Addition of streptomycin causes an inhibition of RNA synthesis not appreciably different from cultures not containing added nucleosides. Under these conditions, cells lose viability as in the absence of the nucleosides.

^{14}C-labeled streptomycin accumulation in CP78 and CP79. Table 1 shows the amounts of streptomycin taken up by stringent and relaxed cells in 5 min. The levels present in relaxed cells in the presence or absence of amino acids is several times the levels present in stringent cells under the same condition. During this time turbidity measurements indicate that both strains increase in size slightly. Turbidity and viability experiments show that the cells from both strains are the same size.

DISCUSSION

The nature of streptomycin lethality in bacteria has interested investigators for many years. An interesting observation, but one which is mentioned infrequently, is that of the stimulation by streptomycin of RNA synthesis in stringent bacteria in which an essential amino acid is withheld (6, 13, 14). This stimulation, in all previous cases studied, has been shown to coincide with the onset of cell death. This paper is the first report of a situation in which such a relationship between cell death and RNA synthesis does not appear to hold. The relationship of this observation to the nature of streptomycin lethality is not clear.

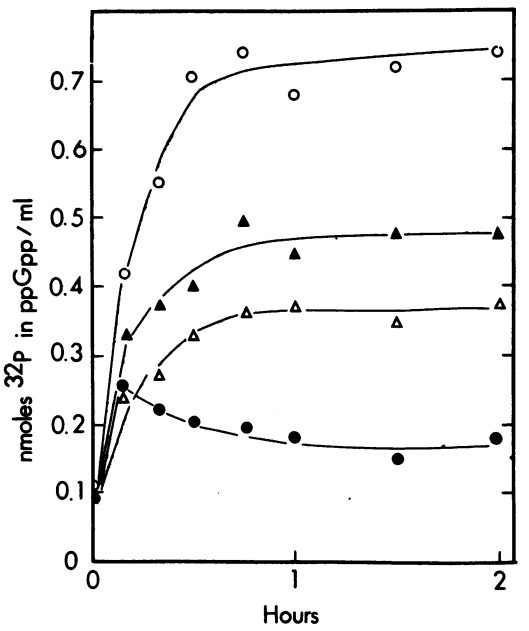

FIG. 6. *Accumulation of ppGpp in CP78 in the absence of leucine (○,●) and histidine (△,▲) in the absence (○,△) and presence (●,▲) of 80 μg of streptomycin per ml.*

TABLE 1. *Uptake of ^{14}C-labeled streptomycin by CP78 and CP79*

Strains	Culture (nmol/ml)	Molecules/cell
CP78 (complete)	0.81	4.43×10^6
CP78 – arginine	0.88	4.82×10^6
CP78 – histidine	0.92	4.77×10^6
CP79 (complete)	1.93	1.28×10^7
CP79 – arginine	1.50	1.81×10^7
CP79 – histidine	1.52	1.18×10^7

Most of the data presented have confirmed the earlier findings from this laboratory on 15 TAU and THU: (i) in the absence of required amino acids, the relaxed strain, CP79, is more susceptible to streptomycin than the stringent strain, CP78; (ii) streptomycin causes a stimulation of RNA synthesis in the stringent strain, CP78, in the absence of any of the required amino acids, except one; and (iii) this stimulation of RNA synthesis is coincident with the onset of cell death. The greater susceptibility of CP79 may be due to a greater initial uptake of the antibiotic by CP79 as compared to CP78.

In contrast to these data, if histidine is withheld from CP78, streptomycin does not stimulate RNA synthesis and cell death still occurs. That this histidine effect is not general

is suggested by the results obtained with 15 $T^-H^-U^-$. Apparently, the effects seen upon histidine deficiency are not attributable to purine depletion, since addition of exogenous purine nucleosides has little effect on cell death and only a slight effect on RNA synthesis.

One explanation for this histidine effect in CP78 is that the mutation for histidine requirement is leaky and that some histidine synthesis is occurring, in effect producing a partially relaxed condition. That this may be occurring is suggested by the higher level of RNA accumulation in the absence of histidine compared to RNA accumulation in the absence of arginine, leucine, or threonine (Fig. 2B versus Fig. 1B). Also, streptomycin has the initial effect of depressing RNA accumulation relative to the control (no streptomycin) levels in the absence of histidine (Fig. 2), as is seen in relaxed strains treated with streptomycin. However, ppGpp levels in CP78 in the absence of histidine suggest a true stringent response. Higher concentrations of streptomycin produce a slight stimulation of RNA synthesis, as is seen when the other amino acids are withheld, although the levels remain below that of the control. Thus, it appears that low levels of streptomycin initially make the cell more stringent and higher concentrations allow some relaxation of RNA synthesis.

The greater susceptibility of relaxed strains to streptomycin than the isogenic stringent strains may be due to greater permeability of the relaxed strain (Table 1). How this difference in uptake relates to the mechanism of cell death is not clear, although damage to the membrane may be a simple explanation. The data in the table show that there are more than enough molecules of streptomycin to bind all ribosomes in a cell, assuming 10^4 ribosomes per cell. Thus, the possibility that streptomycin binds to and affects other macromolecules and structures should be considered.

The levels of ppGpp in CP78 treated with streptomycin in the absence of amino acids seem to correlate well with the stringency of the cultures. Cultures in which streptomycin causes a stimulation of RNA synthesis show a marked decrease in ppGpp levels, whereas ppGpp levels remain fairly high when no stimulation of RNA synthesis is occurring, as is seen when histidine is withheld.

The ppGpp data are not consistent with the observation of Pederson et al. (12) that streptomycin, in an in vitro ppGpp-synthesizing system, stimulates the formation of ppGpp, perhaps, as they suggest (12), by altering the conformation of the transfer RNAs (tRNA's) involved. Our in vivo data show that streptomycin inhibits formation of ppGpp, suggesting that the effects of streptomycin are more complex than alteration of tRNA conformation.

The synthesis, or control of synthesis, of RNA appears to play an important role in streptomycin-induced lethality. The data support, or do not contradict, a role for ppGpp in which it provides at least a measure of the stringency of a cell. The data also suggest that streptomycin interacts with cellular components in a way or ways that ultimately affect the synthesis of stable RNA. Conceivably, this is mediated through the formation and activity of ppGpp, although such a role of ppGpp has yet to be proven rigorously. Streptomycin may interact with ribosomes by binding to its presumed site of action and thus effect the ribosome-associated stringent factor apparently involved in the control of RNA synthesis (1, 9). Alternatively, streptomycin may affect the binding of tRNA to ribosomes and thus affect the control mechanism, since one condition for the stringent response is the unavailability of aminoacylated tRNA (4, 5). It is known that streptomycin can prevent binding of fMet-tRNA and can induce the release of fMet-tRNA from initiation complexes (10, 11), presumably by distortion of the ribosomal A and P sites, and possibly by altering the conformation of tRNA. It seems possible that other tRNA's are also affected. The degree to which each of these possibilities contributes to the effects of streptomycin is not known; however, with the amount of streptomycin found in the cell, it seems likely that each interaction is playing at least some role.

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