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Genome-wide screen identifies *Escherichia coli* TCA-cycle-related mutants with extended chronological lifespan dependent on acetate metabolism and the hypoxia-inducible transcription factor ArcA

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Summary

Single-gene mutants with extended lifespan have been described in several model organisms. We performed a genome-wide screen for long-lived mutants in *Escherichia coli*, which revealed strains lacking tricarboxylic acid (TCA)-cycle-related genes that exhibit longer stationary-phase survival and increased resistance to heat stress compared to wild-type. Extended lifespan in the *sdhA* mutant, lacking subunit A of succinate dehydrogenase, is associated with the reduced production of superoxide and increased stress resistance. On the other hand, the longer lifespan of the lipoic acid synthase mutant (*lipA*) is associated with reduced oxygen consumption and requires the acetate-producing enzyme pyruvate oxidase, as well as acetyl-CoA synthetase, the enzyme that converts extracellular acetate to acetyl-CoA. The hypoxia-inducible transcription factor ArcA, acting independently of acetate metabolism, is also required for maximum lifespan extension in the *lipA* and *lpdA* mutants, indicating that these mutations promote entry into a mode normally associated with a low-oxygen environment. Because analogous changes from respiration to fermentation have been observed in long-lived *Saccharomyces cerevisiae* and *Caenorhabditis elegans* strains, such metabolic alterations may represent an evolutionarily conserved strategy to extend lifespan.

Key words: acetate; *Escherichia coli*; hypoxia; lifespan; stress resistance; superoxide.

Introduction

The existence of a germ line that is distinct from somatic tissue has been proposed as a prerequisite for the evolution of senescence (Partridge & Barton, 1993). A key prediction of this theory was disproved by the observation that one of the two daughter cells that result from the morphologically symmetrical division of an individual *Escherichia coli* cell displays reduced growth rate with successive generations, which is the hallmark of reproductive senescence (Stewart *et al.*, 2005). At the population level, the growth rate of an *E. coli* batch culture (maintained in the same medium without addition or removal of any material) gradually declines and proliferation eventually ceases, despite the presence of extracellular nutrients that could support a further production of biomass (our unpublished observation), marking the onset of stationary phase. As stationary phase progresses, an increasing fraction of the *E. coli* population becomes unable to resume growth upon transfer to fresh nutrient medium, subsequently loses membrane integrity assessed using fluorescent dyes and is therefore considered dead (Ericsson *et al.*, 2000; Finkel, 2006).

Using the fluorescent nucleic acid stain propidium iodide, a good correlation was found between the loss of proliferating potential and the loss of membrane integrity (Ericsson *et al.*, 2000); thus, there does not seem to be a substantial fraction of a stationary-phase *E. coli* population in Luria Bertani (LB) medium that loses culturability but maintains viability. We therefore decided to use the formation of colonies from cells sampled from a stationary-phase population (colony-forming units, CFU) as a measure of the viability of that population. The progressive loss of culturability/viability observed in stationary phase results in the loss of 90–99% of the initial population and is reminiscent of the stationary-phase survival of the budding yeast *Saccharomyces cerevisiae*, which has been introduced by our laboratory as a model system for the study of aging and lifespan in higher organisms (Fabrizio & Longo, 2003). For consistency with our work in yeast and to distinguish it from reproductive lifespan, we call survival in stationary phase 'chronological lifespan'.

Most of the previous work on stationary-phase *E. coli* has focused on the characterization of the organism's physiology compared to log-phase cells. Particular focus has been given to the σ^S subunit of the RNA polymerase, encoded by *rpoS*, which is the master regulator of several stationary-phase-inducible genes and phenotypes, such as resistance to heat and oxidative stress (Hengge-Aronis, 2002). Numerous strains with a more rapid loss of stationary-phase viability than that of wild-type (wt) have been described (Groat *et al.*, 1986; Visick *et al.*, 1998), but there have been a few reports of mutants with extended survival. Loss of the toxin–antitoxin cell death system encoded

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by *hipBA* causes resistance to hydrogen peroxide and extended stationary-phase survival (Kawano *et al.*, 2009). A microscopy-based screen of a transposon-mutagenized collection of *E. coli* mutants revealed that a strain lacking the response regulator RssB has a reduced proportion of dead cells during stationary phase and is also resistant to heat, oxidative and osmotic stress (Fontaine *et al.*, 2008). The authors attributed these results to the stabilization of RpoS in the *rssB* strain. Finally, addition of ethanol has been shown to delay the viability loss of a stationary-phase culture, also in an RpoS-dependent manner (Vulic & Kolter, 2002).

We used the KEIO collection, which consists of single-gene deletion strains of every nonessential protein-coding gene in *E. coli* (Baba *et al.*, 2006), to comprehensively screen for long-lived mutants by a spectrophotometric method. The screen revealed three mutants with extended chronological lifespan that are also stress resistant. Our results provide evidence for the role of novel pathways in the regulation of prokaryotic survival and suggest that some fundamental metabolic processes lie at the center of survival regulation in organisms on either side of the line separating prokaryotes from eukaryotes.

Results

Genome-wide screen reveals three long-lived, heat shock-resistant strains

We employed a screening strategy similar to the one used in *S. cerevisiae* (Powers *et al.*, 2006) to identify *E. coli* strains with increased stationary-phase survival (Fig. S1 and Data S1). Briefly, the mutants of the KEIO collection were maintained in batch culture in 96-well plates and were used to inoculate fresh cultures at two different time-points when more than 90% of a wild-type population is no longer viable (data not shown). The values of the optical density at 600 nm obtained after outgrowth of these fresh cultures normalized by each strain's growth rate were used as a proxy measure of the number of cells that were still alive at these time-points. All strains were thus ranked for both sampled time-points, and top-ranking strains were then individually tested for stationary-phase survival (Table S1). Most of the top-ranking strains displayed delayed entry into stationary phase (gradual increase in biomass several hours after the wt has ceased proliferation) and not extended stationary-phase survival; these strains were not further tested. The four strains shown in Fig. 1A reach stationary phase at approximately the same time as wt but maintain 100% survival for longer periods than wt.

We performed all experiments in LB, a peptide-rich, complex nutrient medium that contains traces of glucose (present in the yeast extract found in LB) that are consumed by a wt strain within the first 90 minutes of incubation (Baev *et al.*, 2006). The metabolism of amino acids in this medium is accompanied by the excretion of ammonia that results in an extracellular stationary-phase pH of 8.5–9 (Pruss *et al.*, 1994; Farrell & Finkel, 2003). The majority of the long-lived strains have a slower growth rate (Fig. S2D) and lower saturation density than wt and also exhibit a slightly acidic pH at stationary phase (Table S2). We compared the survival of these strains to wt in several different ways, shown in Fig. 1. All strains survive longer than wt when no adjustments are made (Fig. 1A). To dissect the effect of pH and lower cell density on the observed lifespan extension, we equalized the cell density of all strains to approximately 1.5×10^9 cells per milliliter of culture and adjusted the pH upon stationary-phase entry to either nine or 7.5 using the biological buffers 3-([1,1-dimethyl-2-hydroxyethyl] aminol-2-hydroxypropanesulfonic acid (AMPSO) or 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), respectively. Only strains that lived longer in both alkaline and neutral conditions were investigated further (Fig. 1B,C). The CFU titers for the survival experiments shown in Fig. 1A–C are shown in Fig. S2A–C. The extended survival of the *sdhA*, *lipA* and *lpdA* mutants was confirmed in the commonly used wt strain MG1655 (Fig. 1D).

To rule out the possibility that the hypoxic conditions generated by extended incubation in an orbitally shaking test-tube play a role in the observed lifespan extension, we compared the survival of wt and the longest lived strain, *lipA*, in 10-mL cultures maintained in 125-mL flasks with loose-fitting caps, which provides a more thorough aeration of the cultures. The survival extension of the *lipA* strain is not diminished under these conditions (Fig. S2E). We also found that the extended survival of the *lipA* mutant is largely unaffected by incubation in cell-free conditioned medium obtained from a stationary-phase wt culture (Fig. 1E). Therefore, the observed lifespan extension is to a large extent independent of the potentially retarded utilization of the carbon and energy available in LB by the *lipA* mutant, which grows slowly and saturates at a low cell density compared to wt.

It is important to note that despite slower growth rates and lower saturation densities, the *lipA* and *lpdA* strains appear to reach stationary phase at the same time as wt, because log-phase populations of the three strains reach a plateau at the same time, as shown by the respective growth curves (Fig. S2D). Therefore, the observed lifespan extension is not attributable to delayed entry of the mutants in stationary phase. On the other hand, the survival of wt is extended by incubation in conditioned medium obtained from a stationary-phase *lipA* culture (Fig. 1E), pointing toward the existence of lifespan-enhancing substances generated by the *lipA* mutant during stationary phase, lifespan-shortening substances generated by wt or both. It is unlikely that the observed lifespan extension of wt in *lipA*-conditioned medium is solely attributable to the increased availability of carbon and energy sources in the mutant's medium, because the wt survives even longer when incubated in 0.5% NaCl, without any extracellular carbon or energy source (compare wt in *lipA*-conditioned medium in Fig. 1E to wt 0.5% NaCl in Fig. 6E).

All subsequent experiments were performed in the following way, unless otherwise stated: cultures were incubated for 12 h in test tubes in unbuffered LB, at which point cell density and pH were adjusted to approximately 1.5×10^9 cells per milliliter and

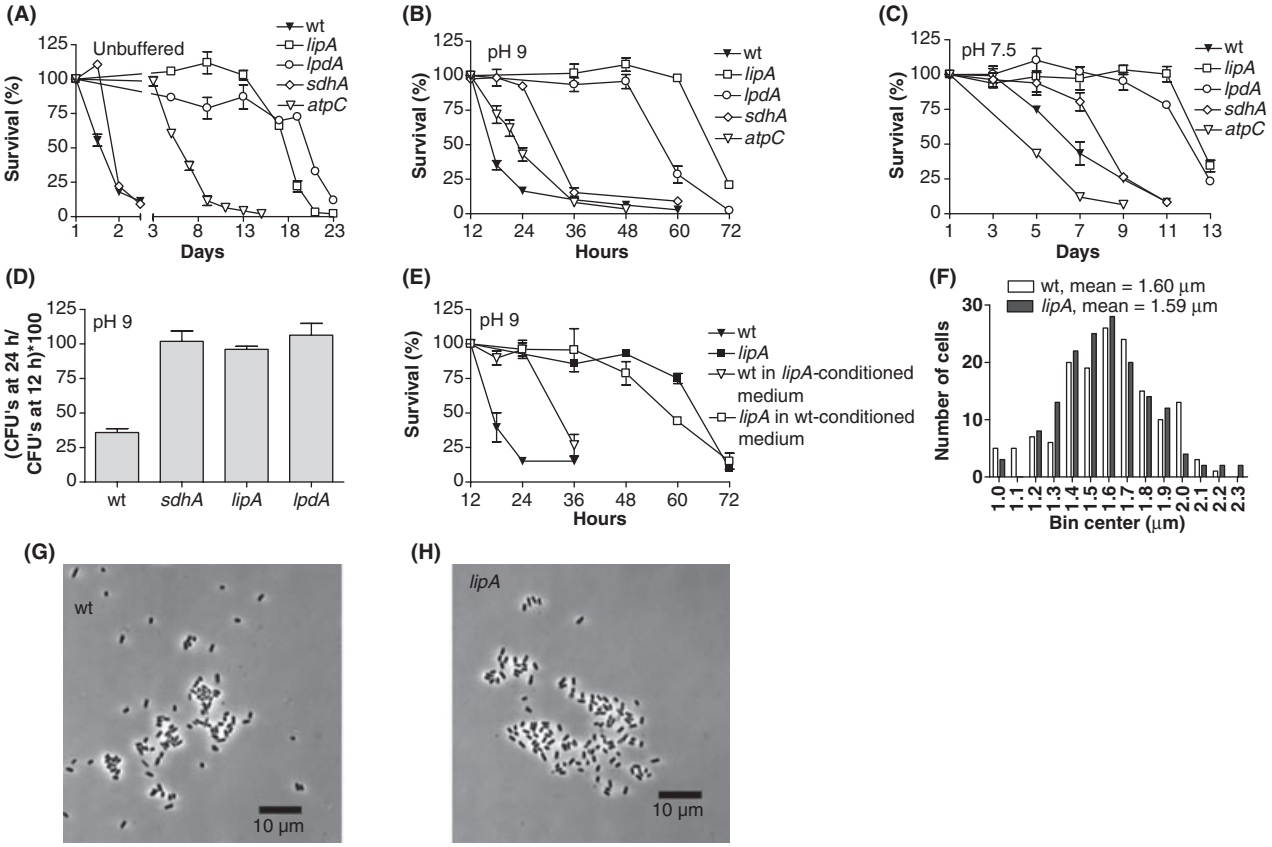

Fig. 1 Survival of *Escherichia coli* mutants recovered from a genome-wide screen for extended lifespan. Stationary-phase survival in unbuffered LB medium (A), LB medium buffered to pH 9 (B) and LB medium buffered to pH 7.5 (C). Cell density was also equalized to approximately 1.5×10^9 cells per milliliter for the buffered survival experiments. (D) Ratio of colony-forming units per milliliter at 24 over 12 h in stationary phase for the indicated strains at pH 9 in the MG1655 genetic background. (E) Survival of wt switched to *lipA*-conditioned medium and of the *lipA* strain switched to wt-conditioned medium. (F) Length distribution of 155 cells of wt and the *lipA* strain (cells outside the shown fields of view were also measured). (G) Phase-contrast image of early stationary-phase cells of wt. (H) Corresponding image for the *lipA* strain. See also Fig. S2(Supporting information).

pH 9, respectively, simulating the stationary-phase conditions generated by wt in this medium. We define this as the zero time-point in all experiments, and all time periods denoted in the figures refer to the time elapsed after this point. Therefore, in the text, the 1-h time-point is referred to as 'early stationary phase' and the 12-hour time-point is referred to as 'late stationary phase'. No loss of viability is observed in wt during this 12-h period.

Because long-lived mutants in other model organisms are typically resistant to heat and oxidative stress (Miller, 2009, Longo & Finch, 2003), we tested the survival of the three mutants after treatment with the superoxide-generating agent paraquat or after a high-temperature incubation. All long-lived strains were more resistant than wt to heat shock (Fig. 2A), and the *sdhA* strain is more resistant than wt to the viability loss caused by treatment with paraquat (Fig. 2B). Fumarate reductase, the protein that functionally replaces succinate dehydrogenase under anaerobic conditions (Maklashina *et al.*, 1998), has been shown to react with paraquat (Jones & Garland, 1977). It is therefore possible that SdhA is also an electron donor in the redox cycling catalyzed by paraquat, which would explain the resistance of the strain lacking this protein to the lethal effects of paraquat. However, SdhA may also affect paraquat redox cycling indirectly.

To further investigate the mechanistic basis of the resistance of the *sdhA* mutant to paraquat, we tested the dependence of the phenotype on the proteins that catalyze the conversion of superoxide to oxygen and hydrogen peroxide, the manganese-containing superoxide dismutase SodA and its iron counterpart, SodB. We found that lack of SodA increases the sensitivity to paraquat in both a wt or *sdhA* background. The *sodB* mutant is more sensitive to paraquat than the *sodA* strain; this pronounced sensitivity is reversed by the deletion of *sdhA* (Fig. 2B), consistent with the hypothesis that redox cycling between SdhA and paraquat or other pro-oxidants is responsible for the enhanced sensitivity of the *sodB* mutant to the paraquat-induced viability loss. Long-lived organisms in other model systems are often smaller than their wt counterparts (Longo & Finch, 2003). We measured the length of both wt and the *lipA* mutant in stationary phase using phase-contrast microscopy and found no difference in cell length between the two strains (Fig. 1F–H). In conclusion, the extended lifespan of the three

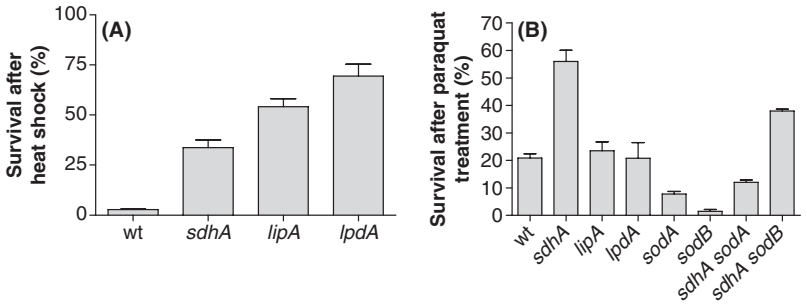

Fig. 2 Stress resistance of long-lived mutants. Survival of stationary-phase cultures after 4 min of incubation at 55°C (A) or 12 h of incubation with 500 μM paraquat at 37°C (B).

mutants we describe is associated with resistance to heat stress and is independent of differences in growth rate, saturation density, external pH and cell size.

Differences in metabolic physiology among long-lived mutants

lpdA encodes lipoamide dehydrogenase, a common component of the 2-ketoglutarate dehydrogenase (2-KGDH) complex of the TCA cycle and of the pyruvate dehydrogenase (PDH) complex (Smith & Neidhardt, 1983a). Its function is to oxidize the protein-bound lipoic acid used during the oxidative decarboxylation of 2-ketoglutarate to succinyl-CoA and of pyruvate to acetyl-CoA. *lipA* encodes lipoic acid synthase, a protein that catalyzes the formation of the carbon–sulfur bonds required to produce the lipoic acid used in the aforementioned reactions (Miller *et al.*, 2000). Lack of either LipA or LpdA therefore results in the inactivation of both the 2-KGDH and the PDH complexes. Lipoic acid is also a cofactor for the glycine cleavage system (Vanden Boom *et al.*, 1991). However, inactivation of the T-component protein of the glycine cleavage system (*gcvT*) has no effect on survival (Fig. S2F). Inactivation of the E1 component of the PDH (*aceE*) or the 2-KGDH (*sucA*) complex individually or in combination also has no positive effect on survival (Fig. S2F). Therefore, although LipA and LpdA are not known to function independently of the PDH, 2-KGDH and glycine cleavage complexes, our data indicate that the lack of either LipA or LpdA extends lifespan by causing metabolic changes that are distinct from those caused by inactivation of the protein complexes in which they participate (see also Discussion).

We first attempted to gain a mechanistic insight into the lifespan extension of the *lipA* and *lpdA* mutants by measuring the oxygen consumption of these strains over time. As shown in Fig. 3A, both strains consume less oxygen than wt at log phase and early stationary phase, and the *lpdA* mutant also respires less at late stationary phase. On the other hand, the *sdhA* mutant displays a profile similar to wt, the only difference being a higher rate of oxygen consumption at late stationary phase. *E. coli* is a facultative anaerobe, able to grow and survive both in the presence of oxygen and also in the absence of any electron acceptors (Clark, 1989). When oxygen respiration is not possible owing to either the lack of oxygen or a genetic block in the respiratory chain, the pyruvate formed by the decarboxylation of amino acids in LB medium is converted to one of four fermentation products, namely lactate, succinate, acetate or ethanol. The relative amount of these by-products is mostly dictated by the need to maintain a physiological NADH/NAD^+ ratio (Clark, 1989).

Because the *lipA* and *lpdA* strains are genetically unable to perform respiratory metabolism at the level of wt, we measured the concentration of extracellular acetate over time. As reported in the literature, we found that the wild-type strain consumes the acetate it initially produced resulting in no detectable acetate at late stationary phase (Kumari *et al.*, 1995), the *sdhA* mutant showing a similar behavior. On the other hand, the *lipA* and *lpdA* strains maintain a high extracellular acetate concentration at late stationary phase (Fig. 3B). It is worth noting that the acetate produced by wt in the presence of oxygen is thought to be the consequence of the inability of the otherwise functional TCA cycle and the respiratory chain to utilize all the available acetyl-CoA produced by PDH (Wolfe, 2005). Therefore, the results presented in Fig. 3 demonstrate that at least two different mechanisms can lead to extended survival in *E. coli*: one, exemplified by the *sdhA* mutant, that involves a wt-like pattern of oxygen consumption and acetate metabolism and another, exemplified by the *lipA* and *lpdA* strains, that is characterized by reduced oxygen consumption and sustained presence of acetate in the extracellular environment.

Decreased superoxide production is associated with extended survival in the *sdhA* mutant

Because the mutant lacking subunit A of succinate dehydrogenase is the only one that is resistant to the superoxide generator paraquat (Fig. 2B) and because this enzyme has also been shown to be a source of superoxide *in vitro*, possibly through its bound flavin adenine dinucleotide cofactor (Messner & Imlay, 2002), we measured superoxide production in wild-type and the *sdhA* mutant by monitoring cyanide-resistant oxygen consumption. Cyanide acts as an inhibitor of the oxygen-consuming cytochrome oxidase, and the oxygen consumption observed in its presence can be used as an approximation for the intracellular

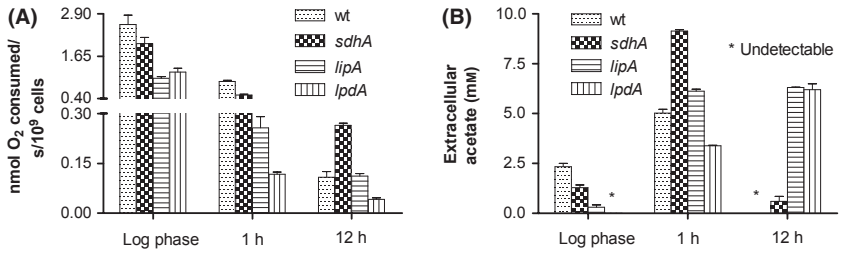

Fig. 3 Characterization of the metabolic physiology of long-lived mutants. Rate of oxygen consumption (A) and extracellular acetate concentration (B) of long-lived mutants and wt over time. '1 h' and '12 h' refer to time-points in stationary phase, the onset of which is defined as 12 h after the inoculation of each culture; '12 h' or 'late stationary phase' is therefore equivalent to 24 h after the cultures' inoculation, and '1 h' or 'early stationary phase' is equivalent to 13 h after the cultures' inoculation.

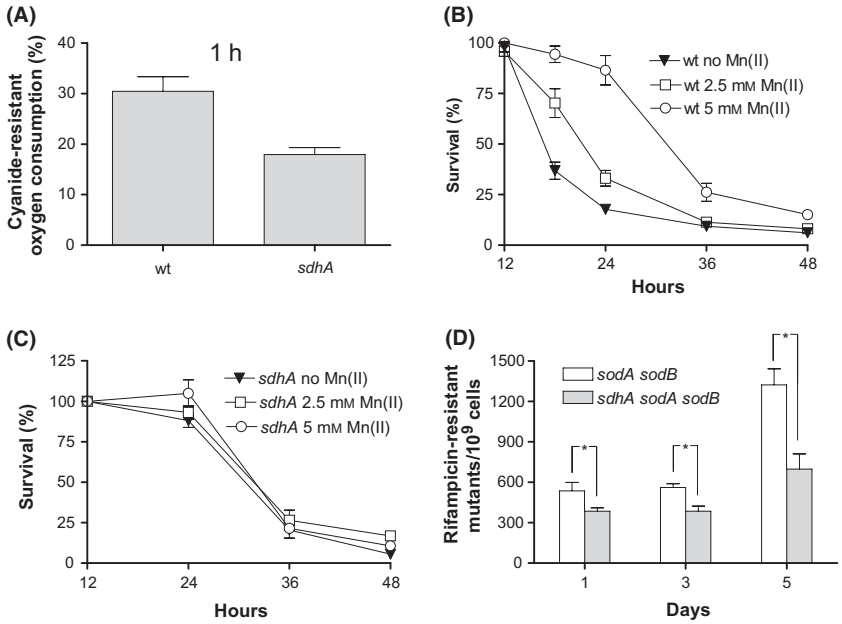

Fig. 4 Extended lifespan is associated with reduced superoxide production in the *sdhA* mutant. Cyanide-resistant oxygen consumption of wt and the *sdhA* mutant at early stationary phase (A). Effect of manganese (II) chloride addition at early stationary phase on the lifespan of wt (B) and the *sdhA* mutant (C). (D) Time-dependent frequency of rifampicin-resistant mutants of the *sodA sodB* and *sdhA sodA sodB* strains during a stationary-phase survival experiment at pH 7.5. Asterisks denote that the shown differences are significant at the $P < 0.05$ level. See also Fig. S3.

production of superoxide (Hassan & Fridovich, 1977). Confirming previous *in vitro* results, the *sdhA* strain produced less superoxide at stationary phase compared to wt (Fig. 4A).

Nystrom *et al.* reported no effect of the overproduction of superoxide dismutase A (*sodA*) on the survival of *E. coli* in minimal glucose medium (Nystrom *et al.*, 1996). We attempted to perform the same experiment in LB medium but observed a strong selection against retention of the SodA-overexpressing plasmid pDT1-5 (Touati, 1983) through stationary phase despite the presence of ampicillin, to which the plasmid confers resistance (data not shown). On the other hand, the *sodB*-overexpressing plasmid pHS1-7 (Carlioz & Touati, 1986) was retained throughout stationary phase but produced no effect on stationary-phase survival (Fig. S3A). Although both SodA and SodB scavenge superoxide generated in the cytosol, it is possible that overexpression of both is required to have an effect on lifespan, as we have previously observed in yeast (Fabrizio *et al.*, 2003). In fact, the SodA- and SodB-deficient mutants display distinct phenotypes (Carlioz & Touati, 1986), consistent with the possibility that the contribution of these two enzymes on the physiology of *E. coli* is not identical. As an alternative method, we tested the effect of manganese, a known superoxide scavenger (Chang & Kosman, 1989), on the survival of wt and the *sdhA* strain. Stationary-phase addition of manganese (II) chloride produced a dose-dependent lifespan extension in wild-type but did not further increase the lifespan of the *sdhA* mutant (Fig. 4B,C).

Because superoxide has been shown to enhance DNA mutations (Benov & Fridovich, 1996), we investigated the effect of succinate dehydrogenase on stationary-phase mutation frequency, by measuring the occurrence of rifampicin-resistant mutants over time. Rifampicin is a drug that inhibits bacterial growth by binding to the β subunit of RNA polymerase, encoded

by *rpoB*. Mutations in *rpoB* allow proliferation in the presence of this drug, and the occurrence of such mutants has been used as a measure of mutation frequency in *E. coli* (Garibyan *et al.*, 2003). No increase in mutation frequency was observed for both wt and the *sdhA* strain (Fig. S3B). However, deletion of *sdhA* attenuated the time-dependent increase in the occurrence of rifampicin-resistant mutants in the strain devoid of both SodA and SodB (Fig. 4D), consistent with the possibility that SdhA-produced superoxide contributes to the increased DNA damage observed over time in *sodA sodB* mutants. Note that the decreased mutation frequency in *sdhA sodA sodB* compared to *sodA sodB* is not growth-rate-dependent because the two strains have similar growth curves (Fig. S3C) and also rifampicin plates were checked for several days for the appearance of resistant colonies (see also Experimental procedures). Although these data are consistent with a role for SdhA-produced superoxide in promoting aging and death in *E. coli*, further studies are required to rule out the possibility that the lifespan extension caused by Mn (II) is superoxide-independent.

ArcA is required for the fully extended lifespan of the *lipA* and *lpdA* mutants

ArcA is a transcription factor that suppresses the expression of genes involved in respiration and activates genes involved in fermentative metabolism, thus contributing to the adaptation of *E. coli* to hypoxic conditions (Iuchi & Lin, 1991). Lack of ArcA causes reduced stationary-phase survival under glucose starvation conditions, which was attributed to the deregulation of cellular redox balance and the uncontrolled drainage of the cells' endogenous energy reserves (Nystrom *et al.*, 1996). Because the PDH and 2-KGDH complexes, in which LipA and LpdA participate, are among the most drastically down-regulated in response to oxygen shortage (Smith & Neidhardt, 1983b), and both the *lipA* and *lpdA* strains consume less oxygen compared to wt (Fig. 3A), we tested whether the hypoxia transcription factor ArcA becomes essential in these strains, even under relative abundance of environmental oxygen. We therefore tested the effect of ArcA deletion on the survival of these mutants and found that ArcA is required for maximum lifespan extension in both (Fig. 5B). ArcA is also required for the extended survival of the mutants at an extracellular pH of 7.5 (Fig. S4C). In contrast, the survival of both the wt and the *sdhA* strain was unaffected by the loss of ArcA (Fig. 5A).

ArcA forms a typical two-component signal transduction module, along with the membrane protein ArcB, which is activated in response to hypoxia and then activates ArcA by phosphorylation (Georgellis *et al.*, 2001). Replacement of the wt *arcB* gene with an allele that is constitutively active, because of its fusion with Tar [a methyl-accepting chemotaxis protein for sensing aspartate (Kwon *et al.*, 2003)], results in an ArcA-dependent lifespan extension of a smaller magnitude compared to the one observed in the *lipA* and *lpdA* mutants (Fig. 5C). Thus, ArcA is necessary for the fully extended lifespan of the *lipA* and *lpdA* strains, and its constitutive activation is sufficient to extend the lifespan of wt.

The fully extended lifespan of the *lipA* mutant is entirely dependent on pyruvate oxidase and partly dependent on acetyl-CoA synthetase

ArcA has been shown to contribute to acetate formation (Vemuri *et al.*, 2006). We found that the extended survival of the *lipA* and *lpdA* strains is associated with a sustained presence

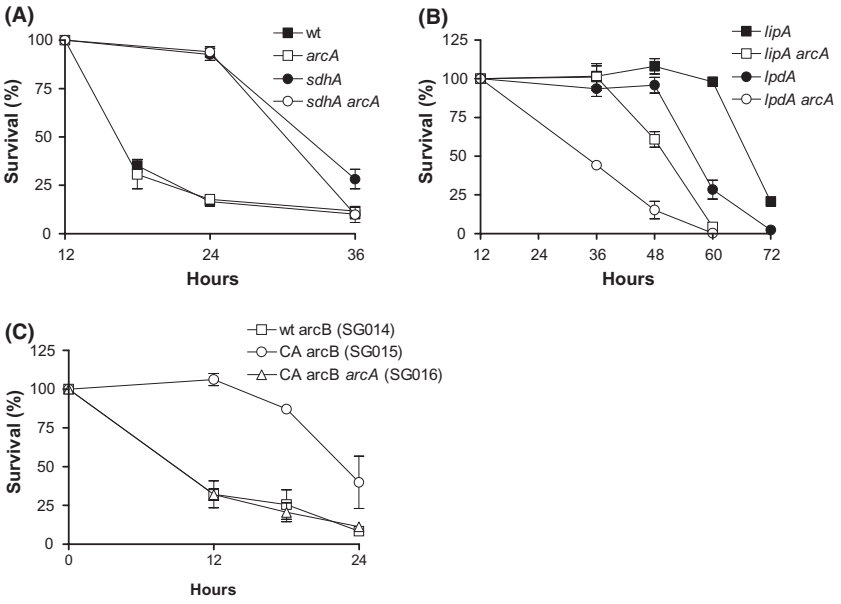

Fig. 5 ArcA is necessary and sufficient for lifespan extension. (A) Effect of *arcA* deletion on the survival of the *lipA* and *lpdA* strains. (B) Effect of *arcA* deletion on the survival of wt and the *sdhA* strain. (C) Effect of constitutive activation (CA) of arcB on the survival of wt and its dependence on *arcA*. The genotype of the shown strains is shown in Table S2.

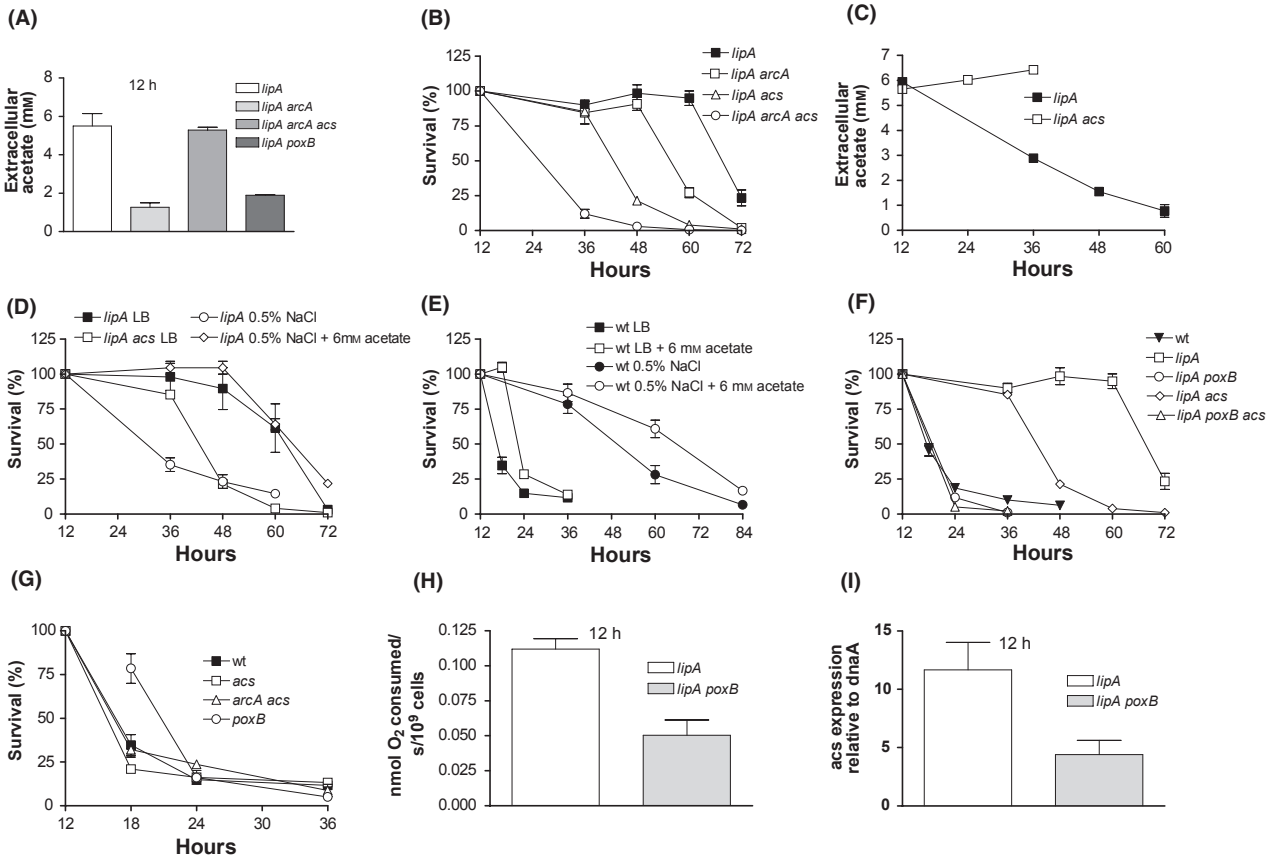

Fig. 6 Acetate production and uptake are required for the extended lifespan of the *lipA* mutant. (A) Extracellular acetate concentration of the *lipA*, *lipA arcA*, *lipA arcA acs* and *lipA poxB* strains at late stationary phase. (B) Effect of deletion of *arcA*, *acs* or both on the survival of the *lipA* mutant. (C) Extracellular acetate concentration of the *lipA* and *lipA acs* strains over time. No data were collected after 36 h for the latter because of loss of viability. (D) Survival of the *lipA* and *lipA acs* strains in LB and of the former strain in 0.5% NaCl with or without the addition of 6 mM acetate. (E) Survival of wt in LB and in 0.5% NaCl with or without the addition of 6 mM acetate. (F) Effect of the deletion of *poxB*, *acs* or both on the survival of the *lipA* strain. (G) Stationary-phase survival of the *acs*, *arcA acs* and *poxB* strains. (H) Effect of *poxB* deletion on the rate of oxygen consumption of the *lipA* strain at late stationary phase. (I) Expression of *acs* in the *lipA* and *lipA poxB* strains at late stationary phase. See also Figs S4 and S5(Supporting information).

of extracellular acetate during stationary phase (Fig. 3B) and that the extended lifespan of these mutants is partially dependent on *arcA* (Fig. 5B). Based on these results, we sought to determine the effect of *arcA* deletion on the extracellular acetate concentration of these mutants. Lack of ArcA results in a ~sixfold decrease in the concentration of acetate in the medium of the *lipA* mutant (Fig. 6A) but has no effect on extracellular acetate in the *lpdA* strain (Fig. S4A).

Extracellular acetate can be converted to acetyl-CoA via two pathways, one catalyzed by the acetate kinase/phosphotransacetylase (AckA/Pta) enzyme pair and the other by acetyl-CoA synthetase (Acs) (Wolfe, 2005). Measurement of extracellular acetate concentration over time reveals that the conversion of acetate to acetyl-CoA by Acs is responsible for the gradual disappearance of the metabolite from the extracellular environment of the *lipA* strain (Fig. 6C), whereas the AckA/Pta enzyme pair is not (Fig. S4F). This difference might be related to the lower K_m of Acs for acetate, which renders it more suitable for acetate concentrations lower than 10 mM (Kumari *et al.*, 1995). The observed trend of decreased expression of both *ackA* and *pta* in the *lipA* strain (Fig. S4G–H) might also be a contributing factor for the nonparticipation of this enzyme pair in acetate uptake in the *lipA* strain. We ruled out the possibility that the disappearance of acetate from the medium of the *lipA* strain is attributable to degradation or conversion to another substance by recovering acetate added at 10 mM from spent medium of the *lipA* mutant after several days of incubation at 37°C (data not shown).

Prompted by the correlation between extracellular acetate concentration and lengthened survival revealed by the deletion of ArcA, we tested the effect of the lack of *acs* on the survival of the *lipA* strain. Lack of Acs shortens the survival of the *lipA* mutant (Fig. 6B), whereas lack of Pta does not (Fig. S4E). Acs mediates the gradual uptake of acetate from the medium in the *lipA* mutant also at pH 7.5 (data not shown), but it is not required for extended survival under these conditions (Fig. S4D). Although the gradual uptake of acetate and its conversion to acetyl-CoA is required for extended survival at basic pH, the availability of extracellular acetate is not limiting for the lifespan of the *lipA* strain, as addition of acetate at a time-point when it

has been almost depleted from the medium does not produce a further lifespan extension (Fig. S4B). Deletion of *acs* from the *lipA arcA* mutant restores extracellular acetate to the level observed in the *lipA* strain (Fig. 6A). However, the additive detrimental effect of *arcA* and *acs* deletion on the lifespan of the *lipA* mutant (Fig. 6B) shows that ArcA and Acs independently contribute to the extended lifespan of this strain.

To confirm the dependence of the extended survival of the *lipA* strain on acetate by nongenetic means, a stationary-phase population of the strain grown in LB was washed once with a solution of 86 mM NaCl (to remove all extracellular metabolites, including acetate) and subsequently maintained in 86 mM NaCl (the concentration used in LB). No acetate was detectable in a saline-resuspended culture of the *lipA* mutant both immediately after transfer to NaCl and 12 h after the transfer (data not shown). The survival of the *lipA* mutant in NaCl is similar to that of the *lipA acs* strain in LB (Fig. 6D). Adding back extracellular acetate at the concentration found in a late stationary-phase culture of the *lipA* mutant maintained in LB (6 mM) right after transfer to NaCl restores the lifespan of the mutant to the level observed in LB (Fig. 6D).

On the other hand, as reported previously (Vulic & Kolter, 2002), the survival of wt is extended in 86 mM NaCl compared to incubation in LB (Fig. 6E). Addition of 6 mM acetate to a wt culture maintained in NaCl causes a small survival increase, bringing the lifespan of wt close to the level of the *lipA* mutant under the same conditions. Hence, transfer of stationary-phase populations of wt and the *lipA* strain from LB to 86 mM NaCl has opposite effects in terms of survival. The lifespan of wt increases, possibly owing to the removal of death-accelerating substances present in spent LB medium, whereas the lifespan of the *lipA* strain is diminished owing to the removal of the survival-extending effect of extracellular acetate present in spent LB medium of that strain.

Individual deletion of several genes encoding proteins known to utilize acetyl-CoA as a substrate had no effect on the survival of the *lipA* strain (Fig. S4E). Lack of Acs causes a major reduction in the rate of oxygen consumption of wt, whereas the *lipA acs* strain does not consume less oxygen than the *lipA* strain (Fig. S5A). These observations are consistent with the explanation that the acetyl-CoA formed by the uptake of extracellular acetate is used by the TCA cycle producing reducing equivalents that are subsequently fed in the electron transport chain in the wt, but not in the *lipA* strain. Thus, conversion of extracellular acetate to intracellular acetyl-CoA by Acs is required for the extended lifespan of the *lipA* mutant, but our genetic analysis could not identify the downstream effect of the produced acetyl-CoA.

Next, we tested the individual contribution of the known acetate-producing proteins to the lifespan of the *lipA* strain. Lack of phosphotransacetylase has no effect on the survival (Fig. S4E) and the extracellular acetate concentration (Fig. S4F) of the *lipA* mutant. We therefore turned our attention to pyruvate oxidase (PoxB), a lipid-activated enzyme that converts pyruvate to acetate and carbon dioxide and in the process supplies electrons to the electron transport chain (Koland *et al.*, 1984). In the absence of PoxB, the acetate concentration of the *lipA* strain is halved and its lifespan is reduced to that of wt (Fig. 6A,F), while the *poxB* strain does not live shorter than wt (Fig. 6G). The presence of the biological buffer AMPSO in the survival experiments shown in Fig. 6F maintains the pH at 9 despite any differences in the concentration of extracellular acetate among certain strains (data not shown); therefore, the observed effects on survival are not attributable to differences in the extracellular pH. Note, however, that lack of PoxB has no effect on the survival of the *lipA* mutant at pH 7.5 (Fig. S4D).

Finally, we sought to determine whether acetate production by PoxB and acetate uptake by Acs function in the same pathway to extend the lifespan of the *lipA* mutant. The epistasis results shown in Fig. 6F support this hypothesis, because the *lipA poxB acs* strain has a similar lifespan as the *lipA poxB* strain. Prompted by the finding that the transcription of *acs* is reduced in the absence of *poxB* (Kumari *et al.*, 2000), we measured the level of *acs* expression by RT–PCR in the *lipA* and *lipA poxB* strains. Similar to published findings (Kumari *et al.*, 2000), the expression of *acs* shows a 2.5-fold reduction in the *lipA poxB* mutant compared to the *lipA* mutant (Fig. 6I). Because pyruvate oxidase is part of the electron transport chain and the *lipA poxB* mutant consumes less oxygen than the *lipA* strain (Fig. 6H), we tested the contribution of PoxB to the level of adenosine triphosphate (ATP) found in the *lipA* mutant, using a luciferase-based assay, but found no difference between the *lipA* and *lipA poxB* strains (Fig. S5B). The results presented in this section demonstrate that pyruvate oxidase and acetyl-CoA synthetase function in the same pathway to extend the lifespan of the *lipA* mutant via the metabolism of acetate.

Discussion

Our genome-wide screen for *E. coli* mutants with prolonged stationary-phase survival revealed three strains, which live longer independently of the alkaline conditions that incubation in LB medium normally generates and also independently of reduced growth rates and saturation densities. Lack of subunit A of succinate dehydrogenase leads to increased stress resistance and extended lifespan, possibly linked to reduced superoxide generation. On the other hand, the extended lifespan of the longest-lived mutant, *lipA*, is entirely dependent on the conversion of pyruvate to acetyl-CoA via acetate by the pyruvate oxidase/acetyl-CoA synthetase (PoxB/Acs) enzyme pair. The hypoxia transcription factor ArcA contributes to the extended lifespan of the *lipA* strain, but it does so independently of acetate metabolism (Fig. 7).

Succinate dehydrogenase is a tetrameric protein complex catalyzing the conversion of succinate to fumarate in the TCA cycle coupled to electron transport to the ubiquinone pool (Yu & Yu, 1980). The enzymatically active part of the complex, subunit A encoded by *sdhA*, is a well-established source of superoxide in the electron transport chain of *E. coli* through its covalently bound flavin cofactor, which is an efficient single-electron donor

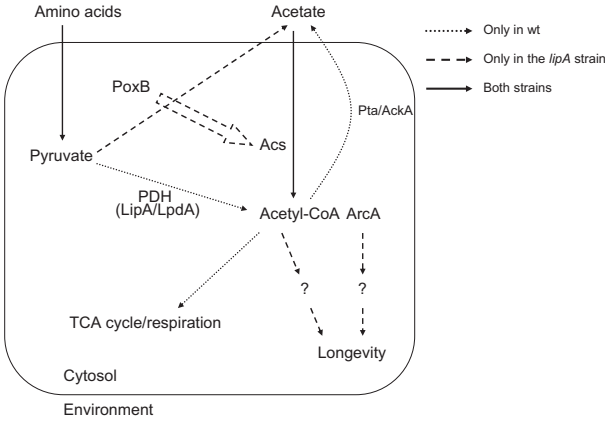

Fig. 7 Model for lifespan regulation by LipA in *Escherichia coli*. In the wt strain, pyruvate generated from the metabolism of amino acids present in LB medium is converted to acetyl-CoA by the PDH complex; acetyl-CoA is subsequently consumed by the TCA cycle or converted to acetate by Pta/AckA as the capacity of the TCA cycle to consume acetyl-CoA becomes limiting. In the *lipA* strain, the PDH complex is inactive and pyruvate is instead converted to acetate and carbon dioxide by PoxB. In both strains, extracellular acetate is taken up and converted to acetyl-CoA by Acs. The PoxB/Acs bypass of the PDH complex is required for lifespan extension in the *lipA* strain. The hypoxia-inducible transcription factor ArcA contributes to the extended lifespan of the *lipA* strain independently of the PoxB/Acs bypass. The block arrow denotes the activation of *acs* transcription by PoxB. PDH: pyruvate dehydrogenase complex, Pta/AckA: phosphotransacetylase/acetate kinase, PoxB: pyruvate oxidase, Acs: acetyl-CoA synthetase, LipA: lipoic acid synthase, LpdA: lipoamide dehydrogenase.

to molecular oxygen (Messner & Imlay, 2002). We are showing that lack of this enzyme results in a reduced rate of superoxide production in early stationary phase, which is accompanied by extended stationary-phase survival (Fig. 4). The accumulation of oxidatively damaged macromolecules in the form of protein carbonyls has been reported in stationary-phase *E. coli* (Dukan & Nystrom, 1998). More recently, these damaged proteins were shown to preferentially accumulate in cells that are about to lose viability and show lower expression of both cytosolic superoxide dismutases (Desnues *et al.*, 2003). Mutants lacking proteins that provide defense against oxidative stress such as superoxide dismutases and catalases have reduced lifespan (Eisenstark *et al.*, 1992), and incubation of stationary-phase *E. coli* in the absence of oxygen results in a slower rate of viability loss (Conter *et al.*, 2001). Observations of this kind implicate oxidative stress as a possible causative factor in the deterioration of stationary-phase *E. coli* and are consistent with our finding of extended lifespan in the *sdhA* strain. We have previously shown that increased scavenging of superoxide extends the lifespan of *S. cerevisiae* (Fabrizio *et al.*, 2003). However, the overexpression of both *SOD1* and *SOD2* caused a 30% lifespan extension versus the threefold extension observed in mutants lacking signal transduction genes (Fabrizio *et al.*, 2003) in agreement with the longer lifespan of the *lipA* and *lpdA* mutants compared to that of the *sdhA* mutant reported in this study.

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 & 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 & Lin, 1996). Furthermore, a microarray analysis of the response of *E. coli* to oxygen limitation also placed *lpdA* among the most down-regulated genes in response to the absence of oxygen (Salmon *et al.*, 2005). We found that the *lipA* and *lpdA* mutants consume less oxygen than wt (Fig. 3A) and that their fully extended lifespan is dependent on ArcA (Fig. 5B). Taken together, these observations suggest that maximum lifespan extension in these mutants is dependent on physiological changes that are normally induced by hypoxic conditions in wt. Inactivation of the protein complexes LipA and LpdA participate in, individually or in combination, had no positive effect on survival (Fig. S2F). It is therefore possible that lack of LipA or LpdA is specifically required to induce the ArcA-dependent physiological changes leading to extended survival and that inactivation of components of the PDH, 2-KGDH or glycine cleavage systems is not sufficient to induce these changes.

Nystrom *et al.*, (1996) reported that the strain lacking ArcA survives poorly during glucose–starvation–induced stationary phase. This transcription factor was shown to be required for the down-regulation of TCA cycle genes including *sdhA* and *lpdA* upon stationary-phase entry (Nystrom *et al.*, 1996). The *arcA* mutant also consumed more oxygen than its wt counterpart. The requirement of ArcA for survival under these conditions was attributed to the minimization of oxidative damage caused by unchecked respiration and possibly to the regulation of the utilization of endogenous carbon reserves, such as membrane lipids (Nystrom *et al.*, 1996). We found ArcA to be required only for the extended survival of the *lipA* and *lpdA* mutants, but not for the survival of wt (Fig. 5A,B). The difference between our results and those of Nystrom *et al.* may be due to the different medium used, minimal glucose in their study versus LB in ours. Similar to anaerobiosis, glucose, which is virtually absent from LB medium, is well known to cause the suppression of enzymes of the TCA cycle (Halpern *et al.*, 1964), because energy can be produced solely through the glycolytic Embden–Meyerhof–Parnas pathway. On the other hand, incubation of wt (and the *sdhA* mutant) in LB is expected to elicit the activation of the TCA cycle for the generation of energy using amino acids, whereas TCA cycle genes are down-regulated in the *lipA* and *lpdA* mutants owing to the combined loss of the PDH and 2-KGDH complexes (Li *et al.*, 2006). Hence, the results of that study (Nystrom *et al.*, 1996) are consistent with ours in showing that ArcA is required for survival under metabolic conditions that do not rely on the function of the TCA cycle for energy generation (wt in glucose minimal medium and *lipA* and *lpdA* strains in LB).

The transcriptional regulator hypoxia-inducible factor 1 (HIF-1) mediates changes in gene expression in response to hypoxia in organisms as diverse as humans and nematode worms (Semenza, 2000; Shen *et al.*, 2005), and it can therefore be considered as the functional homolog of ArcA, although the two proteins do not display significant sequence similarity. Three different groups recently reported the involvement of HIF-1 in the regulation of the lifespan of *Caenorhabditis elegans*. Mehta *et al.*, (2009) reported that loss of VHL-1, the protein responsible for the degradation of HIF-1 under normoxic conditions, leads to extended lifespan. Zhang *et al.*, (2009) found that HIF-1 overexpression also leads to extended lifespan. Lastly, Chen *et al.*, (2009) showed that loss of HIF-1 causes lifespan extension under rich nutrient conditions but failed to show lifespan extension under dietary restriction. These studies reveal complex nutrient-dependent interactions between HIF-1 and lifespan regulation in *C. elegans* and, along with our results, point toward the adaptive response to oxygen shortage as a novel, evolutionarily conserved mechanism of lifespan extension.

Oxygen shortage in *E. coli* results in the production of acetate (Phue *et al.*, 2005). *E. coli* maintained in batch culture in LB undergo what has been described as the acetate switch, whereby the acetate initially produced by the culture is subsequently taken up and utilized by the TCA cycle for energy generation and biosynthesis (Wolfe, 2005). The phosphotransacetylase/acetate kinase enzyme pair converts acetyl-CoA to acetate (Yang *et al.*, 1999), and the excreted acetate is then taken up by acetyl-CoA synthetase (Kumari *et al.*, 1995). Importantly, acetate metabolism has no effect on the survival of wt, because both the *pta* mutant, which makes no acetate (Hahm *et al.*, 1994) and the *acs* mutant, which cannot take up acetate (Kumari *et al.*, 1995), survive as long as wt (Figs S5E and 6G, respectively). The PDH complex, which converts pyruvate to acetyl-CoA in the wt strain is not functioning in the *lipA* mutant, and as a consequence, Pta is not involved in acetate production in this mutant (Fig. S4F).

We are showing that this mutant bypasses the PDH complex through the function of the PoxB/Acs enzyme pair, which converts pyruvate to acetyl-CoA via an acetate intermediate. Furthermore, this metabolic adaptation is fundamental for the extended lifespan of the *lipA* mutant (Fig. 6B,F). Note that the PoxB/Acs bypass of the PDH complex has been previously described in growing cultures (Abdel-Hamid *et al.*, 2001; Wolfe, 2005). The much smaller magnitude of the survival-extending effect of extracellular acetate in the wt compared to the *lipA* strain (Fig. 6D,E) might be attributable to the differential utilization of the molecule by the two strains: consumption by the TCA cycle, producing reducing equivalents used in the electron transport chain in the wt versus an unidentified, yet non-TCA-cycle-dependent utilization in the *lipA* mutant.

Thus, although the metabolism of acetate in the *lipA* mutant is broadly similar to that of wt (initial production from pyruvate followed by assimilation by acetyl-CoA synthetase), the extended lifespan of the *lipA* mutant is dependent on the PoxB–Acs bypass of the PDH complex (Fig. 6B,F), whereas neither the production of acetate by Pta–AckA nor its uptake by Acs affect the survival of wt (Figs 6G and S5C). The slower pace of acetate uptake in the *lipA* strain, as well as its nonutilization by the TCA cycle, are also likely factors favoring the lengthened survival of this mutant. Note that the elimination of extracellular acetate was recently shown to be partly responsible for dietary restriction-induced lifespan extension in wild-type *S. cerevisiae* (Burtner *et al.*, 2009).

In conclusion, we found that mutants that genetically promote aspects of hypoxic metabolism extend the stationary-phase survival of *E. coli*, in an ArcA- and acetate-dependent manner. Our laboratory recently reported the metabolic switch from TCA cycle/respiration to glycolysis and glycerol production as a central component of the lifespan extension observed in the *S. cerevisiae Tor1*Δ and *Sch9*Δ mutants (Wei *et al.*, 2009). Such mechanisms of lifespan extension are expected to be dependent on a broad metabolic repertoire that confers the ability to grow and survive both in the presence and in the absence of oxygen. A metabolic model for lifespan extension in the nematode worm *C. elegans*, which cannot survive in the absence of oxygen, also invoked the reduced use of aerobic respiration in favor of fermentative malate dismutation, producing acetate and succinate, as a common metabolic adaptation of most long-lived mutants described for this species (Rea & Johnson, 2003). Also, several studies recently implicated HIF-1, the functional homolog of ArcA found in metazoans in lifespan regulation in *C. elegans* (Chen *et al.*, 2009; Mehta *et al.*, 2009; Zhang *et al.*, 2009). Thus, the metabolic alterations leading to extended stationary-phase survival in the bacterium *E. coli* might reveal an evolutionary conservation of lifespan-regulating mechanisms that is not purely phenomenological.

Experimental procedures

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 latter reference also describes the pedigree of BW25113, a K-12 derivative. 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, for example *lipA*::FRT in Table S3), as described in (Datsenko & Wanner, 2000), with the only difference that the nonselective 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 in (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' web site (http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp). All strains used in the study are shown in Table S3.

Survival experiments

LB medium consisted of 1% bacto tryptone, 0.5% yeast extract and 0.5% NaCl w/v. 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 per mL by resuspending a pellet containing the desired number of cells in cell-free spent medium of the same strain for the long-lived strains with reduced saturation cell density. AMPSO or HEPES was added to 100 mM to achieve a stationary-phase pH of 9 or 7.5, respectively. Because of the different stationary-phase pH reached spontaneously by each strain, the pH of the buffers had to be adjusted accordingly for each strain. 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 colony-forming units (CFU) 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 'high aeration' experiment shown in Fig. S2E (Supporting information), cultures were grown and maintained in 10-mL volume in orbitally shaking 125-mL Erlenmeyer flasks. For experiments shown in Fig. S4D–E(Supporting information), medium collected after centrifugation of an early stationary-phase (12 h of incubation) wt culture was filter-sterilized by passing through a 0.22-μm filter and its pH adjusted to 9 by the addition of 100 mM AMPSO. Early stationary-phase cultures of wt and the *lipA* mutant were centrifuged and washed once with 0.5% NaCl, and the washed pellet was then resuspended in 30 μL of 0.5% NaCl and transferred to the conditioned medium prepared as mentioned earlier. For survival experiments in 0.5% NaCl, the same washing procedure was followed, and cells were then resuspended in 2 mL 0.5% NaCl to which appropriately buffered AMPSO was added to 100 mM.

Phase-contrast microscopy

Cultures of wt and the *lipA* strain were processed as described earlier with AMPSO. One hour after the AMPSO processing, cultures were washed once with 0.5% NaCl and 5 μL were spread onto a glass slide and air-dried. The dried spots were incubated for 5′ with 100% methanol to fix the cells and then washed three times with phosphate-buffered saline, pH 7.4. Images were obtained under 100 × magnification, and cell length was measured using the ImageJ software (Freeware, available at rsbweb.nih.gov/ij/).

Stress resistance

For the heat shock experiment, cultures were processed as described earlier with AMPSO, returned to the incubator and, 4 h later (16 h after inoculation), were subjected to a 4-min incubation in a 55°C water bath without shaking and CFU enumerated before and after the treatment.

For the paraquat experiment, cultures were processed as described earlier with AMPSO, and paraquat (methyl viologen dichloride hydrate obtained from Sigma-Aldrich, St.Louis, MO, USA) was added to 500 μM at the time of processing (12 h after inoculation). CFU were enumerated before and after a 12-h incubation at 37°C.

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 carried out by assuming that the liquid culture contains the same amount of oxygen as water equilibrated with 21% oxygen in one atmosphere pressure, which is 5.02 μL mL^{-1} (manufacturer's manual) and was further normalized by the number of CFU present. Cyanide-resistant respiration was measured after a 5-min incubation in the 37°C water bath with 1 mM sodium cyanide. 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 R-Biopharm (Darmstadt, Germany) acetic acid kit (catalog number 10148261035) according to the manufacturer's instructions.

acs mRNA quantification

One-milliliter aliquots of late stationary-phase cultures (24 h after inoculation) were added to a 95 μL ethanol + 5 μL 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, catalog number MC85200) according to the manufacturer's instructions. Three micrograms of RNA were 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. Fifty nanograms of reverse-transcribed RNA were then 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 both the *lipA* and *lipA poxB* 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.

Mutation frequency measurement

Cultures were processed as described earlier with HEPES (pH 7.5), with the only difference that cultures were maintained in a volume of 25 mL in 125-mL Erlenmeyer flasks. Rifampicin-resistant mutants were quantified by plating on LB plates containing

the antibiotic at 120 μg mL^{-1} after washing once with 0.5% NaCl. The colonies grown in rifampicin were enumerated and normalized by the total number of CFU at each time-point. Note that rifampicin plates were checked for 3 days after plating and late-appearing colonies (the rifampicin resistance of which was confirmed) were counted.

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Author contributions

SG designed and performed experiments, analyzed data and wrote the manuscript; SEF designed experiments and wrote the manuscript; and VDL designed experiments and wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Schematic representation of the absorbance-based, genome-wide screen for extended stationary-phase survival in *E. coli*.

Fig. S2 Related to Fig. 1. (A–C) colony-forming units (CFU) titers of experiments shown in Fig. 1A–C. (D) Growth curves as CFU

counts of wt and the long-lived strains. (E) Survival of 10-mL cultures of wt and the *lipA* strain in 125-mL flasks. (F) Survival of the shown strains.

Fig. S3 Related to Fig. 4. (A) Effect of the overexpression of SodB (pHS1-7) on the survival of wt (pBR322 empty vector). (B) Time-dependent frequency of rifampicin-resistant mutants in wt and the *sdhA* strain. (C) Growth curves as colony-forming units counts of the shown strains. (D) Stationary-phase survival of all strains included in the mutation frequency experiment.

Fig. S4 Related to Fig. 6. (A) Extracellular acetate concentration at late stationary phase of the shown mutants. (**B**) Effect of sodium chloride or sodium acetate added to 6 mM at the time-point designated by the arrow on the survival of the *lipA* mutant. (C, D) Stationary-phase survival of the shown mutants at pH 7.5. (E) Stationary-phase survival of the shown mutants at pH 9. (F) Extracellular acetate concentration of the shown mutants over time. (G, H) Expression of *pta* and *ackA* in wt and the *lipA* strain over time.

Fig. S5 (A) Rate of oxygen consumption of the shown mutants at early stationary phase. (B) ATP levels of the shown strains, measured using firefly luciferase. (C) Survival of the wt and *pta* strains.

Table S1 Stationary-phase regrowth ratios of strains of the KEIO collection (see supplementary experimental procedures for details).

Table S2 Stationary-phase pH and CPII density of wt and long-lived mutants.

Table S3 List of strains used in this study.

Data S1 Supplementary materials and methods.

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