

Recovery from long-term stationary phase and stress survival in *Escherichia coli* require the L-isoaspartyl protein carboxyl methyltransferase at alkaline pH

Wade M. Hicks,[†] Matthew V. Kotlajich[‡] and Jonathan E. Visick

Department of Biology, North Central College, Naperville, IL 60540, USA

Correspondence

Jonathan E. Visick

jevisick@noctrl.edu

Received 16 December 2004

Revised 2 March 2005

Accepted 24 March 2005

The L-isoaspartyl protein carboxyl methyltransferase (PCM) can stimulate repair of isoaspartyl residues arising spontaneously in proteins to normal L-aspartyl residues. PCM is needed in *Escherichia coli* for maximal long-term survival when exposed to oxidative stress, osmotic stress, repeated heat stress or methanol. The effect of pH on a *pcm* mutant during long-term stationary phase was examined. PCM was not required for long-term survival of *E. coli* subjected to pH stress alone; however, PCM-deficient cells showed impaired resistance to paraquat and methanol only at elevated pH. The mutant also showed stress-survival phenotypes in minimal medium buffered to pH 9.0. Accumulation of isoaspartyl residues was accelerated at pH 8.0 or 9.0 *in vivo*, though PCM-deficient cells did not show higher levels of damage. However, the *pcm* mutant displayed an extended lag phase in recovering from stationary phase at pH 9.0. Protein repair by PCM thus plays a key role in long-term stress survival only at alkaline pH in *E. coli*, and it may function primarily to repair damage in cells that are recovering from nutrient limitation and in those cells that are able to divide during long-term stationary phase.

INTRODUCTION

The spontaneous formation of isoaspartate from aspartyl (Asp) and asparaginyl (Asn) residues via a succinimide intermediate (Fig. 1) can adversely affect the function (e.g. Clarke *et al.*, 1992) and stability (Szymanska *et al.*, 1998) of many proteins. This form of protein damage can be recognized and repaired by the L-isoaspartyl protein carboxyl methyltransferase (PCM; EC 2.1.1.77), a highly conserved enzyme. PCM specifically methylates isoaspartyl (isoAsp) residues, stimulating regeneration of the unstable succinimide and net repair to normal Asp (Visick & Clarke, 1995; see Fig. 1). Protein repair by PCM has been linked to successful ageing, long-term viability and stress survival in bacteria (Visick *et al.*, 1998a), nematodes (Kagan *et al.*, 1997), plants (Mudgett & Clarke, 1994), fruit flies (Chavous *et al.*, 2001) and mice (Kim *et al.*, 1997). However, much remains to be learned about this repair enzyme's *in vivo*

role(s), including where and when its activity is crucial and the identities of its key substrates.

As nutrients are exhausted during exponential growth, *Escherichia coli* enters stationary phase and activates specific genetic programmes, such as the RpoS regulon (Kolter *et al.*, 1993). If nutrient deprivation continues, an initial loss of viability (traditionally termed 'death phase') is observed, but the population then stabilizes and enters long-term stationary phase. This is a state of dynamic equilibrium in which individual cells may die or divide but the community as a whole survives indefinitely, due in part to selection for subpopulations that are better adapted to the current nutritional conditions (Finkel & Kolter, 1999). Long-term stationary phase probably represents an important adaptation for survival of *E. coli* in oligotrophic environments such as its environmental reservoirs.

During long-term stationary phase, *de novo* protein synthesis is greatly reduced, and maintenance of existing proteins becomes crucial. To this end, the transition to stationary phase includes increased synthesis of chaperones, trehalose, catalase and other protective factors (Kolter *et al.*, 1993). Long-term stationary-phase cells model many aspects of senescence in higher organisms, including gradual loss of viability of individual cells, decreased function of macromolecules and increased susceptibility to oxidative damage

[†]Present address: Department of Molecular and Cell Biology, Brandeis University, Waltham, MA 02453, USA.

[‡]Present address: Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697, USA.

Abbreviations: Asn, asparaginyl; Asp, aspartyl; GASP, growth advantage in stationary phase; isoAsp, isoaspartyl; PCM, L-isoaspartyl protein carboxyl methyltransferase.

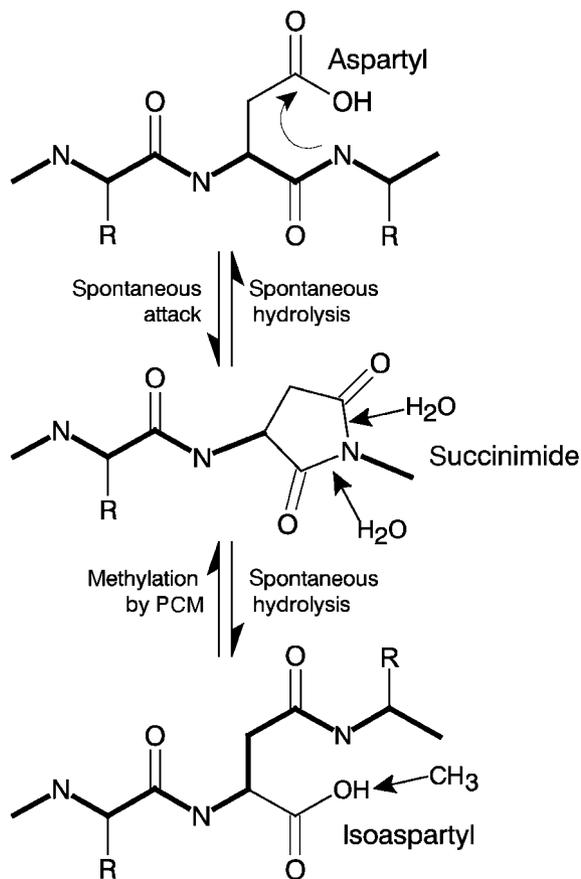


Fig. 1. IsoAsp damage and repair by PCM. IsoAsp residues result from nucleophilic attack (top) of the peptide-bond nitrogen on the side-chain carbonyl group of Asp or Asn, forming an unstable succinimide intermediate (middle) rapidly hydrolysed to either Asp (top) or isoAsp (bottom). Methylation of the isoAsp residue (arrow) by PCM stimulates reformation of the intermediate and results in net repair to normal Asp.

(Kolter *et al.*, 1993; Nyström, 2003; Visick *et al.*, 1998a). Under these conditions, PCM is required for maximal stress resistance: strains carrying *pcm* deletions show reduced long-term survival when exposed to oxidative, osmotic or heat stress, or methanol (Visick *et al.*, 1998a). Based on these results, we have hypothesized that covalent damage (due to isoAsp formation) and conformational damage (due to destabilization of protein structure) synergize to result in impaired stress survival in ageing *E. coli* (Visick *et al.*, 1998b).

Surprisingly, ageing *pcm* mutants are deficient in stress resistance only when grown in rich media such as Luria–Bertani (LB) broth. In glucose minimal medium, no difference in survival between wild-type and mutant strains has been found under conditions tested to date (Visick *et al.*, 1998a). Growth of *E. coli* in rich media such as LB broth exposes the cells to elevated pH as organic acids are consumed (Stancik *et al.*, 2002), whereas glucose minimal

medium would be acidified by acidic fermentation products (Böck & Sawers, 1996), though such media are typically buffered to a near-neutral pH. Alkaline pH increases the rate of isoAsp formation *in vitro* (by sixfold for a model peptide at pH 9.0 compared to 7.5; Brennan & Clarke, 1994), probably favouring succinimide formation (see Fig. 1) by deprotonating the attacking peptide-bond nitrogen. Furthermore, exposure to alkaline pH in rich medium produces or intensifies other known long-term survival phenotypes (Farrell & Finkel, 2003; Lazar *et al.*, 1998; Vulčić & Kolter, 2002; Weiner & Model, 1994). We therefore sought to determine (i) whether PCM is required for survival of pH stress during long-term stationary phase (as it is for other stresses that can affect protein conformation) and (ii) whether elevated pH in LB broth is sufficient to account for the difference in stress survival between rich and minimal media.

METHODS

Bacterial strains. *E. coli* strain MC1000 (λ^- e14⁻ *araD139* Δ [*araA-leu*]7697 *galE15 galK16* Δ [*codB-lac*]3 *rpsL150 mcrB1 relA1 spoT1*; Casadaban & Cohen, 1980) was used as the wild-type strain for all of the experiments described. Construction of the *pcm* mutant derivative JV1068 (Δ *pcm*::Km^r) has been described previously (Visick *et al.*, 1998a).

Growth media and pH buffers. LB broth (Miller, 1972) was used as a rich growth medium for all experiments. Buffers (200 mM final concentration) were added to maintain a specific pH in LB broth as follows (Blankenhorn *et al.*, 1999): pH 5.5 or 6.0, MES; pH 7.0, MOPS; pH 8.0, TAPS; pH 9.0, 3-[(1,1-dimethyl-2-hydroxyethyl)-amino]-2-hydroxypropanesulfonic acid (AMPSO). All buffers were adjusted to the desired pH with KOH. For growth in minimal medium, either MOPS-buffered minimal medium (pH 7.4; Neidhardt *et al.*, 1974) and an AMPSO-buffered derivative (pH 9.0) or M63 medium (pH 7.0; Miller, 1972) and a derivative made by altering phosphate concentrations (pH 9.0; Farrell & Finkel, 2003) were used. All cultures were grown at 37 °C under aerobic conditions.

Stationary-phase and stress-survival assays. Long-term stationary-phase survival was measured as described previously (Visick *et al.*, 1998a). Viable counts were performed to determine the number of viable cells after 24 h of aerobic growth at 37 °C (day 0) and daily for 10 days thereafter. When appropriate, methanol (0.5% final concentration) or paraquat (0.25 mg ml⁻¹ final concentration) was added after the first 24 h growth. Results were normalized by expressing viable counts as percentages of the maximum number of viable cells for a particular culture; maximal density was reached on either day 0 or day 1. In all cases, the maximum number of viable cells ranged from 1×10^9 to 2.5×10^9 cells ml⁻¹, and no significant differences were observed between MC1000 and JV1068.

Growth curves. LB broth buffered to either pH 7.0 or pH 9.0 was inoculated with MC1000 or JV1068, using a 1:100 dilution of a broth culture or a 1:100 dilution of a single resuspended colony from a plate. Inocula consisted of either cells grown overnight at 37 °C in LB broth or on LB plates or ‘aged’ cells incubated at 37 °C for five additional days. Viable counts were used to verify initial c.f.u. ml⁻¹; there was no significant difference in the number of viable cells in the inocula under either condition. Cultures were grown aerobically at 37 °C and growth was monitored spectrophotometrically (Milton-Roy Spectronic 20D spectrophotometer using cuvettes with 1 cm path length) by OD₆₀₀. Length of lag phase and

generation time were calculated for each growth curve according to the Baranyi growth model (Baranyi & Roberts, 1994), using the MicroFit program (www.ifr.ac.uk/microfit). Statistical significance of differences in averaged lag and generation times was evaluated using Student's *t* test.

Measurement of isoAsp damage. IsoAsp content of proteins from crude *E. coli* extracts was measured essentially as described previously (Visick *et al.*, 1998a). Cytosolic extracts were prepared from cells maintained for 24 h, 5 or 10 days after onset of stationary phase in LB broth adjusted to the desired pH. Cells were washed and resuspended in buffer containing 50 mM Tris/HCl (pH 8.0) and 300 mM NaCl, lysed by sonication and centrifuged at 20000 g to pellet debris. Protein concentration was estimated by the Lowry method (Lowry *et al.*, 1951), following precipitation with trichloroacetic acid. Base-labile methyl esters produced by transfer of the methyl group from *S*-adenosyl-L-[methyl-¹⁴C]methionine to isoAsp residues by purified recombinant human PCM were measured as described by Li & Clarke (1992). IsoAsp content was then calculated as picomoles of methyl groups transferred per milligram total protein in the extract.

RESULTS

PCM is not required for survival of pH stress

Maximal survival of *E. coli* in long-term stationary phase in rich growth media (e.g. LB broth) requires PCM under specific stress conditions (oxidative, osmotic or heat stress or methanol) that can affect protein conformation (Visick *et al.*, 1998a). Extremes of pH can also alter protein conformation or even denature proteins (Slonczewski & Foster, 1996); therefore, we initially investigated whether the pH of the medium might affect the long-term survival of a *pcm* deletion mutant. No difference in survival was observed between wild-type (MC1000) and *pcm* mutant (JV1068) strains, using buffered LB broth over a pH range from 5.5 to 9.0 (Fig. 2). Indeed, there was remarkably little change in the long-term survival profile over this range. Replacement of NaCl by KCl in LB broth (LBK; Blankenhorn *et al.*, 1999) did not significantly alter results at any pH (data not shown). Thus, we concluded that any influence of pH on long-term survival must be fundamentally different from that of the stresses tested previously.

Survival of methanol and oxidative stress is PCM-dependent at alkaline pH

We next examined whether the long-term survival defect of *pcm* mutants exposed to stress in rich but not in minimal medium (Visick *et al.*, 1998a) could be explained by the greater alkalinity of rich medium. The pH of long-term stationary-phase cultures in unbuffered LB broth typically increased from 7.5 on day 0 to about 8.0 by day 2 and 9.0 by day 4–5 (Fig. 3a). In the presence of 0.5% methanol, the *pcm* mutant strain JV1068 (triangles) exhibited a distinct survival defect relative to its parent, MC1000 (circles), in this medium (Fig. 3a and Visick *et al.*, 1998a). Buffering the medium to pH 7.0 eliminated the difference between the two strains (Fig. 3b); actual pH remained within 0.5 pH units of 7.0 throughout the 10 day period. In LB

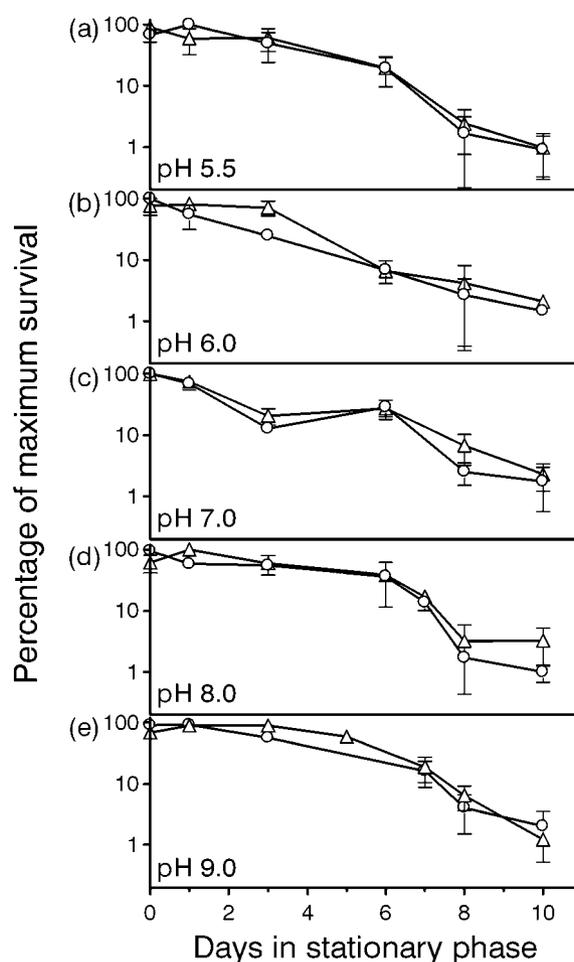


Fig. 2. Long-term survival of pH stress by *pcm* mutants. Cultures of MC1000 (wild-type, ○) and JV1068 (Δpcm , Δ) were grown in LB broth buffered to (a) pH 5.5, (b) pH 6.0, (c) pH 7.0, (d) pH 8.0, or (e) pH 9.0 for 24 h (day 0), and the number of viable cells remaining was determined at intervals for the next 10 days. Data are reported as a percentage of maximum c.f.u. ml⁻¹; the maximum ranged from 1×10^9 to 2.5×10^9 c.f.u. ml⁻¹ for all cultures, and there was no significant difference between the two strains. Means of at least three replicates are shown; error bars represent one standard deviation.

broth buffered to pH 9.0 (Fig. 3c), the *pcm* mutant's survival defect relative to MC1000 reappeared, and the behaviour of the two strains was similar to that observed for unbuffered LB broth. The actual pH of the buffered culture again remained within 0.5 units of the desired value. Similar results were obtained for LB buffered to pH 8.0, though the magnitude of the difference between the two strains was reduced (data not shown).

To determine whether this effect of pH was specific for the methanol survival phenotype, paraquat was used to expose ageing cells to continuous oxidative stress (Hassan & Fridovich, 1979) in a parallel experiment. As was the case for

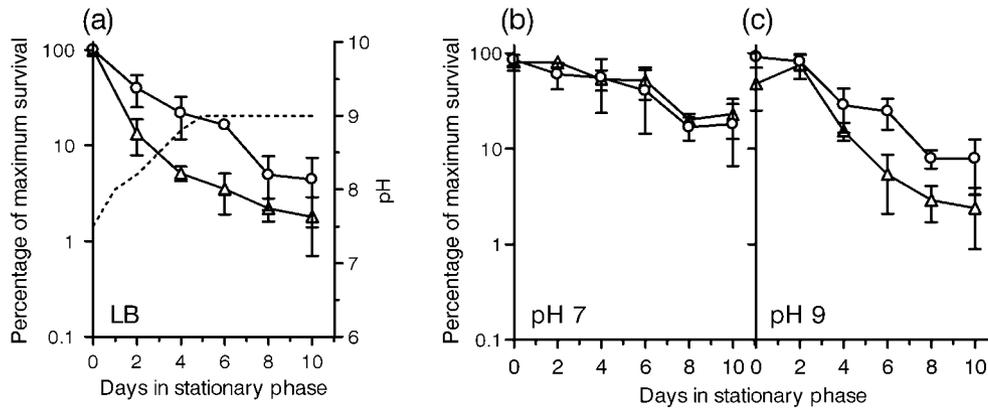


Fig. 3. Effect of pH on long-term stress survival in rich medium. MC1000 (wild-type, ○) and JV1068 (Δpcm , Δ) were grown in unbuffered LB broth (a), or in LB buffered to pH 7.0 (b) or pH 9.0 (c); after 24 h growth (day 0), methanol was added to 0.5%. The number of viable cells is reported as a percentage of maximum c.f.u. ml⁻¹; the maximum ranged from 1×10^9 to 2.5×10^9 c.f.u. ml⁻¹ for all cultures, and there was no significant difference between the two strains. Means of at least three replicates are shown; error bars represent one standard deviation. The dotted line in panel (a) shows the pH of a representative culture in unbuffered medium.

methanol, addition of paraquat resulted in reproducibly impaired survival of the *pcm* mutant strain when grown either in unbuffered LB or in LB buffered to pH 9.0, but no difference in survival was observed between the two strains in LB buffered to pH 7.0 (data not shown). The magnitude of difference between the two strains, however, was smaller in this experiment, probably because paraquat results in a precipitous decline in survival as pH rises even for wild-type *E. coli* (Visick *et al.*, 1998a), and this decline began immediately in the pH 9.0 LB, giving less time for isoAsp damage to accumulate.

While these experiments demonstrate the importance of pH, they do not establish whether the inability to detect survival phenotypes for the *pcm* mutant in minimal medium (Visick *et al.*, 1998a) can be accounted for entirely by the difference in pH. To address this question directly, we compared cells maintained in M63 medium (buffered with potassium phosphate to pH 7.0; Miller, 1972) with those maintained in M63 at pH 9.0 (adjusted by manipulating phosphate concentrations; Farrell & Finkel, 2003). The *pcm* mutant strain JV1068 survived exposure to 0.5% methanol to the same extent as MC1000 at pH 7.0 (open symbols in Fig. 4), but at pH 9, the mutant (closed triangles) exhibited a survival defect relative to its parent (closed circles) comparable to that observed in rich medium (Fig. 3c). The actual pH remained at the target level in the pH 7.0 cultures (dashed line in Fig. 4) but initially decreased somewhat in those buffered to pH 9.0 (dotted line), probably due to the effect of acidic products that resulted from growth on glucose. The appearance of differences between the strains coincided with the point at which the pH reached 8.5. Methanol appeared to have a somewhat stronger deleterious effect on both strains in this medium than in LB broth (compare Figs 3 and 4). Similar results were obtained (data

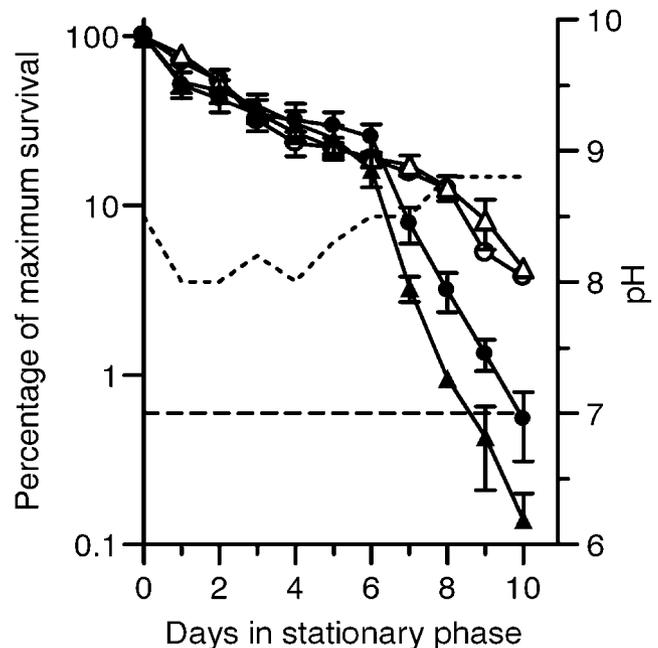


Fig. 4. Effect of pH on long-term stress survival in minimal medium. MC1000 (wild-type, ○) and JV1068 (Δpcm , Δ) were grown in M63 medium buffered to pH 7.0 (○, Δ) or pH 9.0 (●, \blacktriangle). Methanol (0.5%) was added after 24 h growth (day 0). The number of viable cells is reported as a percentage of maximum c.f.u. ml⁻¹; the maximum ranged from 1×10^9 to 2.5×10^9 c.f.u. ml⁻¹ for all cultures, and there was no significant difference between the two strains. Means of at least three replicates are shown; error bars represent one standard deviation. The dashed line shows the actual pH of a representative culture buffered to pH 7.0; the dotted line shows the actual pH of a representative culture buffered to pH 9.0.

not shown) when the strains were exposed to oxidative stress in minimal medium or when stress survival in MOPS-buffered minimal medium (pH 7.4) was compared to AMPSO-buffered minimal medium (pH 9.0).

IsoAsp accumulation is pH-dependent *in vivo*

Based on the increased rate of isoAsp formation previously reported for peptides aged at alkaline pH *in vitro* (Brennan & Clarke, 1994), we hypothesized that PCM is necessary for stress survival at alkaline pH because accelerated formation of isoAsp exacerbates the problem of unrepaired proteins in ageing cells. To determine whether damage would indeed accumulate more rapidly and/or to higher levels under alkaline conditions *in vivo*, we measured methylatable isoAsp residues in crude extracts of *E. coli* cells aged for 1, 5 or 10 days at pH 7.0, 8.0 or 9.0. As shown in Fig. 5(a), accumulation of isoAsp in MC1000 over the first 5 days at pH 8.0 was 1.6-fold that observed at pH 7.0. Ageing at pH 9.0 increased isoAsp content after 5 days by about 4.1-fold relative to pH 7.0. These rates are consistent with *in vitro* data on isoAsp formation (Brennan & Clarke, 1994) showing approximately a 3.5-fold decrease in the

half-life of Asn at pH 8.0 and 10-fold at pH 9.0 as compared to pH 7.0.

After 10 days, however, the differences were smaller: about 1.8-fold more isoAsp was detected at pH 8.0 than at pH 7.0, and about 2.3-fold more at pH 9.0 versus pH 7.0. Fig. 5 shows that while damage continued to increase in a fairly linear fashion over the 10 days at pH 7.0 or 8.0, there was a distinct plateau at pH 9.0; this observation is discussed further below. Similar results were obtained for the *pcm* mutant strain JV1068 (Fig. 5b); there was no statistically significant difference between MC1000 and JV1068 for isoAsp accumulation at any pH. This result is consistent with our earlier findings for unbuffered LB (Visick *et al.*, 1998b), and is a key component of the model for PCM function developed below.

PCM accelerates recovery from long-term stationary phase

In the course of performing the survival assays described above, we noticed that for the *pcm* mutant, the number of c.f.u. ml⁻¹ after 24 h growth at pH 9.0 (day 0) was often lower than after an additional 24 h incubation in stationary phase (day 1), particularly if the culture was started from a colony stored for a few days on a plate, rather than directly from frozen stock. In contrast, wild-type cultures (as well as any culture grown at pH 7.0 or in unbuffered broth) were almost always at their maximum c.f.u. ml⁻¹ on day 0. This phenomenon can be observed, for example, in Fig. 3(c) (compare JV1068 with MC1000 on day 0 and day 1). We therefore investigated whether PCM might be involved in recovery from long-term stationary phase, perhaps affecting either the growth rate (i.e. doubling time) of recovering cells or their lag time before exponential growth begins.

We monitored growth of fresh (grown in unbuffered LB broth overnight) and aged (maintained in LB broth for 5 days) MC1000 and JV1068 after dilution 1 : 100 into fresh LB buffered to pH 7.0 or pH 9.0. Viable counts (data not shown) were used to verify that the initial number of c.f.u. ml⁻¹ was essentially the same for both strains, whether fresh or aged. At pH 7.0, wild-type and *pcm* mutant cells displayed the same growth characteristics regardless of age (Table 1), although aged cells of both types required a slightly (~20 min) longer lag period before resuming exponential growth. Generation time was not affected by age or genotype.

At pH 9.0, however, a different picture emerged. All cultures grown at pH 9.0 showed both a longer lag phase and a longer generation time, consistent with previous reports (Stancik *et al.*, 2002). Aged cells exhibited both a longer generation time and a longer lag phase than fresh cells at pH 9.0 (Table 1). Most strikingly, under alkaline conditions, lag phase for the *pcm* mutant averaged about 90 min longer than for its repair-proficient counterpart, a statistically significant ($P < 0.03$) difference not seen at pH 7.0 or for fresh cells at pH 9.0. Furthermore, no difference was

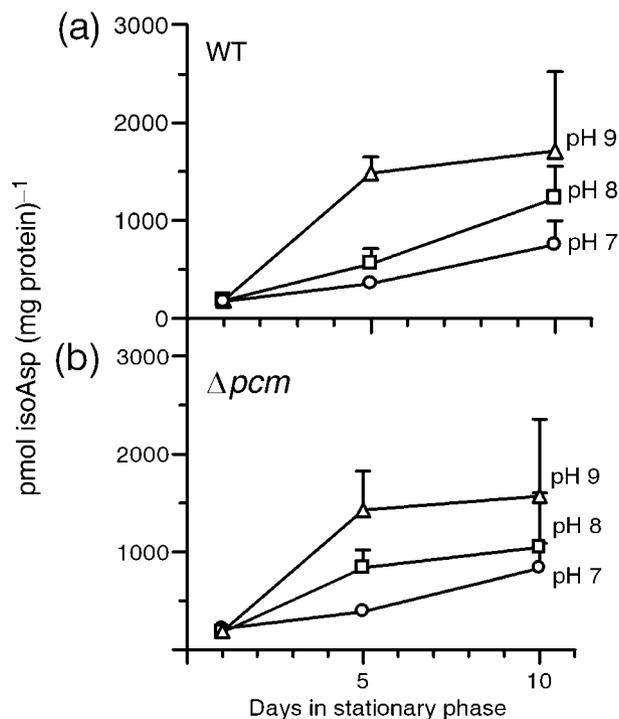


Fig. 5. Effect of pH on accumulation of isoAsp damage. MC1000 (a) or JV1068 (b) was grown overnight in LB broth buffered to pH 7.0 (○), 8.0 (□) or 9.0 (Δ) and sampled after 1, 5 or 10 additional days in stationary phase. Cells were lysed by sonication and the isoAsp content of proteins was determined as described in Methods. Means of at least three replicates are shown; error bars are shown upward only for clarity and represent one-half standard deviation.

Table 1. Recovery of wild-type and *pcm* mutant strains from stationary phase

Strain and age	Recovery at pH 7.0*		Recovery at pH 9.0	
	Lag (min)†	Generation time (min)‡	Lag (min)	Generation time (min)
MC1000 (wild-type)				
Overnight	112 ± 5	28 ± 2	301 ± 28	41 ± 5
Aged 5 days§	134 ± 8	27 ± 2	331 ± 36	65 ± 11
JV1068 (Δpcm)				
Overnight	120 ± 5	26 ± 3	294 ± 24	49 ± 4
Aged 5 days	136 ± 8	28 ± 2	419 ± 39	65 ± 10

*Cells grown in LB broth and diluted into fresh LB buffered to pH 7.0 with MOPS or to pH 9.0 with 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid (AMPSO).

†Calculated time to onset of exponential phase (Baranyi & Roberts, 1994); mean of four trials ± SD.

‡Calculated doubling time during exponential growth; mean of four trials ± SD.

§Cells grown in LB broth and maintained at 37 °C with no added nutrients for 5 days after initial overnight.

||Figures in bold type indicate a significant difference ($P < 0.03$) between MC1000 and JV1068.

observed between the wild-type and mutant strains when aged cells were incubated in unbuffered LB until they began exponential growth and then shifted to pH 9.0 (data not shown). These data suggest a role for PCM during the recovery period between long-term stationary phase and the resumption of exponential growth. PCM decreased this transition period in the absence of stress, but only for aged cells. In all cases, plate-grown inocula gave somewhat longer lag times than broth-grown cultures but displayed the same patterns, and unbuffered LB gave results indistinguishable from pH 7.0 broth (data not shown). The longer lag time does not appear to be due to genetic change, as no difference between wild-type and mutant can be observed if recovered cultures are rediluted in fresh medium at pH 9.0 (data not shown).

DISCUSSION

Role of PCM at alkaline pH

When nutrients are limited – as is very often the case for bacteria living in oligotrophic environments outside vertebrate hosts – the value of preventing or repairing damage to proteins increases, as limitations on both energy and raw materials make cells in long-term stationary phase more dependent on existing proteins than *de novo* synthesis. PCM-catalysed repair of isoAsp damage, however, appears to play a significant role in the long-term survival of *E. coli* only under certain environmental conditions: specifically, when the bacteria are exposed to stresses capable of altering protein conformation (e.g. oxidation, osmotic change, heat, methanol; Visick *et al.*, 1998a). We can now add that stress survival is PCM-dependent only at alkaline pH, a condition that bacteria are likely to encounter frequently, either in inherently alkaline environments or as a result of metabolic activity (Stancik *et al.*, 2002). This pH dependence appears to fully account for the previous observation

(Visick *et al.*, 1998a) that *pcm* mutants show survival phenotypes only in rich media.

Stress survival, including both pH stress (reviewed by Slonczewski & Foster, 1996) and protein-denaturing conditions such as heat and oxidative stress (reviewed by Storz & Zheng, 2000; Yura *et al.*, 2000), has been extensively studied in *E. coli*. Most studies, however, have focused on stress resistance either during exponential growth or shortly after the transition to stationary phase, and little is known about possible combinatorial effects of multiple stresses. Like our *pcm* phenotypes, other long-term effects, including the growth advantage in stationary phase (GASP) phenotype (Farrell & Finkel, 2003), *surA* phenotypes (Lazar *et al.*, 1998), *psp* phenotypes (Weiner & Model, 1994) and viability loss preventable by ethanol (Vulic & Kolter, 2002), also become apparent or more pronounced at alkaline pH, suggesting that pH is a major factor in the ability of ageing *E. coli* to survive stress. It is significant in this regard that even for wild-type, repair-proficient cells, the effect of oxidative stress or methanol on survival was much greater under alkaline conditions than at neutral pH (Figs 3 and 4). The mechanisms by which alkaline pH potentiates loss of viability under stress merit further study: while growing and early stationary cells effectively buffer cytoplasmic pH, it may be that internal pH is less effectively maintained during long-term stationary phase.

Our data suggest that PCM-mediated repair might be more important at high pH due to increased isoAsp damage *in vivo*: damaged proteins accumulated to higher levels sooner under alkaline conditions than at neutral pH (Fig. 5), mirroring previous *in vitro* measurement of isoAsp formation rates (Brennan & Clarke, 1994). Assuming the molecular mass of a typical protein to be 40 000 Da, the amount of methylatable isoAsp we observed at pH 9.0 would represent about one damage site per 17 individual protein

molecules after 5 days, versus one damage site per 70 molecules at pH 7.0. This suggests the potential for the reduced activity of a much larger number of cellular proteins under alkaline conditions, perhaps more rapidly surpassing some threshold of damage beyond which survival is impaired. However, isoAsp content began to plateau between 5 and 10 days of ageing at pH 9.0 (Fig. 5), at a concentration roughly the same as the maximum accumulation observed in PCM-deficient mice (Lowenson *et al.*, 2001). Perhaps most of the susceptible residues have isomerized by this point, but we cannot exclude the possibility of an as-yet-unknown additional physiological mechanism that limits the amount of isoAsp damage. Our previous finding (Visick *et al.*, 1998b) that isoAsp accumulation increases in cells deficient in both PCM and SurE – an acid phosphatase (Zhang *et al.*, 2001) of unknown substrate specificity co-transcribed with *pcm* – favours the second hypothesis.

Effect of PCM on recovery: a model for PCM function *in vivo*

Two of our results seem incongruous with the simple model in which PCM is required to repair isoAsp damage that accumulates during long-term stationary phase due to reduced metabolism and protein synthesis. First, isoAsp levels after 10 days were no higher in the *pcm* mutant than in wild-type cells (Fig. 5), consistent with previous results for unbuffered LB (Visick *et al.*, 1998b). Second, it seems surprising that PCM would play a role during recovery from stationary phase (Table 1), in the presence of plentiful nutrients. Taken together, however, these results may suggest a new model for isoAsp protein repair *in vivo*. While PCM is active in exponential and early stationary phases (Li *et al.*, 1997), its activity may be limited in long-term stationary phase, due to poor expression, instability, limiting concentrations of the methyl donor (*S*-adenosylmethionine) and/or excess of product (*S*-adenosylhomocysteine). If so, then even a repair-proficient cell would be unable to prevent accumulation of isoAsp damage during nutrient limitation, but it may be able to respond more rapidly to the return of favourable conditions when PCM is present to enable repair of existing proteins in addition to *de novo* synthesis.

This hypothesis can also account for the stress-survival phenotypes of *pcm* mutants during long-term stationary phase. It is increasingly clear that 'stationary' phase is really a dynamic state in which some cells (particularly those that have or acquire mutations increasing fitness for current culture conditions) reproduce at the expense of others (Finkel & Kolter, 1999; Vulic & Kolter, 2001). PCM may thus function primarily to repair damaged proteins in those cells that are able to divide: those that undergo a 'recovery' period even in the absence of added nutrients. Our previous finding (Visick *et al.*, 1998a) that aged *pcm* mutants fail to outcompete younger cells – the GASP phenotype (Finkel & Kolter, 1999), which requires the ability to divide during

long-term stationary phase – supports this idea. Future experiments will test this new hypothesis directly.

ACKNOWLEDGEMENTS

We wish to acknowledge the significant contributions of North Central College undergraduates Lyndsey Crampton, Madiha Salim and Megan Peiffer to this work. We are very grateful to Dr Steven Clarke and Dr Jonathan Lowenson of the Department of Chemistry and Biochemistry at UCLA for their assistance with measurement of isoaspartyl damage. This work was supported in part by a summer student fellowship (M.V.K.) from the Council on Undergraduate Research, by a student fellowship (W.M.H.) from the Richter Foundation and by funds administered by the Faculty Professional Development Committee of North Central College.

REFERENCES

- Baranyi, J. & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *Int J Food Microbiol* **23**, 277–294.
- Blankenhorn, D., Phillips, J. & Slonczewski, J. L. (1999). Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. *J Bacteriol* **181**, 2209–2216.
- Böck, A. & Sawers, G. (1996). Fermentation. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 262–282. Edited by F. C. Neidhardt & others. Washington, DC: American Society for Microbiology.
- Brennan, T. V. & Clarke, S. (1994). Deamidation and isoaspartate formation in model synthetic peptides: the effects of sequence and solution environment. In *Deamidation and Isoaspartate Formation in Peptides and Proteins*, pp. 65–90. Edited by D. W. Aswad. Ann Arbor, MI: CRC Press.
- Casadaban, M. J. & Cohen, S. N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J Mol Biol* **138**, 179–207.
- Chavous, D. A., Jackson, F. R. & O'Connor, C. M. (2001). Extension of the *Drosophila* lifespan by overexpression of a protein repair methyltransferase. *Proc Natl Acad Sci U S A* **98**, 14814–14818.
- Clarke, S., Stephenson, R. C. & Lowenson, J. D. (1992). Lability of asparagine and aspartic acid residues in proteins and peptides. In *Stability of Protein Pharmaceuticals, part A: Chemical and Physical Pathways of Protein Degradation*, pp. 1–29. Edited by T. J. Ahern & M. C. Manning. New York, NY: Plenum.
- Farrell, M. J. & Finkel, S. E. (2003). The growth advantage in stationary-phase phenotype conferred by *rpoS* mutations is dependent on the pH and nutrient environment. *J Bacteriol* **185**, 7044–7052.
- Finkel, S. E. & Kolter, R. (1999). Evolution of microbial diversity during prolonged starvation. *Proc Natl Acad Sci U S A* **96**, 4023–4027.
- Hassan, H. M. & Fridovich, I. (1979). Paraquat and *Escherichia coli*: mechanism of production of extracellular superoxide radical. *J Biol Chem* **254**, 10846–10852.
- Kagan, R. M., Niewmierzycka, A. & Clarke, S. (1997). Targeted gene disruption of the *Caenorhabditis elegans* L-isoaspartyl protein repair methyltransferase impairs survival of dauer stage nematodes. *Arch Biochem Biophys* **348**, 320–328.
- Kim, E., Lowenson, J. D., MacLaren, D. C., Clarke, S. & Young, S. G. (1997). Deficiency of a protein-repair enzyme results in the

accumulation of altered proteins, retardation of growth, and fatal seizures in mice. *Proc Natl Acad Sci U S A* **94**, 6132–6137.

Kolter, R., Siegele, D. A. & Tormo, A. (1993). The stationary phase of the bacterial life cycle. *Annu Rev Microbiol* **47**, 855–874.

Lazar, S. W., Almiron, M., Tormo, A. & Kolter, R. (1998). Role of the *Escherichia coli* SurA protein in stationary-phase survival. *J Bacteriol* **180**, 5704–5711.

Li, C. & Clarke, S. (1992). Distribution of an L-isoaspartyl protein methyltransferase in eubacteria. *J Bacteriol* **147**, 355–361.

Li, C., Wu, P. Y. & Hsieh, M. (1997). Growth-phase-dependent transcriptional regulation of the *pcm* and *surE* genes required for stationary-phase survival of *Escherichia coli*. *Microbiology* **143**, 3513–3520.

Lowenson, J. D., Kim, E., Young, S. G. & Clarke, S. (2001). Limited accumulation of damaged proteins in L-isoaspartyl (D-aspartyl) O-methyltransferase-deficient mice. *J Biol Chem* **276**, 20695–20702.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J Biol Chem* **193**, 265–275.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Mudgett, M. B. & Clarke, S. (1994). Hormonal and environmental responsiveness of a developmentally regulated protein repair L-isoaspartyl methyltransferase in wheat. *J Biol Chem* **269**, 25605–25612.

Neidhardt, F. C., Bloch, P. L. & Smith, D. F. (1974). Culture medium for enterobacteria. *J Bacteriol* **119**, 736–747.

Nyström, T. (2003). Conditional senescence in bacteria: death of the immortals. *Mol Microbiol* **48**, 17–23.

Slonczewski, J. L. & Foster, J. W. (1996). pH-regulated genes and survival at extreme pH. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 1539–1549. Edited by F. C. Neidhardt & others. Washington, DC: American Society for Microbiology.

Stancik, L. M., Stancik, D. M., Schmidt, B., Barnhart, D. M., Yoncheva, Y. N. & Slonczewski, J. L. (2002). pH-dependent

expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. *J Bacteriol* **184**, 4246–4258.

Storz, G. & Zheng, M. (2000). Oxidative stress. In *Bacterial Stress Responses*, pp. 47–60. Edited by G. Storz & R. Hengge-Aronis. Washington, DC: American Society for Microbiology.

Szymanska, G., Leszyk, J. D. & O'Connor, C. M. (1998). Carboxyl methylation of deamidated calmodulin increases its stability in *Xenopus* oocyte cytoplasm: implications for protein repair. *J Biol Chem* **273**, 28516–28523.

Visick, J. E. & Clarke, S. (1995). Repair, refold, recycle: how bacteria can deal with spontaneous and environmental damage to proteins. *Mol Microbiol* **16**, 835–845.

Visick, J. E., Cai, H. & Clarke, S. (1998a). The L-isoaspartyl protein repair methyltransferase enhances survival of aging *Escherichia coli* subjected to secondary environmental stresses. *J Bacteriol* **180**, 2623–2629.

Visick, J. E., Ichikawa, J. K. & Clarke, S. (1998b). Mutations in the *Escherichia coli surE* gene increase isoaspartyl accumulation in a strain lacking the *pcm* repair methyltransferase but suppress stress-survival phenotypes. *FEMS Microbiol Lett* **167**, 19–25.

Vulic, M. & Kolter, R. (2001). Evolutionary cheating in *Escherichia coli* stationary phase cultures. *Genetics* **158**, 519–526.

Vulic, M. & Kolter, R. (2002). Alcohol-induced delay of viability loss in stationary-phase cultures of *Escherichia coli*. *J Bacteriol* **184**, 2898–2905.

Weiner, L. & Model, P. (1994). Role of an *Escherichia coli* stress-response operon in stationary-phase survival. *Proc Natl Acad Sci U S A* **91**, 2191–2195.

Yura, T., Kanemori, M. & Morita, M. T. (2000). The heat shock response: regulation and function. In *Bacterial Stress Responses*, pp. 3–18. Edited by G. Storz & R. Hengge-Aronis. Washington, DC: American Society for Microbiology.

Zhang, R. G., Skarina, T., Katz, J. E. & 8 other authors (2001). Structure of *Thermotoga maritima* stationary phase survival protein SurE: a novel acid phosphatase. *Structure* **9**, 1095–1106.