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***E. coli* hypoxia-inducible factor ArcA mediates lifespan extension in a lipoic acid synthase mutant by suppressing acetyl-CoA synthetase**

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Abstract

We have previously shown that both the hypoxia-inducible transcription factor ArcA and the PoxB/Acs bypass of the pyruvate dehydrogenase complex contribute to extended lifespan in *Escherichia coli*. In agreement with studies in higher eukaryotes, we also demonstrated that long-lived *E. coli* mutants, including LipA-deficient cells, are stress resistant. Here, we show that ArcA contributes to the enhanced lifespan and heat shock resistance of the *lipA* mutant by suppressing expression of the acetyl-CoA synthetase (*acs*) gene. The deletion of *acs* reversed the reduced lifespan of the *lipA arcA* mutant and promoted the accumulation of extracellular acetate, indicating that inhibition of carbon source uptake contributes to survival extension. However, Acs also sensitized cells lacking ArcA to heat shock, in the absence of extracellular acetate. These results provide evidence for the role of Acs in regulating lifespan and/or stress resistance by both carbon source uptake-dependent and -independent mechanisms.

Keywords

acetate; ATP; heat shock resistance; oxygen consumption; protein carbonylation

Introduction

When inoculated in the amino acid- and peptide-based LB medium, *Escherichia coli* goes through several phases that are reflected by changes in the number of colony-forming units (CFUs) of the culture over time (Finkel, 2006). The maximum cell density reached by the end of log phase is maintained for a period of 12–36 h depending on the strain and incubation conditions; this period is defined as the stationary phase. The decline in the

number of CFUs marks the onset of the death phase, by the end of which 90–99% of the initial population has lost the ability to proliferate when transferred to fresh nutrient plates. The cell population that survives death phase maintains a fairly constant number of CFUs for several months as a result of successive waves of regrowth of mutants, a phenomenon which is known as the growth advantage in stationary phase (Zambrano et al., 1993). The stationary and death phases of the life cycle of *E. coli* maintained in batch culture are reminiscent of the time-dependent loss of function and death that defines aging in higher organisms.

The accumulation of oxidative damage in the form of protein carbonyls is a common feature of aging both in *E. coli* and in eukaryotic organisms (Nystrom, 2003). Using *in situ* detection of protein oxidation in single *E. coli* cells and a density-gradient centrifugation technique to separate culturable from non-culturable cells, Desnues et al. (2003) showed that proteins in non-culturable cells show increased and irreversible oxidative damage in the form of protein carbonyls. Furthermore, incubation of a stationary phase *E. coli* population in the absence of oxygen causes a lifespan extension compared to incubation in normoxic conditions (Dukan and Nystrom, 1999). Such observations gave rise to the hypothesis that unchecked oxidative damage in the form of protein carbonylation is the proximal cause of the stochastic deterioration of a stationary phase *E. coli* population (Desnues et al., 2003).

There have been two reports of mutant strains surviving longer than wild type (wt) and two environmental interventions that were also shown to lengthen the stationary phase survival of a wt population. Loss of the toxin/antitoxin pair encoded by *hipBA* results in more viable cells during stationary phase quantified using the fluorescent dye SYTO 9 and also in enhanced resistance to a stationary phase treatment with hydrogen peroxide (Kawano et al., 2009); based on their data, the authors concluded that HipBA causes stationary phase cell death through oxidative stress. The other genetic report was based on a microscopic screen of a transposon-mutagenized population for mutants with a reduced amount of cells stained with propidium iodide (which have damaged membranes and are therefore considered dead). Using this approach, the authors found that loss of either FliA, which regulates the transcription of flagellar operons, or RssB, which regulates the stability of the sigma factor RpoS, leads to a reduced ratio of dead stationary phase cells (Fontaine et al., 2008). Both incubation under anaerobic conditions (Dukan and Nystrom, 1999) and stationary phase addition of ethanol, 1-propanol or 1-butanol (Vulic and Kolter, 2002) have been shown to delay the viability loss of a stationary phase population. The latter effect was also attributed to a modulation of the physiological response elicited by RpoS. None of these four reports tested the role of protein carbonylation in the observed lifespan extension.

In a previous study, we used the KEIO collection, which contains single gene knockout strains of all protein-coding genes in *E. coli* (Baba et al., 2006) to perform a genome-wide screen for extended stationary phase survival in this organism. Among the recovered long-lived strains, the one lacking LipA was the longest-lived, its fully extended lifespan being dependent on the hypoxia-inducible transcription factor ArcA (Gonidakis et al., 2010). LipA catalyzes the formation of the carbon-sulfur bonds present in the cofactor lipoic acid (Miller et al., 2000). This cofactor is required for the redox reactions catalyzed by the pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase complexes and also for the function of

the glycine cleavage system (Cronan et al., 2005). Here, we investigate the mechanisms by which ArcA contributes to both the extended lifespan and heat shock resistance of the *lipA* mutant.

Results

Excretion of ammonia resulting from the breakdown of amino acids present in LB (Pruss et al., 1994) results in an extracellular stationary phase pH of 8.5–9 for wt. By contrast, the *lipA* mutant reaches an extracellular stationary phase pH of approximately 7 (Gonidakis et al., 2010). We chose to perform all experiments at an external pH of 7.5 (adjusted by addition of the biological buffer HEPES after entry to stationary phase), to remove the alkaline pH stress generated by incubation of wt in LB medium. The value 7.5 was chosen, because the intracellular pH of *E. coli* is maintained at pH 7.4 over a wide range of extracellular pH values (Slonczewski et al., 1981). As revealed by our genome-wide screen for extended stationary phase survival, the *lipA* mutant survives longer than wt. In alkaline conditions, the hypoxia-inducible transcription factor ArcA contributes to the longevity of the *lipA* strain independently of acetate metabolism (Gonidakis et al., 2010). The finding that lack of ArcA shortens the lifespan of the *lipA* mutant but does not affect the survival of wt is also observed when cultures are maintained at an extracellular pH of 7.5 (Figure 1A).

The transcriptional changes induced by ArcA contribute to the physiological state that allows this organism to survive and proliferate under oxygen shortage (Luchi and Lin, 1991). Four different metabolic byproducts are produced as part of this physiological state, namely acetate, lactate, ethanol and succinate (Clark, 1989). ArcA has been shown to contribute to acetate production (Vemuri et al., 2006). We therefore examined the effect of *arcA* deletion on the extracellular acetate concentration of the *lipA* mutant. As shown in Figure 1B, the *lipA* mutant reaches a maximum extracellular acetate concentration of 6 mM which declines during the course of a survival experiment; lack of ArcA expedites the loss of acetate from the extracellular environment of the *lipA* strain. Addition of 2.5 mM acetate to a *lipA arcA* culture on day 3 does not reverse the shortened lifespan of this mutant (Figure 1C). Hence, the availability of extracellular acetate is not limiting for the survival of the *lipA arcA* strain. Note that no acetate can be detected in the cultures of wt and the *arcA* mutant by day 1 (data not shown).

The reduced extracellular acetate concentration of the *lipA* mutant in the absence of ArcA is consistent with the established role of the transcription factor in the production of acetate. However, we sought to dissect any contribution of acetate uptake in the observed effect of ArcA on the extracellular acetate concentration of the *lipA* mutant. Towards this goal, we tested the effect of lack of Acs, the protein responsible for scavenging extracellular acetate at concentrations lower than 10 mM (Kumari et al., 1995) on the extracellular acetate concentration of the *lipA* and *lipA arcA* mutants. We found that in both mutants deletion of *acs* results in the sustained presence of acetate in the extracellular environment throughout the experiment (Figure 1B). Hence, Acs is responsible for acetate uptake in the *lipA* and *lipA arcA* mutants. We then measured the level of transcription of *acs* by RT-PCR and found that lack of ArcA results in elevated expression of the gene in both the *lipA* and wt strains (Figure 1D). Consequently, contrary to its established role of directly activating acetate

production, ArcA suppresses acetate uptake through the expression of *acs* in the long-lived *lipA* mutant.

Next, we measured the lifespan of the *lipA arcA acs* mutant and found it to be closer to the length of the *lipA* than the *lipA arcA* strain (Figure 1A). In other words, deletion of *acs* from the *lipA arcA* mutant restores the extended lifespan observed in the *lipA* strain. Resistance to heat stress is commonly observed in long-lived organisms of several model systems (Longo and Finch, 2003). We tested the resistance of the *lipA* strain to a 15-min incubation at 54°C and found that the long-lived mutant withstands this treatment better than wt (Figure 2). Similar to stationary phase survival, lack of ArcA causes a partial loss of the heat shock resistance phenotype; this loss is partially reversed by the deletion of *Acs*. Whereas ArcA is not required for the stationary phase survival of wt, we found that it contributes to the heat shock resistance of a stationary phase wt population, because the survival of the *arcA* mutant after heat shock is diminished compared to that of wt. Interestingly, the heat shock survival of the *arcA acs* strain is intermediate between that of wt and the *arcA* strain. These results indicate that ArcA extends the lifespan and heat shock resistance of the *lipA* strain by, among others, suppressing the expression of *acs*.

We then sought to identify the molecular mechanism linking elevated expression of *acs* to reduced lifespan and heat shock resistance in the *lipA arcA* mutant. Towards this purpose, we characterized the effect of ArcA and *Acs* on another aspect of metabolic physiology, namely oxygen consumption. We chose this assay because of the established role of ArcA in the adaptation of *E. coli* to oxygen shortage (Iuchi and Lin, 1991). We found that ArcA suppresses the rate of oxygen consumption both in wt and the *lipA* mutant. Nystrom et al. (1996) have observed a similar effect under glucose starvation conditions. Whereas *Acs* has no effect on the rate of oxygen consumption of the *lipA* strain, its upregulation in the absence of ArcA appears to be largely responsible for the increased oxygen consumption observed in the *lipA arcA* strain, because the *lipA arcA acs* mutant consumed oxygen at a rate only slightly higher than that of the *lipA* strain (Figure 3A).

This observation led to the hypothesis that unregulated conversion of extracellular acetate to acetyl-CoA by *Acs* and the subsequent consumption of acetyl-CoA by the TCA cycle followed by enhanced oxygen consumption by the electron transport chain causes the observed reduction of lifespan in the *lipA arcA* mutant. To test this hypothesis, we genetically blocked consumption of acetyl-CoA by the TCA cycle, by inactivating either citrate synthase (*GltA*) that catalyzes the condensation of acetyl-CoA and oxaloacetate to form citrate or malate synthase (*AceB*), which converts acetyl-CoA to malate as part of the glyoxylate cycle (Cronan and Laporte, 1996). Although both the *lipA arcA gltA* and *lipA arcA aceB* mutants display reduced oxygen consumption compared to the *lipA arcA* mutant, none of them demonstrated a reversion of the shortened lifespan of the latter (Figure 3A,B). We also tested the interaction between rate of oxygen consumption and lifespan by measuring the survival of the *lipA arcA* mutant in the presence of the respiratory inhibitor cyanide (Pudek and Bragg, 1974). At a concentration of 150 μM , this substance reduces the rate of oxygen consumption of the *lipA arcA* strain to the levels of the *lipA* strain (data not shown), but has no effect on the survival of the former mutant (Figure 3B). At this concentration, cyanide also has no effect on the stationary phase survival of wt (data not

shown). The data presented in Figure 3 therefore are in disagreement with a causal association between enhanced oxygen consumption and reduced lifespan in the *lipA arcA* strain.

Acs-mediated conversion of acetate to acetyl-CoA proceeds through an acetyl-AMP intermediate, consuming one molecule of ATP per molecule of acetate converted (Wolfe, 2005). Intracellular ATP concentration has been shown to decrease during stationary phase (Buckstein et al., 2008). Based on these observations, the second hypothesis we tested was whether the enhanced activity of Acs in the *lipA arcA* strain results in diminished ATP concentration which in turn leads to reduced lifespan and heat shock resistance. We used a luciferase-based assay to measure intracellular ATP and found that the *lipA* strain contains approximately three times less ATP than the wt. Consistent with the enhanced activity of Acs in the absence of ArcA, the *lipA arcA* mutant contains less ATP than the *lipA* strain; lack of *acs* restores ATP concentration in the *lipA arcA acs* mutant to the levels of the *lipA* mutant (Figure 4A).

We attempted to restore the diminished ATP content of the *lipA arcA* strain, by making use of a recently published characterization of the ATP content of all single-gene deletion strains present in the KEIO collection (Hara et al., 2009). According to this analysis, the stationary phase ATP content of 21 strains was at least twice that of wt, with the highest ranking-strain, *atpA*, showing a 3-fold elevation. We arbitrarily chose to delete two of the genes that limit ATP content in stationary phase wt cells (*yfgA* and *add*) from the *lipA arcA* mutant. Both triple mutants caused a reversion of ATP concentration to the levels of the *lipA* mutant (Figure 4A). However, the reversion in ATP content did not have a similar effect on lifespan, because both strains survive either similar to or shorter than the *lipA arcA* mutant (Figure 4B). Hence, our data do not support a causal association between decreased ATP concentration and shortened lifespan in the *lipA arcA* mutant.

Our third attempt to gain a mechanistic insight into the regulation of lifespan by ArcA and Acs in the *lipA* strain was based on the published observation of a time-dependent increase in the amount of protein carbonyls during the stationary phase incubation of a wt population (Dukan and Nystrom, 1998). Confirming the published findings, we found a time-dependent increase in protein carbonylation in the wt strain detected after chemical derivatization of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH), forming a stable dinitrophenyl hydrazone product which can be detected by a commercially available antibody (Figure 5B). We also found that protein carbonylation does not increase during stationary phase in the long-lived *lipA* mutant. However, the similarly low amount of protein carbonylation in the *lipA arcA* mutant, as well as the increased carbonylation in the *lipA acs* and *lipA arcA acs* mutants, is not consistent with protein carbonylation being a causative factor for the observed lifespan differences between the *lipA* strain and the strains derived from it.

Two technical considerations are worth stressing: firstly, as we quantified protein carbonylation in cultures maintained in LB buffered to pH 7.5, there is no death of the mutants and a small viability loss of the wt (approx. 20% from day 3 to day 5) during the experiment. Therefore, we show that the accumulation of protein carbonylation can occur independently of cell death in *E. coli*. Secondly, the use of high-resolution mass

spectrometry recently revealed that DNPH does not react exclusively with carbonyl groups, as it was found to also react with sulfenic acids forming a DNPH adduct (Dalle-Donne et al., 2009).

Discussion

A genome-wide screen for extended stationary phase survival in *E. coli* identified the *lipA* strain as the one with the longest lifespan, partially dependent on the hypoxia transcription factor ArcA (Gonidakis et al., 2010). We have described that ArcA contributes to the extended lifespan and the enhanced heat shock resistance of the *lipA* mutant by suppressing the expression of *acs*, which encodes the protein responsible for the uptake of extracellular acetate and its conversion to acetyl-CoA. Therefore, the ArcA-regulated, slow uptake and utilization of extracellular acetate appears to be a central component of the lifespan extension and heat shock resistance observed in the *lipA* strain (Figure 6). We found no evidence for the elevated expression of *acs* causing the decreased lifespan of the *lipA arcA* mutant through effects on oxygen consumption or ATP content. Our measurement of protein carbonylation over time in five different strains also found no correlation between lifespan and the load of protein carbonylation.

The adaptation of the metabolic physiology of *E. coli* to changes in oxygen availability mostly occurs at the level of gene expression through the action of the transcription factors Fnr and ArcA (Iuchi and Lin, 1991). Experiments quantifying the transcriptional and functional changes elicited by varying oxygen tensions led to the current model that Fnr is activated under anaerobic conditions, whereas ArcA is active under microaerobic conditions (Levanon et al., 2005). ArcA suppresses the expression of TCA cycle genes such as citrate synthase (*gltA*), while activating the expression of genes required for energy generation under limited oxygen availability such as the cytochrome d terminal oxidase operon, *cydAB* (Lynch and Lin, 1996). A strain lacking ArcA has been shown to have reduced stationary phase survival under glucose starvation conditions, possibly as a result of the uncontrolled oxidation of endogenous metabolic reserves (Nystrom et al., 1996). We observed that ArcA is required for heat shock resistance but not for the stationary phase survival of wt in LB. These disparate observations with regard to survival probably reflect the requirement for ArcA when fermentation is the predominant means of energy generation (as in glucose minimal medium), but not under conditions where respiration is the major source of energy (as in LB).

By a combined experimental and computational analysis it was concluded that ArcA controls 100–150 operons involved in metabolism, survival and transcriptional regulation (Liu and De Wulf, 2004). The production and uptake of acetate, one of the metabolic byproducts of *E. coli* under hypoxic conditions, is not predicted to be under the direct control of ArcA according to this and another genome-wide study with the same subject (Salmon et al., 2005). Therefore, the previously published effect of ArcA on extracellular acetate accumulation in wt *E. coli* (Vemuri et al., 2006) is related to either the bacterial ‘Crabtree effect’, whereby lack of ArcA increases the capacity of the TCA cycle to consume the pyruvate formed by the oxidation of glucose resulting in the decreased formation of

acetate (Wolfe, 2005), or to the suppression of acetate uptake by ArcA that we are hereby reporting for the *lipA* strain.

We found that ArcA contributes to the extended lifespan and the enhanced heat shock resistance of the *lipA* strain by suppressing the expression of *acs* (Figures 1 and 2). Based on the aforementioned studies examining the targets of ArcA regulation, the ArcA-mediated suppression of the expression of *acs* is most probably indirect. Eight different proteins have been shown to contribute to the expression of *acs* during the transition from log to stationary phase (Kumari et al., 2000); none of them is expected to be an ArcA target. The mechanism by which ArcA suppresses the transcription of *acs* is therefore unclear.

We tested three alternative hypotheses for the lifespan-shortening effect of the overexpression of *acs* in the *lipA arcA* mutant. According to the first hypothesis, the overexpression of *acs* results in increased production of acetyl-CoA which is then consumed by the TCA cycle and the electron transport chain, resulting in the elevated rate of oxygen consumption and the shortened lifespan observed in the *lipA arcA* mutant. Deletion of the two TCA cycle proteins known to consume acetyl-CoA, namely citrate synthase and malate synthase, reduced the rate of oxygen consumption but did not extend the lifespan of the *lipA arcA* mutant (Figure 3). However, it is possible that these deletions are not associated with lifespan extension because they interfere with required energy and biosynthetic outputs. Pharmacological inhibition of oxygen consumption by means of sodium cyanide also had no effect on the survival of the *lipA arcA* strain (Figure 3B). These results are in disagreement with the enhanced rate of oxygen consumption caused by *acs* overexpression being the cause of the shortened survival of the *lipA arcA* mutant.

The second hypothesis is that the elevated consumption of ATP by Acs in the *lipA arcA* mutant causes the observed survival decrease in the latter. Consistent with this hypothesis, a stationary phase population of the *lipA arcA* mutant contains less ATP than the respective *lipA* strain; this difference is reversed by the deletion of *acs* (Figure 4A). However, elevation of the ATP concentration of the *lipA arcA* mutant by deletion of genes other than *acs*, known to limit ATP production in *E. coli*, had no effect on the lifespan of the mutant (Figure 4A,B). Therefore, the ATP hypothesis is not consistent with our data either. However, considering both the elevated rate of oxygen consumption and the reduced ATP content of the *lipA arcA* mutant, the possibility arises that the Acs-dependent, non-energy-generating consumption of oxygen in the *lipA arcA* mutant is linked to the impaired survival of this strain.

Both the ATP and the oxygen consumption hypotheses were formulated and tested based on the assumption that Acs affects survival and heat shock resistance through its known biochemical function, namely the conversion of acetate to acetyl-CoA. The data we obtained against both acetate-based hypotheses, as well as the observation that Acs sensitizes the *arcA* strain to heat shock in the absence of extracellular acetate (Figure 2), raise the possibility that Acs exerts its lifespan-shortening and heat shock-sensitizing effects through an unidentified, acetate-independent mechanism.

Motivated by the observation that protein carbonyls accumulate over time in a stationary phase *E. coli* population (Dukan and Nystrom, 1998), we questioned whether this oxidative modification is the acetate-independent mechanism through which Acs exerts its effect on lifespan. Protein carbonyls are carbon-oxygen double bonds formed at the side chains of the amino acids arginine, lysine, proline and threonine (Nystrom, 2005). Unlike other oxidative modifications such as disulfide bond formation, these modifications are irreversible, rendering the degradation of the affected proteins the only means of limiting the dysfunction caused by their presence. Certain proteins (including, among others, TCA cycle enzymes) show increased susceptibility to the time-dependent accumulation of protein carbonyls (Dukan and Nystrom, 1998). Populations lacking both cytosolic superoxide dismutases display a higher amount of protein carbonylation and also a more rapid loss of viability during stationary phase. By contrast, incubation of a stationary phase population in the absence of oxygen causes a significant lifespan extension (Dukan and Nystrom, 1999). The use of radioselectan equilibrium density-gradient centrifugation allows the separation of a stationary phase population of *E. coli* to a high-density and a low-density fraction. Desnues et al. (2003) showed that the high-density fraction displays increased expression of catalase, enhanced protein carbonylation and preferential loss of viability compared to the low-density fraction. These observations provided credence to the notion that oxidative deterioration of proteins is causally linked to stationary phase death in *E. coli*.

Examination of the protein carbonylation data for wt and the *lipA* and *lipA arcA* mutants shown in Figure 5 provide further support for this theory, because both mutants survive longer and display decreased protein carbonylation load compared to wt. However, the inverse correlation between protein carbonylation and lifespan is not extended to the data obtained for the other strains shown in Figure 5; the *lipA acs* and *lipA arcA acs* mutants survive longer than wt, despite having a similar protein carbonylation load as wt. It is possible that a compensatory, longevity-assuring mechanism is activated in the *lipA* strain to counteract the increased protein carbonylation caused by the lack of Acs. However, the most parsimonious conclusion of the data we present is the lack of a causal association between stationary phase survival and protein carbonylation in *E. coli*.

In conclusion, our results add the regulation of stationary phase heat shock resistance to the cellular phenotypes regulated by ArcA. In light of the aforementioned wide-ranging impact of ArcA on *E. coli* physiology, it is remarkable that most of the effect of this transcription factor on the survival and heat shock resistance of the *lipA* mutant is mediated by a single protein, namely Acs. Our data are consistent with ArcA contributing to the longevity and heat shock resistance of the *lipA* strain by causing a slow, regulated uptake and utilization of extracellular acetate, thus inhibiting the futile consumption of oxygen observed in the *lipA arcA* strain. Finally, the lack of correlation we observed between protein carbonylation and lifespan opposes a causal role of this form of oxidative damage in the stationary phase death of *E. coli*.

Materials and methods

Strains and genetic manipulations

The wild-type *E. coli* strain BW25113 and its respective single-gene knock-outs were provided by the KEIO collection (Baba et al., 2006). The pedigree of BW25113, a K-12 derivative, is described by Baba et al. (2006). To create strains deleted for multiple genes, the kanamycin cassette was excised using FLP-mediated recombination, resulting in deletions carrying only a single FRT site (denoted, e.g., *lipA*::FRT in Table 1), as described previously (Datsenko and Wanner, 2000), whereby the only difference was that the non-selective incubation took place at 37°C and not at 43°C. Kanamycin alleles were transduced by bacteriophage P1 using standard techniques and the correct insertion was verified by PCR using primer K1 described previously (Baba et al., 2006), along with a locus-specific primer annealing to a sequence upstream of the disrupted locus. Genomic sequence information was obtained from the 'Profiling of the *E. coli* Chromosome' website (<http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp>).

Survival and heat shock resistance

LB medium consisted of 1% bacto tryptone, 0.5% yeast extract and 0.5% NaCl w/v (purchased from VWR, West Chester, PA, USA). Cultures were inoculated 1:1000 using an overnight culture created by inoculating 2–3 colonies from an LB plate to 1 ml of LB. Cultures were grown in 3 ml of LB in 16-mm diameter test tubes rotating orbitally at 220 rpm for 12 h, at which point cell density was adjusted to approximately 1.5×10^9 CFU/ml by resuspending a pellet containing the desired number of cells in cell-free spent medium of the same strain. HEPES was added to 100 mM to achieve a stationary phase pH of 7.5. Spontaneous and adjusted pH was quantified using a pH electrode and pH test strips. All cultures were grown and maintained at 37°C and 70% relative humidity and CFUs were enumerated over time by removing an aliquot, serially diluting in 0.5% NaCl, followed by colony enumeration after plating on LB plates that were incubated at 37°C. For the heat shock experiments, cultures were processed as described above with HEPES, returned to the incubator and 12 h later (24 h after inoculation) were subjected to a 15-min incubation in a 54°C water bath without shaking and CFUs were enumerated before and after the treatment.

Oxygen consumption and acetate quantification

Oxygen consumption measurements were performed with 2 ml of culture stirred by a magnetic stir bar in a 37°C water bath using a Clark-type electrode. Conversion to nanomoles of oxygen consumed was done by assuming that the liquid culture contains the same amount of oxygen as water equilibrated with 21% oxygen in 1 atmosphere pressure, which is 5.02 µl/ml (according to the manufacturer's manual), and was further normalized by the number of CFUs present. Data were recorded until a straight line trace was obtained indicating that a steady state of oxygen consumption had been reached. Extracellular acetate concentration was quantified on cell-free samples obtained by centrifugation using the acetic acid kit from R-Biopharm (Darmstadt, Germany) according to the manufacturer's instructions.

acs mRNA quantification

One ml aliquots of late stationary phase cultures (24 h after inoculation) were added to a 95 μ l ethanol+5 μ l water-equilibrated acidic phenol mixture and rapidly centrifuged for 45 s at 4°C. RNA was subsequently extracted using the MasterPure complete DNA and RNA purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. Then, 3 μ g of RNA was used per reverse transcription reaction using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers as primers according to the manufacturer's instructions. Next, 50 ng of reverse-transcribed RNA was used as substrate for real-time PCR. The expression level of three different housekeeping genes was measured (*rpoA*, *frr* and *dnaA*); *dnaA* was found to be highly expressed at similar levels in the wt, *arcA*, *lipA* and *lipA arcA* strains and was therefore used for normalization of the values obtained for *acs*. Standard curves were constructed for each assayed transcript and used for quantification.

Protein carbonyls quantification

To minimize the fraction of culture removed for each sampling, 25-ml cultures were maintained in 125-ml flasks; the survival curves were similar to those obtained from experiments performed as described in the survival section. 1.5-ml samples were collected from all cultures at the indicated time points, flash-frozen by incubating in crushed dry ice for 2' and subsequently stored at -70°C until further processing. Crude protein extracts were obtained by room temperature incubation with the Y-PER protein extraction reagent (Pierce, Rockford, IL, USA). Then, 8 μ g of protein from each strain (quantified using the BCA assay) was used with the protocol suggested by the manufacturer of the oxyblot protein oxidation detection kit (Chemicon, Billerica, MA, USA). Signal intensity was quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>).

ATP quantification

Cell lysis was achieved using a protocol similar to the one proposed by Napolitano and Shain (2005). Culture samples obtained at 24 h post-inoculation were centrifuged, the supernatant removed and the pellet resuspended in 50 mM HEPES, pH 7.4. Then, 5 μ g proteinase K was added and the samples were incubated for 15 min at 50°C (activity maximum for proteinase K) and then for 5 min at 80°C to inactivate the proteinase. Light production was measured kinetically; 50 consecutive readings were obtained at 0.8-s intervals with 100 ms integration time. The area under the resulting curve was calculated using the software accompanying the luminometer, similar to Schneider and Gourse (2004). The background luminescence of a sample without any luciferase was subtracted for each sample and the resulting value was normalized by the amount of protein present in the lysed sample, quantified using the BCA assay.

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Figure 1.

Genetic interaction of ArcA and Acs in the regulation of lifespan.

(A) Stationary phase survival of the strains (n=4). (B) Extracellular acetate concentration of the strains (n=3). (C) Effect of 2.5 mM acetate addition on day 3 on the survival of the *lipA arcA* strain (n=4). (D) Transcription levels of *acs* in the strains obtained by RT-PCR (n=3). Data are presented as mean±SEM throughout; the number of experiments is indicated in parentheses.

Figure 2.
Survival of day 1 cultures of the strains after a 15-min incubation period at 54°C (n=4).

Figure 3.

Genetic interaction of ArcA and Acs in the regulation of oxygen consumption.

(A) Rate of oxygen consumption of the strains (n=3). (B) Survival of the strains and the effect of 150 μM cyanide on the survival of the *lipA arcA* mutant (n=3).

Figure 4.
Genetic interaction of ArcA and Acs in the regulation of ATP content.
(A) ATP concentration of the strains (n=3). (B) Stationary phase survival of the strains (n=4).

Figure 5.

The effect of ArcA and Acs in the time-dependent accumulation of protein carbonyls.

(A) Representative experiment for the quantification of protein carbonylation using derivatization with 2,4-dinitrophenylhydrazine. (B) Average values of three different such experiments (n=3).

Figure 6.
Model for the regulation of lifespan by ArcA and Acs in the *lipA* mutant.

Table 1List of *E. coli* strains used in this study.

Name	Genotype	Source
BW25113	<i>rrnB3 lacZ4787D hsdR514 (araBAD) 567 (rhaBAD) 568 rph-1</i>	Baba et al., 2006
<i>lipA</i>	BW25113 <i>lipA::kan</i>	Baba et al., 2006
<i>acs</i>	BW25113 <i>acs::kan</i>	Baba et al., 2006
<i>arcA</i>	BW25113 <i>arcA::kan</i>	Baba et al., 2006
SG010	BW25113 <i>lipA::FRT arcA::kan</i>	Gonidakis et al., unpublished
SG017	BW25113 <i>lipA::FRT acs::FRT arcA::kan</i>	Gonidakis et al., unpublished
SG019	BW25113 <i>lipA::FRT acs::kan</i>	Gonidakis et al., unpublished
SG020	BW25113 <i>arcA::FRT acs::kan</i>	Gonidakis et al., unpublished
SG031	BW25113 <i>lipA::FRT arcA::FRT gltA::kan</i>	This study
SG032	BW25113 <i>lipA::FRT arcA::FRT aceB::kan</i>	This study
SG033	BW25113 <i>lipA::FRT arcA::FRT yfgA::kan</i>	This study
SG037	BW25113 <i>lipA::FRT arcA::FRT add::kan</i>	This study