

Mutations in the *Escherichia coli surE* gene increase isoaspartyl accumulation in a strain lacking the *pcm* repair methyltransferase but suppress stress-survival phenotypes

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Abstract

The *Escherichia coli surE* gene is co-transcribed with *pcm*, encoding the L-isoaspartyl protein repair methyltransferase, and is highly conserved among both the Eubacteria and the Archaea; however, no biochemical function has yet been identified for this gene. Isoaspartyl accumulation during stationary phase was much higher in a *pcm surE* double mutant than in either single mutant, suggesting that the two genes may represent two parallel pathways by which *E. coli* can respond to protein damage. A null mutation in *surE* also suppressed stress-survival defects previously observed in a *pcm* mutant strain, providing further evidence for an interaction between the two gene products. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Protein repair; Isoaspartate; Protein methyltransferase; Stationary phase; Stress; Survival

1. Introduction

The formation of isoaspartyl residues from aspartate and asparagine amino acids is a common form of spontaneous damage to proteins [1], affecting both conformation and function [2,3]. Isoaspartyl residues are recognized and methylated by L-isoaspartyl protein carboxyl methyltransferase (EC 2.1.1.77), resulting in their net repair to normal aspartyl residues in vitro [4]. In the absence of this repair enzyme, detrimental effects have been observed in vivo in *Escherichia coli* [5], nematodes [6], and mice [7].

The gene encoding the *E. coli* isoaspartyl methyltransferase, *pcm*, is located at 61.5 minutes on the chromosome, in a cluster of nine closely spaced genes with the same orientation. Its transcriptional unit also includes at least *surE*, which is located immediately upstream and overlaps the *pcm* start codon [8,9]. Although we found that *surE* is well-conserved among prokaryotes, nothing is known about its biochemical function or physiological role.

In this paper, we present both genetic and biochemical evidence for a functional relationship between SurE and Pcm based on analysis of mutant strains and accumulation of isoaspartyl damage in *E. coli* proteins during ‘aging’ in stationary phase. We also clarify previous results [9,10] regarding

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surE mutant phenotypes and report that *surE* null mutants have no observable survival defects under the conditions that we have tested thus far.

2. Materials and methods

2.1. Bacterial strains

Strain MC1000 (λ^- e14⁻ *araD139* Δ (*araA-leu*)7697 *galE15 galK16* Δ (*codB-lac*)3 *rpsL150 mcrB1 relA1 spoT1*) was used as the parent strain for these experiments. The construction of strains JV1068 (MC1000 Δ *pcm*::Cm^R [5]) and JKI2010 (MC1000 *surE*::Km^R *rpoS396* [10,11]) has been described. A *surE*::Cm^R derivative, JV1066, was constructed by inserting a chloramphenicol resistance (Cm^R) marker into the *BstEII* site near the midpoint of *surE*, with the marker gene oriented in the same direction as *surE* and *pcm*. Chromosomal gene replacement was then carried out using pKAS46 as described previously [5,12]. Strain JV1070, a *surE*::Km^R Δ *pcm*::Cm^R double mutant, was made by P1 transduction of *surE*::Km^R from JKI2010 into JV1068. A Δ (*surE-pcm*)::Km^R double-deletion construct (deleted from a *PvuII* site upstream of *surE* to a *PvuII* site near the 3' end of *pcm*) was PCR-amplified from the chromosome of strain JKI1200, ligated into pKAS32 [12] and recombined into the MC1000 chromosome as above to yield strain JV1061. Constructions were confirmed by transduction and linkage analysis, PCR amplification, sequencing of *pcm*, *surE* and *rpoS* and measurement of Pcm activity.

2.2. Oligonucleotides, PCR and sequencing

An Oligoassembler apparatus (Pharmacia) was used to synthesize oligonucleotide primers for PCR and sequencing. Taq DNA polymerase (Promega) and an MJR thermocycler (MJ Research) were used for PCR amplifications, and Taq Extender (Stratagene) was added when longer PCR products were required. Chromosomal DNA was PCR-amplified directly from colonies or overnight cultures [13] for sequencing using an Applied Biosystems 373A or 377 automated sequencing apparatus.

2.3. Biochemical assays

Activity of the L-isoaspartyl methyltransferase in extracts of stationary-phase *E. coli* prepared by gentle sonication was determined as described [5] by quantitation of base-labile ¹⁴C-methyl esters transferred to an isoaspartyl-containing peptide substrate. Activity of HPII catalase, used as a reporter for RpoS, was measured spectrophotometrically in extracts in which HPI catalase had been heat-inactivated [11].

Isoaspartyl accumulation was measured in cultures grown to stationary phase in LB broth and aged for 10 days with aeration. All subsequent steps in the preparation of the extracts were performed at 4°C. Cells were harvested by centrifugation at 5000 × g; pellets were washed twice, resuspended in a solution containing 50 mM Tris-HCl (pH 8.0) and 300 mM NaCl at a ratio of 2 ml per gram (wet weight) of cells, and disrupted by two passages through a French pressure cell (American Instrument Co.) at 15000 psi. Unbroken cells and debris were removed from the lysates by centrifugation at 27000 × g for 30 min, and membranes were removed by further centrifugation at 100000 × g for 1 h. Isoaspartyl substrates which could be methylated using purified recombinant L-isoaspartyl methyltransferase from *Thermotoga maritima* were then quantitated. A typical assay consisted of enzyme (23 pmol min⁻¹), 50 mM sodium citrate (pH 6.0), *E. coli* cytosol (approximately 20 µg total protein) and 25 µM S-adenosyl-L-[methyl-¹⁴C]methionine (Amersham) in a final volume of 40 µl. After incubation for 2 h at 37°C, the tube was spun briefly, frozen in dry ice to stop the reaction, rapidly thawed and mixed with 40 µl of 0.2 M NaOH and 1% SDS. A 60-µl aliquot was then spotted onto a piece of filter paper, and volatile methyl esters were quantitated as for the methyltransferase assay [5].

2.4. Survival assays

Resistance to lethal (55°C) heat shock and oxidative and osmotic stresses, as well as long-term stationary-phase survival in the presence or absence of methanol, paraquat or salt were determined for *E. coli* cultures grown in LB broth with aeration as described previously [5].

2.5. Sequence analysis

Sequences similar to *pcm* and *surE* were identified using the gapped Basic Local Alignment Tool (BLAST [14]) to search databases at the National Center for Biotechnology Information, the Institute for Genome Research (TIGR), and the University of Oklahoma's Advanced Center for Genome Technology. In the case of incompletely sequenced genomes (TIGR databases), sequence sections were downloaded and assembled to obtain full-length genes. All nucleotide sequences were translated into amino acid sequences for alignment.

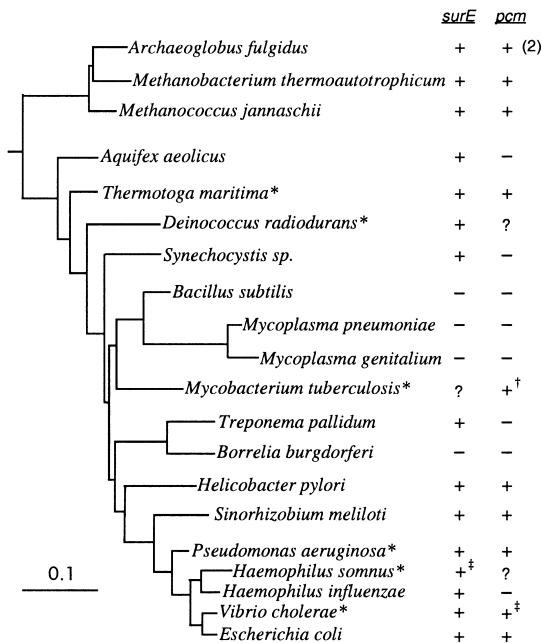


Fig. 1. Comparison of prokaryotic phylogeny with occurrence of *surE* and *pcm*. Phylogenetic tree is adapted from 5S rRNA tree [16], showing only relevant taxa; bar length indicates expected substitutions per site. Sequences similar to *surE* and *pcm* were identified in databases of sequenced genes as described in Section 2; a plus indicates the presence of a sequence with significant amino acid identity, while a minus indicates that no significantly similar sequence was found. Genomes not yet completely sequenced are indicated by asterisks, and question marks in the rows for these organisms indicate that no similar sequence was found but that one might be found in the regions yet to be sequenced. Partial sequences with questionable similarity to *pcm* or *surE* are indicated by a dagger and partial sequences with good identity in the sequenced portion by a double dagger. *A. fulgidus* has two distinct sequences with good identity to *pcm*.

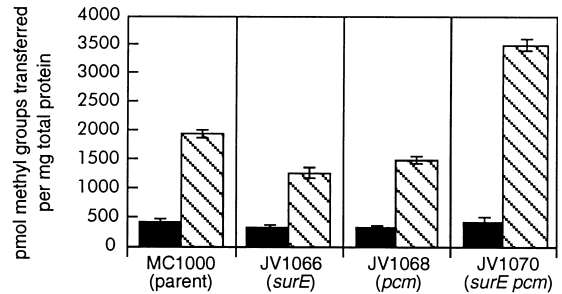


Fig. 2. Accumulation of isoaspartyl damage in stationary phase. The indicated strains were grown in LB broth for 24 h, and the level of isoaspartyl residues was measured as described in the text (solid bars). The cultures were then maintained for 10 additional days, and isoaspartyl damage was again measured (striped bars). Values are given in pmol of ^{14}C -methyl groups transferred to proteins in the cell extract (see Section 2) per mg of total protein and are averages of at least three trials; error bars represent one standard deviation.

3. Results and discussion

As shown in Fig. 1, *surE*, like *pcm*, is both an ancient gene and a well-conserved one. Similarity searches of sequenced and partially sequenced bacterial genomes identified matching sequences among both the Eubacteria and the Archaea, with the notable exception of the Gram-positive bacteria and the mycoplasmas. In addition, the relative positions of *surE*, *pcm* and also *rpoS* (encoding σ^S , an RNA polymerase sigma factor important for stationary phase and stress survival) were frequently, though not universally, conserved (data not shown). Despite this broad prokaryotic distribution, only one eukaryotic sequence with significant similarity to *surE* has been identified, an uncharacterized open reading frame recently sequenced from *Arabidopsis thaliana* (GenBank accession number Z97337 [15]).

None of these putative *surE* homologues has a known or hypothesized biochemical function. We have speculated previously [1,8], however, that the operon organization of *surE* and *pcm* and their proximity to *rpoS* suggest that SurE might participate in some repair or stress-response pathway. Measurement of the accumulation of isoaspartyl residues in 'aged' proteins from *E. coli* cultures subjected to long-term nutrient limitation provided evidence to support this idea. As described in Section 2, extracts were prepared from cultures which had been maintained in LB broth for 24 h or 10 days, and the

ability of purified L-isoaspartyl methyltransferase to methylate proteins in the extracts was used as a measure of the level of isoaspartyl damage present (Fig. 2).

The amount of isoaspartyl damage would be expected to increase over time as these cultures age, particularly in the case of the repair-deficient *pcm* mutant. Isoaspartyl residues did, indeed, accumulate in all strains over the 10-day period (compare solid bars for 24-h cultures to hatched bars for 10-day cultures in Fig. 2). Surprisingly, however, the level of isoaspartyl accumulation for the *pcm* mutant, JV1068, was no greater after 10 days than for the parent strain (compare hatched bars for JV1068 to hatched bars for MC1000 in Fig. 2). Similarly, isoaspartyl residues in the *surE* mutant extracts (JV1066) reached a final level much like that seen for MC1000.

A different result was seen when the *surE pcm* double-mutant strain (JV1070) was used. In extracts from this strain, methylatable isoaspartyl residues accumulated to about twice the level observed for the single mutants. The level of isoaspartyl damage observed for this strain was very similar to that seen for an *rpoS* mutant (JV1012), even though nearly 99% of the σ^S -deficient cells were inviable after 10 days and thus lacked any means of repairing or replacing damaged proteins (data not shown). This result suggests that a functional copy of either *pcm* or *surE* is sufficient to keep isoaspartyl damage down to wild-type levels and that Pcm and SurE may participate in parallel pathways to reduce protein damage in *E. coli*.

A genetic approach was employed to further examine the possible in vivo roles of SurE. Previously [9], we reported that mutations in *surE* resulted in decreased viability in stationary phase (strain

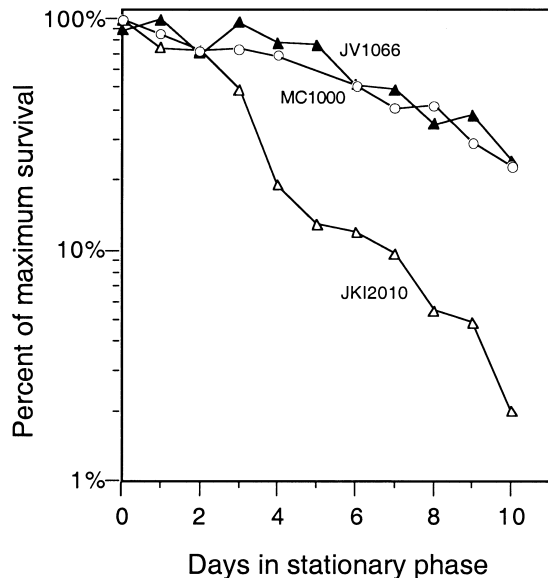


Fig. 3. Survival of *surE* mutants in stationary phase. The parent strain (MC1000, ○), original *surE* mutant (JKI2010, △) and newly constructed *surE* mutant (JV1066, ▲) were grown in M9 minimal medium and maintained in the same medium for 10 days. Survival is shown as the percentage of cells remaining viable and is the average of at least four trials.

JKI2010 in Fig. 3) and when stationary-phase cells were exposed to heat (55°C), osmotic stress (2.5 M NaCl) or peroxide (15 or 30 mM) for short periods (strain JKI2010 in Table 1). However, we recently determined that a nonsense mutation in *rpoS* had been inadvertently introduced into the *surE* mutant strains [11]. In order to distinguish between the effects of the *surE* mutation and the secondary *rpoS* mutation, we constructed a new *surE* null mutant strain, JV1066, and verified that *rpoS* was unmutated in this strain (see Section 2).

As shown in Fig. 3, this newly constructed strain

Table 1

Enzyme activities and stress-survival phenotypes for original and newly constructed *surE* mutant strains

Strain	Genotype	Enzyme activity ^a		Percent survival ^a			
		Pcm ^b	HPH ^c	55°C, 10 min	2.5 M NaCl, 4 h	15 mM H ₂ O ₂ , 1 h	30 mM H ₂ O ₂ , 1 h
MC1000	parent	3.3 ± 0.61	29.0 ± 1.5	4.4 ± 3.7	40.4 ± 12.2	75.0 ± 6.8	52.1 ± 10.1
JKI2010	<i>surE</i> ::Km ^R <i>rpoS396</i>	5.6 ± 0.91	3.0 ± 0.42	0.011 ± 0.1	13.8 ± 4.3	0.6 ± 0.07	0.02 ± 0.01
JV1066	<i>surE</i> ::Cm ^R	0.59 ± 0.05	28.8 ± 2.3	4.4 ± 3.2	36.7 ± 14.2	73.8 ± 10.4	56.3 ± 11.6

^aValues are averages of at least two trials in duplicate; error range represents one standard deviation.

^bpmol methyl groups transferred to an isoaspartyl-containing peptide substrate per min per mg total protein.

^cμmol of hydrogen peroxide decomposed per min per mg total protein.

survived long-term maintenance in stationary phase as well as the parent strain, MC1000. Furthermore, although L-isoaspartyl methyltransferase activity was somewhat reduced in this mutant (presumably due to transcriptional termination after the inserted chloramphenicol-resistance gene), its HPII catalase activity (transcribed under the control of σ^S) and its ability to survive heat, osmotic and oxidative stresses were virtually identical to the parent strain (Table 1). Since the presence of the *rpoS* nonsense mutation in JKI2010 is the only difference in genotype between these strains, it can be concluded that the phenotypes originally attributed to *surE* were in fact caused by the greatly reduced RpoS activity in JKI2010.

Previously, we reported that similar phenotypes previously ascribed to mutations in *pcm* were also due to the lack of RpoS; however, we identified new, specific phenotypes when a *pcm* mutant was exposed to denaturing stresses during long-term maintenance in stationary phase [5]. We tested the newly constructed *surE* mutant to determine whether the absence of SurE might have any of the same effects. Long-term survival in stationary phase was measured for MC1000 (open circles) and JV1066 (closed triangles) in the presence of 0.5% methanol (Fig. 4A); 0.1 mg ml⁻¹ paraquat, a recyclable generator of oxygen radicals (Fig. 4B); or 0.5 M NaCl (Fig. 4C). However, no loss of viability was observed for the *surE* mutant under any of these conditions; JV1066 survived essentially as well as MC1000 in each case. In addition, exponential growth was normal for the *surE* mutant strain either in M9 minimal medium or in LB broth (data not shown). We have not as yet been able to detect any significant differences between this *surE* mutant and its parent strain under any conditions tested.

A *surE pcm* double mutant, JV1070, was also constructed and tested. In view of the apparent physiological well-being of the *surE* mutant, it is surprising that this double mutant (closed diamonds in Fig. 4) did not show the same phenotypes as a *pcm* mutant. Instead, the *surE* mutation appeared to suppress the *pcm* defect, restoring the survival of long-term stationary-phase cultures challenged with methanol (Fig. 4A), paraquat (Fig. 4B) or osmotic stress (Fig. 4C) to a level not significantly different from the parental strain. Survival was significantly better

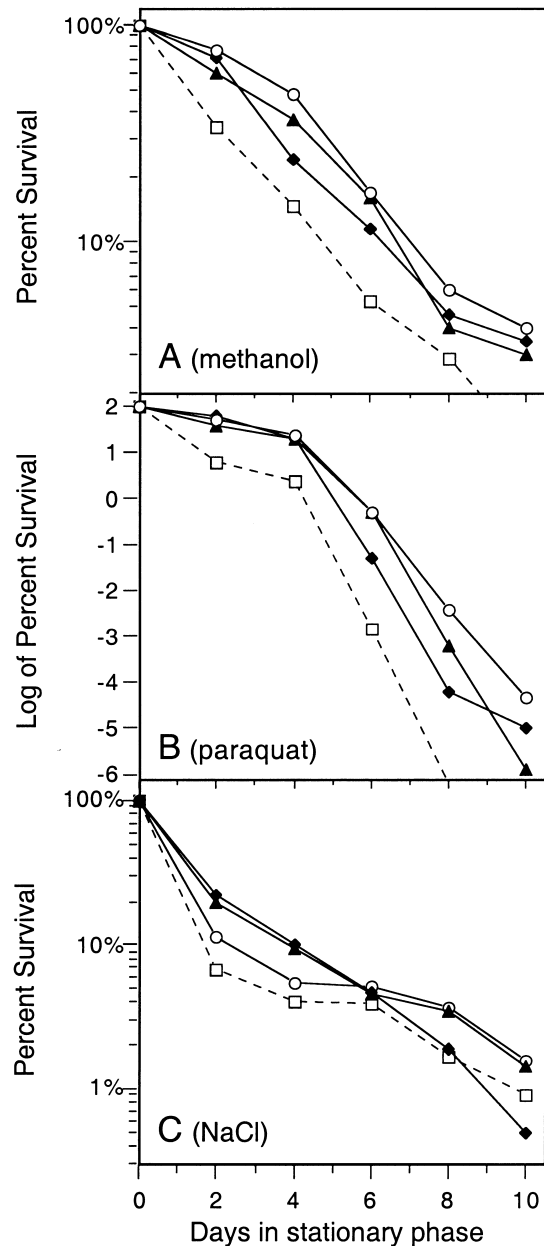


Fig. 4. Survival of stress by *surE* mutants and *surE pcm* double mutants in stationary phase. The parent strain (MC1000, ○), *surE* mutant (JV1066, ▲) and *surE pcm* double mutant (JV1070, ◆) were grown to stationary phase in LB broth, then (A) 0.5% methanol, (B) 0.1 mg ml⁻¹ paraquat, or (C) 0.5 M NaCl was added to the cultures and the cells were maintained an additional 10 days. Values shown are averages of at least two trials, and the survival of a *pcm* mutant under the same conditions [5] is shown by the dashed lines for comparison.

than the *pcm* mutant, JV1068 (dotted line), in each case. These results were confirmed with a strain in which both *surE* and *pcm* were deleted and replaced by a single antibiotic-resistance marker (data not shown).

The *surE* mutation thus appears to be acting as a specific suppressor of the *pcm* mutant phenotypes. Genetic suppression of a mutant phenotype by a mutation at a second site is generally interpreted as evidence that the products of the two genes interact or that both participate in the same cellular process. This would appear to support our hypothesis that both SurE and Pcm might be involved in responding to protein damage.

If there is an interaction between SurE and Pcm, it does not seem to be an entirely straightforward relationship. Given that all the stresses which so far have been shown to reduce viability of *pcm* mutants in stationary phase are capable of denaturing proteins (methanol, paraquat, salt, heat), we hypothesized that the formation of isoaspartyl residues is most detrimental under denaturing conditions, when, for example, the isoaspartyl damage could facilitate unfolding or hinder re-folding [5]. Suppression of these phenotypes by *surE* null mutations, however, would imply that the negative effects of isoaspartyl formation require not only denaturing stresses but also the presence of SurE.

What biochemical function for SurE could account for its apparent ability to exacerbate the effect of isoaspartyl formation and stress during stationary phase? One hypothesis consistent with the results presented here would be a role in the control of protein damage by proteolysis; this would explain the observation that either Pcm or SurE alone is sufficient to limit isoaspartyl accumulation, while damage reaches a higher level in a double mutant. This idea could also account for the apparent detrimental effect of SurE in the absence of Pcm during stationary phase. In the absence of the repair enzyme, attack on damaged proteins by SurE (or a SurE-dependent protease) might eventually become so widespread as to deplete essential proteins, affecting survival. Under normal conditions, repair of damaged proteins by Pcm would limit the activity of SurE. Another possible explanation is that the products of SurE activity are toxic to the cell in the absence of Pcm. Although preliminary results

suggest no observable reduction in bulk degradation in the *surE* mutant (data not shown), further study will be required to determine whether SurE might possess or affect protease or peptidase activity. The details of the relationship between these two proteins and the effects of isoaspartyl accumulation on cell physiology will also be topics for future analysis.

Acknowledgments

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References

- [1] Visick, J.E. and Clarke, S. (1995) Repair, refold, recycle: how bacteria can deal with spontaneous and environmental damage to proteins. *Mol. Microbiol.* 16, 835–845.
- [2] Capasso, S., DiDonato, A., Esposito, L., Sica, F., Sorrentino, G., Vitagliano, L., Zagari, A. and Mazzarella, L. (1996) Deamidation in proteins: the crystal structure of bovine pancreatic ribonuclease with an isoaspartyl residue at position 67. *J. Mol. Biol.* 257, 492–496.
- [3] Sharma, S., Hammen, P.K., Anderson, J.W., Leung, A., Georges, F., Hengstenberg, W., Klevit, R.E. and Waygood, E.B. (1993) Deamidation of HPr, a phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system, involves asparagine 38 (HPr-1) and asparagine 12 (HPr-2) in isoaspartyl acid formation. *J. Biol. Chem.* 268, 17695–17704.
- [4] McFadden, P.N. and Clarke, S. (1987) Conversion of isoaspartyl peptides to normal peptides by coupled enzymatic/non-enzymatic reactions: implications for the cellular repair of damaged proteins. *Proc. Natl. Acad. Sci. USA* 84, 2595–2599.
- [5] Visick, J., Cai, H. and Clarke, S. (1998) The L-isoaspartyl protein repair methyltransferase enhances survival of aging *Escherichia coli* subjected to secondary environmental stresses. *J. Bacteriol.* 180, 2623–2629.
- [6] Kagan, R.M., Niewmierzycka, A. and 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.
- [7] Kim, E., Lowenson, J.D., MacLaren, D.C., Clarke, S. and 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. USA* 94, 6132–6137.
- [8] Fu, J.C., Ding, L. and Clarke, S. (1991) Purification, gene cloning, and sequence analysis of an L-isoaspartyl protein carboxyl methyltransferase from *Escherichia coli*. *J. Biol. Chem.* 266, 14562–14572.
- [9] Li, C., Ichikawa, J.K., Ravetto, J.J., Kuo, H.-C., Fu, J.C. and

- Clarke, S. (1994) A new gene involved in stationary-phase survival located at 59 minutes on the *Escherichia coli* chromosome. *J. Bacteriol.* 176, 6015–6022.
- [10] Li, C. and Clarke, S. (1992) A protein methyltransferase specific for altered aspartyl residues is important in *Escherichia coli* stationary-phase survival and heat-shock resistance. *Proc. Natl. Acad. Sci. USA* 89, 9885–9889.
- [11] Visick, J.E. and Clarke, S. (1997) RpoS- and OxyR-independent induction of HPI catalase at stationary phase in *Escherichia coli* and identification of *rpoS* mutations in common laboratory strains. *J. Bacteriol.* 179, 4158–4163.
- [12] Skorupski, K. and Taylor, R.K. (1996) Positive selection vectors for allelic exchange. *Gene* 169, 47–52.
- [13] Gussow, D. and Clackson, T. (1989) Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucleic Acids Res.* 17, 4000.
- [14] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- [15] Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R., Dirkse, W., Van Staveren, M., Stiekema, W., Drost, L., Ridley, P., Hudson, S.A., Patel, K., Murphy, G., Piffanelli, P., Wedler, H., Wedler, E., Wambutt, R., Weitzenegger, T., Pohl, T.M., Terryn, N., Gielen, J., Villarroel, R., De Clerck, R., Van Montagu, M., Lecharny, A., Auborg, S., Gy, I., Kreis, M., Lao, N., Kavanagh, T., Hempel, S., Kotter, P., Entian, K.D., Rieger, M., Schaeffer, M., Funk, B., Mueller-Auer, S., Silvey, M., James, R., Montfort, A., Pons, A., Puigdomenech, P., Douka, A., Voukelatou, E., Milioni, D., Hatzopoulos, P., Piravandi, E., Obermaier, B., Hilbert, H., Duesterhoft, A., Moores, T., Jones, J.D.G., Eneva, T., Palme, K., Benes, V., Rechman, S., Ansoerge, W., Cooke, R., Berger, C., Delseny, M., Voet, M., Volckaert, G., Mewes, H.W., Klosterman, S., Schueller, C. and Chalwatzis, N. (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature* 391, 485–488.
- [16] Olsen, G.J. and Woese, C.R. (1993) Ribosomal RNA: a key to phylogeny. *FASEB J.* 7, 113–123.