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Filamentation in protein repair-deficient *Escherichia coli*

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Abstract

In *Escherichia coli*, PCM is an enzyme that repairs spontaneous isoaspartyl damage to proteins. Preliminary data suggest that under stressful conditions, particularly low salt and oxidative stress, *pcm*-deletion mutants undergo filamentation. Filamentation is the elongation of cells during replication, instead of their physical division. We found both under no stress and oxidative stress conditions, that *pcm*-deletion mutants showed a higher rate of filamentation compared to wild-type. Cell division in *E. coli* can be inhibited by the induction of the *sulA* gene. SulA is associated with the SOS regulatory system, which responds to DNA damage by arresting the cell cycle to allow for the response of DNA repair factors. We hypothesized that *sulA*-deletion mutants under oxidative stress would show a decrease in filamentation relative to wild-type and that the lack of the *pcm* and *sulA* genes would result in a change in the amount of filamentation relative to the *pcm*-deletion mutant. We found that both *sulA*- and *sulA*- *pcm*-deletion mutants showed a decrease in filamentation relative to wild-type and *pcm*-deletion mutants, both under no stress and oxidative stress conditions. These results indicate that filamentation may not only occur by a SulA-dependent mechanism, but by a SulA-independent mechanism.

Keywords: Protein repair, filamentation, oxidative stress

1. Introduction

Molecules vital for cellular function can become damaged by environmental influences, spontaneous chemical reactions, free radicals, and genetic changes[(1)]. Although DNA damage and its modes of repair have been categorized quite thoroughly[(2)], changes can also occur in proteins, as they are not entirely stable even under favorable conditions[(3)]. Two types of protein damage can occur; either conformational or covalent damage. Conformational damage is a change that modifies the three-dimensional structure of the protein without changing its chemical composition, while covalent damage is covalent bond breakage or formation that alters the primary structure of the protein[(3)]. The need for repair of damaged proteins may be especially crucial in cells which are not dividing or in those in which protein turnover is decreased due to environmental stress or insufficient nutrient levels. Although proteolysis and replacement is an important response to protein damage[(3)], protein synthesis is significantly decreased in bacteria without an adequate nutrient supply[(4)] and reduced when bacteria are exposed environmental stress[(5)].

One type of protein damage, known as isoaspartyl damage, arises as a result of the spontaneous isomerization of either the amino acid aspartate or asparagine, as shown in Figure 1. The formation of isoaspartate causes a “kink” in the protein backbone (heavy line in Figure 1) and may detrimentally affect protein conformation and enzymatic

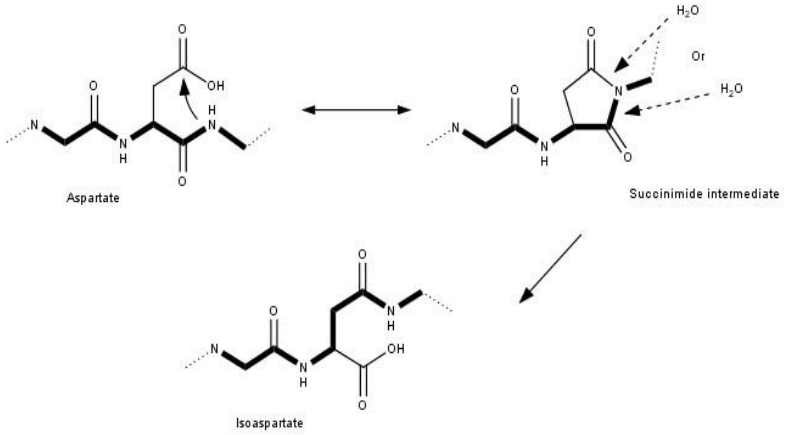


Figure 1. Isoaspartyl Formation

The spontaneous chemical reaction that results in the formation of isoaspartate from aspartate (or asparagine) occurs by an intramolecular nucleophilic attack of the peptide-bond nitrogen on the side-chain carbonyl carbon, which produces a succinimide intermediate that then undergoes spontaneous hydrolysis to yield either isoaspartate or the reformation of aspartate.

activity[3]. The formation of isoaspartate occurs by an intramolecular nucleophilic attack of the peptide-bond nitrogen on the side-chain carbonyl carbon, which produces a succinimide intermediate. This succinimide intermediate can then undergo spontaneous hydrolysis to either produce isoaspartate or reform aspartate. Isoaspartyl residues can be detected and repaired in numerous organisms by the L-isoaspartyl protein carboxyl methyltransferase (PCM; EC 2.1.1.77)[6-9], which in *Escherichia coli* is the product of the *pcm* gene[10, 11]. PCM is able to catalyze the net repair of isoaspartyl damage by methylating the hydroxyl group of the isoaspartate (Figure 2), facilitating reformation of the succinimide intermediate which can then be hydrolyzed to yield either normal aspartate or isoaspartate (which would then be re-methylated). The net result is thus the molecular conversion of isoaspartyl residues back to normal aspartate.

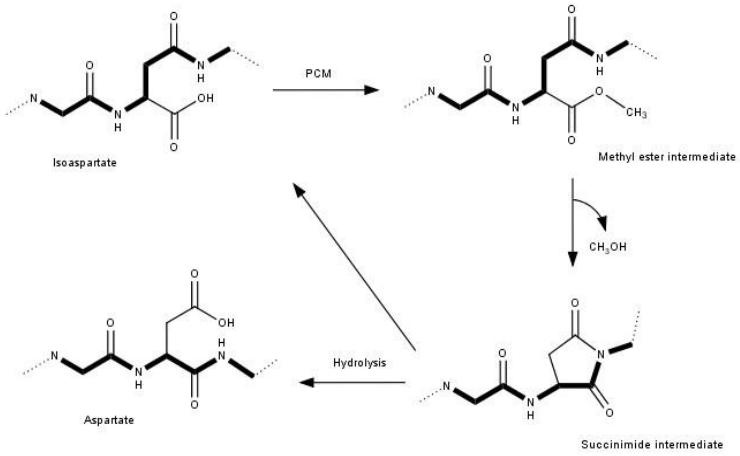


Figure 2. PCM-Mediated Repair

Methylation by PCM to the hydroxyl group of isoaspartate facilitates the reformation of a succinimide intermediate, which can then be spontaneously hydrolyzed to yield either isoaspartate (which can then be re-methylated) or normal aspartate (or asparagine).

In a 2003 study, it was found that the accumulation of isoaspartyl residues in protein repair-deficient mice generated a strong autoimmune response in the form of autoantibody production and T cell hyperproliferation[12]. It has also been found that repair-deficient mice die at an early age from a seizure disorder similar to epilepsy[8]. PCM deficiency may also contribute to several other diseases, including cancer and Alzheimer's disease[13, 14]) and PCM has been found to be important for aging and long-term survival in various organisms[6-8, 15]. In *Escherichia coli*, mutants that lack PCM and are consequently unable to repair proteins demonstrate growth and survival that is comparable to wild-type cells in long-term stationary phase (when the growth rate and death rate of the bacterial cells are equal), but under environmental stress, the long-term survival of these mutants decreases[15]. Stressors such as heat, high salt, or oxidative stress are optimal conditions for protein denaturation, which may explain why these stresses cause cellular survival to become PCM-dependent.

The present study examines the effect of protein repair on filamentation during stationary phase, based on a preliminary observation suggesting that a mutant strain in which the *pcm* gene has been deleted (Δ*pcm*) showed increased filamentation under low-salt conditions. Filamentation is cellular elongation without physical division[16] and has been described as a response to DNA damage. Activation of the SOS regulatory system in response to damaged DNA inhibits DNA replication and promotes repair[17]. During this process, cell division is inhibited by activation of SulA[18, 19] until repair is complete. SulA binds to FtsZ[20, 21] and blocks its polymerization[22, 23], preventing formation of the FtsZ ring that initiates division[20] until SulA is degraded by the Lon protease. Filamentation has also been observed as a response of pathogens to the host's innate immune system or to predators[24], as well as to antibiotics[24], or changes in nutrient levels[25]. Filamentous bacteria were previously considered the sick and dying members of a bacterial population, but recent studies suggest that they may result from a specific survival response[24, 25].

Our laboratory hypothesizes that PCM is important in the maintenance of functional protein conformation, and we have observed increased filamentation in Δ*pcm* mutants under some conditions. We therefore investigated filamentation in repair-deficient cells under conditions where PCM is important, hypothesizing that there may be a link between unstable, unrepaired protein and activation of a filamentation response. Secondly, we investigated the mechanism of filamentation, hypothesizing that since SulA inhibits cellular division under some stress conditions, SulA may be responsible for the increased filamentation seen in our repair-deficient strain. Our results suggest that Δ*pcm* mutants increase filamentation, especially in response to oxidative stress, in a manner dependent both on SulA and some unidentified, possibly novel, factor.

2. Methodology

2.1 Bacterial Strains and Culture Conditions

E. coli strains used in this study are described in Table 1. All strains were grown in Luria-Bertani (LB) medium supplemented with 50μg/ml kanamycin where necessary. The Δ*sulA* mutation in strains JV1187 and JV1188 was verified by PCR.

Table 1.Genotyes of *E. coli* strains employed for this study

Strain or Plasmid	Relevant Genotype	Source or Reference
MGAY[a]	ibpA::YFP	François Taddei[26]
pJV132	Δ*pcm*	Visick, unpublished
JV1166	MGAY Δ*pcm*	Recombination of pJV132 with MGAY
JW0941-1	Δ*sulA*773::Km^R	Coli Genetic Stock Center
JV1187	MGAY Δ*sulA*773::Km^R	Transduction from JW09411-1[27]
JV1188	JV1166 Δ*sulA*773::Km^R	Transduction from JW0941-1

[a] Used as wild-type strain for all experiments.

For microscopy, cultures were grown to stationary phase ("Day 0", approximately 16 hours) at 37°C with aeration and incubated five additional days in long-term stationary phase with continued aeration. Oxidative stress was induced where appropriate by addition of paraquat on Day 0 to 1mg/ml.

2.2 Microscopy and Measurements

Light microscopy and image capture were done using a DM 2500 microscope, DFC 295 camera, and Leica Application Suite software (Leica Microsystems). At least two random fields of view were photographed for every sample; samples were taken from duplicate cultures daily over the five-day period in long-term stationary phase.

2.3 Data Analysis Cell-length was measured using Leica Application Suite software (Leica Microsystems) and used to determine the frequency of cell-lengths for each strain and condition. The most frequently occurring cell - length was determined to be 2.5µm in wild-type (WT) *E. coli* cultures not exposed to oxidative stress (Figure 3): a filamentation threshold was thus established at 3µm, and all cells found to be at or above 3µm were counted as filamented, allowing the percentage of filamented cells in each condition to be calculated and graphed using Excel (Microsoft). MATLAB software (Math Works) was also utilized to analyze average cell-length values for statistical significance using an F-test for equal variance and piecewise t-tests with unequal variance (asterisks in figure 6).

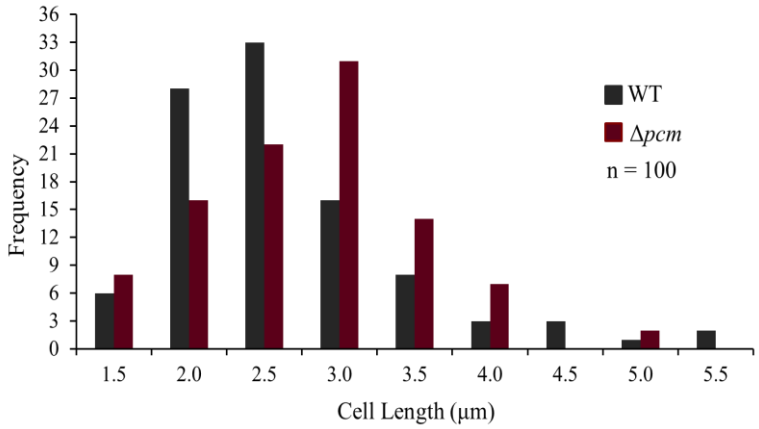


Figure 3. Distribution of Cell-Lengths at Day 0

Frequencies of cell-lengths for wild-type (MGAY; gray bars) and Δ*pcm (*JV1166; red bars) strains at stationary phase (Day 0), determined by measuring cell-lengths before the addition of paraquat. The most frequently occurring cell-length for wild-type (WT) *E. coli* was 2.5µm; therefore, a filamentation threshold was established at 3µm.

3. Results

3.1 Increased filamentation Was Observed For Δ*pcm* Mutants

Preliminary observations (DeRosa and Visick, unpublished) suggested that in low-salt conditions, a mutant strain in which the *pcm* gene had been deleted (Δ*pcm*) showed noticeable filamentation. Since PCM has been observed to contribute to maximum long-term survival in protein repair-deficient *E. coli* under environmental stress, we studied filamentation in long-term stationary phase and under oxidative stress conditions to assess the possible relationship between damaged protein and the initiation of a bacterial filamentation response. In order to test this link, cell-length data were collected over a five-day period from wild-type (Figure 4 A) and Δ*pcm* mutant (Figure 4 B) strains in long-term stationary phase, both under normal conditions (open symbols in Figures 4A and B) and after induction of oxidative stress with paraquat (closed symbols in Figures 4 A and B). Filamentation levels (percentage of cells > 3µm

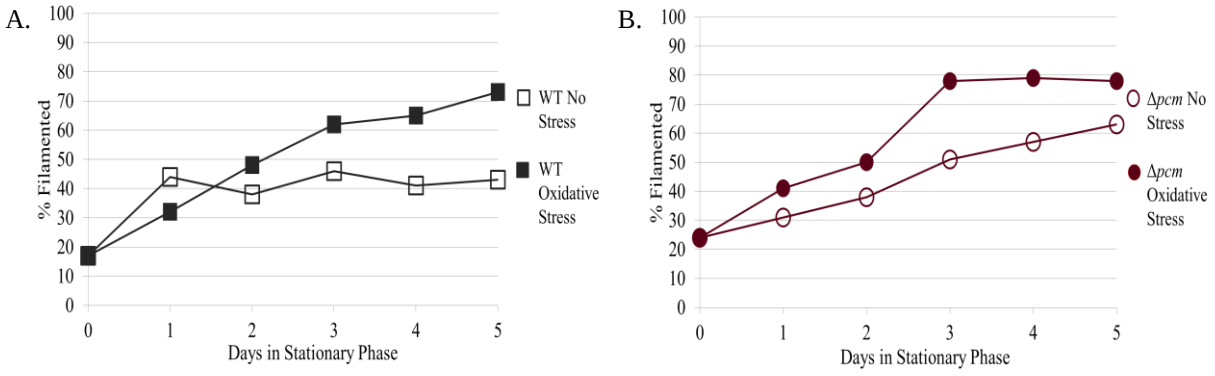


Figure 4. Effect of Oxidative Stress and PCM Deficiency on Filamentation

Percentage of filamented cells determined by measuring cell-lengths on Days 0-5 of growth. Cells were considered filamented if they exceed our threshold (see section 2.3) of 3µm. To induce oxidative stress, 1mg/ml paraquat was added on Day 0 (approximately 16 hours of growth). N = 100. (A) Effect of oxidative stress on filamentation of the WT strain MGAY; (B) Effect of oxidative stress on filamentation of the Δ*pcm* mutant JV 1166.

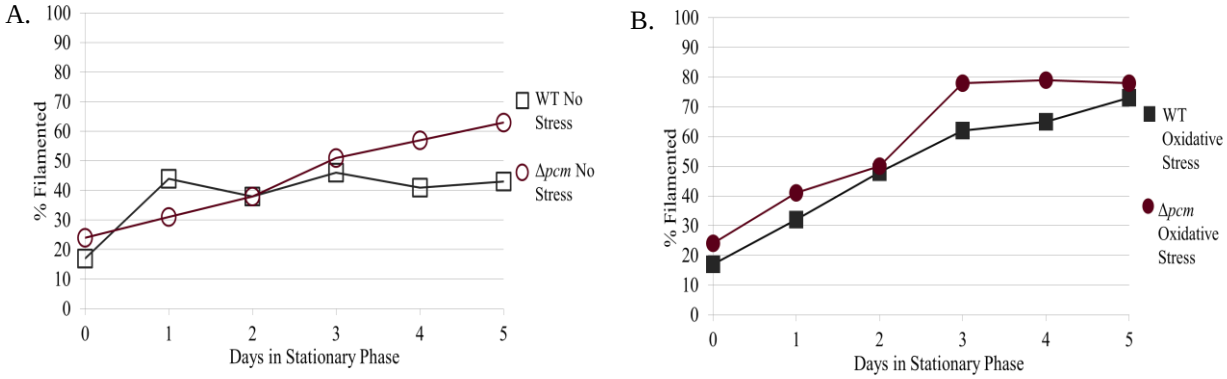


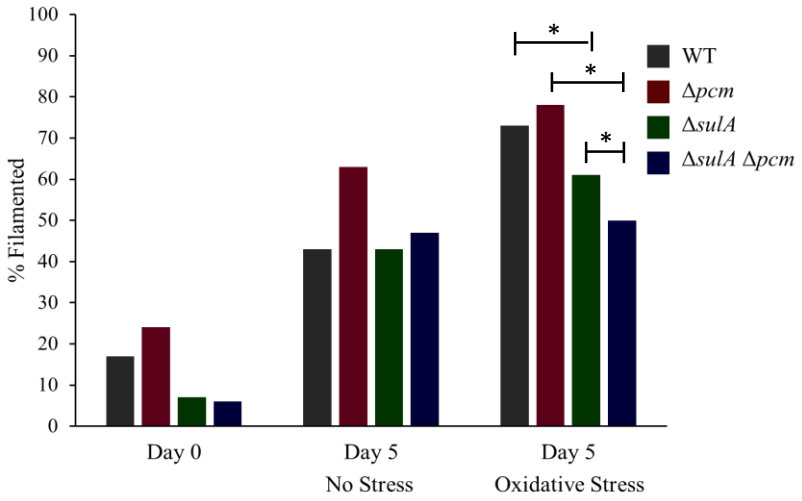
Figure 5. Comparison of the Effect of Oxidative Stress and PCM Deficiency on Filamentation

Percentage of filamented cells determined and cellular stress induced as described in figure 4. N=100. (A) Comparison of filamentation of WT vs. Δ*pcm* mutant (data from Figures 4 A and B) without stress; (B) Effect of oxidative stress on filamentation of WT vs. Δ*pcm* mutant (data from Figures 4 A and B).

long; see section 2.3) were higher for wild-type and Δ*pcm* mutants exposed to oxidative stress relative to those not exposed to stress. When these data were rearranged so that the wild-type and mutant strains could be directly compared (Figure 5 A and B), we observed higher levels of filamentation for the Δ*pcm* mutant relative to wild-type in unstressed cultures (Figure 5 A) and a more rapid rise in the percentage of filamentation under oxidative stress conditions (Figure 5 B), even though the final percentage of filamented cells was similar in the latter case.

3.2 Deletion of *sulA* Reduces Filamentation of WT and Δ*pcm* Mutant Strains

Due to the ability of the SulA protein to inhibit cellular division as part of the SOS response (see Introduction), we made *sulA*-deletion mutants (*ΔsulA* and Δ*sulA* Δ*pcm*; JV1187 and JV1188, respectively) in order to determine if the increased filamentation observed for our Δ*pcm* mutant was due to SulA activation. Cell-length data were collected as previously described, again under no stress and oxidative stress conditions (Figure 6).



6. Effect of SulA on Filamentation

Percent filamented cells for WT (MGAY; grey bars), Δ*pcm* (JV1166; red bars), Δ*sulA* (JV1187; green bars), and Δ*sulA* Δ*pcm* (JV1188; blue bars) at stationary phase (first group of bars) and after 5 days in long-term stationary phase without (second group of bars) and with (third group of bars) oxidative stress as described in Figure 4. N = 100. Asterisks represent average cell-lengths that are statistically different ($p<0.05$).

At Day 0, less than 10% of the filamentation seen in the wild-type strain and less than 15% of the filamentation in the Δ*pcm* mutant strain was observed for the *sulA*-deletion mutants. At this time point, it would appear that SulA is responsible for nearly all filamentation and accounts for all of the increase in filamentation in the Δ*pcm* mutant.

At Day 5 under unstressed conditions, a marked level of filamentation was present even in the SulA-deficient strains, indicating that stationary-phase cells activate some filamentation mechanism other than SulA. However, filamentation in the Δ*sulA* Δ*pcm* double mutant was reduced to the level of the wild-type strain, suggesting that the increased filamentation in the repair-deficient mutant may be due to SulA activation. A similar pattern was observed under oxidative stress conditions, though under these conditions, the Δ*sulA* mutation also reduced filamentation in otherwise wild-type cells, suggesting that oxidative stress may trigger SulA-dependent filamentation in both wild-type and PCM-deficient cells. We found that the average cell-lengths of both the filamented population and the total population were statistically different in the Δ*sulA* mutants vs. their wild-type parents (asterisks in Figure 6).

4. Discussion

4.1 A Δ*pcm* Mutant Shows Increased Filamentation

We set out to determine if there is a connection between unrepaired protein in cells lacking the protein-repair type enzyme PCM and the formation of filaments. The evidence presented here shows that Δ*pcm* mutants do filament

during long-term stationary phase (Figure 4 B) and indeed show increased levels of filamentation compared to wild-type (Figure 5 A) or, under oxidative stress conditions (Figure 5 B), a higher rate of filamentation. We are presently working to automate length measurement in order to obtain a larger sample size from more independent trials and establish the statistical significance of this observation.

Filamentation is known to occur in the SOS response to DNA damage[24]. It may be that Δ*pcm* mutants filament due to damaged DNA, perhaps because replication or repair proteins are sensitive to isoaspartyl damage, or due to isoaspartyl damage in cell-division proteins. However, recent research indicates that filamentation may also be activated under other conditions as a survival response[24, 25]. Uropathogenic *E. coli* (UPEC), for example, utilize filamentation to resist neutrophil phagocytosis, and their recovery from filamentation results in the rapid invasion of epithelial cells[24]. Additional work must be done in order to determine whether the filaments that occur in our repair-deficient mutants are viable, whether they have multiple chromosomes, and whether they are capable of dividing into multiple cells upon nutrient re-addition and protein turnover. Such findings would support the possibility that filamentation is a specific survival response in this case.

4.2 Filamentation In Long-Term Stationary Phase Occurs By Both A Sula-Dependent And A Sula-Independent Mechanism

In the SOS response, the SulA protein triggers filamentation by inhibiting cellular division[24]. We therefore sought to determine whether the increase in filamentation observed in the Δ*pcm* mutant is SulA-dependent. We found that at Day 0 the levels of filamentation for the *sulA*-deletion mutants were only 10% and 15% of the levels seen in wild-type and Δ*pcm* mutants, respectively (Figure 6), which implicates the SulA protein in filamentation and suggests that almost all of the filamentation at this point, including the increased levels of filamentation in the Δ*pcm* mutant strain, can be attributed to SulA.

In the unstressed condition at Day 5, however, high levels of filamentation were observed for all strains (Figure 6), including the SulA-deficient mutants, suggesting that cells initiate some SulA-independent mechanism of filamentation in stationary-phase. Interestingly, however, filamentation under these conditions for the wild-type and the Δ*sulA* Δ*pcm* double mutant were at about the same level, implying that the increased levels of filamentation in the PCM-deficient strain may be a result of SulA activation. In cells subjected to oxidative stress (Figure 6), a reduced level of filamentation was observed for the Δ*sulA* mutant, which suggests that SulA-dependent filamentation may be triggered by oxidative stress in both wild-type and repair-deficient cells.

The increase in filamentation levels for the *sulA*-deletion mutants from Day 0 to Day 5, even though filamentation induced by SulA could not occur, leads us to conclude that filamentation results not only from SulA activation by the SOS system, but also by means of a second, undetermined (and potentially novel) mechanism. Further study will be necessary to gain a more comprehensive understanding of filamentation in repair-deficient *E. coli*, but our data point to its potential significance as a cellular response to unrepaired isoaspartyl damage.

5. Acknowledgements

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