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Inorganic Polyphosphate Supports Resistance and Survival of Stationary-Phase *Escherichia coli*

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The *Escherichia coli* mutant (*ppk*) lacking the enzyme polyphosphate kinase, which makes long chains of inorganic polyphosphate (poly P), is deficient in functions expressed in the stationary phase of growth. After 2 days of growth in a medium limited in carbon sources, only 7% of the mutants survived compared with nearly 100% of the wild type; the loss in viability of the mutant was even more pronounced in a rich medium. The mutant showed a greater sensitivity to heat, to an oxidant (H_2O_2), to a redox-cycling agent (menadione), and to an osmotic challenge with 2.5 M NaCl. After a week or so in the stationary phase, mutant survivors were far fewer in number and were replaced by an outgrowth of a small-colony-size variant with a stable genotype and with improved viability and resistance to heat and H_2O_2; neither polyphosphate kinase nor long-chain poly P was restored. Suppression of the *ppk* feature of heat sensitivity by extra copies of *rpoS*, the gene encoding the RNA polymerase sigma factor that regulates some 50 stationary-phase genes, further implicates poly P in promoting survival in the stationary phase.

Inorganic polyphosphates (poly P), linear polymers of P_i linked by phosphoanhydride bonds, are ubiquitous in nature, having been found in all organisms examined (31, 32, 57). Microbes, including bacteria, cyanobacteria, fungi, and algae, accumulate poly P, which may amount to 10 to 20% of their dry weight. Chain length may vary from 3 to 1,000 P_i residues, depending on the organism, its growth, and physiological conditions (45, 53, 54). Despite its ubiquity and conservation in evolution, the physiological roles of poly P remain unknown or ignored. Poly P provides a substitute for ATP for sugar and adenylate kinases (8, 13, 22, 42), a pH-stat to counterbalance alkaline stress (43, 44), a phosphate reservoir with osmotic advantages (31, 33), an energy source (31, 57), a chelator of divalent metal ions (4, 14), a membrane component for DNA entry and transformation (46), and a possible regulator in transcription. Poly P appears to have a multiplicity of roles in its many locations (30). To elucidate the functions of poly P in *Escherichia coli*, we examined mutants that fail to synthesize polyphosphate kinase (PPK) and lack the long-chain poly P. These mutants failed to survive in stationary phase and were less resistant to heat and oxidants (10). In further studies reported here, the mutants, after a few days in the stationary phase, were replaced by a small-colony variant that was genetically stable, more viable, and stress resistant. Still other results implicate poly P in the complex responses that promote survival in the stationary phase.

MATERIALS AND METHODS

Reagents. The sources of the reagents used in this study were as follows: ATP, creatine kinase, catalase, DNase I, and RNase, Boehringer Mannheim; creatine phosphate, 3-(*N*-morpholino) propanesulfonic acid (MOPS), menadione, kanamycin, ampicillin, and bovine serum albumin, Sigma; H_2O_2, Fisher Scientific Co.; Immobilon-N membrane, Millipore; [γ-^{32}P]ATP, Amersham Corp.; [^{3}H-methyl]thymidine, ICN Biomedicals Inc.; and polyethylene imine-cellulose F thin-layer chromatography plates, Merck. rPPX1 used in the poly P assay was prepared and assayed as described previously (59).

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Bacterial strains and plasmids. The following *E. coli* K-12 derivatives were used: JM101 {Δ(*lac-proAB*) *supE thi-1*/F' [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ M15]}; CA10, the *ppk ppx* mutant constructed by transfer of a disrupted *ppk* gene, insertionally inactivated with a gene for kanamycin resistance, into JM101 by P1 transduction (10); CA10 pMM*kat*F3, constructed by transformation of CA38 (CA10 *recA*) with pMM*kat*F3 (40) (obtained from A. Matin, Stanford University, Stanford, Calif.); and pBC29, a multicopy plasmid bearing the gene *ppk*, which was used for poly P overexpression (2). *rpoS*::Tn*10* derivatives of CA10 and the CA10 small-colony phenotype were made by transduction with P1 grown on MC8301 [MC4100 F$^-$ *araD139* Δ (*arg-lac*) *u169 rpsL150 relA1 flb B5301 deoC ptsF25 rpsR rpoS*::Tn*10*].

Survival assays. Long-term survival of *E. coli* was assayed as described earlier (10) by growing cells either in MOPS-buffered minimal medium (MOPS medium [41]) containing glucose (0.1%) as the carbon source and K_2HPO_4 (2 mM) as the P_i source or in a rich medium, Luria-Bertani (LB) liquid medium. Viable cell counts were determined by plating of cells onto LB agar plates; kanamycin (50 μg/ml) was added to the medium for plating of CA10.

To test the sensitivity of the stationary-phase cells to H_2O_2, JM101 and CA10 were grown overnight (about 20 h) in LB medium and LB medium with kanamycin (50 μg/ml), respectively; the medium for CA10 pBC29 contained 50 μg of ampicillin per ml. The cells were washed and resuspended in 0.85% NaCl to an optical density at 540 nm (OD_{540}) of 1.0; H_2O_2 was added to a final concentration of 42 mM. At the times indicated, 0.1-ml samples were withdrawn, diluted immediately in 0.85% NaCl, and plated on LB plates to determine viable cell counts. For actively growing cells, JM101 and CA10 were grown aerobically at 37°C to the mid-exponential phase (ca. 4×10^8 cells per ml) in MOPS medium containing glucose (0.4%) and K_2HPO_4 (2 mM). Two-milliliter samples of these cultures were exposed for 15 min to H_2O_2 (0 to 40 mM) in air at 37°C. To study the lethal effect of H_2O_2, the exposure was terminated by addition of 2 μg of catalase per ml, followed by dilution into 0.85% NaCl. Cells were then plated onto LB plates to determine the viable cell count.

Starvation-induced protection against osmotic challenge in JM101 and CA10 cells was assessed by comparison of the viabilities of cultures osmotically challenged during mid-log growth or at 3 h after entering the stationary phase. Cultures were grown at 37°C in M9 medium (8 mM NaCl) supplemented with either 0.025% glucose (for starvation at an OD_{540} of about 1.0 [4×10^8 cells per ml]) or in 0.4% glucose for sampling during exponential growth at a similar cell density. Cultures were osmotically challenged by addition of solid NaCl to the medium to reach a final concentration of 2.5 M (27). Survival was determined by colony counts on LB plates compared with those of the cultures just before the osmotic challenge.

For the heat shock survival assay, cells were grown overnight in LB medium. Stationary-phase cells were washed once and diluted in 0.85% NaCl to a density of about 5×10^3 cells per ml. Samples (2 ml) were put into prewarmed glass tubes (55°C); at the times indicated, 0.1-ml portions were plated directly onto LB plates to determine viable cell numbers.

Growth studies. JM101, CA10 small-colony phenotype, and CA10 big-colony phenotype cells were grown overnight in MOPS medium containing glucose (0.4%) and K_2HPO_4 (2 mM). The cultures were then diluted in fresh, pre-

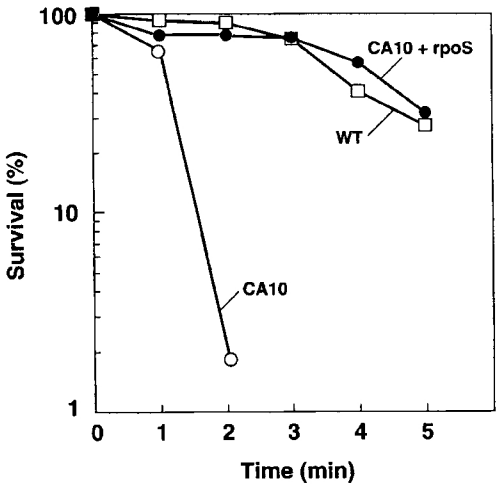

FIG. 1. Heat resistance. Wild-type (WT), CA10, and CA10 pMM*katF*3 cells grown overnight (~20 h) at 37°C in LB medium were exposed to 55°C, and their viability was determined by plating on LB agar, with antibiotics wherever necessary. Percent survival is determined as the viable cell number at each time point divided by the viable cell number before exposure to heat.

warmed (37°C) medium, and samples were removed at hourly intervals to monitor growth by measurement of the turbidity at 540 nm.

[^{3}H]thymidine uptake. Cells were grown to the stationary phase in LB medium aerobically at 37°C and then incubated further for assay of long-term survival (10). The number of surviving cells was determined each day by plating on LB agar. From the 3rd to 10th days, cells were labeled by addition of [^{3}H]thymidine to a final concentration of 1 μM in 100 μl of culture medium and incubation for 60 min at 37°C with shaking. Cells were collected in a microcentrifuge and washed once with 0.85% NaCl. Cold 10% trichloroacetic acid (1 ml) was added to the pellet; the suspension was vortexed and collected on a Whatman GF/C Millipore glass fiber filter, washed first with cold 10% trichloroacetic acid and then with ethanol. Radioactivity was measured in a liquid scintillation counter.

Resistance to menadione. For growth-inhibition measurements, 0.1 ml of an overnight culture grown in LB medium was added to 3 ml of molten F top agar (39) and poured on a K medium agar plate (12). A solution (100 μl) of menadione (2 mg/100 μl of dimethyl sulfoxide) was allowed to saturate a 13-mm-diameter filter disc (Whatman no. 1). The disc was then placed in the center of the solidified top agar plate and allowed to incubate overnight (18 to 24 h). The zone of inhibition was determined by measurement of the diameter of the zone, less that of the disc.

Extraction and assay of poly P. Cells were grown in LB medium to the stationary phase (OD_{540}, ~4.5). Poly P was extracted, and the concentration was estimated by a rapid radioassay technique (36).

Other methods. PPK and exopolyphosphatase (PPX) activities in cell extracts of wild-type and CA10 (small- and big-colony phenotype) cells were assayed as described previously (1, 2).

Catalase activities were measured in cells grown to the stationary phase in LB medium. Cells permeabilized by toluene were used in the assay, which measures the disappearance of peroxide spectrophotometrically at 240 nm (5).

Visualization of catalases (HPI and HPII) on acrylamide gels. Typical hydroperoxidase I (HPI) and HPII bands were visualized by running whole-cell extracts on an 8% polyacrylamide gel and then staining them with a 50:50 solution of 2% $K_3Fe(CN)_6$ and 2% $FeCl_3$ (9, 26). Bovine serum catalase (Sigma) was used as a standard.

RESULTS

Effect of poly P deficiency on long-term survival in the stationary phase: heat resistance of the *ppk* mutant (CA10). During survival in the stationary phase or upon starvation, cells acquire *rpoS*(*katF*)-mediated resistance to multiple stresses, including heat and oxidants (3, 6, 16, 19, 27, 33–35, 56). When poly P-deficient cells (CA10) were held at 55°C for 2 min, only 2% survived compared with about 90% of the corresponding wild-type cells (JM101) (10). The mutant complemented with multiple copies of the plasmid (pMM *katF3*) bearing *rpoS*, the stationary-phase RNA polymerase sigma factor, proved to be as heat resistant as wild-type cells (Fig. 1). Similar results were obtained when the mutant was complemented with a plasmid which contained *ppk* (data not shown).

TABLE 1. Menadione sensitivity of *E. coli* with and without poly P

Strain	Genotype	Killing zone (mm^2)[a]
JM101	*ppk*$^+$	31 ± 13
CA10	*ppk*	200 ± 74
CA38	*ppk recA*	210 ± 24
CA38 pUC18	*ppk recA*	284 ± 6
CA10 pBC29	*ppk*$^+$	13 ± 6

[a] The killing zone measurements represent the average of six separate experiments (see Materials and Methods).

Resistance to oxidative stress. Preliminary studies had noted that poly P-deficient cells in the stationary phase were more sensitive than wild-type cells to 42 mM H_2O_2 (10). When the viability of exponentially growing cells was determined after exposure to various levels of H_2O_2 for 15 min, both wild-type and mutant cells showed similar degrees of sensitivity to low levels (2.5 to 10 mM) of H_2O_2 (Fig. 2). However, the mutants were far more sensitive to higher concentrations of H_2O_2 (15 to 45 mM) than the wild type. Complementation of the mutant in the stationary phase with a plasmid bearing the *ppk* gene restored H_2O_2 resistance to near wild-type levels (data not presented here).

Exposure of cells to the quinone menadione leads to oxidative stress due to the production of oxygen radicals (51); cells lacking catalase, peroxidase, and superoxide dismutase show increased sensitivity (11, 17). The *ppk* mutant in the stationary phase also proved to be highly sensitive to menadione toxicity and could be restored to resistance by complementation with the *ppk* gene (Table 1).

Resistance to osmotic challenge. Stationary-phase cells show enhanced osmotic resistance compared with cultures in the exponential growth phase or preadapted to osmotic stress (27). The levels of osmotic resistance of stationary-phase cells 4 h after they were depleted of a carbon source (glucose) were compared with those of cells in the mid-log phase by exposure to 2.5 M NaCl. Viability in the stationary-phase cultures con-

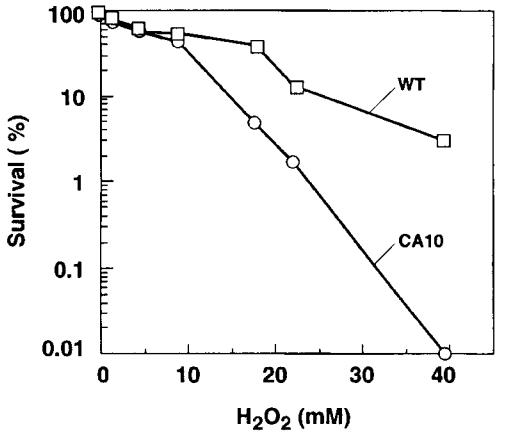

FIG. 2. Resistance to H_2O_2. Wild-type (WT) and CA10 cells were grown exponentially in MOPS medium. The cells were exposed to various concentrations of H_2O_2 for a period of 15 min at 20°C, and the viable cell numbers were determined by plating onto LB agar. Survival of 100% corresponds to the viable cell number determined immediately before the addition of H_2O_2.

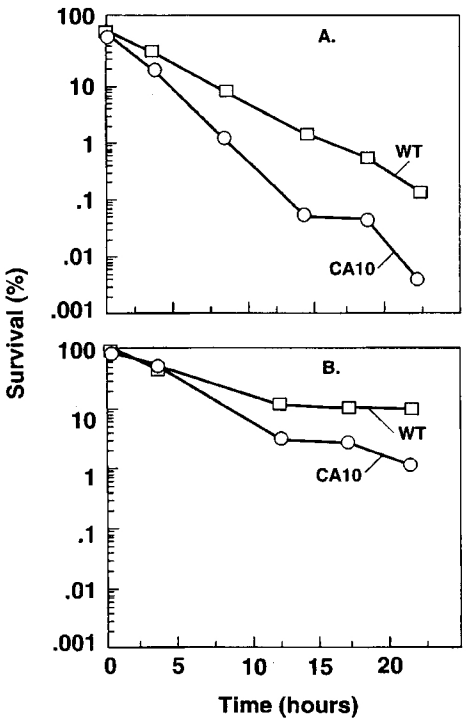

FIG. 3. Osmotic resistance to 2.5 M NaCl. Wild-type (WT) and CA10 cells were examined in the exponential phase (A) or in the stationary phase after 3 h of starvation for glucose (B).

sistently indicated higher osmotic resistance than did that in the cultures challenged during the log phase of growth (Fig. 3). However, the level of protection conferred on the mutant by carbon starvation was lower than that observed in the wild-type cells. After exposure of the starving stationary-phase cells to 2.5 M NaCl for 21.5 h, about 10% of the wild-type cells and only about 1.0% of the mutant cells survived (Fig. 3B). The sensitivity towards osmotic challenge was more pronounced in the exponential cultures, and the absence of long-chain poly P in the *ppk* mutant resulted in even lower levels of viability (Fig. 3A).

Mutants fail to survive in the stationary phase and are replaced by a small-colony variant. Wild-type and CA10 mutant cells were grown to the stationary phase in a synthetic MOPS medium limited in glucose (0.1%) and incubated aerobically at 37°C for several days. Viable cell counts measured after 1 day in the stationary phase indicated a 93% loss for the mutant compared with no loss in the wild type (Fig. 4A). After the second day, the mutant showed equal numbers of novel small colonies and the standard larger type. Both the small- and big-colony phenotypes of the mutant decreased in number upon prolonged incubation. About 3% of the *ppk* cells (both small- and big-colony phenotypes) and 20% of the wild-type cells survived after 10 days in the stationary phase (Fig. 4A). Both phenotypes of the mutant exhibited a growth lag when overnight (~20-h) cultures were diluted and grown in a rich medium (LB); they were also more heat sensitive (10).

In LB medium, survival of the mutant was 0.001 to 1.0% within 4 days of incubation, whereas the wild-type cell number remained about 20% of the initial value (Fig. 4B). As in the minimal medium, a small-colony phenotype appeared in the mutant cultures. Mutants lost about 99% of their viability during the first 4 days of the stationary phase, dropping to values as low as 100 CFU/ml of culture. At this point, the big-colony phenotype predominated, but when cultures grew back to about 5×10^7 CFU/ml, 90% or more of the population were of the small-colony phenotype. Despite variations in loss of viability in several trials (Fig. 4B), over 90% of the mutants were of the small-colony variety after 5 to 6 days. The viability of the wild type remained constant after 3 days of incubation with no visible change in colony morphology.

Thymidine uptake and stationary-phase survival. The dynamic nature of stationary-phase cultures incubated for prolonged periods is manifested in the appearance of revertants and suppressors (52, 61). In order to study the rapid takeover of the mutant population by a small-colony stable phenotype, both the wild type and the mutant were grown to the stationary phase in LB medium and incubated aerobically at 37°C. The viability of the cultures was determined daily for 10 days (Fig. 5). Starting from the third day of incubation, when the viability of both the wild type and the mutant started to decline, the cultures were pulse-labeled for 1 h with [^{3}H]thymidine. A loss in viability of the wild-type cells was observed until the third day of incubation, and then viability remained almost unchanged thereafter; the uptake of [^{3}H]thymidine was not significant (Fig. 5A). The viability of CA10 cells decreased considerably with time to about 10^6 CFU/ml on the seventh day of incubation (Fig. 5B). The drop in viability of the mutant on the seventh day coincided with a burst of thymidine uptake and also coincided with a rapid increase in the number of cells of

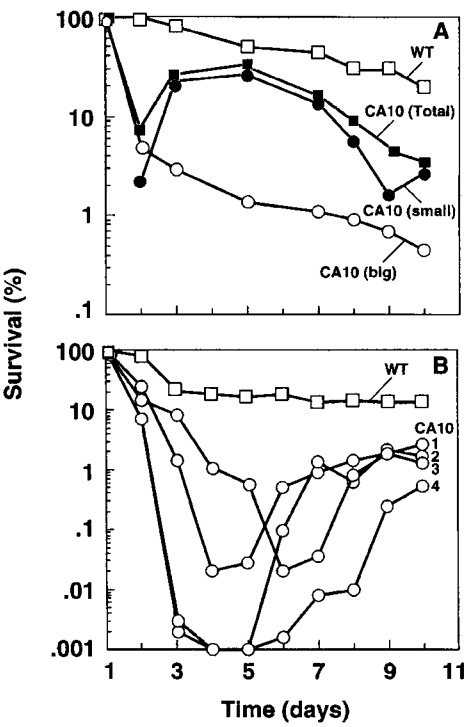

FIG. 4. Long-term survival. Wild-type (WT) and CA10 cells (small and big) were incubated in MOPS medium with limited glucose (0.1%). (A) Percent survival is expressed as the viable cell number at each time point divided by the viable cell number of the same culture after 24 h after initial inoculation of the culture. Similar results were obtained in three independent experiments. (B) Wild-type and CA10 cells were kept in LB medium. Curves 1, 2, 3, and 4 represent four different CA10 cultures showing fluctuations in survival due to time of appearance of the small-colony variant. Percent survival is calculated as for panel A.

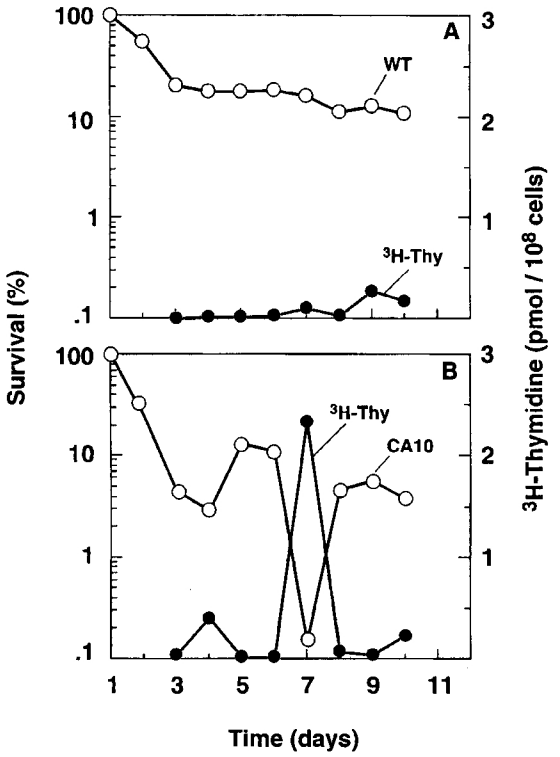

FIG. 5. Thymidine (Thy) uptake during long-term incubation of stationary-phase wild-type (WT) (A) and CA10 (B) cells. The cells were pulse-labeled with [^{3}H]thymidine from the 3rd to 10th days. Both survival of the cells and the incorporation of [^{3}H]thymidine into cells were measured (Materials and Methods).

the small-colony phenotype. The viable cell number increased to about 5×10^7 CFU/ml after 9 days; most of the cells (~90%) observed in four separate experiments were the small-colony variants. Takeover of the cell population by the small-colony phenotype was invariably accompanied by a burst of thymidine uptake.

Characteristics of the small-colony variant. (i) Stable phenotype. CA10 cells were grown to the stationary phase in LB medium with kanamycin, incubated aerobically at 37°C for 5 days, and plated on LB plates containing kanamycin. The stationary-phase culture contained small- and big-colony phenotypes. The big colonies and the more slowly growing small colonies were isolated and maintained as stable phenotypes. The colony size was well defined for these phenotypes. When grown in MOPS-buffered medium for 48 h at 37°C, the colony diameters were 1.0 ± 0.5 mm for the small-colony phenotype and 3.0 ± 0.5 mm for the big-colony phenotype. A few of the characteristics of these variants were studied relative to the role of poly P during survival in the stationary phase.

(ii) Growth lag. When wild-type cells and the small- and big-colony variants of CA10 were grown in MOPS medium containing 2 mM K_2HPO_4 and 0.4% glucose, the small-colony phenotype cells consistently showed a longer growth lag (Fig. 6). Generation times were 56 min for the wild-type and the CA10 big-colony phenotype cells; the CA10 small-colony cells grew with a generation time of 60 min.

(iii) Stationary-phase survival. The big- and small-colony variants of CA10 were grown in LB medium to the stationary phase and incubated aerobically at 37°C for 10 days (Fig. 7).

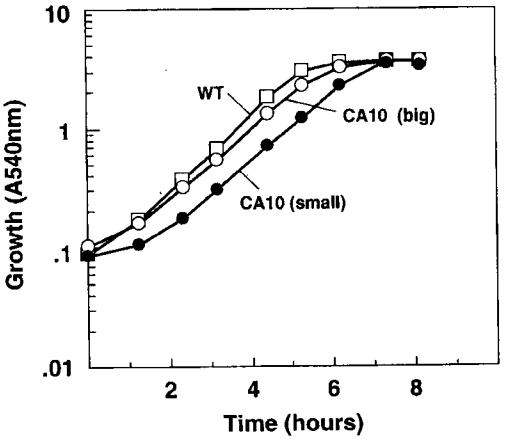

FIG. 6. Growth of wild-type (WT) cells and CA10 cells exhibiting either the big-colony phenotype or the small-colony phenotype. Prewarmed (37°C) MOPS medium was inoculated with overnight (~20 h) cultures (diluted 1 to 100); growth was monitored by measuring the turbidity at OD_{540}.

The big-colony phenotype, like its parent strain, showed a considerable decrease in viability; after 5 days, only about 1% of the original population had survived. On the other hand, the small-colony phenotype showed an improved viability with long-term incubation; about 10% of the population survived until 8 days (Fig. 7).

(iv) Heat resistance. Upon entry into the stationary phase, wild-type cells develop a pronounced tolerance to heating at 55 to 57°C (28). Stationary-phase cells of the wild-type and big- and small-colony phenotypes of CA10 were exposed to 55°C, and their viability was assessed (Fig. 8). The survival profile of the big-colony phenotype was similar to that of the parent CA10 strains; less than 1% of the cells survived heating for 2 min compared with over 90% of the wild-type cells (JM101). The small-colony variant showed a significantly higher level of resistance towards heat shock than did the big-colony phenotype; about 60% of the small-colony cells survived the 2-min heating.

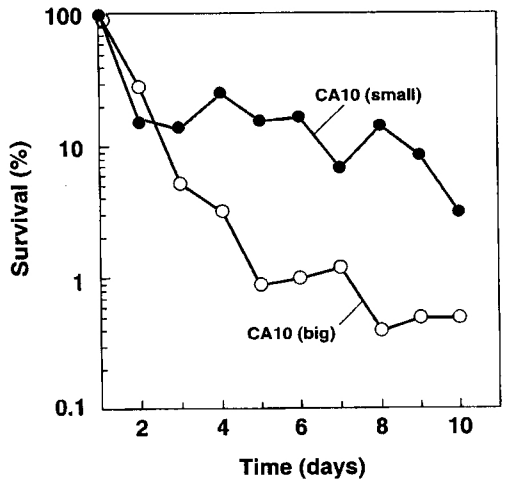

FIG. 7. Comparison of rates of long-term survival of big- and small-colony phenotypes of CA10 cells in the stationary phase. The cells were grown and incubated aerobically in LB medium at 37°C. Percent survival is calculated as described in the legend to Fig. 1.

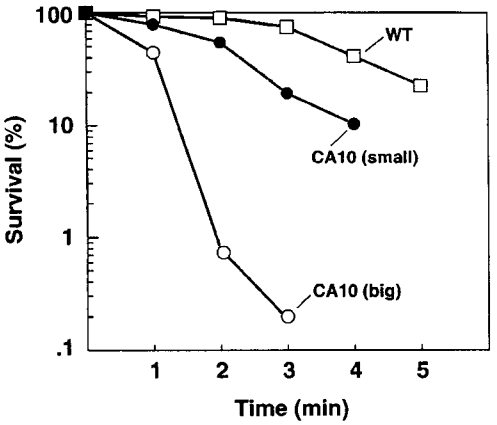

FIG. 8. Heat resistance. Wild-type (WT) cells and big- and small-colony phenotypes of CA10 were grown overnight (~20 h) at 37°C in LB medium and exposed to 55°C; percent survival is calculated as described in the legend to Fig. 1. No viable cells were detected beyond the 3-min time point for the CA10 big-colony phenotype.

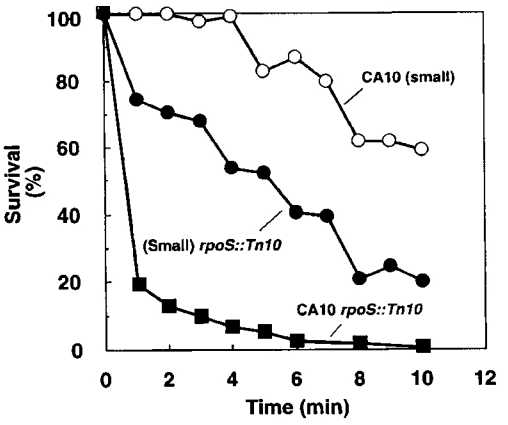

FIG. 9. Heat resistance. CA10 small-colony phenotype, CA10 small-colony phenotype *rpoS*::Tn*10*, and CA10 *rpoS*::Tn*10* cells were grown overnight (~20 h) at 37°C in LB medium and exposed to 55°C; percent survival is calculated as described in the legend to Fig. 1.

(v) PPK and PPX activities. To determine whether the enhanced survival of the small-colony phenotype was due to a reversion or suppression of the PPK and PPX mutant phenotype, the enzyme activities were compared (Table 2). CA10 cells still showed very low levels of both PPK (~2% of the wild-type level) and PPX activity. The residual PPX activity (about 6% of the wild-type level) in the mutant could be attributed to the guanosine pentaphosphate poly Pase (29). No significant difference was observed in the enzyme activities of the mutant strains showing either the big-colony or the small-colony phenotype (Table 2). To confirm that the PPX protein was not being expressed in the mutants, particulate fractions obtained after lysis of the cells by lysozyme were analyzed by immunoblotting with an anti-PPX antibody. Neither the big-colony phenotype nor the small-colony phenotype of CA10 showed the presence of PPX (data not shown). Similarly, a Western blot (immunoblot) analysis of a trichloroacetic acid precipitate of the total cell protein of the mutant phenotypes of CA10 failed to indicate the presence of PPK (data not shown).

(vi) Poly P content. The poly P content of the stationary-phase wild-type cultures was 678 ± 19 pmol/mg of protein; the levels for the CA10 big- and small-colony phenotypes were at the limit of detection (about 100 pmol/mg of protein). Poly P levels in an *rpoS* knockout mutant were compared with those of a corresponding isogenic wild-type strain, during both the exponential and stationary phases of growth. No significant difference in poly P contents was noticed between the wild type and the *rpoS*::Tn*10* strain, especially in cells during stationary-phase growth (data not shown).

(vii) Effect of *rpoS* on heat resistance. The enhanced heat resistance acquired by the stationary-phase small-colony phenotype might be attributed to a secondary mutation in *rpoS*. In order to test this hypothesis, *rpoS* mutant derivatives of the small-colony phenotype, as well as that of the original *ppk* strain (CA10), were constructed. The stationary-phase cells of these *rpoS* knockout mutants and the cells of the small-colony phenotype were tested for their heat resistance (Fig. 9). Introduction of the *rpoS* mutation to the small-colony *ppk* mutant resulted in reduced heat tolerance at 55°C, but this effect was less pronounced than that observed with the *rpoS* mutant of the CA10 cells, thus failing to identify an *rpoS* locus for the small-colony phenotype; *rpoS* knockout mutant derivatives of both the small-colony phenotype and the CA10 cells were found to be starvation sensitive, and only about 10% of the stationary-phase cells survived after 20 h of incubation in LB medium.

(viii) Catalase activity. Stationary-phase cells exhibit strong *rpoS*-dependent resistance to H_2O_2. Catalase HPII, the *rpoS*-controlled *katE* gene product, is induced in stationary-phase cells as a defense against H_2O_2 (37). Catalase activities of the wild type and of the small- and big-colony phenotypes of CA10 cells were measured in cells grown to the stationary phase in LB medium (Table 3). The small-colony phenotype contained about 60% of the activity seen in the wild-type cells; the big-colony phenotype contained only 35%. When the small-colony variant was complemented with a plasmid, pBC29, which carries the *ppk* gene, the catalase activity was restored to the wild-type level. However, these catalase activity measurements

TABLE 2. PPK and PPX activities

Strain (phenotype)	Enzyme activity (U/mg of protein)[a]	
	PPK	PPX
JM101 (wild type)	424	248
CA10-s (small colony)	8.0	7.5
CA10-b (big colony)	12	6.0

[a] The PPK assay measured the production of acid-insoluble [^{32}P]poly P from [γ-^{32}P]ATP (1). One unit of enzyme is defined as the amount incorporating 1 pmol of phosphate into acid-insoluble poly P per min at 37°C. The assay for PPX measured the loss of acid-insoluble [^{32}P]poly P. One unit of enzyme is defined as the amount that decreases the acid-insoluble poly P by 1 pmol of phosphate per min at 37°C.

TABLE 3. Catalase activities of strains used in this study

Strain	Phenotype	Activity (U/mg of protein)[a]
JM101	PPK^+ (wild type)	32
CA10	PPK^- (big colony)	11
CA10	PPK^- (small colony)	19
CA10pBC29	PPK^{+++} (small colony)	31

[a] The activities of permeabilized stationary-phase cells were measured. One unit is equal to 1 μmol of hydrogen peroxide decomposed per min at 25°C.

fail to differentiate between the *rpoS*-independent HPI and the *rpoS*-dependent HPII activities. Therefore, extracts of stationary-phase cells, analyzed qualitatively on a nondenaturing polyacrylamide gel, were stained for catalase activity in the HPI and HPII bands. No significant difference in HPI activity was observed between the wild-type and mutant cells (both small- and big-colony phenotypes). The stationary-phase wild-type cells possessed a high level of HPII activity; however, the mutants showed only trace amounts of this enzyme activity. Complementation of either CA10 cells or the CA10 small-colony phenotype with the *ppk* gene (pBC29) or an *rpoS* plasmid (pMM*kat*F3) elevated synthesis of HPII to the wild-type level (data not shown).

DISCUSSION

The stationary phase of growth studied in the laboratory can resemble the stressful and deprived state that characterizes the natural habitat of most bacteria (47). To cope with adversity, cells entering the stationary phase undergo drastic changes in cell physiology and morphology (48). Many genes not required for exponential growth are induced in order that cells may survive in the stationary phase (6, 19, 20, 34, 50). Mutations in these genes in *E. coli* decrease viability during the prolonged periods of nutritional deprivation in a stressful environment. Among them, as the present studies show, is *ppk*, the gene that encodes PPK, the enzyme responsible for making long-chain poly P.

Stresses that *E. coli* responds to or anticipates include oxidants, heat, and high salt levels (27, 28). Aerobic metabolism produces reactive byproducts such as superoxide and hydrogen peroxide (15, 17) that are involved in the generation of cytotoxic hydroxyl radicals (7, 23, 24). In log-phase cultures, genes induced to cope with these oxidants are found in the *soxR* and *oxyR* regulons (11, 17, 18). We report here that the *ppk* mutant, lacking poly P, is more sensitive to hydrogen peroxide than the wild type (Fig. 2). Also, in stationary-phase cultures, the heightened resistance to hydrogen peroxide is diminished in the *ppk* mutant (10). This resistance to hydrogen peroxide is largely restored to the wild-type level when the mutant is complemented with a plasmid bearing the *ppk* gene.

Another example of oxidant stress is the toxicity of menadione, the one-electron redox agent that generates cytotoxic oxidants as in the redox cycling of respiratory chain components during aerobic metabolism (58). Catalase activities induced to scavenge oxidants such as hydrogen peroxide include HPI in log-phase cells and HPII in stationary-phase cells (37). The *ppk* mutant proved to be hypersensitive to menadione (Table 1), presumably because of a deficiency in catalase activities (Table 3), particularly that of HPII because of the lack of its induction in the stationary phase (17, 26).

In addition to increased sensitivity to oxidants, responses to heat and high salt levels were also examined in the *ppk* mutant, and resistance to these agents was found to be greatly diminished. After exposure to 55°C for 2 min, only 2% of the mutant cells in the stationary phase survived compared with about 90% of the wild-type cells (Fig. 1). With regard to the challenge of a medium with 2.5 M NaCl, the mutants lost viability to a greater extent than wild-type cells in both the log and stationary phases (Fig. 3).

The most striking phenotype of the *ppk* mutant is its poor rate of survival in stationary-phase cultures (10) (Fig. 4). Viable cell counts decreased drastically within a few days upon aerobic incubation, the more so in a rich medium. Of mutants in a medium limited in carbon source (i.e., glucose), about 1% survived after 9 days, whereas in a rich medium only about 1 in 10^6 cells survived after 4 days. A similar influence of growth medium on the long-term survival of wild-type cells has also been observed (52).

Although the bases for the dramatic poly P effects in promoting resistance to stressful agents and prolonging survival in the stationary phase are still unexplained, some insight may be gained from the suppressive influence of *rpoS* on the *ppk* mutation. The product of *rpoS* is the σ^{38} subunit of RNA polymerase responsible for the expression of nearly 50 genes involved in adjustments in the stationary phase to nutrient deprivations, high osmolarity (21), and other stressful agents. When multiple-copy *rpoS* plasmids were introduced into the *ppk* mutant, heat resistance was elevated to the wild-type level (Fig. 1). RpoS plays a central role in the development of stress resistance in *E. coli*. Modulation of the concentration of this sigma factor brought about either by the exposure of the wild-type cells to various stresses or by the presence of a plasmid bearing *rpoS* in the *ppk* mutant may be responsible for the induction of proteins essential for heat tolerance.

Less direct, but suggestive of a relationship between *rpoS* and poly P, is the emergence of a novel small-colony phenotype in *ppk* mutants (10). After maintenance of the *ppk* mutant for several days in the stationary phase, the colonies of normal size are largely replaced by small colonies which are genetically stable. Their emergence coincides with a burst of DNA synthesis indicated by thymidine incorporation (Fig. 5). The small-colony variant demonstrates increased resistance to heat (Fig. 8) and an improved rate of survival (Fig. 7). Inasmuch as the levels of PPK activity and poly P in the variant remain at the mutant cell levels, some other genetic alteration can be inferred.

The altered gene in the small-colony variant was initially assumed to be allelic to *rpoS*, since this locus is involved in adaptive mutations (49, 52, 61). Besides, *rpoS* plays a major regulatory role during survival in the stationary phase (25, 38, 55, 60). The *rpoS*-negative mutants did not lack poly P during both exponential and stationary-phase growth. Introduction of *rpos*:Tn*10* into the stable, small-colony variant did not result in the complete restoration of heat sensitivity in stationary-phase cells (Fig. 9). It appears that the second site suppressor mutation in the small-colony phenotype may act independently of *rpoS*.

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