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Heat Shock and Hydrogen Peroxide Responses of *Escherichia coli* Are Not Changed by Dinucleoside Tetraphosphate Hydrolase Overproduction

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In *Escherichia coli* strains overproducing dinucleoside tetraphosphate hydrolase, the accumulation of dinucleoside tetraphosphates (AppppN, with N = A, C, G, or U) during heat shock or H_2O_2 treatment was reduced about 10-fold as compared with a control strain. This accumulation neither modified the pattern of the proteins induced by a temperature shift or H_2O_2 nor reduced the protection against oxidative damage induced by moderate H_2O_2 levels.

Diadenosine 5′,5‴-P[1],P[4]-tetraphosphate (AppppA) is an unusual nucleotide occurring in a wide variety of cells from bacteria to humans (1, 3, 4, 9, 14, 22, 23, and references therein). Ames and co-workers observed that AppppA accumulated when bacteria were exposed to heat shock or to various oxidizing agents (3, 11, 12). In parallel, the other dinucleoside tetraphosphates (AppppN, with N = C, G, or U) or triphosphates (ApppN, with N = A, C, G, or U) also accumulated in bacteria (3; P. Plateau, A. Brevet, and S. Blanquet, *in* A. M. Krstulovic, ed., *Handbook of Nucleic Acids and Related Compounds*, in press). These authors proposed that dinucleoside polyphosphates may be alarmones signaling the onset of the stress and, thus, may be responsible for the cellular adaptation (3). This hypothesis gained support with the demonstration that a similar relationship between stress and dinucleoside polyphosphate metabolism occurred in other cellular types such as *Drosophila* (4), mouse (1), and *Physarum* (9) cells.

The gene for the *Escherichia coli* enzyme responsible for AppppN catabolism (AppppN hydrolase) has been recently isolated (2, 13). This enzyme is also capable of hydrolyzing ApppN (20). In this work, *E. coli* strains overproducing AppppN hydrolase were used to investigate the putative role of the AppppN nucleotides in triggering the heat shock response. In these strains, AppppN concentration was decreased by a factor of about 10, as compared to the wild type (13), and AppppN accumulation upon heat shock treatment was markedly reduced. The same strains were also used to examine the role of AppppN in protection against oxidative damage by H_2O_2 (6, 8).

Heat shock response. To determine whether the cellular AppppN concentration influences heat shock protein (HSP) synthesis in *E. coli*, strain SC122 was transformed with plasmid pUC1247 carrying *apaH*, the gene encoding the *E. coli* AppppN hydrolase (13). AppppN hydrolase specific activity was measured (20) in this strain and in the control strain transformed with pUC12 plasmid. The activities were 380 and 20 U/mg, respectively. To identify the HSP, the *htpR* mutant K165 (7, 17) was used after transformation with the same plasmids. The amounts of AppppN hydrolase activity in K165 transformants were identical to those in the corresponding SC122 transformants.

The variations of AppppN concentration were followed after exposure to heat shock from 30 to 46°C. For this purpose, cells were grown at 30°C in 3-(*N*-morpholino)propanesulfonic acid (MOPS) medium (16) containing 0.4% glucose, 0.1 mg of tryptophan per ml, and 50 μg of ampicillin per ml. When cultures reached an optical density of 0.3 to 0.5 at 600 nm, heat shock was applied to a 10-ml sample by transferring the flask to a water bath at 46°C. After the temperature shift, 0.1-ml samples were withdrawn from the culture at various times and mixed with perchloric acid (final concentration, 10% [wt/wt]). The cellular extract was centrifuged, and the supernatant was neutralized with 5 M potassium carbonate. Each sample was then diluted threefold in a 20 mM Tris hydrochloride buffer (pH 7.8) containing 1 mM $MgCl_2$ and incubated for 30 min at 37°C with 10 U of alkaline phosphatase (from Boehringer) per ml to remove ATP. AppppN concentration was determined by biolumine-

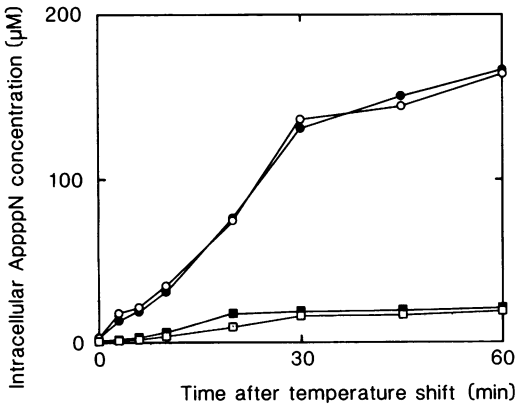

FIG. 1. AppppN concentration during heat shock in *E. coli* cells. The cellular concentrations of nucleotides were calculated assuming that 1 unit of optical density at 600 nm corresponded to 0.4 μl of intracellular volume (21). Symbols: Strain SC122 transformed with pUC12 (○) or pUC1247 (□); K165 with pUC12 (●) or pUC1247 (■).

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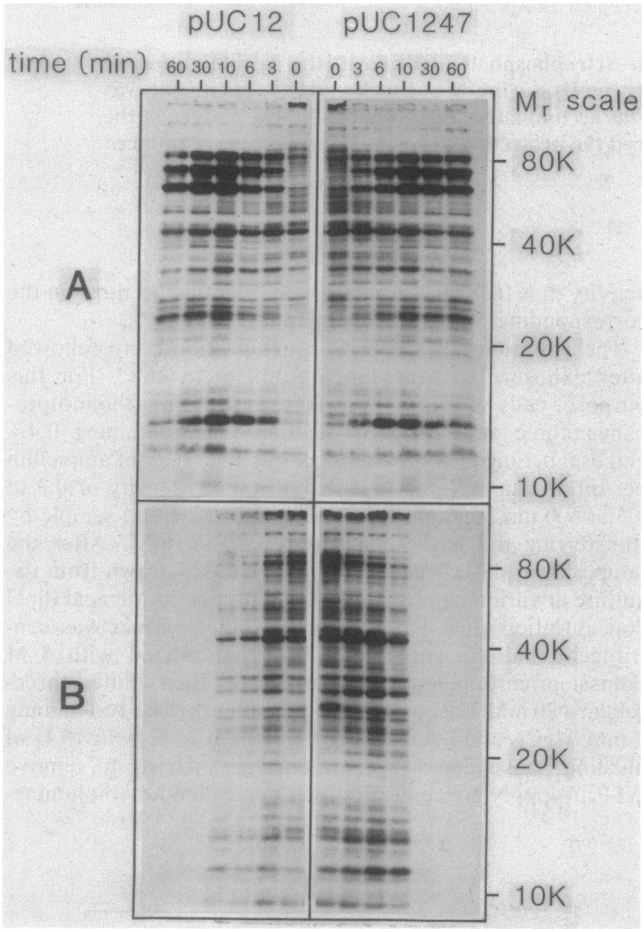

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of HSP synthesis. Strains SC122 (A) and K165 (B), with either pUC12 or pUC1247, were heat shocked as described in the text. Samples applied in the slots were measured for labeled trichloroacetic acid-precipitable material: 100,000 cpm ± 30% in all slots, except for times 6 and 10 min in the left side of panel B (50,000 cpm). Gels were treated for fluorography with sodium salicylate (5) and autoradiographed.

scence with a luciferin-luciferase-phosphodiesterase assay (19; Plateau et al., in press).

The pUC12 control strains accumulated ApppppN in a linear fashion from an initial value of 3.2 μM. A plateau value of 150 to 160 μM was reached 30 min after the heat shock treatment (Fig. 1). The strains showed identical kinetics of ApppppN variation, thus confirming the previous conclusion that ApppppN accumulation is not under the control of the *htpR* allele (11). In the strains with the pUC1247 plasmid, the initial ApppppN concentration was less than 1 μM, due to the overproduction of the ApppppN hydrolase. Although the ApppppN concentration increased somewhat upon heat shock, it never exceeded 20 μM.

In this experiment, 0.5-ml portions of the culture were also pulse-labeled for 4 min with [^{35}S]methionine (0.45 μM, 74 kBq/ml). Labeling was stopped by isotopic dilution with 30 μl of 1.5 mM methionine, and the samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (10). Upon heat shock, the synthesis of proteins of M_r 83,000 (83K), 75.5K, 66K, and 13.6K strongly increased in the SC122 strains (Fig. 2A), in agreement with previous reports (24). As expected, this was not seen in the K165 mutant (Fig. 2B). Also, neither the kinetics nor the intensities of HSP expression were sensitive to an overproduction of the ApppppN hydrolase. With both plasmids, HSP induction was seen about 3 min after the temperature shift. At this time, in the SC122(pUC1247) cells, ApppppN concentration was less than 1.5 μM, a value smaller than that in the nonstressed control SC122(pUC12) cells (3.2 μM). Therefore, ApppppN accumulation beyond a resting threshold concentration value is not required for HSP induction.

Adaptation to H_2O_2. The second part of this study asked whether ApppppN nucleotides could play a role in the adaptation of *E. coli* to oxidative damage by hydrogen peroxide. For this purpose *E. coli* SC122 cells containing pUC12 or pUC1247 were exposed for 10 min to 1 mM H_2O_2, and newly synthesized proteins were analyzed by two-dimensional gel electrophoresis (18). Cellular ApppppN concentrations were measured in the same strains.

H_2O_2 stimulated the synthesis of a minimum of 20 polypeptides in strain SC122(pUC12) (Fig. 3A and B). The ApppppN concentration increased from 3.2 to 11.6 μM.

Spots 1 and 2 in Fig. 3A and B depended on the presence of either plasmid and probably correspond to the β-lactamase and its precursor (15). Spot 3 in Fig. 3C (pUC1247) is absent from panel A (pUC12) and was identified as the ApppppN hydrolase.

Figure 3B (pUC12) and D (pUC1247) show the effect of ApppppN hydrolase overproduction on the response to H_2O_2. No differences were seen. Because the ApppppN concentration in the ApppppN hydrolase-overproducing strain after the H_2O_2 treatment was smaller (<1 μM) than that in the nonstressed control strain (3.2 μM), it is unlikely that the changes observed in the protein pattern could have been caused by an ApppppN accumulation beyond a resting threshold concentration value.

However, at this stage, it could not be excluded that the resistance to killing by H_2O_2 conferred by pretreatment with nonlethal levels of H_2O_2 (6, 8) was caused by an interaction of ApppppN with an unknown protein(s). To test this possibility, the cell viability of *E. coli* SC122(pUC1247) was measured as described elsewhere (8), i.e., after 5 mM H_2O_2 challenge with or without pretreatment with 30 μM H_2O_2. At various times, samples (100 μl) were withdrawn from the H_2O_2-treated cultures and diluted in MOPS buffer. Portions

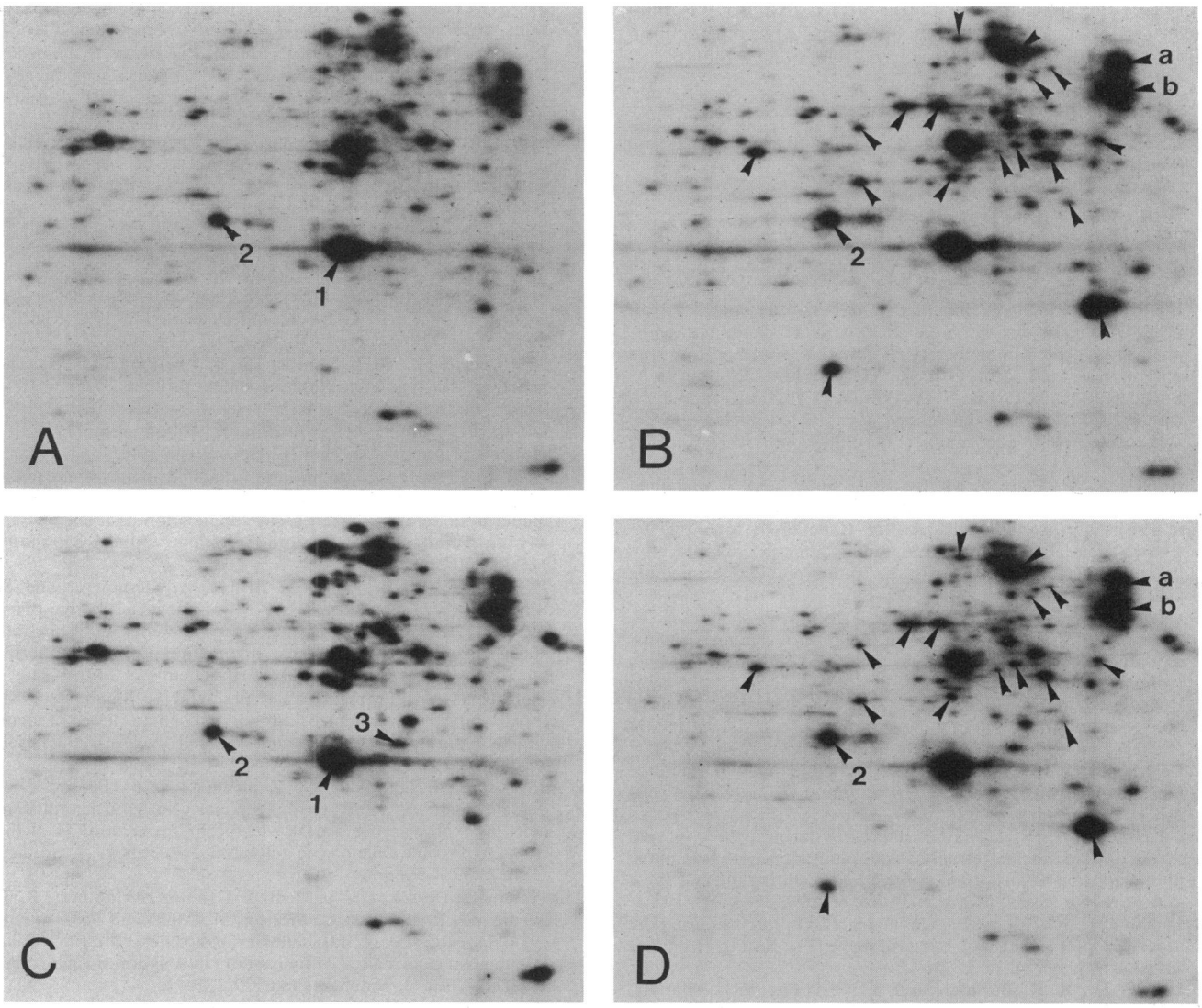

FIG. 3. Protein synthesis in strain SC122 with pUC12 or pUC1247, before or after H_2O_2 treatment. Cells were grown at 37°C in MOPS medium and pulse-labeled for 4 min with [^{35}S]methionine. Proteins were then analyzed by two-dimensional gel electrophoresis. Gels were treated for fluorography and autoradiographed. Strain SC122(pUC12): panel A, without H_2O_2 treatment; panel B, after 10 min of treatment with 1 mM H_2O_2. H_2O_2-specific polypeptides are indicated by arrows. Spots a and b correspond to *dnaK* and *groEL* gene products, respectively. Strain SC122(pUC1247): panel C, without H_2O_2 treatment; panel D, after 10 min of treatment with 1 mM H_2O_2.

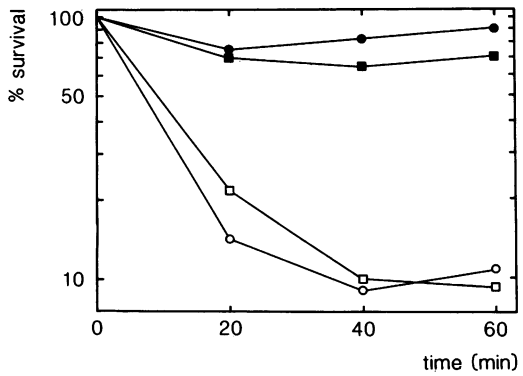

FIG. 4. Influence of AppppN hydrolase overproduction on induced resistance to H_2O_2. Strain SC122 carrying pUC12 (○, ●) or pUC1247 (□, ■) was grown at 37°C in MOPS medium. In the exponential growth phase, the culture was divided, and either no addition was made (○, □) or 30 μM (final) H_2O_2 was added (●, ■). Thirty minutes later, 5 mM H_2O_2 was added to each culture. At the times indicated, portions were removed to determine the number of CFU.

were plated on LB agar with 3 ml of molten soft agar. AppppN hydrolase overproduction neither prevented adaptation to hydrogen peroxide nor modified the viability after the 5 mM H_2O_2 challenge (Fig. 4).

In conclusion, this study indicates that the heat shock response in *E. coli* is not dependent on a high intracellular concentration of diadenosine tetraphosphate. In addition, high levels of dinucleoside tetraphosphates are unnecessary for adaptation to hydrogen peroxide stress. An abnormally small AppppN concentration (i) neither modifies the pattern of the protein induced by H_2O_2, (ii) nor reduces the resistance to H_2O_2 induced by moderate H_2O_2 levels, (iii) nor

decreases the survival of bacteria after a strong H_2O_2 challenge. Under our experimental conditions, AppppN accumulation during the H_2O_2 stress has no beneficial effect.

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