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Phosphate-Enhanced Stationary-Phase Fitness of *Escherichia coli* Is Related to Inorganic Polyphosphate Level▽

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We found that *Escherichia coli* grown in media with >37 mM phosphate maintained a high polyphosphate level in late stationary phase, which could account for changes in gene expression and enzyme activities that enhance stationary-phase fitness.

Polyphosphate (poly-P) is a long-chain polymer composed of many orthophosphates linked together by high-energy ATP-like bonds. Studied mainly in prokaryotes, poly-P plays an important role as an energy source, as a regulator of gene expression, as a store of inorganic phosphate, and as a chelator of heavy metals (8, 10). The main enzymes associated with poly-P metabolism in bacteria are the polyphosphate kinase (PPK, encoded by *ppk*) and the exopolyphosphatase (PPX, encoded by *ppx*) (1, 2). The *ppk ppx* double mutant exhibits greatly reduced synthesis of poly-P, is deficient in stationary-phase functions, and lacks resistance to different stresses (6, 17).

Previously, we found that expression of several respiratory (*ndh*, *sdhC*, *ubiC*, *nuoAB*, and *cydA*) and defense (*katG* and *ahpC*) genes was maintained in late stationary phase when the medium's phosphate concentration was above 37 mM (24, 25). Furthermore, *Escherichia coli* cells grown in medium containing this critical phosphate concentration had high viability, low oxidative damage, and elevated resistance to external H_2O_2 stress in late stationary phase (25).

We examined the relationship between the medium's phosphate concentration and intracellular poly-P levels in the wild-type and *ppk ppx* mutant strains to see if the previously observed effects on gene expression, enzyme activity, and tolerance to H_2O_2 were correlated with elevated poly-P levels.

Measurements of poly-P level in whole cells. Intracellular poly-P was measured in cell suspensions by using a DAPI (4′,6-diamidino-2-phenylindole)-based fluorescence approach (3). Cells were washed and resuspended in buffer T (100 mM Tris HCl [pH 7.5]). DAPI (Sigma) was added to 10 μM in cuvettes containing cell suspensions in buffer T at an optical density at 560 nm of 0.02. After 5 min of agitation at 37°C, the DAPI fluorescence spectra (excitation, 415 nm; emission, 445 to 650 nm) were recorded using an ISS PCI spectrofluorometer (Champaign, IL). The fluorescence (in arbitrary units) of the DAPI-poly-P complex at 550 nm was used as a measure of intracellular poly-P because fluorescence emissions from free DAPI and from DAPI-DNA are minimal at this wavelength (3).

We first compared various conditions for preparing the cell samples, using exponential-phase cells grown aerobically in LB medium at 37°C (Fig. 1). Untreated cells (buffer T wash only) with or without exposure to heat for 10 min at 60°C gave essentially identical spectra, similar to those of heat-treated competent cells (3) (Fig. 1). In contrast, competent cells without heat treatment did not exhibit a poly-P-DAPI fluorescence signal (Fig. 1). Thus, it appeared that poly-P levels could be directly measured in untreated cells, and we used this method for subsequent experiments.

The poly-P level was determined for cells of wild-type and *ppk ppx* mutant strains (Table 1) grown in LB medium (6 mM phosphate) and in minimal medium M9 (64 mM phosphate) (23) or MT (2 mM phosphate) (26) (Fig. 2). All media were supplemented with 0.5% glycerol, and minimal medium was also supplemented with 0.1% tryptone. The poly-P level in LB medium-grown wild-type cells was always low, whereas it was high in M9 medium-grown cells even at 72 h (Fig. 2a and b). However, the poly-P level in MT medium-grown cells was high up to 24 h but decreased at 48 and 72 h (Fig. 2c). The *ppk ppx* strain exhibited low poly-P levels regardless of the culture medium or growth stage, confirming that the fluorescence sig-

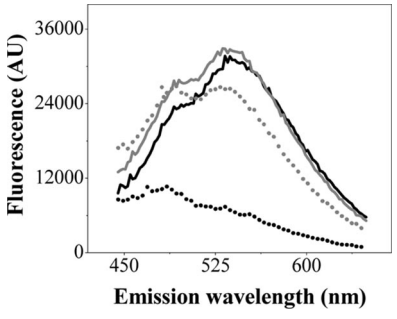

FIG. 1. DAPI-poly-P complex fluorescence in live *E. coli* cells. Fluorescence emission spectra of DAPI-poly-P were measured in untreated (solid black line), untreated and heated (solid gray line), competent (dotted black line), and heated competent (dotted gray line) exponential MC4100 cells. Data are representative of results of at least three separate experiments. AU, arbitrary units.

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TABLE 1. Strains used in this work

Strain	Relevant genotype	Reference or source
MC4100	*araD* Δ*lac rpsL flbB deoC ptsF rbsR relA1*	4
TSDH00	MC4100 λ[Φ(*sdhC-lacZ*)]	16
MC4100(λMO2)	MC4100 λ[Φ(*ubiC-lacZ*)]	13
NR629	MC4100 λ[Φ(rpoS477′-′lacZ)]	22
CA10	Δ(*lac-proAB*) *supE thi-1*/F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] (*ppk ppx*)::Km	6
LSB022	MC4100 (*ppk ppx*)::Km	P1(CA10) × MC4100
LSB023	MC4100 λ[Φ(*sdhC-lacZ*)] (*ppk ppx*)::Km	P1(CA10) × TSDH00
LSB024	MC4100 λ[Φ(*ubiC-lacZ*)] (*ppk ppx*)::Km	P1(CA10) × MC4100(λMO2)
LSB025	MC4100 λ[Φ(rpoS477′-′lacZ)] (*ppk ppx*)::Km	P1(CA10) × NR629

nals were specific for poly-P (Fig. 2d to f). The basal level of DAPI-poly-P fluorescence in the mutant strain could be produced by short-chain poly-P synthesized through an alternative pathway (5) or by some lack of specificity of the DAPI stain.

A high phosphate concentration maintains a high poly-P level in stationary phase. To determine if the extracellular phosphate concentration in the growth medium influenced the poly-P level in stationary phase, we measured poly-P levels at 48 h in the wild-type and mutant strains grown in MT medium with the addition of different phosphate concentrations. In the wild type, the poly-P level was maintained only when the phosphate concentration was >37 mM (Fig. 3). This effect could be a consequence of PPX inhibition by high phosphate concentrations in the medium, as described for *Saccharomyces cerevisiae* (9). It is important to note that even in MT+P medium (defined as MT medium with added 40 mM buffer phosphate [pH 7]) (24), the poly-P level was low in the *ppk ppx* mutant (Fig. 3).

Unusual P_i-dependent stationary-phase events are correlated with poly-P level. Using the *ppk ppx* mutant strain, we studied some of the stationary-phase physiological events that are triggered by growth at high phosphate concentrations (25). By using chromosomal *lacZ* transcriptional fusions (Table 1), the expression of *sdhC* (encoding succinate dehydrogenase) and *ubiC* (chorismate pyruvate-lyase) was assayed as β-galactosidase activity (15) in MT+P medium-grown cells (Fig. 4a and b). Expression of *rpoS* (encoding sigma S factor), which is known to be positively regulated by poly-P levels (22), was measured similarly to a control (Fig. 4c). The *sdhC*, *ubiC*, and *rpoS* gene expression remained high in the wild type, but in the *ppk ppx* mutant strain, expression decreased as the cells progressed into stationary phase. We cannot yet say whether poly-P directly regulates expression of these respiratory genes, but Kusano and Ishihama (12) have reported that poly-P physically interacts with RNA polymerase and under certain conditions activates *rpoS* transcription, although the precise mechanism is still unclear.

In MT+P medium-grown cells, membrane NDH-2 and succinate dehydrogenase activities (18, 21, 24) decreased through stationary phase in the mutant strain, whereas they remained high in the wild-type strain (Table 2). In addition, catalase

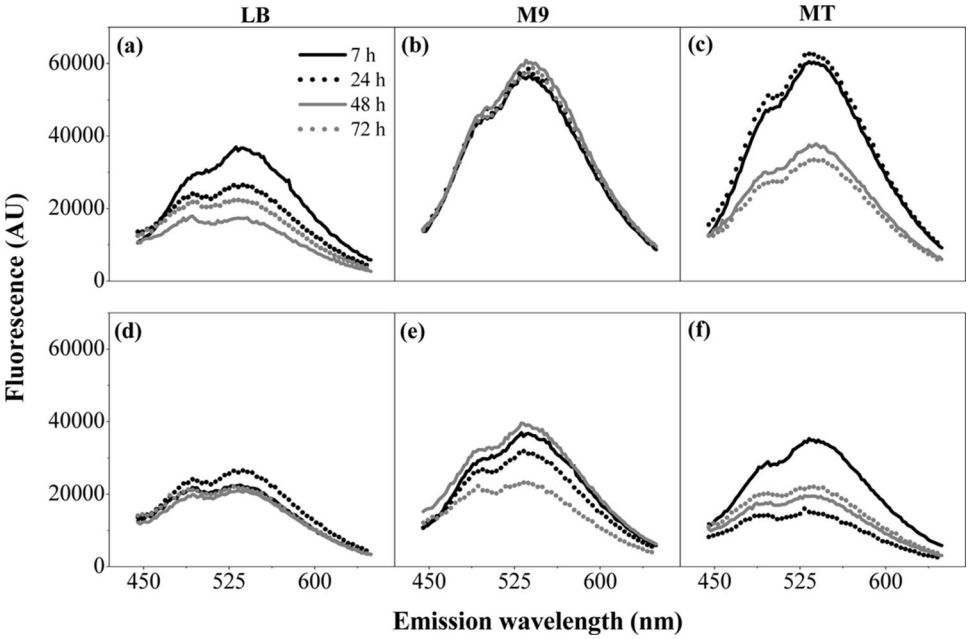

FIG. 2. DAPI-poly-P fluorescence in a wild-type and a *ppk ppx* mutant strain. Fluorescence emission spectra of DAPI-poly-P were measured in untreated MC4100 (a to c) or LSB022 (d to f) cells grown in LB, M9, or MT medium for different periods of time as indicated. Data are representative of results of at least three separate experiments. AU, arbitrary units.

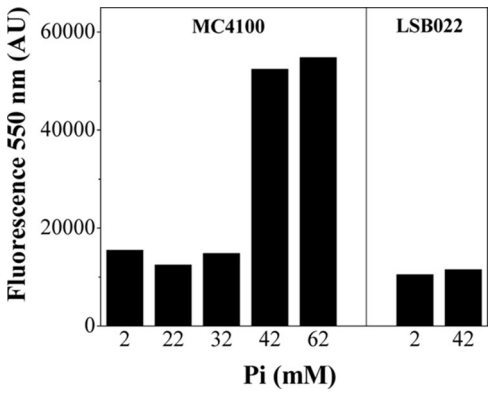

FIG. 3. poly-P level in stationary phase versus initial phosphate concentration. DAPI-poly-P fluorescence was measured in stationary MC4100 or LSB022 cells grown in MT medium with the indicated phosphate concentrations. Data are representative of results of at least three separate experiments. AU, arbitrary units.

activity (7, 25) increased in the wild-type strain only during stationary phase (Table 2). These unusual enzyme activities in stationary phase require a persistent protein synthesis. In high-phosphate media, an elevated poly-P level may prolong the amino acid supply required for de novo synthesis, because it promotes ribosomal protein degradation by the Lon protease (11).

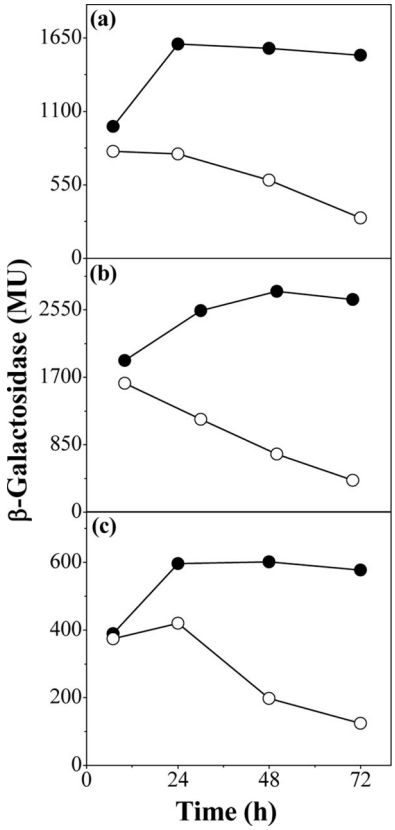

FIG. 4. Gene expression. *sdhC* (a), *ubiC* (b), and *rpoS* (c) expression was measured as β-galactosidase activity in the wild type (filled symbols) and the *ppk ppx* mutant (open symbols), with corresponding fusions, at different times of growth in MT+P. Data are representative of results of at least four separate experiments performed in duplicate. MU, Miller units.

TABLE 2. Enzymatic activities in a wild-type and a *ppk ppx* mutant *E. coli* strain[c]

Length of culture (h)[a]	Rate of MTT[b] reduction (nmol min^{-1} mg $protein^{-1}$)				Rate of H_2O_2 decomposition (μmol min^{-1} mg $protein^{-1}$)	
	NADH dehydrogenase-2		Succinate dehydrogenase			
	MC4100	LSB022	MC4100	LSB022	MC4100	LSB022
7	1,284 ± 65	1,230 ± 68	602 ± 22	611 ± 44	16 ± 3	15 ± 1
48	1,257 ± 54	257 ± 48	624 ± 28	188 ± 21	26 ± 1	14 ± 2

[a] Cells were grown in MT+P.
[b] MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.
[c] Values are averages ± standard deviations.

The ability to tolerate the exogenous oxidative stress generated by H_2O_2 was also studied. After 1 h of incubation with 10 mM H_2O_2, the numbers of CFU on LB agar plates incubated for 24 h at 37°C were determined. The wild-type cells were highly tolerant to exogenous peroxide, as evidenced by the fact that the viability was maintained, whereas the viability of the *ppk ppx* mutant strain decreased around 2 orders of magnitude. These results for MT+P could be explained by the high poly-P level and the induction of several antioxidant enzymes (e.g., catalase).

A phosphate concentration of around 30 mM (similar to the critical concentration used here) could be present in the human intestinal lumen in healthy adults consuming an average Western diet (14, 27). The PPK sequence was found in several human pathogens in which the role of poly-P is related to growth advantages, motility, quorum sensing, biofilm formation, and virulence (19, 20). Therefore, our observation of the relationship between exogenous phosphate concentration and poly-P level may help to elucidate the importance of high phosphate as a physiological signal.

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