Polyphosphate Accumulation in Escherichia coli in Response to Defects in DNA Metabolism

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Phenol-chloroform extraction of [32P]orthophosphate-labeled Escherichia coli cells followed by alkaline gel electrophoresis revealed, besides the expected chromosomal DNA, two non-DNA species that we have identified as lipopolysaccharides and polyphosphates by using a combination of biochemical and genetic techniques. We used this serendipitously found straightforward protocol for direct polyphosphate detection to quantify polyphosphate levels in E. coli mutants with diverse defects in the DNA metabolism. We detected increased polyphosphate accumulation in the ligA, ligA recBCD, dut ung, and thyA mutants. Polyphosphate accumulation may thus be an indicator of general DNA stress.

Materials and Methods

Bacterial strains, growth conditions, labeling, and isolation of phosphate-containing species. All strains are described in Table 1. Cells were grown with shaking at 30°C in MOPS (morpholinepropanesulfonic acid) low-phosphate medium (25) supplemented with 0.2% Casamino Acids to an optical density at 600 nm (OD600) of 0.2 to 0.4. Cultures were further incubated at 30°C, 37°C, or 42°C with shaking at 30°C in MOPS (morpholinepropanesulfonic acid) low-phosphate containing species.

All strains are described in Table 1. Cells were grown with agarose because there are other molecules in larger amounts in

DNA replication intermediates, also known as Okazaki fragments, have classically been detected by pulse labeling thymine-limited thyA mutant cells with [3H]thymidine, a DNA-specific label (27). However, when limited for thymidine, thyA mutants are known to undergo thymine-less death (1), a phenomenon during which chromosomal DNA suffers single-strand breaks (24). The products of this nicking could be mistaken for Okazaki fragments, compromising DNA synthesis studies that rely on [3H]thymidine labeling (28, 37). Cavets were also raised against interpreting [3H]thymidine labeling as an accurate reflection of DNA synthesis in cells of higher eukaryotes, on the basis of differences with [32P]orthophosphate DNA labeling (10, 15, 30).

To avoid the possibility of thymine starvation in our experiments, we also attempted to visualize Okazaki fragments by using the [32P]orthophosphate label which we routinely employ to label chromosomal DNA for pulsed-field gel electrophoresis (17, 36). Since we expected that the bulk of the [32P] label will be deposited into RNA, we removed RNA altogether by separating chromosomal DNA from replication intermediates in alkaline agarose gels. We found, however, that Okazaki pieces cannot be detected using [32P]orthophosphate even by alkaline agarose gel because there are other molecules in larger amounts in the cells that take in [32P]-label and mask the replication intermediates. We report on the identification and quantification of two of the “masking species” in wild-type Escherichia coli, as well as in several mutants.

Materials and Methods

Bacterial strains, growth conditions, labeling, and isolation of phosphate-containing species. All strains are described in Table 1. Cells were grown with shaking at 30°C in MOPS (morpholinepropanesulfonic acid) low-phosphate medium (25) supplemented with 0.2% Casamino Acids to an optical density at 600 nm (OD600) of 0.2 to 0.4. Cultures were further incubated at 30°C, 37°C, or 42°C and labeled with [32P]orthophosphate (5 to 10 μCi/ml) for the amount of time indicated in the figures. Samples were processed by spinning down the cells and resuspending them in 50 μl of 20% sucrose in Tris-EDTA. Three hundred fifty

microliters of 2% sodium dodecyl sulfate (SDS) were added, and after thorough mixing, the cells were lysed by incubation at 70°C for 10 min. Isolation of nucleic acids was achieved by subsequent extraction with 400 μl of phenol, followed by 400 μl of phenol-chloroform, and finally, 400 μl of chloroform, with two ethanol precipitations (18). Alternatively, the Wizard genomic DNA purification kit (Promega) was used.

Enzymatic reactions. DNase I, exonuclease I, and calf intestinal phosphatase (CIP) were purchased from New England Biolabs. RNase A is from Boehringer. All reactions were performed at 37°C in a 50- to 60-μl final volume for 1 h, unless indicated otherwise. Afterwards, the DNA samples were ethanol precipitated, dried, and dissolved in 20 μl of alkaline or neutral loading buffers for alkaline or neutral agarose gels, respectively.

Gel electrophoresis. For alkaline gel electrophoresis, 1.1% agarose was prepared in water, cooled to 60°C, and supplemented with NaOH to 50 mM and EDTA (pH 8.0) to 1 mM before pouring a gel. [32P]-labeled samples were ethanol precipitated, vacuum dried, dissolved in 10 μl of a 50 mM NaOH-1 mM EDTA solution, and mixed with 10 μl of 1× alkaline loading dye (21). Gels were run for 600 to 650 min at 1.5 V/cm and were either vacuum dried onto a Whatman paper after neutralization for 30 min with Tris-HCl or vacuum transferred and UV cross-linked to a positively charged nylon membrane (Hybond-N+; Amersham Biosciences). Before vacuum transfer, gels were treated with 0.2 M HCl for 40 min, then with 0.5 M NaOH for 45 min, and finally with 1 M Tris-HCl (pH 8.0) for 40 min. Nylon membranes were UV irradiated to cross-link the DNA and, where indicated, prehybridized for 1 hour at 65°C in hybridization solution (5% SDS, 0.5 M sodium phosphate [pH 7.4], 1 mM EDTA). Neutral gels were also 1.1% agarose and were run in TAE buffer (21): 2 μl of 1% neutral dye (21) and 18 μl of Tris-EDTA were used to dissolve the DNA samples. Membranes and dried gels were autoradiographed or quantified by phosphorimaging.

Comparison of polyphosphate accumulation in different E. coli strains. Since polyphosphate accumulation is increased in cells under stressful conditions, we grew a series of E. coli strains at 30°C to an OD600 of 0.2 and then heat stressed them by switching to 42°C for 2 hours before labeling with [32P]orthophosphate for 10 minutes. Total [32P] radioactivity in the polyphosphate species was determined from dried alkaline gels by use of a phosphorimager and normalized to the OD of the corresponding cultures. The normalized values for every strain were then divided by the normalized value of their background strain from the same gel.

RESULTS

Rationale. To eliminate the possibility of chromosomal DNA fragmentation due to thymine starvation, inherent to the classic protocols of detection of Okazaki fragments (24, 27, 38), we tried detecting Okazaki fragments by labeling cells with [32P]orthophosphate, extracting total DNA and running it in alkaline agarose gels. We chose the alkaline conditions for two reasons: (i) to separate single-stranded Okazaki fragments from chromosomal-length DNA strands; (ii) to hydrolyze and
From this study

Sp1 is present in phenol-extracted total DNA preparations from all [32P]orthophosphate-labeled *E. coli* strains and has a molecular size equivalent to 3 to 4 kb of DNA in alkaline gels (Fig. 1C), running faster in a neutral gel (Fig. 1A, lane 3), broadly agreeing with the behavior of Okazaki fragments. However, our investigation of Sp1 revealed characteristics that are inconsistent with this interpretation. (i) Sp1 has a narrow molecular size distribution around 3 to 4 kb, while Okazaki fragments have a broader distribution, appearing as a smear with a mode around 1 to 2 kb (3). (ii) Sp1 is abundant in wild-type cells, where Okazaki fragments are generally undetectable due to their rapid maturation (13). (iii) Unlike true Okazaki fragments (3), Sp1 could not be chased into large-molecular-size species (not shown), suggesting that it represents final products, rather than (replication?) intermediates. (iv) Sp1 could not be UV cross-linked to a nylon membrane regardless of the doses of UV applied (Fig. 1C), indicating the absence of purine/pyrimidine bases, required for cross-linking nucleic acids to nylon (8). (v) Treatment with DNase eliminated the chromosomal DNA signal near the gel origin but had no effect on the Sp1 signal (Fig. 1D, lane 3), proving that Sp1 is not DNA. However, treatment with a phosphatase (CIP) removed most of the label from Sp1 without decreasing its molecular size (not shown), suggesting that it represents phosphorolipids because, not only are they too small, but also the ester bonds would be thus remove RNA, which absorbs the bulk of the [32P] label and becomes the major labeled species if the samples are run in neutral agarose gels (Fig. 1A). Although initial results looked encouraging, as we have indeed detected [32P]-labeled species with molecular sizes lower than those of chromosomal DNA (Fig. 1A and B), subsequent analysis revealed the non-DNA nature of these species. Apparently, the small amount of true replication intermediates inside the cell (13, 27) does not allow them to compete out other phosphorus-containing species with similar mobilities in agarose gels. Instead of pursuing elusive Okazaki fragments, we took advantage of our robust separation protocol and identified the two major 32P-labeled species that were interfering with Okazaki fragment detection.

**Detection of nucleic acids and Sp1.** As already mentioned, neutral gel electrophoresis shows that the bulk of the label in the nucleic acid preparations from [32P]orthophosphate-labeled cells is found in RNA (Fig. 1A, lane 2). This RNA signal is eliminated upon RNase treatment (Fig. 1A, lane 3) or by running the samples under alkaline conditions (Fig. 1B), which unmasks a minor small-molecular-size species (Sp1). 32P-labeled chromosomal DNA of wild-type *E. coli* in alkaline gels forms a tight band near the origin of the gel (thus, in all our gels, the “origin” signal represents “chromosomal DNA”), regardless of the temperature at which bacterial cultures were grown (Fig. 1B, lanes 1 and 2). In contrast, the signal from chromosomal DNA in a *ligA* (Ts) strain labeled at 42°C is barely visible (Fig. 1B, lane 4), indicating a gross defect in DNA synthesis due to the absence of maturation of Okazaki fragments.

Sp1 is present in phenol-extracted total DNA preparations from all [32P]orthophosphate-labeled *E. coli* strains and has a molecular size equivalent to 3 to 4 kb of DNA in alkaline gels (Fig. 1C), running faster in a neutral gel (Fig. 1A, lane 3), broadly agreeing with the behavior of Okazaki fragments. However, our investigation of Sp1 revealed characteristics that are inconsistent with this interpretation. (i) Sp1 has a narrow molecular size distribution around 3 to 4 kb, while Okazaki fragments have a broader distribution, appearing as a smear with a mode around 1 to 2 kb (3). (ii) Sp1 is abundant in wild-type cells, where Okazaki fragments are generally undetectable due to their rapid maturation (13). (iii) Unlike true Okazaki fragments (3), Sp1 could not be chased into large-molecular-size species (not shown), suggesting that it represents final products, rather than (replication?) intermediates. (iv) Sp1 could not be UV cross-linked to a nylon membrane regardless of the doses of UV applied (Fig. 1C), indicating the absence of purine/pyrimidine bases, required for cross-linking of nucleic acids to nylon (8). (v) Treatment with DNase eliminated the chromosomal DNA signal near the gel origin but had no effect on the Sp1 signal (Fig. 1D, lane 3), proving that Sp1 is not DNA. However, treatment with a phosphatase (CIP) removed most of the label from Sp1 without decreasing its molecular size (Fig. 1D, lane 4), indicating the presence of terminal phosphates in Sp1.

**Sp1 is LPS.** We considered the possibility that Sp1 is derived from the cell membrane, which carries a significant phosphate pool. Still, Sp1 could not represent phospholipids because, not only are they too small, but also the ester bonds would be

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**TABLE 1. Bacterial strains used in this study**

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<td>ΔrecBCD::kan</td>
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<tr>
<td>PhoU-29</td>
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* Complete genotype of the background is F′ Δλ rafA1 spoT1 thr-1 ρ. (strR).
* Complete genotype of the background is F′ traD36 pro4AΔ B+ lacI Δ(lacZ)M15/Δ(lac-proAB) glnV thi.
* Complete genotype of the background is His relA1 spoT1 thr-1 λ−.
* Complete genotype of the background is Δutr-1 ΔargG689::Cm.
* Complete genotype of the background is Δthr his metE tyrA sup-126.
* Complete genotype of the background is F′ Δλ ibG rfb-30 ρf-1.
hydrolyzed at the alkaline pH of our gels, removing their fatty acid tails. At the same time, the outer leaflet of the outer membrane is made of a very different lipid, lipid A, connected to the core antigen (26). Altogether, this assembly is known as the bacterial lipopolysaccharide (LPS), which coisolates with nucleic acids by the phenol extraction procedure (11). The core antigen of LPS is phosphorylated at several positions (31), which would be consistent with the phosphate labeling that we observed. In fact, not only is $^{32}$Porthophosphate used to label LPS, but this label can be removed with alkaline phosphatase treatment (34), just like in the case of Sp1. Lipid A has a total of six fatty acid tails (31), and while the four ester-linked fatty acid tails should be hydrolyzed off at an alkaline pH, the two amide-linked tails would be stable. Indeed, when SDS was added to the alkaline gels, the Sp1 signal disappeared (Fig. 2A), suggesting that Sp1 contains fatty acids, allowing it to partition into the SDS micelles, which would make it run much faster and out of the gel. Sp1 also disappeared when we used a commercial kit for DNA isolation that specifically removes phosphatases, or RNase for 1 h at 37°C.

**Detection of Sp2.** The experiments described above used 5- to 10-min labeling with $^{32}$Porthophosphate at 42°C and most of the time revealed a single small-molecular-size labeled species (Sp1, which is LPS), but sometimes a second species also appeared (Fig. 2B), which we named “Sp2.” Sp2 was more diffuse than Sp1 and ran below it (Fig. 2B and 3A). A similar signal was observed by others in $^{32}$P-labeled DNA preparations from mammalian cells and was assumed to represent Okazaki fragments (30). When we used longer labeling periods (30 to 120 min at 42°C), Sp2 became quite apparent in preparations of six fatty acid tails (31), and while the four ester-linked fatty acid tails should be hydrolyzed off at an alkaline pH, the two amide-linked tails would be stable. Indeed, when SDS was added to the alkaline gels, the Sp1 signal disappeared (Fig. 2A), suggesting that Sp1 contains fatty acids, allowing it to partition into the SDS micelles, which would make it run much faster and out of the gel. Sp1 also disappeared when we used a commercial kit for DNA isolation that specifically removes phosphatases, or RNase for 1 h at 37°C.

**Sp2 represents polyphosphates.** Polyphosphates are long chains of orthophosphates (up to 1,000 units) linked by high-energy phosphoanhydride bonds (16, 19, 20) and especially since polyphosphate accumulation in response to inhibition of DNA replication was known for a long time (12), we suspected that Sp2 could be these mysterious polyphosphate molecules.
nondetectable during growth phase, accumulating upon entry into the stationary phase and during cell stress (16, 32). To verify if Sp2 in fact represents polyphosphates, we purified 32P-labeled “nucleic acids” material from a known polyphosphate-overproducing mutant of *E. coli*, which has a defect in the *phoU* gene (22), and performed a series of enzymatic treatments of it. We saw a dramatic increase in the Sp2 signal in the *phoU* mutant (Fig. 4B, lane 1) and also showed that Sp2 is not sensitive to RNase A (Fig. 5A, lane 3) and proteinase K (Fig. 5B, lane 3). At the same time, Sp2 turned out to be partially sensitive to incubation in the DNase I buffer, either in the presence or absence of the enzyme (Fig. 5A, lane 4, and Fig. 5C, lanes 2, 4, and 5), apparently reacting to the presence of Mg^{2+} ions, known to cause polyphosphate degradation (12).

The following characteristics of Sp2 are consistent with those of polyphosphates: (i) resistance to alkali, RNase, DNase, and phenol (12, 29); (ii) overexpression in the *phoU* mutants (22); and (iii) overexpression during general cell stress (16). As a final test, we extracted DNA samples from three strains; one has a mutational inactivation of both *ppk* and *ppx* genes, the second one overexpresses only the *ppk* gene (*ppk*/H11001), and the third one overexpresses both *ppk* and *ppx* (*ppk*/H11001/H11001 and *ppx*/H11001/H11001) (2, 14). 32P-labeled preparations from the *ppk*-overexpressing strain showed significant accumulation of Sp2 (Fig. 6, lane 3). At the same time, the strain that overproduced both *ppk* and *ppx* showed little signal, confirming that Sp2 corresponds to polyphosphate species (Fig. 6, lane 4).

We were also able to detect both Sp1 and Sp2 in neutral agarose gels when the samples were treated with RNase

FIG. 2. Sp1 identification. (A) The Sp1 signal disappears in SDS gels. GR523 DNA labeled samples were run in an alkaline gel supplemented with 0.1% SDS. Some samples were digested with DNase or RNase. (B) Sp1 disappears if an LPS-removing DNA extraction protocol is used. Nucleic acids from wild-type (WT; GR523) labeled cells were purified by either phenol extraction (lane 3) or the Wizard genomic DNA purification kit from Promega (lane 2). (C) Sp1 is almost absent in *rfaP* mutants. WT (GR523) and *rfaP* (LA18) labeled DNA was digested with DNase or RNase for 2 h at 37°C. Undigested controls are also shown.

FIG. 3. Appearance of Sp2 signal. (A) Accumulation of Sp2 with time of incubation. *ligA251* cells (GR501) were grown to an OD 600 of 0.4 at 30°C, switched to 42°C, and labeled with [32P]orthophosphate for the indicated amount of time. (B) Accumulation of Sp2 in pulse-labeling experiments. The *ligA251* mutant (AK148) was grown at 30°C to an OD 600 of 0.4, then incubated for 10 min at the indicated temperatures and labeled for 10 min with 3 μCi/ml of [32P]orthophosphate. The same was done for the *recBCD* single mutant (AK147). MWM, molecular size markers. (B) Comparison of the expression of Sp2 in *ligA251* (GR501) and *phoU29* mutants. The *ligA251* mutant was incubated for 10 min at 42°C, while the *phoU* mutant was incubated at 30°C. Label was added, and after 40 min of incubation, nucleic acids were purified by phenol extraction. Note almost no chromosomal DNA material in the *ligA* mutant preparation.

FIG. 4. Sp2 abundance under metabolic stress and in a *phoU* mutant. (A) Expression of Sp2 in a *ligA recBCD* double mutant strain. The *ligA251* Δ*recBCD* mutant (AK148) was grown at 30°C to an OD 600 of 0.4, then incubated for 10 min at the indicated temperatures and labeled for 10 min with 3 μCi/ml of [32P]orthophosphate. The same was done for the Δ*recBCD* single mutant (AK147). MWM, molecular size markers. (B) Comparison of the expression of Sp2 in *ligA251* (GR501) and *phoU29* mutants. The *ligA251* mutant was incubated for 10 min at 42°C, while the *phoU* mutant was incubated at 30°C. Label was added, and after 40 min of incubation, nucleic acids were purified by phenol extraction. Note almost no chromosomal DNA material in the *ligA* mutant preparation.
Polyphosphates run in a much tighter distribution in the neutral gel (which has been noted before [29]), probably reflecting their more uniform original length and higher stability. Without RNase treatment, both species are obscured by the RNA signal (Fig. 7, lane 1). In this respect, alkaline agarose gels are more convenient for the visualization of both LPS and polyphosphate species, obviating the need for enzymatic treatments of the samples, although they somewhat destabilize polyphosphate.

Quantification of polyphosphate accumulation in different *E. coli* mutants. While current methods of polyphosphate detection tend to be laborious and somewhat indirect (16), we realized that our protocol offers a simpler and direct alternative, if not for quantitative, but at least for comparative studies. Noticing that some of our strains from the Okazaki fragment study (3) accumulate more polyphosphates than others, we used our method of polyphosphate quantification to compare polyphosphate accumulations in a series of mutants of *E. coli* with various defects in the DNA metabolism (Fig. 8). Our positive controls for polyphosphate accumulation, the phoU mutant and the ppk/H11001/H11001/H11001 strain, identified the upper limit of polyphosphate accumulation over the wild-type cells as be-
tween 5- and 50-fold. We tested some 50 various strains for levels of polyphosphate accumulation relative to those of the wild-type cells and found that most of them have no more than threefold deviations from the wild-type levels. We observed a uniformly significant polyphosphate accumulation in three different ligA mutants at the nonpermissive temperature (42°C) (Fig. 8). We confirmed that the ligA recBCD double mutant shows the second-most accumulation of polyphosphate, second only to the pppk++ strain (Fig. 8). Finally, we found that the dut ung, ΔdnaQ, and ΔthyA mutants also accumulate significant amounts of polyphosphates (Fig. 8). We conclude that polyphosphate accumulation may be a general indicator of DNA stress, but the specific aspects of this stress that trigger polyphosphate production remain unclear.

**DISCUSSION**

While looking for DNA replication intermediates in *E. coli* by pulse-labeling or chronically labeling cells with [35P]orthophosphate, we detected two very different alkali-resistant species, Sp1 and Sp2. Both species do not have nucleic acid bases (cannot be cross-linked to nylon membranes) and are resistant to DNase and RNase treatments and to phenol extraction, with or without proteinase K. We think that Sp1 is LPS, because (i) it is produced continuously, (ii) it has only terminal phosphates, (iii) it is extracted with phenol, (iv) it is greatly reduced in *radF* mutants deficient in LPS phosphorylation, and (v) it is absent in preparations by a DNA isolation protocol that specifically removes LPS. We think that Sp2 represents polyphosphates, because (i) it is overproduced in stressed cells, (ii) it is overproduced in known polyphosphate-overproducing mutants, and (iii) it is degraded in vivo by polyphosphatase and in vitro in the presence of Mg2+.

Our facile detection of non-DNA 32P-containing species in the bona fide “DNA preparations” should serve as a warning to anyone using 32P labeling to monitor DNA. Sometimes the differences in DNA labeling between [3H]thymidine and [32P]orthophosphate are interpreted to mean that the thymidine labeling of DNA is unreliable (10, 15, 30). At the same time, eukaryotic cells readily synthesize polyphosphates (29), and whenever “low molecular DNA species” do not match all the characteristics of DNA, especially if they cannot be labeled with [3H]thymidine (15, 30), the polyphosphate contribution needs to be considered. In gram-negative bacteria, LPS is an additional complication, as our work demonstrates.

The current methods of polyphosphate detection include (i) an enzymatic reaction in which orthophosphate from the polyphosphate molecule is transferred to ADP to make ATP, while the latter molecule is detected by a standard enzymatic assay (7); (ii) label release by Ppx from a filter-bound 32P-labeled polyphosphate (33); (iii) nuclear magnetic resonance detection (14); and (iv) cell staining with 5-bromo-4-chloro-3-indolylphosphate (XP) (22). The various limitations of these procedures include the use of enzymes that are not commercially available or complex protocols that give only qualitative and/or indirect results. Our protocol of polyphosphate detection is both direct and quantitative for comparative purposes.

Although the presence of polyphosphates inside the cell has been reported for decades, their role is still unclear. Polyphosphates have been always implicated in the storage of energy and phosphate inside the cell (12, 16, 32). Other proposed roles of polyphosphates (16, 32) include acquisition of cell competence by perturbing the conformation of the lipid matrix when in complex with polyhydroxybutyrate and Ca2+, chelation of divalent cations, detoxification of cells from heavy metals as they enter the cell, enhancement of the SOS induction, and stimulation of biofilm formation. Our results implicate polyphosphates in the cellular response to DNA stress in general and to DNA replication problems in particular. In fact, observations to this effect were discussed as far back as the 1960s (12) but have apparently been forgotten since then. On a separate note, our data are mute on the demonstrated importance of RelA in the polyphosphate dynamics (33); although some of our ligA mutants were in relA+ cells, while others were in relA- mutant cells, arguing for the nonimportance of RelA in polyphosphate accumulation in ligA mutants, other background differences likely invalidate such a simple comparison. However, it would be informative to run our protocol on other known possible regulators of polyphosphate production to see if they are required in the replication stress induction of polyphosphates.

In conclusion, we found that Okazaki fragments cannot be detected by a whole-cell 32P-labeling procedure since these small-molecular-size DNA species are masked by more-abundant phosphate-containing molecules, such as LPS and polyphosphates. On the other hand, we found a direct and quantitative (for comparative purposes) protocol for LPS and polyphosphate detection in *E. coli* by labeling cultures with [32P]orthophosphate, followed by the standard “genomic DNA extraction” protocol (18) with subsequent alkaline gel electrophoresis. The 32P-labeled species are quantified by a phosphor-
imager. By normalizing these signals to the OD of the culture, it is possible, within the same gel, to compare levels of polyphosphate accumulation between various mutants and growth conditions.

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