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A protein methyltransferase specific for altered aspartyl residues is important in *Escherichia coli* stationary-phase survival and heat-shock resistance

(protein L-isoaspartyl methylation/repair/spontaneous protein degradation/aging)

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ABSTRACT **Proteins are subject to spontaneous degradation reactions including the deamidation, isomerization, and racemization of asparaginyl and aspartyl residues. A major product of these reactions, the L-isoaspartyl residue, is recognized with high affinity by the protein-L-isoaspartate(D-aspartate) *O*-methyltransferase (EC 2.1.1.77). This enzyme catalyzes the methyl esterification of the L-isoaspartyl residue in a reaction that can initiate its conversion to the normal aspartyl configuration. To directly study the physiological role of this methyltransferase, especially with respect to the potential repair of isomerized aspartyl residues in aging proteins, we examined the ability of the bacterium *Escherichia coli* to survive in the absence of its activity. We utilized gene disruption techniques to replace the chromosomal copy of the *pcm* gene that encodes the methyltransferase with a kanamycin-resistance cassette to produce mutants that have no detectable L-isoaspartyl methyltransferase activity. Although no changes in exponential-phase growth were observed, *pcm*$^-$ mutants did not survive well upon extended culture into stationary phase or upon heat challenge at 55°C. These results provide genetic evidence for a role of the L-isoaspartyl methyltransferase in the metabolism of altered proteins that can accumulate in aging cells and limit their viability.**

Although genetically encoded information can be transcribed and translated with high fidelity into protein sequences, newly synthesized proteins are immediately subjected to a variety of spontaneous chemical degradation reactions (1, 2). For example, L-aspartyl and L-asparaginyl residues are susceptible to nucleophilic attack on the side chain carbonyl carbon by the neighboring peptide bond nitrogen. This reaction results in the formation of an unstable five-membered succinimide ring structure that can both racemize and hydrolyze to generate a mixture of DL-isoaspartyl and DL-aspartyl residues, with the predominant product being the L-isoaspartyl residue (3, 4). Since L-isoaspartyl-containing proteins or peptides may not perform their biological functions as efficiently as the native forms (5–7), the accumulation of these altered species would be expected to have deleterious effects upon cells.

Interestingly, an enzyme exists that can catalyze the transfer of the methyl group from *S*-adenosylmethionine to the α-carboxyl group of L-isoaspartyl residues and the β-carboxyl group of D-aspartyl residues (8–11). *In vitro*, enzymatic methylation of synthetic peptides containing L-isoaspartyl residues facilitates a subsequent nonenzymatic conversion of L-isoaspartyl residues to normal L-aspartyl residues via a succinimide intermediate (12–14). This enzyme, the protein-L-isoaspartate(D-aspartate) *O*-methyltransferase (EC 2.1.1.77), can recognize L-isoaspartyl residues in most amino acid sequence contexts (5, 9) and is found in a broad spectrum of both eukaryotic and prokaryotic organisms (15–18). The protein sequence of this methyltransferase is highly conserved—there is 31% identity between the human and *Escherichia coli* enzymes (19, 20). These facts point to a general housekeeping role of this enzyme in the repair or degradation of damaged proteins. If this is true, the methyltransferase may be particularly important in cells with limited abilities to otherwise replace their aged proteins (21, 22).

Bacterial cells are noted for their ability to grow rapidly under favorable conditions. However, upon the depletion of nutrients, bacteria are faced with the prospect of surviving under conditions where accumulating age-damaged proteins cannot be diluted. In natural habitats, nutrients are generally limiting and starvation is often the rule rather than the exception (23, 24). Some bacteria undergo sporulation or other morphological differentiation in restricted conditions, but others, such as *E. coli*, simply alter their metabolism in stationary phase to delay cell death until conditions improve (24–26). Since the accumulation of altered aspartyl residues may be a contributing factor to the loss of viability, we decided to test the hypothesis that enzymes that recognize abnormal residues play a role in stationary-phase survival.

An L-isoaspartyl protein carboxyl methyltransferase (the product of the *pcm* gene) has been identified in *E. coli* (20), *Salmonella typhimurium* (15), and other enteric species (18). The recent characterization of the *E. coli pcm* gene (20) has now allowed us to initiate genetic approaches to determine the function of its product in intact cells. We report here the construction and analysis of *E. coli* strains with deletions of the *pcm* gene. The resulting *pcm*$^-$ mutants were found to survive poorly upon heat treatment or during stationary-phase culture. This evidence provides direct support for a role of the methyltransferase in ameliorating the potentially harmful accumulation of isoaspartyl-containing proteins and peptides in aging cells and suggests that the bacterial system may be a useful model for understanding these processes in mammalian cells.

MATERIALS AND METHODS

Construction of *pcm* Deletion Mutants in *E. coli* by Gene Replacement. Gene replacement was performed by either the insertion or replacement of *E. coli* chromosomal DNA with DNA encoding kanamycin resistance (Km^R) (27, 28). We first modified plasmids containing the *E. coli pcm* gene and its neighboring chromosomal DNA (Fig. 1 and Table 1). As illustrated in Fig. 2, we replaced a 0.6-kilobase (kb) *Mlu* I–*Cla* I fragment (containing 89% of the *pcm* coding region) from plasmid pCL1 or a 1.3-kb *Mlu* I–*Sna*BI fragment (containing 98% of the *pcm* coding region) from plasmid pMMkatF1 with

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Abbreviation: Km^R, kanamycin resistance.

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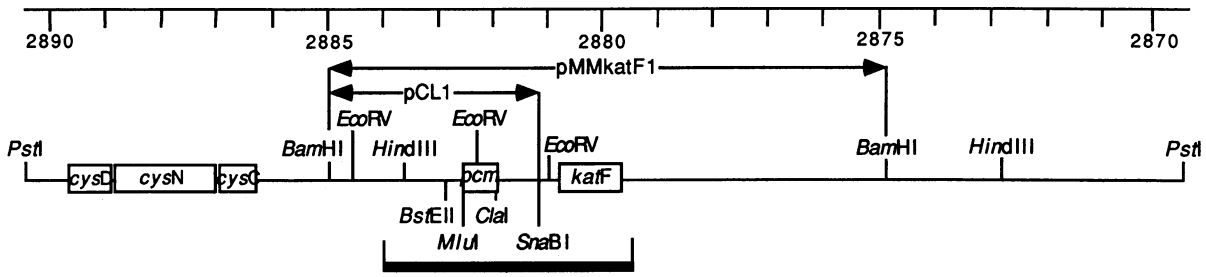

FIG. 1. Map of the 59-min region of the *E. coli* chromosome containing the *pcm* gene. The Kohara coordinates for the *E. coli* chromosome (in kb from the *thr* locus) are indicated on the top line (29). Restriction sites that are labeled below the line were utilized for strain constructions and those that are labeled above the line were used in Southern blot analysis. The regions of genomic DNA that are inserts in pMMkatF1 or pCL1 are also shown. The restriction map is based on refs. 20 and 29–31 and unpublished work of C.L., J. K. Ichikawa, J. Fu, and S.C. The region that is marked by the thick line is enlarged and shown in Fig. 2.

a 1.3-kb *Sma* I Km^R cassette from pUC4KIXX (Pharmacia). As controls, the Km^R cassette was also inserted into a *Bst*EII site (≈350 base pairs upstream of *pcm*) in pCL1 or into a *Sna*BI site (≈750 base pairs downstream of *pcm*) in pMMkatF1 (Fig. 2). The orientation of the Km^R element inserted by blunt-end ligation was determined by restriction endonuclease mapping. Linear DNA prepared from the modified plasmids was used to transform strain JC7623, a strain capable of incorporating linear DNA into the chromosome by homologous recombination (32). Each of the mutations in JC7623 background was then introduced into strain MC1000 by P1 transduction (35).

Preparation of *E. coli* Cytosolic Extracts and the *pcm* Methyltransferase Assay. *E. coli* cells grown in LB medium (Difco; 1% Bacto tryptone/0.5% Bacto yeast extract/1% NaCl) at 37°C overnight were harvested and washed, and the pelleted cells were stored at −20°C. Cells were disrupted by French press treatment and cytosol was prepared at 4°C by centrifugation at 5000 × *g* to remove the cell debris, followed by centrifugation at 100,000 × *g* for 90 min to remove the membrane fraction (18). The activity of L-isoaspartyl protein methyltransferase was determined in a 40-μl reaction mixture containing about 500 μg of cytosolic proteins as the enzyme source, a methyl acceptor (100 μM L-Lys-L-Ala-L-Ser-L-Ala-L-isoAsp-L-Leu-L-Ala-L-Lys-L-Tyr or ovalbumin at 40 mg/ml), the methyl donor *S*-adenosyl-L-[*methyl*-^{14}C]methionine (10 μM; ICN; 52 mCi/mmol; 1 Ci = 37 GBq), and 0.1 M sodium citrate (pH 6.0). After incubation at 37°C for 20–40 min, [^{14}C]methyl esters were quantitated as base-labile volatile radioactivity as described (11).

Physiological Tests for *pcm*$^-$ and Related Mutants. Long-term survival cultures were started by adding aliquots of overnight cultures to 30 ml of freshly supplemented M9 medium (35) [0.2% glucose/leucine (40 μg/ml)/thiamine (0.2 μg/ml)] in a 125-ml Erlenmeyer flask to give an OD_{600} of ≈0.025. The flasks were then shaken at 200 rpm in a New Brunswick Scientific gyrotory water bath at 37°C. Serial dilutions of the culture with 0.9% NaCl were plated on LB plates (1.5% Bacto agar) at ≈24-h intervals. Survival was determined by counting colonies on the plates after 20 h at 37°C. If the strain showed Km^R, the antibiotic at 50 μg/ml was included in the medium throughout the experiment.

Resistance of cells to heat challenge was measured using overnight cultures in LB medium that were diluted to 3 × 10^5 cells per ml with 0.9% NaCl and transferred to 13 × 100 mm glass tubes prewarmed in a 55°C water bath. At each time point, 10–200 μl of cells were directly plated as described above. Survival to an H_2O_2 challenge was measured with overnight cultures that were pelleted, washed, and resuspended in 0.9% NaCl to 5 × 10^8 cells per ml in 18 × 150 mm glass tubes and shaken in a 37°C water bath. H_2O_2 was added to a final concentration of 15 mM. Aliquots of cells were diluted for plating as described above.

RESULTS

Loss of L-Isoaspartyl Protein Methyltransferase in *E. coli pcm*$^-$ Mutants. We constructed two strains (CL1010 and CL1020) where the chromosomal *pcm* gene was replaced by a Km^R cassette and two other strains (CL2010 and CL3010) where this cassette was inserted into adjoining upstream (*orf1*) and downstream (*orf2*) open reading frames, respectively (Table 1 and Fig. 2). The results of Southern blot analysis of *Eco*RV, *Hin*dIII, and *Pst* I endonuclease-digested chromosomal DNA of each of these strains with a Km^R DNA probe were consistent with homologous recombination of the mutated DNA fragments at the 59-min region of the *E. coli* chromosome (data not shown).

For *pcm* deletion strains CL1010 and CL1020, we could detect no methyltransferase activity in cytosolic extracts upon the addition of exogenous substrates such as an L-isoaspartyl-containing peptide or ovalbumin (Table 2). These results indicate that the *pcm* gene was in fact removed and

Table 1. *E. coli* strains and plasmids

Strain/plasmid	Description/genotype	Ref(s).
JC7623	F$^-$ *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE37 recB21 recC22 sbcB15 sbcC201*	32
MC1000	F$^-$ *araD139* Δ(*araABC-leu*)7679 *galU galK* Δ(*lac*)X74 *rpsL thi*	33
CL1010	MC1000, Δpcm (Δ*Mlu* I–*Cla* I)::Km^R	This study
CL1020	MC1000, Δpcm (Δ*Mlu* I–*Sna*BI)::Km^R	This study
CL2010	MC1000, Km^R::*orf1*	This study
CL3010	MC1000, Km^R::*orf2*	This study
CL4010	MC1000, Km^R::*glnG* (P1 transduction, YMC12 × MC1000)	This study, 41
UM122	*thi-1* HfrH, *katF13*::Tn*10*	34
pMMkatF1	10.2-kb *Bam*HI *E. coli* chromosomal fragment in pAT153	20, 31
pCL1	Religation of a 7.5-kb *Eco*RI–*Sna*BI fragment from pMMkatF1	This study

JC7623 was obtained from K. Oden (University of Illinois), MC1000 was obtained from C. Ball (University of California, Los Angeles), and UM122 and pMMkatF1 were obtained from P. Loewen (University of Manitoba, Winnipeg, MB, Canada).

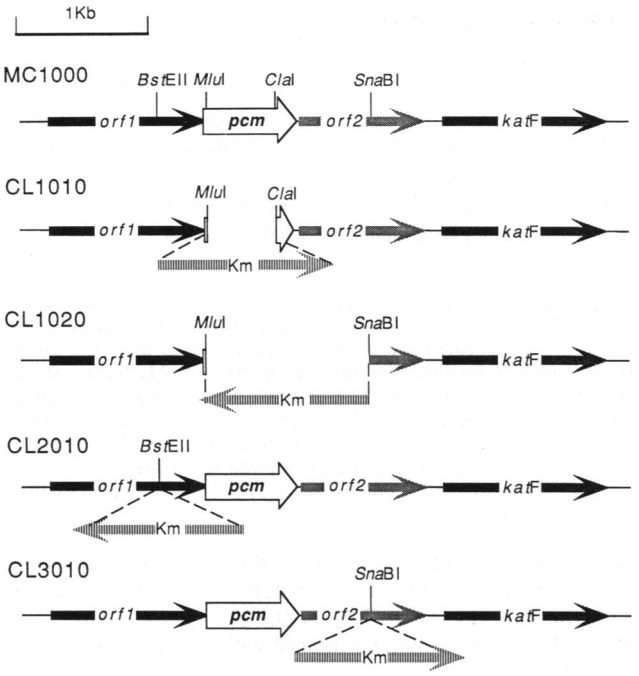

FIG. 2. Construction of *pcm* mutants. Arrows represent open reading frames and indicate the direction of expression. *orf1* and *orf2* represent two open reading frames identified from sequence analysis (C.L., J. K. Ichikawa, J. Fu, and S.C., unpublished results).

that other methyltransferases with similar activities are not present. To confirm that the loss of methyltransferase activity and related cellular physiology was not due to the interferences of the neighboring genes in *pcm* deletions but the loss of the *pcm* gene itself, we directly interrupted the two open reading frames adjacent to *pcm* to make two other strains. In strain CL2010, containing the Km^R cassette inserted in the upstream *orf1* gene with a direction of transcription opposite of that of *pcm* (Fig. 2), the L-isoaspartyl-dependent methyltransferase activity was ≈18% of the pcm^+ parent strain MC1000 (Table 2). Since it is possible that the *orf1* and *pcm* genes are present together in an operon, the activity detected may result from read-through transcription from the original promoter before *orf1* or from a second weak promoter before the *pcm* gene. On the other hand, when the Km^R cassette was inserted ≈750 base pairs downstream of *pcm* in the *orf2* gene (strain CL3010, Fig. 2), the expression of the methyltransferase gene was not affected (Table 2).

Long-Term Survival in Stationary Phase at 37°C Is Defective in pcm^- Mutants. Since we obtained pcm^- deletion mutants, loss of this gene in *E. coli* is not lethal. No differences in growth rate could be detected for the pcm^+ parent and pcm^- mutants during logarithmic-phase growth in either rich medium (LB) or glucose/M9 minimal medium (data not shown). We then examined the survival of the parent and each of the mutant strains in long-term cultures.

The viable cell numbers of the parent pcm^+ *E. coli* MC1000 gradually decreased from 3×10^9 colony forming units/ml to 7.5×10^8 colony forming units/ml (≈25% of the original value) after 10 days in stationary phase in glucose/M9 minimal medium (Fig. 3). Although the pcm^- mutants CL1010 and CL1020 grew to the same original density as the parent strain in stationary phase, the number of viable cells dropped much more rapidly upon prolonged incubation. For example, a 10-day stationary-phase culture of CL1010 or CL2010 contained only 10^7 colony forming units/ml, a value that is <0.5% of the cells originally present in stationary phase and ≈1.3% of that of the parent strain at 10 days. In the

Table 2. L-Isoaspartyl protein methyltransferase activity in cytosolic extracts of *E. coli pcm* deletion mutants and related strains

Strain	Methyltransferase activity, pmol per min per mg		
	L-Isopeptide substrate	Ovalbumin substrate	Endogenous substrates
MC1000	1.57 ± 0.08	0.37 ± 0.07	0.32 ± 0.02
CL1010	0.01 ± 0.02	0.02 ± 0.02	0.05 ± 0.01
CL1020	0.00 ± 0.03	0.00 ± 0.02	0.12 ± 0.01
CL2010	0.28 ± 0.04	0.05 ± 0.04	0.07 ± 0.01
CL3010	1.59 ± 0.13	0.29 ± 0.06	0.15 ± 0.02

All values are the average ± standard deviation from four experiments for cells grown 13 h. Endogenous substrates represent the methyl-accepting species present in the cytosolic extract itself. The L-isopeptide substrate was L-Lys-L-Ala-L-Ser-L-Ala-L-isoAsp-L-Leu-L-Ala-L-Lys-L-Tyr used at a final concentration of 100 μM. The final concentration of the ovalbumin methyl-accepting substrate was 40 mg/ml. Activities with endogenous substrates reflect both background methylation and Pcm methyltransferase activity and have been subtracted from the values shown for the L-isopeptide or ovalbumin substrates.

pcm^- strains, we also observed some additional minute colonies not found in the pcm^+ strain.

Strain CL2010, containing a Km^R insertion in the upstream gene *orf1* (Fig. 2), retained ≈18% methyltransferase activity and demonstrated a survival curve similar to those of the pcm^- deletion mutants (Fig. 3). It is not clear at this time whether the product of the *orf1* gene is itself important for long-term survival or whether the 82% loss of methyltransferase activity in this strain compromises its ability to survive. On the other hand, strain CL3010, containing a Km^R insertion in the downstream gene *orf2*, retained full methyltransferase activity and survived markedly better than the pcm^- mutants although not as well as the parent MC1000 strain upon extended culture (Fig. 3). Whether the small difference in survival between strains CL3010 and MC1000 was due to the Km^R of CL3010 was determined by comparing the survival of strain MC1000 and a derivative strain (CL4010) with a Km^R cassette inserted in the *glnG* gene at 87 min. No difference in the survival in these two strains in

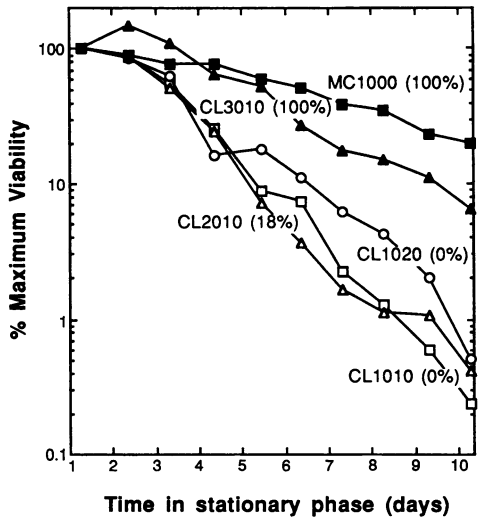

FIG. 3. Long-term survival of *E. coli* cells at 37°C in M9/glucose medium. Percent maximum viability is expressed as the viable cell number at each time point divided by the viable cell number of the same culture after 30 h in stationary phase, corresponding to 50 h after the initial inoculation of the culture. Similar results were obtained in three experiments. The percentage values in parentheses reflect the relative L-isoaspartyl-dependent methyltransferase activity.

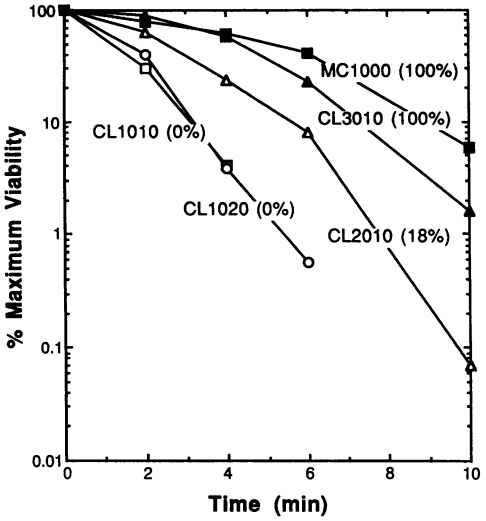

FIG. 4. Survival of stationary-phase *E. coli* cells upon a 55°C heat challenge. Percent maximum viability is determined as the viable cell number at each time point divided by the viable cell number before heating. No viable cells were detected beyond the 4-min time point for strain CL1010 or the 6-min time point for strain CL1020. The percentage values in parentheses reflect the relative L-isoaspartyl-dependent methyltransferase activity.

stationary phase was detected (data not shown), suggesting that the product of the *orf2* gene is itself important for survival.

Survival of *pcm*⁻ Mutants Is Defective Upon Heat or Oxidative Challenge. We used stationary-phase cells in these experiments to allow not only for the possible accumulation of L-isoaspartyl residues but also for the expression of other potentially protective stationary-phase-specific proteins (36, 37), so that cell death would be more directly related to the *pcm* mutation itself. In Fig. 4, we show the survival of *E. coli* strains at 55°C. Although ≈60% of the parent cells (MC1000) and the *orf2* insertion mutant cells (CL3010) could survive after 4 min of exposure, only 5% of the *pcm*⁻ deletion mutant cells (CL1010 and CL1020) survived. The survival of the *orf1* insertion mutant (CL2010) with 18% of the methyltransferase activity was intermediate between that of the *pcm*⁺ and *pcm*⁻ cells. We then tested the viability of *E. coli* cells exposed to an oxidizing agent, 15 mM H_2O_2. We found that the *pcm*⁻ mutants were also less-resistant to this stress than the parent cells, although the effect was much smaller than that seen with thermal stress; 40–70% of the *pcm*⁻ cells were viable after a 40-min treatment compared with ≈100% of the parent cells (Fig. 5).

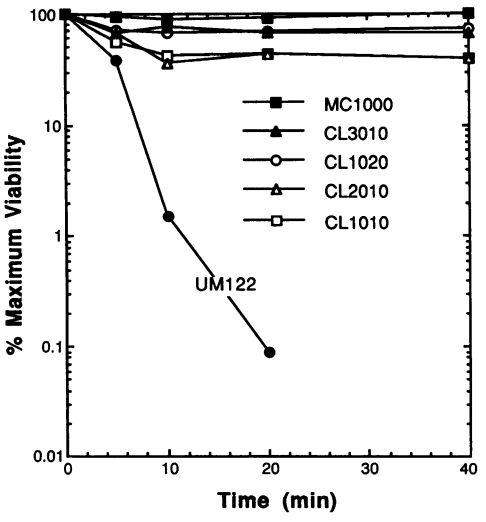

FIG. 5. Survival of stationary-phase *E. coli* cells upon 15 mM H_2O_2 challenge. Percent maximum viability is determined as the ratio of the viable cell number at each time point to the viable cell number before the addition of H_2O_2.

Table 3. L-Isoaspartyl methyltransferase activity in *E. coli* cytosolic extracts at various growth stages

		Methyltransferase activity	
OD_{600}	Growth stage	Endogenous substrates	L-Isopeptide substrate
0.32	Logarithmic (3.5)	0.13 ± 0.01	1.53 ± 0.06
0.60	Logarithmic (4)	0.18 ± 0.02	1.95 ± 0.04
0.95	Late logarithmic (4.75)	0.24 ± 0.04	2.08 ± 0.17
1.23	Late logarithmic (6)	0.36 ± 0.02	2.50 ± 0.08
2.43	Stationary (24)	0.83 ± 0.08	2.55 ± 0.10

MC1000 cells were harvested at the indicated OD_{600} values and cell extracts were prepared. Numbers in parentheses after the growth stage represent the time after inoculation in hours. Methyltransferase activity is expressed as pmol per min per mg. Values from triplicate determinations are given as the mean ± standard deviation. Methyltransferase activity was assayed in the absence (endogenous substrates) or presence of added L-isopeptide substrate (L-Lys-L-Ala-L-Ser-L-Ala-L-isoAsp-L-Leu-L-Ala-L-Lys-L-Tyr at a final concentration of 100 μM).

As a control, we also tested a mutant strain (UM122) defective in the *katF* (identical to *rpoS*) gene product (a central regulator for stationary-phase gene expression) and susceptible to oxidative stress (38–40). Less than 0.1% of the *katF*⁻ cells could survive this oxidative challenge at 20 min (Fig. 5). Interestingly, *katF* mutants share the poor stationary-phase survival and impaired thermal resistance seen in the *pcm*⁻ cells (39). Mutations in these genes are distinguished, however, by their differential sensitivity to H_2O_2 (Fig. 5).

These results suggest that stationary phase *pcm*⁻ cells are more sensitive to environmental challenges than the *pcm*⁺ parent cells. If the absence of methyltransferase results in the inability of cells to metabolize L-isoaspartyl-containing proteins, the levels of these abnormal species would be expected to increase. Although such proteins may function to some extent under nonstress conditions, they may be more susceptible to thermal or oxidative denaturation than their L-aspartyl or L-asparaginyl-containing counterparts.

Expression of L-Isoaspartyl Methyltransferase at Various Growth Stages. Since the mutation of *pcm* gene affects stationary-phase survival, we asked whether the activity could be induced at the end of logarithmic-phase growth. As shown in Table 3, there is at most a 1.7-fold increase in enzyme activity from logarithmic phase to stationary phase. These results indicate that the enzyme may also play a role during rapid growth. Interestingly, we noted a 6-fold increase in the methylation of endogenous cytosolic substrates in stationary phase vs. logarithmic-phase extracts (Table 3), consistent with a time-dependent generation of L-isoaspartyl-containing proteins.

DISCUSSION

We have shown that *E. coli pcm*⁻ mutants that lack the L-isoaspartyl methyltransferase appear to grow normally in logarithmic phase but do not survive well in extended stationary-phase culture or under thermal and oxidative stresses. These results are consistent with the proposed physiological role of the enzyme in recognizing altered proteins containing L-isoaspartyl residues for reactions leading to either repair or degradation, although alternative functions cannot be excluded at this point. In logarithmic-phase growth of *E. coli*, newly synthesized molecules can replace proteins

inactivated by the spontaneous generation of L-isoaspartyl or other types of damaged residues, even in the absence of methyltransferase. However, when cells enter stationary phase and protein synthesis is limited, the presence of the methyltransferase may be crucial to limit the accumulation of potentially labile or inactive proteins containing altered aspartyl residues.

The *E. coli* L-isoaspartyl methyltransferase shares 31% amino acid sequence identity with the human enzyme (19, 20). This conservation in sequence suggests a conservation of function as well. Although the *E. coli* methyltransferase has not been shown to methylate D-aspartyl residues in addition to L-isoaspartyl residues, the catalytic properties of these enzymes are otherwise similar (20). These results also suggest that the metabolism of damaged proteins in stationary-phase bacterial cells may be a model for reactions that occur in the aging process of mammalian cells.

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