

# Targeted Gene Disruption of the *Caenorhabditis elegans* L-Isoaspartyl Protein Repair Methyltransferase Impairs Survival of Dauer Stage Nematodes<sup>1</sup>

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The methylation of abnormal L-isoaspartyl residues by protein L-isoaspartate (D-aspartate) O-methyltransferase (EC 2.1.1.77) can lead to their conversion to L-aspartyl residues. For polypeptides damaged by spontaneous reactions that generate L-isoaspartyl residues, these steps represent a protein repair pathway that can limit the accumulation of potentially detrimental proteins in the aging process. We report here the construction and the characterization of an animal model deficient in this methyltransferase. We utilized Tc1-transposon-mediated mutagenesis in the nematode *Caenorhabditis elegans* to construct a homozygous excision mutant lacking exons 2–5 of the *pcm-1* gene encoding this enzyme. Nematodes carrying this deletion exhibited no detectable L-isoaspartyl methyltransferase activity. These worms demonstrated normal morphology and behavior and adult mutant nematodes exhibited a normal lifespan. However, the survival of dauer-phase mutants was diminished by 3.5-fold relative to wild-type dauers after 50 days in the dauer phase. The fitness of the *pcm-1* deletion nematodes was reduced by about 16% relative to that of wild-type nematodes as measured by the ability of these mutants to compete reproductively against a wild-type population. We found that the absence of the functional methyltransferase gene leads to a modest accumulation of altered protein substrates in aged dauer worms. However, in the viable fraction of these dauer worms, no differences were seen in the levels of altered substrate proteins in the parent and methyltransferase-deficient worms, suggesting that the en-

zyme in wild-type cells does not efficiently catalyze the repair of spontaneously damaged proteins. © 1997

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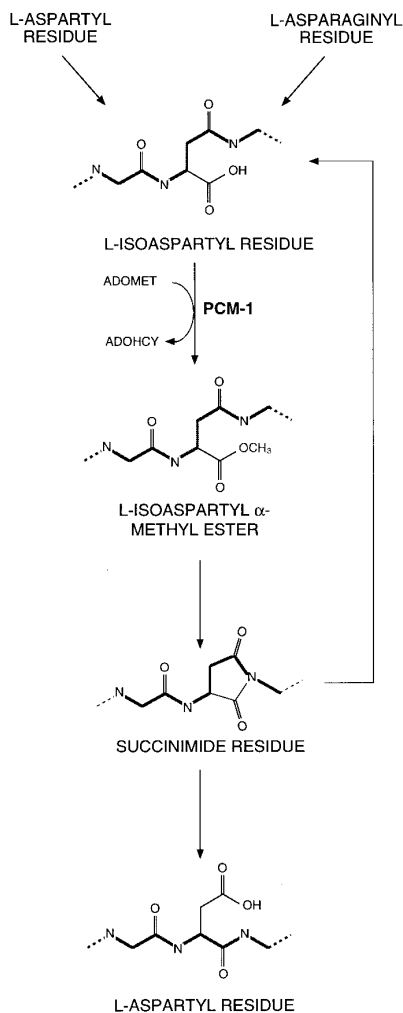
**Key Words:** methyltransferases; protein aging; protein repair.

Protein L-isoaspartate (D-aspartate) O-methyltransferases (EC 2.1.1.77) catalyze the methylation of the free carboxyl groups of L-isoaspartate and D-aspartate residues but not of normal L-aspartate residues (1–4). These abnormal residues derive from the spontaneous degradation of proteins, and their methylation can lead to their conversion to L-aspartyl forms (Fig. 1; Refs. 5–9). These results have suggested that the L-isoaspartyl methyltransferase functions in cells to “repair” proteins damaged in the biological aging process. This methyltransferase is widely distributed in nature with forms specific for L-isoaspartyl residues distributed in bacteria (10, 11), plants (12, 13), and nematodes (14), and forms specific for both L-isoaspartyl and D-aspartyl residues in mammalian tissues (15–17).

Although a role in protein repair is consistent with the biochemical evidence presented so far, the power of genetic techniques now allows us to determine the phenotype of organisms deficient in this enzyme. We wished to develop an animal model to study the role of the L-isoaspartyl methyltransferase in aging organisms. The nematode worm *Caenorhabditis elegans* is a well-characterized species whose short life span, well-defined life cycle, and amenability to genetic and biochemical analysis make it a good model for the study of aging (18). Of special relevance to the study of protein damage in nematodes is that they also have a specialized larval form with a much longer life span than adult worms. The dauer larval stage of *C. elegans* is a non-aging, developmentally arrested third-stage larva spe-

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**FIG. 1.** Role of the *pcm-1* gene product in the repair of spontaneously damaged proteins. The PCM-1 L-isoaspartyl methyltransferase can recognize the major L-isoaspartyl product of the nonenzymatic degradation of protein as aspartyl and asparaginyl residues and initiate a process which leads to the conversion of isomerized aspartyl residues to normal L-aspartyl residues. Both the formation and the hydrolysis of the succinimide intermediate are spontaneous steps under physiological conditions.

cialized for long-term survival and dispersal (19, 20). Dauer larvae are capable of surviving for several months without feeding and exhibit reduced metabolic rates (20, 21) and RNA polymerase II transcription levels (22). Superoxide dismutase, an enzyme that protects cells from oxidative damage, is detected at 4.5- to 5-fold higher levels in dauer larvae (23, 24) and the mRNA coding for the heat shock protein hsp90 is present at 15-fold higher levels (22).

In previous work we have shown that in *C. elegans* there is a twofold increase in the specific activity of the L-isoaspartyl methyltransferase in the dauer stage and have proposed that the increased levels of this methyl-

transferase may help minimize the accumulation of isomerized aspartate residues during the extended dauer stage (14). We have also shown that there is a single gene (*pcm-1*) on chromosome V that encodes the methyltransferase and have characterized its intron-exon structure (14). In this work, we have constructed a strain of *C. elegans* in which the *pcm-1* gene encoding the L-isoaspartyl methyltransferase has been inactivated by Tc1-transposon-mediated insertion and deletion (25) and we have characterized the effects of this deletion in both adult and dauer-phase nematodes.

## MATERIALS AND METHODS

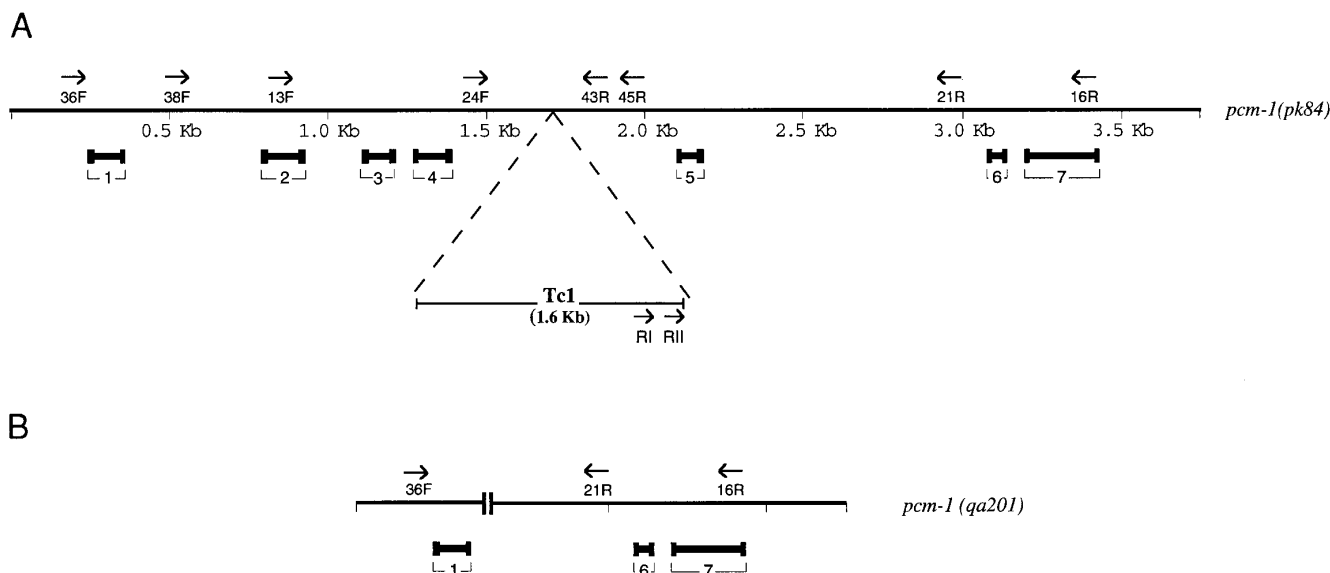
**Construction of a *pcm-1::Tc1* insertion mutant.** Tc1 transposon insertion into the *pcm-1* gene was detected by PCR screening a Tc1 insertion mutant library in the *C. elegans* strain MT3126 (25–27) in the laboratory of Dr. Ronald H. A. Plasterk (Netherlands Cancer Institute, Amsterdam). The first screen was done with the *pcm-1*-specific primer in exon 7, 16R (5' CGCGACTTGTCAATGGCACCAGT 3') and the Tc1-specific primer RI (5' TCACAAGCTGATCGACTCGATGATGCCACGTCG 3') and a 1600-bp product was obtained (Fig. 2A). The secondary screen was done with primers nested with respect to the first set, the *pcm-1*-specific primer in intron 5, 21R (5' GCCTCTCATAATTTTGGGAAGTCG 3') and the Tc1-specific primer RII (5' GATTTTGTGAACACTGTGGTGAAG 3') and a 1300-bp product was obtained (Fig. 2A). One viable homozygous *pcm-1::Tc1* nematode [*pcm-1(pk84)*] was obtained by further screening in the Plasterk Lab (Netherlands Cancer Institute, Amsterdam) and propagated. This new strain was designated NL729.

**Construction of a *pcm-1* deletion mutant.** Further screening of the Tc1 insertion strain NL729 (25) was conducted to detect a Tc1 excision event in the *pcm-1* gene. Nematode populations or single nematodes were lysed for 2 h at 60°C in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.45% (v/v) NP-40<sup>3</sup> (Sigma), 0.45% (v/v) Tween 20 (Sigma), 0.01% (w/v) gelatin (ICN), and 200 μg/ml proteinase K (Sigma) for 2 h at 60°C and screened by PCR (95°C, 30 s; 56°C, 1 min; 72°C, 1 min 45 s) with primers 36F (5' CGACTTCAGTTTCGTGTCAACT 3') and 16R (above) flanking the Tc1 insertion (Fig. 2A). A putative Tc1 excision and deletion event as evidenced by the detection of a 750-bp PCR product was obtained. Single nematodes carrying the *pcm-1* deletion allele designated *qa201* were out-crossed eight times to the male-producing *him-8(e1489)* strain CB1489 (28) and then selfed, utilizing CB1489 males and *pcm-1(qa201)* hermaphrodites in order to eliminate the MT3126 parent strain mutator background. The isogenic and homozygous *qa201* strain thus obtained was designated XA201.

**Growth and harvesting of nematodes.** Nematodes were grown at 20°C on a lawn of *Escherichia coli* strain OP50 cells on NG agar plates as described (29) with the addition of the anti-mycotic agent nystatin (Sigma) at a final concentration of 50 units/liter. Liquid cultures of nematodes grown on *E. coli* OP50 cells in S media (29) were inoculated from 5-day-old saturated NG plates and incubated with shaking at 200 rpm at 20°C as described (29) with the addition of nystatin as above. Dauer worms were prepared by incubating aged nematodes in 1% SDS for 20 min at 22°C. Nematodes were harvested by washes and sucrose floatation as described (14, 29).

**Preparation of *C. elegans* cytosol.** Nematodes were harvested and homogenized by grinding in a liquid-nitrogen-cooled mortar and pestle, and cellular debris was pelleted in a microfuge as described (14). Protein concentrations were determined after precipitation in 10% trichloroacetic acid using a modification of the Lowry method (32).

<sup>3</sup> Abbreviations used: NP-40, Nonidet P40; NG, nematode growth.



**FIG. 2.** PCR mapping of the Tc1 insertion into the *C. elegans pcm-1* gene and the spontaneous excision product. (A) *C. elegans* L-isoaspartyl methyltransferase Tc1 insertion allele *pcm-1(pk84)*. The locations of the PCR primers are indicated above the scale and the positions of exons 1–7 (14) are indicated below. (B) Tc1 excision product *pcm-1(qa201)*. Exons 2–5 have been deleted.

**Methyltransferase activity assay.** Methyl group transfer from Ado-Met to an L-isoaspartyl-containing peptide was determined using a vapor diffusion assay (33). The reaction mixture contained cytosolic extract in final concentrations of 10  $\mu$ M S-adenosyl-L-[methyl- $^{14}$ C]-methionine (ICN, 52 mCi/mmol), 0.44 mM Val-Tyr-Pro-L-isoAsp-His-Ala, and 0.1 M sodium citrate, pH 6.0, in a final volume of 50  $\mu$ l. Reactions were incubated at 30°C for 30 min and then quenched with 50  $\mu$ l 0.2 M NaOH, 1% (w/v) SDS. A 70- $\mu$ l aliquot was spotted on a 1.5  $\times$  8-cm piece of thick filter paper (Bio-Rad 165-090) that had been prefolded in an accordion pleat. The filter paper was wedged into the neck of a 20-ml scintillation vial containing 6 ml of Safety-Solve counting fluor (RPI), which was then capped and allowed to equilibrate at room temperature for 2 h to allow the [ $^{14}$ C]methanol produced by methyl ester hydrolysis to diffuse into the fluor. The filter paper was then removed and the vials were counted. Each sample was assayed in duplicate and a no-enzyme blank, generally representing less than 0.014 pmol methyl groups transferred per minute, was subtracted as the background. The presence of a cytosolic thiol methyltransferase activity was assayed in the same manner in the absence of a methyl-accepting peptide but with 10 mM 2-mercaptoethanol serving as an exogenous substrate. The methylated thiol product was detected by the vapor diffusion assay described above.

**Survival, fertility, and aging studies of male and hermaphrodite nematodes.** CB1489 and XA201 eggs were prepared by the alkaline hypochlorite method as described (29) and hatched on seeded NG plates. After 3 days, male nematodes were transferred to 10 seeded NG plates (four or five per plate) and incubated at 20°C. The nematodes were observed and counted every 1 or 2 days. Approximately four or five individual hermaphrodite nematodes obtained from the 3-day-old synchronized culture described above were transferred to each of 10 seeded plates, incubated at 20°C, and counted and transferred to fresh plates every 2 days to separate them from their progeny. The number of offspring per nematode was determined by transferring two approximately 4-day-old hermaphrodite nematodes from the fourth outcross between the mutant and CB1489 strains to each of 10 seeded plates and then transferring them daily to fresh plates. The offspring on each plate were counted after incubating the plates for 2 days at 20°C.

**Survival and aging studies of dauers.** CB1489 and XA201 dauer nematodes from 200-ml liquid cultures were prepared by treating nematodes with 1% SDS to kill any remaining non-dauers followed by washes in M9 buffer (19). Dauers were then transferred to duplicate flasks containing 50 ml of M9 media (29) and shaken at 20°C. At 2- to 5-day time points, 5- to 20- $\mu$ l aliquots of nematodes were removed from the cultures and transferred to six seeded NG plates. The plates were incubated at 20°C for 2 days in order to allow for dauer recovery up to the L4-larval to young adult stage and counted. Due to the low concentration of worms at later time points (>40 days), it was necessary to concentrate 1–6 ml of the cultures by centrifugation followed by resuspension in 0.1 ml M9 buffer.

**Population genetics of mixed populations of nematodes.** Ten each of 3-day-old CB1489 and XA201 hermaphrodite nematodes were transferred to each of six seeded 9-cm NG plates and incubated at 20°C until the plates were saturated. The nematodes were then washed off the plate, and several hundred nematodes were transferred to six new seeded plates. These transfers were repeated every 6–8 days for 195 days, or ca. 65 generations. Nematodes from plates contaminated with mold or bacteria were treated with an alkaline bleach solution to sterilize the eggs prior to transfer (29). At each time point, 72 single worms were picked and analyzed via multiplex PCR reactions to screen for the wild-type and *pcm-1(qa201)* alleles in single nematodes for the presence of the mutant or wild-type alleles. The primers used were 36F (see above) and 16R (see above), which yield a 750-bp PCR product for the *pcm-1(qa201)* allele, but no product for the wild-type allele under the conditions utilized in the amplification (Figs. 2A, 2B), along with the *pcm-1*-specific primer pair 24F (5' ATAGCTGTACTCACCTAGGACTG 3') and 45R (5' GGTCGAATGTTTCTGGTTTTTCGAC 3'), which yield a 550-bp product for the wild-type allele, but no product for the *pcm-1(qa201)* allele (Figs. 2A, 2B).

**Quantitation of methyl-accepting proteins in cytosolic extracts.** Purified preparations of the human and nematode L-isoaspartyl methyltransferase were used to measure the level of proteins containing the L-isoaspartyl (and for the human enzyme, D-aspartyl) residues. For general quantitation, nematode extracts were mixed in final concentrations of 10  $\mu$ M S-adenosyl-L-[methyl- $^{14}$ C]-methionine (ICN, 52 mCi/mmol) and 0.1 M sodium citrate, pH 6.0, in a final

**TABLE I**  
Methyltransferase Activity in Wild-Type  
and Mutant Nematodes

Strain <sup>a</sup>	Methyltransferase activity <sup>b</sup>		
	Endogenous	L-Isoaspartyl peptide	Thiol
wt (CB1489)	0.025 ± 0.03	1.88 ± 0.08	6.35 ± 0.12
<i>pcm-1(pk84)</i> (NL729)	0.043 ± 0.03	2.09 ± 0.1	NA <sup>c</sup>
<i>pcm-1(qa201)</i> <sup>d</sup> (XA201)	0.056 ± 0.03	0.050 ± 0.05	13.1 ± 3.3

<sup>a</sup> The cytosol from duplicate preparations of mixed-stage nematodes were prepared and assayed as described under Materials and Methods.

<sup>b</sup> Activity is expressed as pmol methyl groups transferred per minute per milligram. The values in the table were obtained after subtracting the values for the no-enzyme blank as described under Materials and Methods. The methyl-accepting peptide used for the L-isoaspartyl methyltransferase assay was VYP(isoD)HA at a final concentration of 0.44 mM, some 22 times greater than the 19.4  $\mu$ M  $K_m$  of the *C. elegans* PCM-1 enzyme for this peptide (14). 2-Mercaptoethanol at a final concentration of 10 mM was used to assay the thiol methyltransferase.

<sup>c</sup> NA, Not assayed.

<sup>d</sup> Three independent preparations of XA201 [*pcm-1(qa201)*] nematodes were assayed, and the values were averaged and are given  $\pm$  SD.

volume of 50  $\mu$ l containing either recombinant human protein L-isoaspartate (D-aspartate) O-methyltransferase (1  $\mu$ l of a preparation with an activity of 1.8 pmol/min/ $\mu$ l; Ref. 35) or a partially purified preparation of the nematode protein L-isoaspartate O-methyltransferase (4  $\mu$ l of a preparation with an activity of 0.65 pmol/min/ $\mu$ l; Ref. 14). After 60 to 90 min at either 22°C for the worm enzyme or 37°C for the human enzyme, the level of methyl esters formed was assayed by the vapor-phase assay described above.

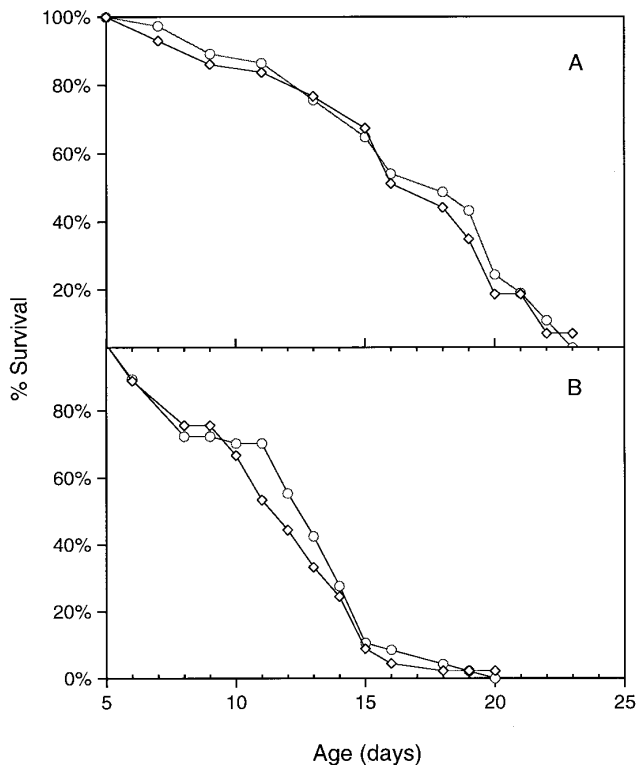
## RESULTS

*Identification and mapping of a Tc1 transposon insertion mutant in the pcm-1 gene.* A Tc1 transposon insertion mutant bank in *C. elegans* strain MT3126 (25) was screened in the Laboratory of Dr. Ronald H. A. Plasterk (Netherlands Cancer Institute, Amsterdam) by PCR with two sets of *pcm-1*-specific and Tc1-specific primers. A single isolate of nematodes homozygous for a Tc1 insertion in the *pcm-1* gene encoding the L-isoaspartyl O-methyltransferase was obtained and the allele was designated [*pcm-1(pk84)*] (Fig. 2A). PCR amplification and sequencing of the genomic DNA flanking the Tc1 insertion site determined that the transposon was inserted in the fourth intron of the *pcm-1* gene between nucleotide positions 1676 and 1677 (14). The insertion of the transposon in an intron did not affect the L-isoaspartyl methyltransferase activity in the cytosol of the homozygous insertion mutant (Table I), suggesting that the Tc1 insert was excised along with the rest of intron IV in an RNA splicing reaction.

*Generation and analysis of a Tc1 excision product of the pcm-1 gene.* Spontaneous excision of the Tc1 transposon often results in the deletion of up to several kilobases of flanking DNA (25). In order to isolate a worm carrying a germline deletion of the *pcm-1* gene, we screened by PCR nematode lysates from 56 plate cultures started with 10–20 nematodes that were allowed to propagate for about 12 days until the plates were nearly saturated. As the spontaneous excision of the transposon has been observed to occur at a frequency of about  $10^{-3}$  per gene per generation, this number of nematode cultures was likely to allow the detection of at least one excision event. The PCR primers utilized in the screen flanked the Tc1 insertion site and were separated by 4.6 kb, a span that was unlikely to be amplified under normal PCR conditions unless an excision event had occurred (Fig. 2A). A single lysate gave a 750-bp PCR product consistent in size with the deletion of the 1.6-kb Tc1 element and 2.2–2.3 kb of flanking DNA. Secondary, tertiary, and quaternary PCR screens (“sib selection”; Ref. 34) of increasingly smaller numbers of progeny from positive cultures successfully isolated single nematodes carrying the deletion allele *qa201* (Fig. 2B). In order to obtain homozygous deletion mutants in a clean genetic background lacking the mutator (*mut-2*) gene on linkage group I and the expected Tc1 insertion and deletion mutations in other genes of the parent MT3126 strain, we exploited the independent assortment of the *pcm-1(qa201)* allele to linkage group V and outcrossed *pcm-1(qa201)* hermaphrodite nematodes into the male-producing strain CB1489 [*him-8(e1439)*] for a total of eight crosses, utilizing two sets of PCR primers to distinguish between heterozygous and homozygous cross progeny, and the resultant strain was designated XA201. PCR amplification and DNA sequencing of the genomic DNA spanning the deletion site established that 2307 bp of genomic sequence was deleted from nucleotide position 384 in intron I to nucleotide position 2691 in intron V, thereby encompassing two-thirds of the L-isoaspartyl methyltransferase coding region (Ref. 14; Fig. 2B).

*Characterization of methyltransferase-deficient nematodes.* Methyltransferase assays of three separate cytosolic extracts from XA201 homozygous *pcm-1* deletion nematode preparations showed that L-isoaspartyl methyltransferase activity was completely absent in these nematodes under conditions that give high methyltransferase activity in cytosol from wild-type nematodes (Table I). A control assay of a distinct thiol methyltransferase activity in these same cytosolic extracts with 2-mercaptoethanol as a substrate showed no decrease in the activity of this enzyme in the deletion mutant cytosol (Table I) indicating that the cytosolic preparations from both strains were equivalent.

Nematodes lacking the PCM-1 activity and nema-



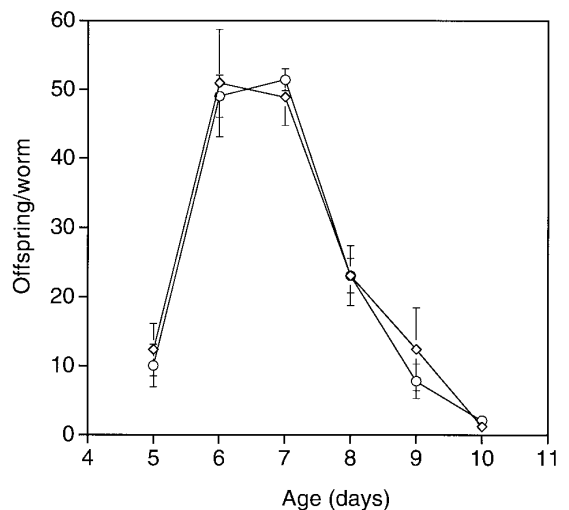
**FIG. 3.** Survival of methyltransferase-deficient nematodes. (A) 43 Wild-type parent CB1489 and 37 methyltransferase-deficient XA201 4-day-old hermaphrodites were placed on seeded NG plates at 20°C. Surviving nematodes were transferred to fresh plates every 2 days to separate them from their offspring and were counted. (B) 45 CB1489 and 47 XA201 4-day-old males were placed on seeded NG plates and survivors were counted every 1–2 days. CB1489 control strain, diamonds; XA201 *pcm-1* deletion strain, circles.

todes of the control strain were observed on seeded NG plates using a stereomicroscope at magnifications of 30 $\times$  and 60 $\times$ . No apparent differences in morphology, coloration, size, or motility were observed. Apparently normal egg production by hermaphrodites and mating behavior of male nematodes of these two strains was also observed.

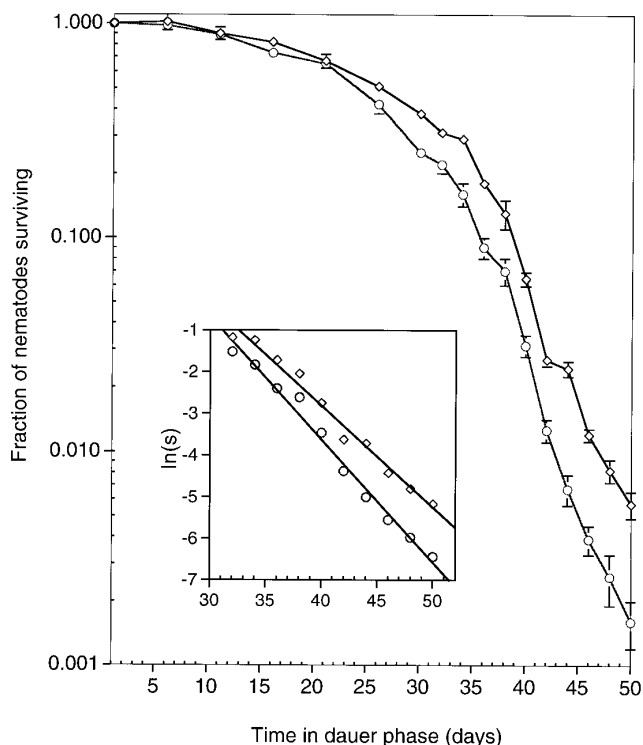
**Lifespan and fertility of methyltransferase-deficient nematodes.** In order to ascertain whether the deletion of the L-isoaspartyl methyltransferase gene decreases nematode lifespan, we monitored the survival of hermaphrodite and male nematodes growing on seeded NG plates (Fig. 3). The maximum measured lifespan of CB1489 control and XA201 methyltransferase-deficient hermaphrodites was 23 days for both strains and the mean lifespans were 16.5 and 17.5 days, respectively (Fig. 3A). The maximum lifespan of CB1489 and XA201 male nematodes was 20 days for both strains and the mean lifespans were 11.5 and 12.5 days, respectively (Fig. 3B). Thus, the absence of an L-isoaspartyl methyltransferase gene

in strain XA201 does not appear to affect nematode lifespan under these laboratory culture conditions. The number of offspring produced by both strains also showed no difference either in the number of offspring produced per hermaphrodite ( $149 \pm 13$  SE for CB1489 and  $142 \pm 5$  SE for XA201), or in the duration of the reproductive span (Fig. 4). For reasons that are unclear, the number of observed offspring per hermaphrodite in both strains was approximately 50% of the number reported previously (28).

**Survival of methyltransferase-deficient dauer larvae.** We exploited the extended lifespan of dauer stage nematodes to determine whether the effects of inactivating the *pcm-1* gene become evident only after a much longer incubation time than is attainable with adult nematodes. Figure 5 compares the survival curves of control and methyltransferase-deficient dauer larvae isolated by SDS treatment from a liquid culture. The survival rate for the XA201 nematodes began to decrease more rapidly than the curve for the CB1489 controls only after about 21 days (Fig. 5). The mean survival time in dauer of the XA201 nematodes was 24.5 days, compared to 27 days for the CB1489 control (Fig. 5). Statistical comparison of the survival curves using the Mantel–Haenzel log-rank test (36) applied to either the data in its entirety or only the fourth-quartile survival data showed that the curves were significantly different ( $P < 0.001$ ). The relative risk, repre-



**FIG. 4.** Fertility of methyltransferase-deficient hermaphrodites. Twenty 4- to 5-day-old hermaphrodites from the wild-type parent CB1489 strain and 20 4- to 5-day-old hermaphrodites from the fourth cross of the *pcm-1* (*qa201*) mutants with the parent strain were placed on 10 seeded NG plates and incubated at 20°C. The nematodes were transferred to fresh plates daily until no new eggs appeared. Following transfer, the previous sets of plates were incubated at 20°C for 2 days and then the 2-day-old offspring were counted. The error bars represent  $\pm 1$  standard error. CB1489 control strain, diamonds; XA201 *pcm-1* deletion strain, circles.



**FIG. 5.** Daurer-stage survival of methyltransferase-deficient nematodes. Daurer nematodes were prepared after 2 weeks as described under Materials and Methods and shaken at 20°C in duplicate flasks for each strain containing 50 ml of M9 buffer. Nematodes were plated out in triplicate every 2–5 days and surviving nematodes were counted after a 2-day incubation at 20°C to allow for dauer recovery. The fraction of surviving nematodes is plotted on a logarithmic scale. Error bars are shown for the standard deviation; where the error is smaller than the width of the plot symbol, no bar is seen. Inset: natural log-linear plot of fourth-quartile survival data. CB1489 control strain, diamonds; XA201 *pcm-1* deletion strain, circles.

senting the ratio between the relative death rates of the methyltransferase-deficient mutants and the controls, was only 1.38 (95% confidence interval: 1.32–1.45; Ref. 36), suggesting no overall increase in mortality of the methyltransferase deficient dauers. In fact, the mortality rate doubling time calculated from the slopes of the log-linearized mortality curves for both strains is not significantly different ( $11.8 \pm 1.6$  days for CB1489 and  $11.5 \pm 1.7$  days for XA201). However, the relative risk in the fourth quartile increased to 2.33 (95% confidence interval: 1.96–2.76), suggesting that late-stage methyltransferase-deficient dauers survive less well than the control strain. After 50 days, when only 0.16% of the XA201 nematodes remained alive, 3.5 times as many of the controls were still viable (0.57%; Fig. 5) and a natural log-linear plot of the fourth-quartile survival rates (Fig. 5, inset) showed that the survival rate of the methyltransferase-deficient mutant in the fourth quartile was decreasing more rapidly than that of the control strain ( $P < 0.01$ ; Student's *t* test, Ref. 37).

*Fitness of methyltransferase-deficient nematodes.* We monitored the change in the frequencies of the wild-type and the null *pcm-1* allele over 65 generations in a mixed and interbreeding population of nematodes that was derived from an equal number of CB1489 and XA201 hermaphrodites. In two independent trials, we found that the frequency of the wild-type allele increased to nearly 13 times that of the null allele in the first trial and 6.5 times the null allele in the second trial (Table II). Assuming no decrease in fitness for heterozygous nematodes that have one active copy of the methyltransferase gene, these results indicate a significant decrease in the fitness of the homozygous null mutants. We calculated a selection coefficient (38) of 0.22 from the data for the first trial and a value of 0.10 from the data for the second trial. On average, therefore, these results show that the fitness of the homozygous null mutants is only 84% of that of the wild-type nematodes.

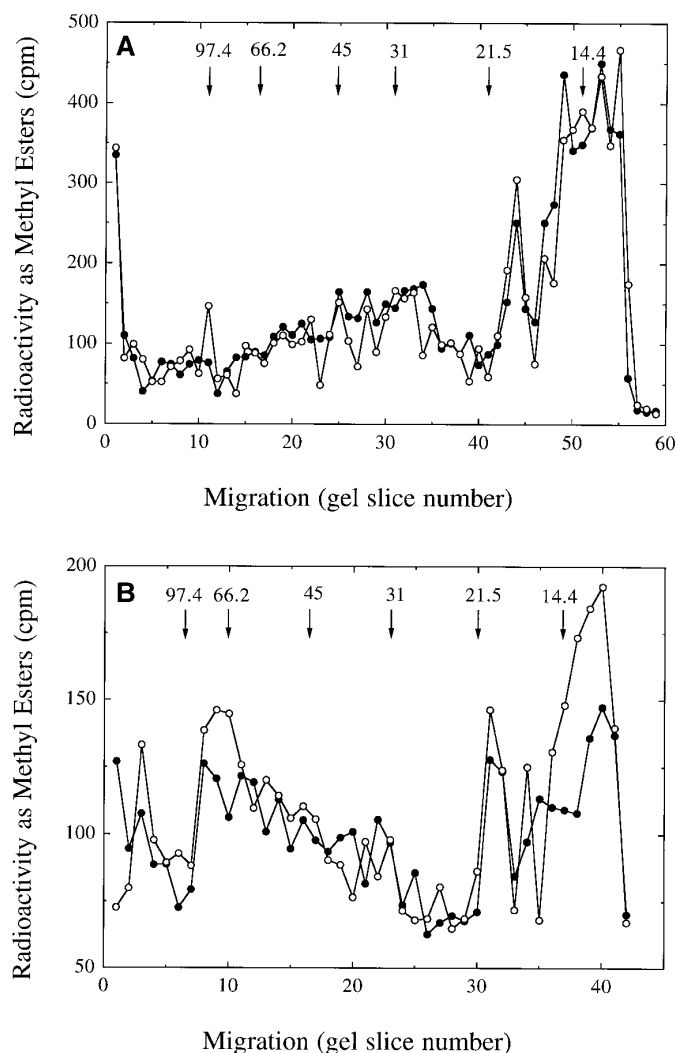
*Accumulation of altered proteins in methyltransferase-deficient nematodes.* In order to determine whether nematodes lacking the L-isopartyl methyltransferase accumulate isomerized protein substrates of this enzyme,

**TABLE II**  
Changes in wt and null *pcm-1* Allele Frequencies in a Mixed Population of wt and L-Isoaspartyl Methyltransferase-Deficient Nematodes<sup>a</sup>

Day	Generation <sup>b</sup>	wt	null
Trial 1			
1	0	0.500	0.500
4	1	0.509	0.491
13	4	0.467	0.533
27	9	0.739	0.261
37	12	0.600	0.400
59	19	0.653	0.347
77	25	0.620	0.380
121	40	0.888	0.112
163	54	0.875	0.125
195	65	0.928	0.072
Trial 2			
1	0	0.500	0.500
6	2	0.591	0.409
23	7	0.729	0.271
50	16	0.797	0.203
70	23	0.854	0.146
120	40	0.853	0.147
159	53	0.892	0.108
195	65	0.866	0.134

<sup>a</sup> Trial 1 and Trial 2 represent two independent experiments. Several hundred mixed-stage nematodes were transferred at each time point, and PCR analyses were done on 72 worms at each time point as described under Materials and Methods.

<sup>b</sup> The generation time was 3 days.



**FIG. 6.** Distribution of methyl-accepting polypeptides in cytosolic extracts of control and methyltransferase-deficient aged dauers. (A) Substrates for the recombinant human protein L-isoaspartate (*D*-aspartate) *O*-methyltransferase. Nematodes were incubated at 22°C for 34 days on NG agar plates with a lawn of OP50 *E. coli* cells. Dauers were collected, washed, and enriched for live dauers by centrifugation through a 40% (v/v) Percoll gradient. Fifty micrograms of cytosolic protein prepared as described under Materials and Methods was incubated with 0.8  $\mu$ M *S*-adenosyl-L-[methyl-<sup>3</sup>H]methionine (Dupont NEN, 55.1 Ci/mmol) and 1  $\mu$ l of purified human recombinant enzyme (Ref. 35; activity is 1.8 pmol/min/ $\mu$ l using Val-Tyr-Pro-L-isoAsp-His-Ala as a substrate) in 100 mM sodium citrate, pH 6.0 (final volume 25  $\mu$ l), for 60 min at 37°C. Protein (25  $\mu$ g) of each sample was separated on a 10% polyacrylamide gel containing 1% SDS in a pH 2.4 phosphate buffer system (13). Gels were stained with Coomassie brilliant blue and destained in 5% (v/v) methanol/10% (v/v) acetic acid. Gel slices (3 mm) were then assayed for the incorporation of base-labile [<sup>3</sup>H]methyl esters by placing each slice into a 1.5-ml microcentrifuge tube, adding 500  $\mu$ l of 1.5 M Na<sub>2</sub>CO<sub>3</sub>, and then placing the open tube into a 20-ml vial containing 5 ml of scintillation fluid (Safety-Solve, Research Products International). The vial was tightly capped and incubated at 60°C for 24 h to promote the diffusion of [<sup>3</sup>H]methanol into the fluor for counting. The open symbols show the radioactivity for extracts of wild-type dauer worms; the closed symbols are for the methyltransferase-deficient worms.

we prepared cytosolic extracts from 5-day-old cultures of CB1489 control and XA201 methyltransferase-deficient nematodes. We utilized the recombinant human L-isoaspartyl methyltransferase to assess the level of methyl-accepting substrates in these extracts. No significant difference in methyl accepting substrates was observed between the control strain CB1489 ( $120 \pm 7$  pmol methyl groups/mg extract protein) and the methyltransferase-deficient strain XA201 ( $110 \pm 2$  pmol methyl groups/mg extract protein). Methyl-accepting substrate levels increased markedly in extracts prepared from 14-day-old dauer cultures ( $453 \pm 33$  pmol/mg protein for controls and  $511 \pm 96$  pmol/mg protein for mutants) and in extracts prepared from dauer nematodes cultured for an additional 17 days for a total of 31 days ( $1115 \pm 193$  and  $1890 \pm 70$  pmol/mg protein for mutants). Further fractionation of comparably aged dauers (34 days) on a Percoll gradient to obtain only the viable fraction of the dauer population (30) yielded similar levels of methyl-accepting substrates for the control and mutant extracts ( $1444 \pm 177$  pmol/mg protein and  $1383 \pm 93$  pmol/mg protein, respectively).

The accumulation of methyl-accepting substrates in dauer nematodes suggests that methylation may not be occurring to a significant extent in dauer phase, perhaps due to the depletion of ATP (39), the biosynthetic precursor of the methyl group donor AdoMet. Electrophoretic fractionation of the polypeptide substrates methylated with either the human or the nematode recombinant enzyme show a pronounced accumulation of substrates in the low-molecular-weight range for both the control and the mutant strains, suggesting that altered proteins in dauers may undergo protein degradation rather than repair (Fig. 6).

## DISCUSSION

In this work, we have established an animal model for the elucidation of the biological function of the pro-

The positions of marker proteins (Bio-Rad low-range molecular weight standard) electrophoresed in a parallel lane are indicated by arrows. (B) Substrates for the purified recombinant *C. elegans* protein L-isoaspartate *O*-methyltransferase. Nematodes were transferred to seeded plates and incubated at 22°C for 28 days. Dauers were collected, washed, and enriched for live dauers as described above. Fifty-eight micrograms of cytosolic protein prepared as described under Materials and Methods was incubated with *S*-adenosyl-L-[methyl-<sup>3</sup>H]methionine and 4  $\mu$ l of the purified nematode recombinant enzyme (Ref. 14; activity is 0.65 pmol/min/ $\mu$ l using Val-Tyr-Pro-L-isoAsp-His-Ala as a substrate) in 100 mM sodium citrate, pH 6.0 (final volume 25  $\mu$ l), for 60 min at 22°C. The entire sample was then loaded on a polyacrylamide gel and processed as described above, except that the volume of the sodium carbonate solution was reduced to 100  $\mu$ l and the gel was vacuum dried for 90 min at 65°C before cutting. The open symbols show the radioactivity for extracts of wild-type dauer worms; the closed symbols are for the methyltransferase-deficient worms. The positions of marker proteins are indicated as above.

tein repair enzyme L-isoaspartyl *O*-methyltransferase. We have shown that transposon-mediated homozygous deletion and inactivation of the *pcm-1* gene encoding the L-isoaspartyl methyltransferase in the nematode *C. elegans* results in viable animals that have a normal adult lifespan and fertility rate but no detectable L-isoaspartyl methyltransferase activity.

Under conditions of crowding and diminished food supply, *C. elegans* L1 larvae enter a developmentally arrested dauer larval stage that allows for extended survival for up to several months, significantly longer than the normal 20-day adult lifespan (19, 20). Dauer nematodes also exhibit an increased resistance to oxidative damage, possibly due to the significantly elevated levels of protective enzymes such as superoxide dismutase (23, 24). The first indication that the L-isoaspartyl methyltransferase may have a similar protective role in dauer nematodes came when we discovered that the levels of the enzyme were elevated two-fold in dauer worms (14). In this work, we examined the effect of disrupting the gene for this enzyme on the dauer life span. We found that the loss of methyltransferase activity in the mutant strain significantly decreased their survival at time points beyond the maximum life span of adults. Furthermore, we found that mutant worms competed poorly with wild-type worms in mixed cultures. These results suggest that optimal survival of worms is dependent upon the presence of an active protein repair methyltransferase. The *pcm-1* null mutants used in this study were outcrossed to the CB1489 nonmutator strain to remove unlinked mutations. However, we cannot yet exclude the possibility that the reduced dauer survival and/or out-competition in mixed culture experiments may have resulted from a closely linked mutation. Nevertheless, the observed dauer survival affect is consistent with a role for the protein repair methyltransferase in facilitating long-term survival. The dauer life span is significantly longer than that of adult nematodes. In fact, indications that the survival of the mutant dauers was affected were only apparent after 21 days in the dauer phase, at which time the age of the nematodes was 25 days or more. This age already surpasses the maximum life span of adult nematodes observed in this study.

Perhaps the most surprising result from this study was our observation that the altered substrates for the methyltransferase accumulated at roughly equal rates in the wild-type and methyltransferase-deficient worms under a variety of conditions. If in fact this enzyme was initiating the conversion of L-isoaspartyl residues to normal L-aspartyl residues as demonstrated *in vitro* (5–9), we would expect that the level of the L-isoaspartyl-containing substrates would be lower in the wild-type worms that contain methyltransferase activity. In fact, this is exactly what has been recently shown to occur in a transgenic mouse model in which the gene

for the corresponding enzyme was knocked out (40). In this case, cytosolic extracts of brain, heart, liver, and erythrocytes from mice lacking the L-isoaspartyl methyltransferase had levels of substrates 4- to 8-fold higher than those of wild-type mice (40). Why a similar result is not seen in the worm system is not clear. One possibility is that the level of the methyl-donating substrate for the methyltransferase, AdoMet, is low in dauer-phase worms as discussed under Results. Another possibility is that the lower affinity of the worm L-isoaspartyl methyltransferase for peptide and protein substrates compared with the mammalian enzyme results in a much slower repair reaction. For example, the  $K_m$  values of the human enzyme for a series of four L-isoaspartyl-containing synthetic peptides are 18- to 67-fold lower than those for the nematode enzyme (14). Mathematical simulations have suggested that each 10-fold increase in the  $K_m$  of an L-isoaspartyl-containing substrate can result in up to a 10-fold decrease in the conversion of the isoaspartyl residues to the normal aspartyl residue (16, 17). Thus, it is possible that protein repair in nematodes is much less efficient than that in longer-lived mammals. Additional studies are needed to clarify this issue.

In many cases, gene disruptions in higher organisms give unexpected results, ranging from a normal phenotype to a minimal one (41). Null mutants, it has been suggested, "are not animals without that protein, but are 'reactionisms,' organisms that respond to the mutation" (42). However, the maintenance of a gene throughout evolution is evidence in itself that its absence would occasion a decrease in fitness (43). Indeed, we have shown that the absence of the L-isoaspartyl methyltransferase in this organism does result in a decline in the fitness of the population of mutants as measured by the ability of this population to compete against a wild-type population. The decline in fitness of the mutant population found here would lead to the near-complete disappearance of animals lacking this enzyme in as little as 400 generations (computer simulation on PopGen 2.0; Ref. 44). Although we were unable to measure the direct cause of this reduction in fitness in the deletion mutants, we hypothesize that the added metabolic cost of degrading and/or resynthesizing isomerized proteins may lead to a moderate decline in the fitness of the mutant.

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