The EMBO Journal vol.15 no.13 pp.3219–3228, 1996

Bacterial defense against aging: role of the *Escherichia coli* ArcA regulator in gene expression, readjusted energy flux and survival during stasis

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Using two-dimensional gel electrophoresis and N-terminal amino acid sequencing analysis, we demonstrate that a mutant of the global regulatory protein ArcA fails to decrease the synthesis of the TCA cycle enzymes malate dehydrogenase, isocitrate dehydrogenase, lipoamide dehydrogenase E3 and succinate dehydrogenase in response to stasis, while the increased production of the glycolysis enzymes phosphoglycerate mutase and pyruvate kinase is unaffected. Microcalorimetric and respiratory measurements show that the continued production of TCA cycle enzymes in the Δ*arcA* mutant is manifested as an elevated rate of respiration and total metabolic activity during starvation. The Δ*arcA* mutant is severely impaired in surviving prolonged periods of exogenous carbon starvation, a phenotype that can be alleviated by overproducing the superoxide dismutase SodA. In addition, flow cytometry demonstrates that starving Δ*arcA* mutant cells, in contrast to wild-type cells, fail to perform reductive division, remain large and contain multiple chromosomal copies. We suggest that the ArcA-dependent reduced production of electron donors and the decreased level and activity of the aerobic respiratory apparatus during growth arrest is an integral part of a defense system aimed at avoiding the damaging effects of oxygen radicals and controlling the rate of utilization of endogenous reserves.

Keywords: aging/ArcA/*Escherichia coli*/glucose starvation stimulon/SodA

Introduction

While multicellular organisms and their component cells are intrinsically mortal, unicellular prokaryotes, like *Escherichia coli*, appear to be immortal as long as the environment supports self-replication, and no equivalent of the commitment to death found in whole macrobes can be observed (Postgate, 1976). Yet, unicellular prokaryotes do die if circumstances arrest multiplication, and it has been suggested that specific genetic programs have evolved to prolong the survival of non-growing prokaryotes. Cells of non-differentiating bacteria continue to synthesize proteins during carbon–energy starvation, and starvation-specific proteins can be grouped in different classes according to their time of appearance during starvation (Groat *et al.*, 1986; Nyström *et al.*, 1990; Nyström and Neidhardt, 1994). Matin and co-workers (Reeve *et al.*, 1984) suggested that the synthesis of one or several proteins belonging to the 'early' class of starvation proteins is/are required for long-term survival because adding protein synthesis inhibitors for a brief period at the onset of carbon starvation dramatically decreases long-term survival of *E.coli*; adding inhibitors later has little effect. This was later shown to be true also for *Salmonella typhimurium* (Spector and Foster, 1993) and the *Vibrio* S14 strain (Nyström *et al.*, 1990, 1992). Recent work in several laboratories has confirmed the notion of Matin and co-workers, and survival-related networks and individual genes have been genetically identified (Jenkins *et al.*, 1988; Spence *et al.*, 1990; Tormo *et al.*, 1990; Lange and Hengge-Aronis, 1991; McCann *et al.*, 1991; Li and Clark, 1992; Spector and Cubitt, 1992; Nyström, 1994a; Nyström and Neidhardt, 1994). Thus, we know that utilizable energy derived from endogenous material allows proteins to be synthesized in stationary phase cells and that this protein synthesis is important for stasis survival. But what endogenous materials and central metabolic pathways are used for generating the utilizable energy supporting continued translation, and how does the cell regulate the degradation of these reserves to satisfy the requirements of maintenance energy? While these questions remain largely unanswered, the regulatory systems that allow the *E.coli* cell to exploit any given growth-promoting situation are beginning to be understood.

In our attempts to achieve a holistic understanding of the physiological adjustments required by *E.coli* to survive in the absence of a growth-supporting carbon and energy source, we have studied starvation-induced gene expression at the whole cell level (Nyström and Neidhardt, 1992; Nyström, 1993, 1994a,b, 1995a,b) using the 'proteome' (the protein complement expressed by a genome) research approach (Pedersen *et al.*, 1978; VanBogelen *et al.*, 1992; Kahn, 1995). This approach has demonstrated that the response of *E.coli* to starvation for exogenous carbon and energy includes an increased synthesis of the glycolysis enzymes, and pyruvate formate lyase, phospho-transacetylase and acetate kinase concomitantly with a reduced production of TCA cycle enzymes (Nyström, 1994a). Thus, the modulation of the synthesis of catabolic enzymes during aerobic carbon–energy starvation is remarkably similar to the response of cells shifted to anaerobiosis (Smith and Neidhardt, 1983a,b).

Escherichia coli can grow under aerobic and anaerobic conditions, deriving energy from a number of different respiratory pathways or from fermentation. Under aerobic conditions, *E.coli* uses the aerobic respiratory chain with oxygen as the terminal electron acceptor. In the absence of oxygen, anaerobic respiratory pathways using alternative electron acceptors such as fumarate, nitrate, nitrite,

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dimethylsulfoxide or trimethylamine-*N*-oxide, can replace the aerobic respiratory pathway. In the absence of any of these electron acceptors, the cell has to rely on fermentation to generate utilizable energy. A number of genetic regulatory programs in *E.coli* coordinately direct the cells' selection of the most efficient metabolic system in a particular environment. This selection ensures that electrons are channeled from donors to a terminal acceptor such that the drop in Gibbs energy is the maximal allowed under the particular growth condition (Iuchi and Lin, 1993). This regulation, presumably, optimizes the metabolic systems used to maximize growth rate in a given environment. Several global regulatory systems, including the ArcA–ArcB, NarL–NarX, Fnr (e.g. Iuchi and Lin, 1993) and FruR systems (Ramseier *et al.*, 1995) are involved in this metabolic regulation. The ArcA–ArcB pair makes up a two-component regulatory system which is activated when the environment contains no electron acceptors or only poor ones; ArcA being the regulator and ArcB the sensor component (Iuchi and Lin, 1988). When phosphorylated during anaerobiosis, ArcA becomes active and acts as a repressor of genes encoding dehydrogenases of the flavoprotein class, several enzymes of the TCA cycle and the cytochrome *o* oxidase complex (Iuchi and Lin, 1991). In other cases, ArcA acts as an activator; the cytochrome *d* and pyruvate formate lyase (*pfl*) operons are positively regulated by ArcA (Iuchi and Lin, 1988; Sawers and Suppman, 1992).

Table I. N-terminal amino acid sequence of protein F030.2

Organism	N-terminal sequence
E.coli G027.1	AVTKLVLVRH
E.coli Gpm	MAVTKLVLVRH
S.cerevisiae Gpm	PKLVLVRH
R.norvegicus Gpm	AAYKLVLIRH
H.sapiens Gpm	AAYKLVLIRH
Streptomyces coelicolor Gpm	ADAPYKLILLRH

The six best matches of the databases searched are depicted. The N-terminal sequence of malate dehydrogenase (Mdh) is extremely conserved, as expected, considering that this part harbors a dinucleotide binding domain.

In this study, we show that the ArcA regulator is a key player also in starvation-induced modulations of gene expression and that an Δ*arcA* mutant is impaired in several activities associated with the *E.coli* starvation response and survives poorly during long-term stasis. We report that the ArcA modulon is involved in checking the rate of catabolic degradation of endogenous biomolecules and in mitigating potentially harmful effects of aerobic respiration during stasis.

Results

Identified members of the glucose starvation stimulon

We have observed previously that the synthesis of proteins G027.1 and F030.2 (VanBogelen *et al.*, 1990; VanBogelen and Neidhardt, 1991) were markedly affected by aerobic glucose starvation; the kinetics of synthesis of G027.1 and F030.2 are similar to those of the glycolysis and TCA cycle enzymes, respectively (Nyström, 1994a). N-terminal sequencing identified G027.1 as phosphoglycerate mutase (Gpm) of the glycolysis pathway (Table I). While most glycolysis enzymes increase only moderately during starvation (Nyström, 1994a), the rate of Gpm synthesis increased steadily and became one of the 10 proteins having the highest rate of synthesis during long-term starvation (Figure 1). Coomassie brilliant blue-stained gels demonstrated that the product was stable and accumulated in growth-arrested cells (not shown). The F030.2 protein was identified as the TCA cycle enzyme malate dehydrogenase (Mdh; Figure 1; Table II). The rate of Mdh synthesis decreased drastically during carbon starvation, as does the rate of synthesis of other TCA cycle enzymes analyzed (Nyström, 1994a).

In addition to Gpm and Mdh, a large number of enzymes involved in central metabolism have been identified as members of the glucose starvation stimulon (Figure 2; Nyström, 1994a). The levels of several of these enzymes are known to be controlled by the ArcA–ArcB regulatory system; anaerobically ArcA represses the expression of the genes of the TCA cycle enzymes *icd*, *lpd*, *sucB*, *sucC*, *sdhA* and *mdh* but activates *pfl* (Figure 2; Lin and Iuchi, 1991). Therefore, we examined whether ArcA is also

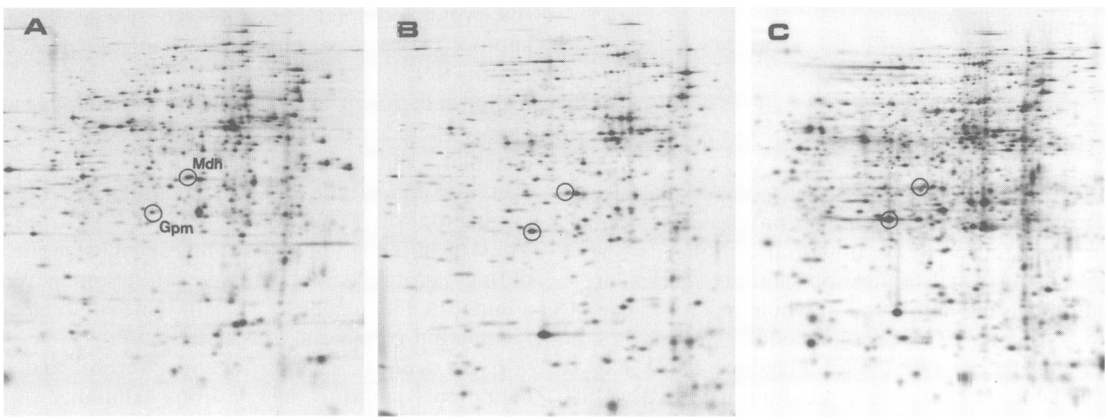

Fig. 1. Autoradiograms of 2-D polyacrylamide gels of extracts of *E.coli* W3110 growing aerobically in glucose minimal MOPS at 30°C (**A**) or glucose starved for 3 h (**B**) and 7 days (**C**). Cells were labeled for 5 min with [^{35}S]methionine during exponential growth, for 15 min after 3 h of starvation and for 1 h after 7 days of starvation. Abbreviations: Mdh, malate dehydrogenase, Gpm, phosphoglycerate mutase. For pI and molecular weight designations of protein spots in the autoradiograms, consult the reference gels of the 'Gene–protein database of *Escherichia coli*' (VanBogelen *et al.*, 1990).

Table II. N-terminal amino acid sequence of protein G027.1

Organism	N-terminal sequence
E.coli F030.2	MKVAVLGAAGIG
E.coli Mdh	MKVAVLGAAGIG
H.influenzae Mdh	MKVAVLGAAGIG
Citrullus lanatus Mdh	MKVAVLGAAGIG
Eucalyptus gunnii Mdh	MKVAVLGAAGIG
S.typhimurium Mdh	MKVAVLAAAGIG
Photobacterium sp. Mdh	MKVAVIGAAGIG

The five best matches of the databases searched are depicted. The amino acid sequencing data suggest that the initiator methionine is absent in the mature phosphoglycerate mutase (Gpm) protein.

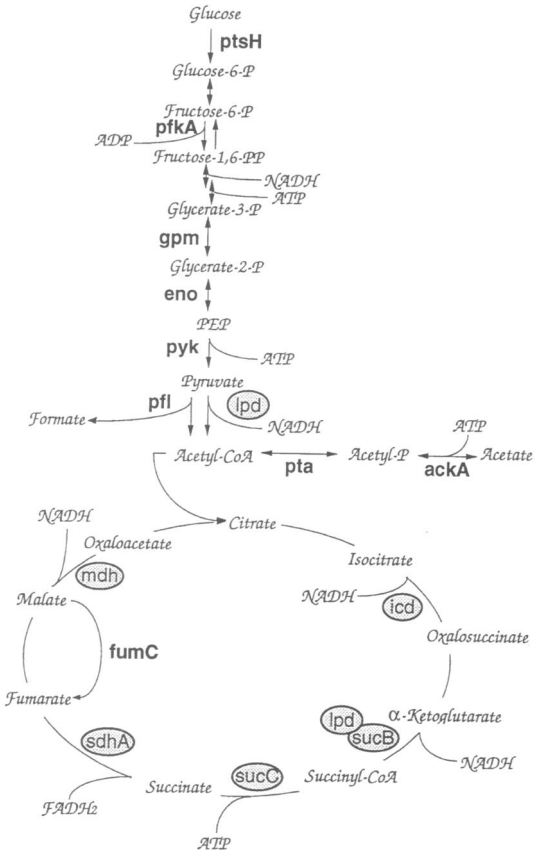

Fig. 2. Some principal routes for carbon metabolism with enzymes found to be members of the glucose starvation stimulon. Symbols in bold face denote enzymes induced by glucose starvation. Circled symbols denote enzymes repressed by glucose starvation. In general, gene symbols are used to identify enzymes. Abbreviations: ackA, acetate kinase; eno, enolase; gpm, phosphoglycerate mutase; icd, isocitrate dehydrogenase; lpd, lipoamide dehydrogenase E3; mdh, malate dehydrogenase; pfkA, phosphofructokinase; pfl, pyruvate formate lyase; pta, phosphotransacetylase; ptsH, phosphohistidinoprotein–hexose phosphotransferase; pyk, pyruvate kinase; sdhA, succinate dehydrogenase; sucB, α-ketoglutarate dehydrogenase, E2; sucC, succinyl-CoA synthetase, β subunit.

required for the regulation of the flow of carbon in central metabolism during aerobic starvation by determining the effect of an Δ*arcA* mutation on (i) the rate of synthesis of glycolysis and TCA cycle enzymes, (ii) rates of respiration and total metabolic activity, and (iii) the cells ability to survive.

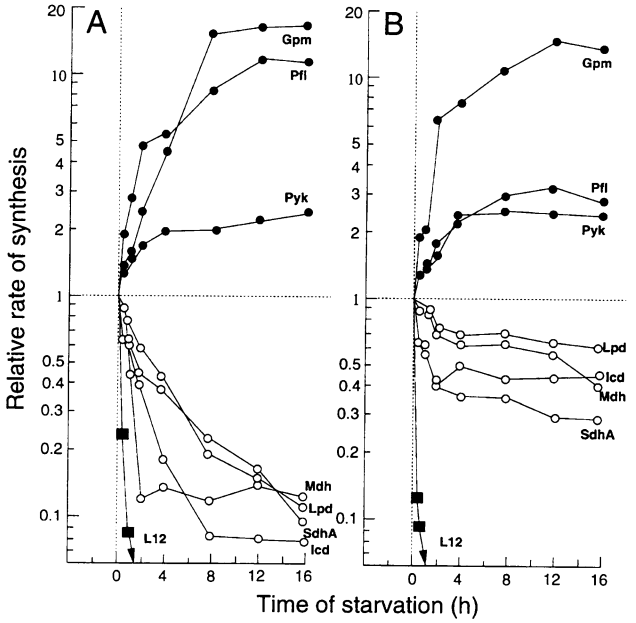

Fig. 3. Relative rates of synthesis of individual proteins at various times after glucose depletion in the wild-type W3110 strain (**A**) and the isogenic Δ*arcA* mutant (**B**). All values are normalized to the measured pre-shift rate of synthesis. Abbreviations: Mdh, malate dehydrogenase; Icd, isocitrate dehydrogenase; Lpd, lipoamide dehydrogenase E3; SdhA, succinate dehydrogenase; Gpm, phosphoglycerate mutase; PykA, pyruvate kinase; Pfl, pyruvate formate lyase; L12, ribosomal protein large subunit 12.

ArcA is involved in controlling the levels of TCA cycle enzymes but not glycolysis enzymes during aerobic glucose starvation

The Δ*arcA* mutant and the wild-type parent were pulse-labeled with [^{3}H]leucine during exponential growth and after growth ceased due to glucose depletion. Analysis by two-dimensional gel electrophoresis revealed that the decreased synthesis of the TCA cycle enzymes selected for analysis, Mdh, isocitrate dehydrogenase (Icd), lipoamide dehydrogenase subunit E3 (Lpd) and succinate dehydrogenase (SdhA), was much less pronounced in the Δ*arcA* mutant while the kinetics of synthesis of glycolysis enzymes Gpm and pyruvate kinase (Pyk) were essentially identical in the mutant and the wild-type strains (Figure 3). In addition, the synthesis of pyruvate formate lyase (Pfl) was only weakly induced in Δ*arcA* as compared with the parent (Figure 3), consistent with the role of ArcA as an activator of *pfl* (Sawers, 1993). Thus, the reduced and increased synthesis during glucose starvation of the TCA cycle enzymes and Pfl respectively is, in part, the result of activation of the ArcA regulator. However, the increased synthesis of glycolysis enzymes during starvation is apparently governed by another regulatory system activated in parallel with the ArcA modulon. The result with respect to TCA enzyme synthesis in the Δ*arcA* mutant cannot be explained simply by a slow or delayed starvation response, since the rate of synthesis of ribosomal protein L12 decreased instantly in both the wild-type and Δ*arcA* mutant strain. In addition, synthesis of ribosomal proteins L12, L7 and S6 could not be detected by 2-D analysis in either the wild-type or Δ*arcA* strain after 2 h of starvation, showing that no growth occurred in any fraction of the starving population.

Table III. Total metabolic and respiratory activity in wild-type and Δ*arcA* mutants during growth and starvation

Strain	Total activity[a] [J/(OD×l×h)$^{-1}$]		Respiratory activity[b] [J/(OD×l×h)$^{-1}$]		Respiratory activity/total activity	
	Log	Starvation[c]	Log	Starvation[c]	Log	Starvation[c]
W3110 (wt)	1323	350	1072	140	0.81	0.40
TN616 (Δ*arcA*)	1248	1087	960	881	0.77	0.81

[a]The total activity data are derived from direct microcalorimetric measurements. The data are normalized to the optical density (420 nm) of the culture.
[b]The heat production rate derived from respiration was calculated as described in Materials and methods.
[c]The data in this table are from cells starved for 12 h.

An arcA mutant exhibits an elevated rate of respiration and total metabolic activity during starvation

The results described above suggest that wild-type *E.coli* cells deprived of exogenous glucose reduce the levels of enzymes generating electron donors for respiration and that this response is much less pronounced in Δ*arcA* mutant cells. To examine whether this alteration in gene expression is manifested in measurable differences in the cell's catabolic activities, we applied non-disruptive respirometric measurements in combination with microcalorimetric measurements of heat production in growing and starving cells. This approach allows us to measure total metabolic activity and to calculate the fraction of the total activity which is derived from aerobic respiration. We found that the total metabolic activities of wild-type and Δ*arcA* mutant cells were indistinguishable during exponential growth (Table III) but differed significantly during glucose starvation (Figure 4; Table III). As expected from the 2-D analysis, the respiratory activity per unit mass was significantly higher in the Δ*arcA* mutant than in the wild-type parent, as was the total metabolic activity and the fraction of total activity derived from aerobic respiration (Table III; the fraction of heat production that is derived from aerobic respiration is calculated as described in Materials and methods).

Next the metabolic capacity of starving populations was determined by measuring heat production and respiratory activity after giving the starved cells a pulse of glucose. The glucose-starved wild-type cell exhibited a reduced capacity for aerobic respiration, while the Δ*arcA* mutant maintained a high capacity for aerobic respiration during starvation (Tables III and IV). In addition, the glucose taken up by the cells was catabolized via respiration to a higher extent in the Δ*arcA* mutant than in the wild-type (Table IV). As shown in Figure 5 and Table IV, the wild-type cells instantly increased their respiratory (4.8-fold) and total metabolic activity (3-fold) in response to glucose addition. In contrast, the starved Δ*arcA* mutant cells appeared to perform at a near maximal rate since only a marginal increase in respiration (1.2-fold) and total activity (1.3-fold) occurred in response to exogenously added glucose (Figure 5 and Table IV). These measurements corroborate the results of the 2-D analysis demonstrating that wild-type *E.coli* cells subjected to aerobic starvation for exogenous glucose decrease their levels of enzymes involved in generating reducing power and reduce their respiratory capacity, while cells lacking functional ArcA do not. The starved wild-type cell also regulates its catabolic activities by controlling the activity of the existing catabolic systems (and/or the rate of substrate production made available for catabolism) because the increase in total metabolic activity occurred before the cells could accumulate new proteins (within seconds after the glucose pulse). The Δ*arcA* mutant cell, however, fails to perform this control.

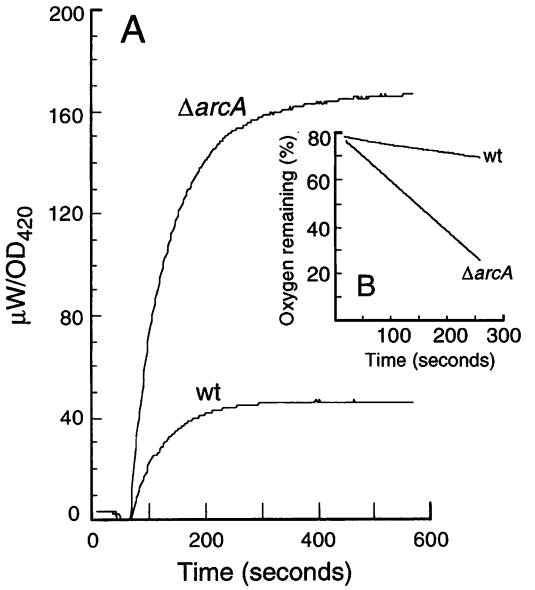

Fig. 4. Heat production (**A**) and oxygen consumption (**B**) of 12 h glucose starved wild-type and Δ*arcA* mutant cells. Heat production was measured as μW dissipated per unit time and volume (0.46 ml) cell suspension and subsequently was normalized to the optical density (420 nm) of the cell culture. Rates of respiration were measured as oxygen consumed per time and subsequently were normalized as described. The heat production rate derived from respiration was then calculated as described in Materials and methods.

The arcA mutant is greatly impaired in surviving aerobic glucose starvation

Since the Δ*arcA* mutant is defective in regulating its catabolic activity, we next asked if this response is important for the longevity of carbon-starved cells? We approached this question simply by determining the ability of Δ*arcA* mutant cells to form colonies after different times of aerobic glucose starvation. As depicted in Figure 6A, the Δ*arcA* mutant was found to be severely impaired in its ability to survive glucose starvation. The colony-forming capacities of the wild-type and Δ*arcA* strains were indistinguishable during the first 12 h of starvation, after which the rate of die-off increased markedly in the Δ*arcA* mutant (Figure 6A).

Table IV. Total metabolic and respiratory activity of 12 h starved wild-type and Δ*arcA* cells after a pulse of glucose (0.2% glucose)

Strain	Total activity [J/(OD×1×h)$^{-1}$]	Respiratory activity		Glucose consumption[b] [mmol/(OD×h)$^{-1}$]	Relative increase[c]	
		[J/(OD×1×h)$^{-1}$]	(O_2/glucose)[a]		Total activity	Respiratory activity
W3110 (wt)	1050	675	1.00	1.40	3.0	4.8
TN616 (Δ*arcA*)	1448	1057	1.46	1.95	1.3	1.2

[a]Respiratory activity is expressed as mmole of oxygen respired per mmol of glucose taken up by the cells.
[b]Expressed as mmol/(OD×h)$^{-1}$.
[c]The relative increase is calculated from the activity measured after the addition of glucose divided by the activity measured prior to the glucose pulse.

The poor survival of the arcA mutant can be alleviated by overproducing the superoxide dismutase SodA

Next we asked whether the ArcA-dependent response during starvation may serve to minimize production of harmful species of oxygen. The superoxide radical arises as a by-product of all aerobic respiration and can be scavenged by at least two superoxide dismutases in *E.coli*, SodA and SodB (Carlioz and Touati, 1986). We approached the question by transforming the Δ*arcA* mutant with plasmid pDT1-19 (gift from D.Touati) which is pBR322 carrying *lacI*^{q} and an operon fusion between the *tac* promoter and the *sodA* gene. The wild-type and Δ*arcA* control cells were transformed with pKK223-3 which is pBR322 carrying the *tac* promoter alone. Cells were grown with 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG) in glucose-limited M9 medium and samples were taken for colony-forming units (c.f.u.) determinations throughout a period of 9 days of glucose starvation. As shown in Figure 6B, overproduction of SodA markedly mitigated the harmful effects of the Δ*arcA* mutation and no significant difference could be observed between the wild-type and the Δ*arcA* mutant overexpressing *sodA* during the first few days of starvation. However, the ability of Δ*arcA*/pDT1-19 cells to form colonies dropped drastically after ~4 days of starvation and eventually reached the same value as the Δ*arcA* control cells (Figure 6B). The Δ*arcA*/pDT1-19 cells that had been grown without IPTG also exhibited an enhanced survival capacity as compared with the Δ*arcA* control during the first days of starvation but died-off more rapidly than the cells cultivated in the presence of IPTG (not shown). Presumably, the leakiness of the *tac* promoter and the high copy number of the gene provided the Δ*arcA*/pDT1-19 cells with an elevated level of SodA even without the addition of inducer.

The arcA mutant does not complete reductive cell division after glucose exhaustion

The final yield of cells was found to be lower in the Δ*arcA* mutant population, and microscopic analysis revealed that the size of starving Δ*arcA* cells markedly exceeded that of the wild-type. This was found to be due to the failure of Δ*arcA* cells to perform reductive division [continued cell division after completion of ongoing DNA replication during conditions that do not allow for cell growth (Nyström and Kjelleberg, 1989)] after the depletion of the carbon source (Figure 7). Flow cytometer analysis demonstrated that the cell size and chromosome content of exponentially growing wild-type and Δ*arcA* cells were very similar (not shown) but differed significantly during starvation (Figure 8). The median cell size of 24 h starved Δ*arcA* mutants exceeded that of the wild-type parent by a factor of 3 (Figure 8A). While the 24 h starved wild-type cells contained either one or two chromosomes (Figure 8B), most starved Δ*arcA* cells contained two or four chromosomes (Figure 8B), as did both wild-type and mutant cells growing exponentially in glucose minimal medium (μ = 61 min in wild-type and 69 min in Δ*arcA*). Reductive division in wild-type *E.coli* follows immediately after normal division when the exogenous carbon source is depleted. Thus, the results suggest that the Δ*arcA* mutant encounters severe difficulties very early during starvation, long before a significant die-off can be observed (Figure 6A). The data showing that many stationary phase wild-type *E.coli* cells may contain two chromosomes (Figure 8B) demonstrate that each initiation of chromosome replication does not lead to cell division during the phase of reductive division at least during the growth and starvation conditions analyzed. This is in accord with the results of Åkerlund *et al.* (1995).

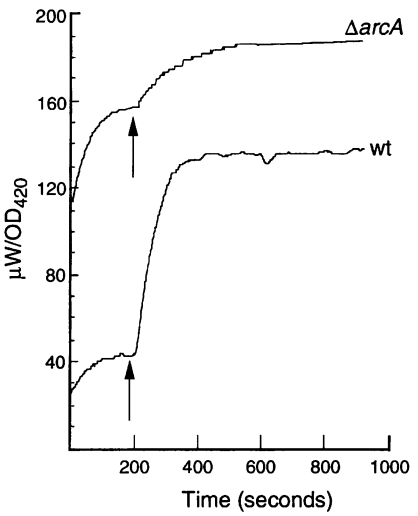

Fig. 5. Heat production of 12 h glucose-starved wild-type and Δ*arcA* mutant cells after receiving a pulse of glucose (0.2%). Arrows indicate the time at which glucose was added. Consult Figure 4 for methodology.

Discussion

We report that the glucose starvation stimulon (Neidhardt *et al.*, 1990) of *E.coli* encompasses the ArcA modulon (Iuchi and Lin, 1988). This modulon was found to be

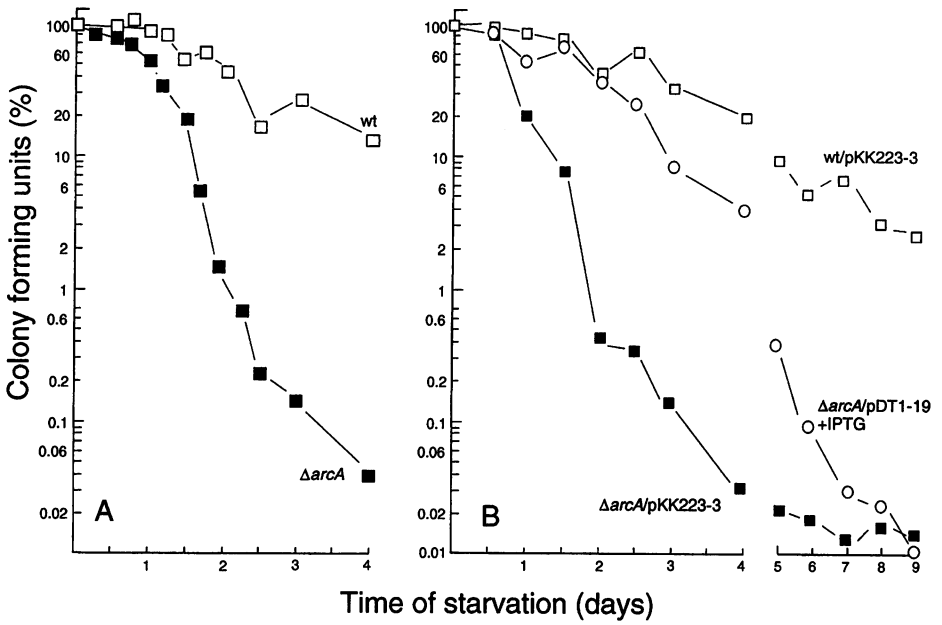

Fig. 6. (**A**) Survival of the wild-type (W3110; □) and Δ*arcA* (TN616; ■) mutant strains during glucose starvation. Strains were grown in glucose minimal M9 medium containing 0.04% glucose. After growth arrest commenced, incubation was continued for 4 days under the same conditions. Viable cells were counted as colonies plated on LB after appropriate dilutions. 100% viability corresponds to the number of viable cells (W3110, 2.4×10^9; TN616, 1.0×10^9) counted 1 h after growth was arrested due to the depletion of glucose. (**B**) Survival of Δ*arcA* cells overproducing SodA. Viability was determined for TN616 cells carrying pDT1-19 (○) or pKK223-2 (■) and wild-type W3110 cells carrying pKK223-3 (□). Cells were grown and starved in the presence of ampicillin (50 μg/ml) and IPTG (500 μM). 100% viability corresponds to the number of viable cells (W3110/pKK223-3, 2.1×10^9; TN616/pKK223-3, 1.0×10^9; TN616/pDT1-19, 1.1×10^9) counted 1 h after growth was arrested due to the depletion of glucose.

involved in adjusting the cell's catabolic activity during depletion of the exogenous carbon source. Specifically, ArcA is required for the observed decrease in the synthesis of NADH/$FADH_2$-generating TCA cycle enzymes and presumably other proteins known to be under control of ArcA, including cytochrome *o*. The ArcA-dependent response of glucose-starved wild-type cells is manifested in a reduced respiratory *activity* which may dictate the total metabolic activity, measured here as heat production. In addition, the decreased aerobic respiratory *capacity* of starving cells is dependent on a functional *arcA* gene. These results are in accordance with the data of Poole and Ingledew (1987) showing that the amount of the intermediate respiratory-component ubiquinone, predominantly used during aerobic respiration, decreased several-fold during stasis while the amount of menaquinone, predominantly used during anaerobic respiration, increased. In other words, the pattern of gene expression during aerobic stasis and anaerobiosis are conspicuously similar. Clearly, the ArcA-dependent modulation of the pattern of enzyme synthesis and flux of energy is extremely important, since cells lacking ArcA fail to perform reductive division early during starvation and lose viability rapidly after a few days of starvation. Why is the elevated rate of aerobic respiration in the starving Δ*arcA* cells so detrimental during growth arrest? We believe that several features of the ArcA-dependent response are of importance for the longevity of growth-arrested cells.

Firstly, the reduced production of respiratory substrate and components of the aerobic respiratory apparatus during starvation may be a defense mechanism mustered by the cell to protect itself against oxidative stress. The superoxide radical arises as a by-product of all aerobic respiration and, if not scavenged, this radical can damage critical biomolecules. In *E.coli*, two superoxide dismutases, encoded by *sodA* and *sodB*, protect the cells by removing superoxide radicals by catalyzing the reaction $O_2^- + O_2^- \rightarrow O_2 + H_2O_2$. The produced hydrogen peroxide can be removed subsequently by catalases, and the gene, *katE*, encoding one such catalase is specifically induced in stationary phase (von Ossowski *et al.*, 1991). Cells lacking functional superoxide dismutase activity (*sodA*, *sodB*

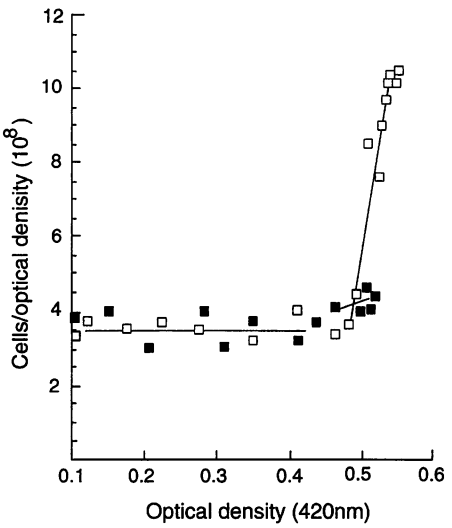

Fig. 7. Cell number in wild-type (□) and Δ*arcA* mutant (■) cultures during exponential growth and transition to glucose starvation-induced stationary phase. Cells were grown in glucose (0.02%)-limited M9 medium. In this medium, glucose is depleted at an optical density (420 nm) of 0.5 in the wild-type and 0.45 in the Δ*arcA* cultures.

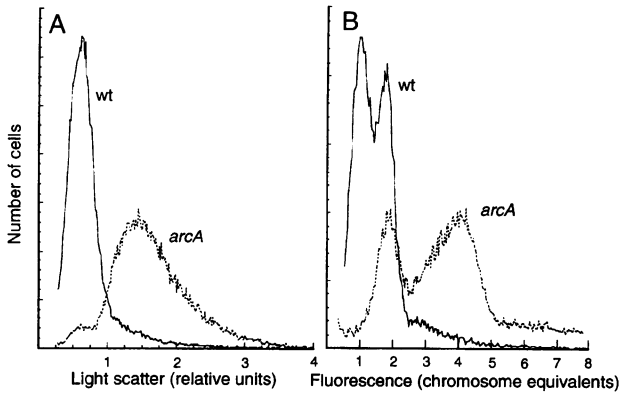

Fig. 8. Flow cytometry profiles of stationary (12 h) phase W3110 (wild-type) and TN616 (Δ*arcA*) cultures. Overnight cultures were diluted into fresh glucose (0.02%)-limited M9 medium and grown at 30°C until depletion of the glucose. The light scatter (**A**) and the fluorescence parameters (**B**) represent the relative cell size and the number of chromosome equivalents, respectively.

double mutant) can grow aerobically when supplemented with specific amino acids (Carlioz and Touati, 1986) but are greatly impaired in their ability to survive, aerobically but not anaerobically, in stationary phase (Benov and Fridovich, 1995). It may be argued that the sensitivity of *sodA*, *sodB* double mutants to stasis is related to the accumulation of inactive 'radical-damaged' biomolecules. Such damaged molecules may be diluted rapidly during growth but not during growth arrest. Obviously, it is critical that the long-term growth-arrested cell, which has little ability to produce new polypeptides, maintains a pool of functional proteins. Thus, the ArcA-dependent starvation response may be an integral component of a defense system aimed at minimizing potential damaging effects of oxygen. The effect on starvation survival of overproducing SodA in the Δ*arcA* mutant confirms this notion. Interestingly, *sodA* transcription is repressed by ArcA during anaerobiosis (Tardat and Touati, 1993). However, we have found that *sodA* is increasingly expressed during entry of cells into stationary phase, and thus transcriptional activators of *sodA* must override ArcA repression during these conditions (our unpublished data).

Secondly, the reduced production and activity of the aerobic respiratory apparatus during starvation may prevent an uncontrolled drainage of endogenous reserves. The degradation of endogenous membrane phospholipids is an integral part of the starvation process of non-differentiating bacteria (Hood *et al.*, 1986; Lonsmann-Iversen, 1987). This degradation is proposed to provide the cell with carbon and energy for maintenance requirements when no exogenous carbon source is available. However, the degradation of membrane constituents must be tightly regulated since an unrestrained degradation would seriously debilitate the membrane, resulting in loss of cell integrity. It is likely that the rate of degradation of endogenous carbon–energy reserves, like membrane lipids, is feed-back regulated by the activity of the catabolic apparatus. An uncontrolled respiratory activity, as seen in the Δ*arcA* mutant, would presumably drain this reserve causing loss of integrity and cell death. Overproduction of SodA would presumably not obliterate this effect of the Δ*arcA* mutation. Consequently, the Δ*arcA* mutant is expected to be moribund even in the presence of elevated levels of SodA, as shown in Figure 6B.

Besides membrane lipids, ribosomes are suggested to provide the starved cell with carbon and energy for maintenance. Again, an unchecked respiratory activity may cause an unrestrained degradation of the ribosomes which eventually may result in the irreversible loss of function of an essential component of the protein-synthesizing machinery. The loss of function of translational initiation factors has been suggested to be the cause of cell death in phosphate-starved *E.coli* cells (Davis *et al.*, 1986).

Thirdly, the ArcA-dependent starvation response may serve in regulating cellular redox balance and energetic status. The ArcA-dependent increased synthesis of formate transacetylase (Pfl) and decreased production of NADH-generating enzymes during glucose starvation is consistent with this notion. In contrast to pyruvate dehydrogenase, Pfl provides acetyl-CoA from pyruvate without producing NADH. [However, Pfl is presumably inactive in the presence of oxygen (Knappe and Schmitt, 1976) making its synthesis during aerobic, glucose starvation conditions rather obscure.] The inability of the Δ*arcA* mutant to complete reductive division at the onset of glucose starvation may be the result of a failure to regulate the energy and/or redox balance. An inbalance in the phosphorylation potential and/or redox state may block biosynthetic activities, such as septum formation and cell division, initiated before the depletion of the carbon source. On the other hand, the uncontrolled drainage of endogenous reserves and the possible accumulation of radical damaged proteins in the Δ*arcA* mutant as discussed above would not affect the cell's metabolic activities at the onset of starvation, such as reductive division, but may eventually cause irreversible damage to the cell.

Is the stimulus for ArcA activation the same during anaerobiosis and aerobic carbon starvation? It is clear that oxygen itself is not the signal for the ArcA–ArcB system (Iuchi and Lin, 1988). Rather, it has been proposed that the signals activating ArcA via ArcB during shifts in oxygen availability may be a reduced form of either an intermediate in the electron transport chain or an Fe^{2+} complex (Lin and Iuchi, 1991), or small metabolites, such as pyruvate, acetate or NADH (Iuchi, 1993). Any of these mechanisms may well operate also during a sudden exhaustion of the exogenous carbon source. However, the results shown here do not imply that the same signal must be operating during anaerobiosis and carbon starvation. It is possible that a sensor component other than ArcB is responsible for cross-activation of ArcA during carbon starvation or that starvation-induced elevation in levels of low molecular weight phosphorylated compounds, such as acetyl phosphate or carbamoyl phosphate, enhance autophosphorylation of ArcA. It has been demonstrated recently that ArcA can be activated *in vitro* by carbamoyl phosphate (Drapal and Sawers, 1995). In addition, perturbations in the production of acetyl phosphate have been shown to affect the *in vivo* expression of the phosphate (Pho) and nitrogen (Ntr) two-component regulatory pathways of *E.coli* (Feng *et al.*, 1992; Wanner and Wilmes-Riesenberg, 1992), and phosphorylation of the regulatory protein of the Ntr regulon occurs *in vitro* with acetyl phosphate as phosphate donor, bypassing the need for

autophosphorylation of the sensor protein (Feng *et al.*, 1992). It has been shown previously that the acetyl phosphate–acetate production pathway (encoded by *pta* and *ackA*) is induced concomitantly with the repression of TCA cycle enzymes during glucose starvation and that the production of acetyl phosphate is indispensable for the survival of glucose-starved cells (Nyström, 1994a). Further work will be aimed at determining whether the *pta–ackA* pathway and the ArcB sensor have a role in the ArcA-dependent response described in this work.

In conclusion, it has been argued that reduced oxygen species may be one of the causal factors underlying the aging process in multicellular organisms and that, in some cases, overproduction of superoxide dismutase alone, or together with a catalase, may significantly increase the maximal life span of a variety of species (e.g. Tyler *et al.*, 1993; Warner, 1994). In addition, the variations in the rates of aging in different species, that are otherwise closely related phylogenetically, have been suggested to be due to differences in the rates of O_2^- and H_2O_2 production (Sohal and Orr, 1992). Also, it has been suggested that the rates of oxidant generation are a better correlate of the rates of aging than are the levels of antioxidant defenses (Sohal and Orr, 1992). Although this work does not show that the life span of non-growing wild-type *E.coli* populations is limited by oxygen-derived damage, it demonstrates that an integral and important part of the stasis response is devoted to avoiding the damaging effects of oxygen radicals and controlling the rate of utilization of endogenous reserves.

Materials and methods

Bacterial strains, media and growth conditions

The W3110 and MC4100 strains of *E.coli* were used as noted. Strain GC4468 Δ*arcA*::Tn*10* was kindly provided by Dr Danielé Touati. The Δ*arcA*::Tn*10* mutation was transduced into W3110 and MC4100 by standard P1 transduction (Miller, 1972). Cultures were grown in liquid MOPS (morpholine-propanesulfonic acid; Neidhardt *et al.*, 1974) or M9 (Sambrook *et al.*, 1989) medium supplemented with glucose (0.4% w/v) and thiamine (10 mM) for minimal medium at 30°C. For analysis of metabolic activities and patterns of protein synthesis during glucose starvation, cells were grown aerobically in glucose minimal MOPS or M9 medium with 0.02 or 0.04% glucose as described (Nyström and Neidhardt, 1992).

Resolution of proteins on two-dimensional polyacrylamide gels

Culture samples were processed to produce extracts for resolution on two-dimensional polyacrylamide gels by the methods of O'Farrell (1975) with modifications (VanBogelen and Neidhardt, 1990). The 2-D gel apparatus used for the O'Farrell methodology was that of Hoefer Corporation. Alpha-numeric (A-N) designations and/or *x–y* coordinates were assigned protein spots after matching them to the reference 2-D images of the gene–protein database of *E.coli* (VanBogelen *et al.*, 1990).

N-terminal sequencing

After electrophoresis, the gels were soaked in transfer buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11] for 10 min to reduce the amount of glycine and Tris contaminations. PVDF Immobilon (Millipore) membranes were rinsed with 100% methanol and stored in transfer buffer. The gels, sandwiched between sheets of PVDF membrane and several sheets of blotting paper (Whatmann), were assembled into a blotting apparatus and electroeluted for 1 h at 50 V (170–100 mA) in transfer buffer. The PVDF membranes were washed in sterile deionized water for 30 min, stained with Amido Black (0.1% Amido Black, 1% acetic acid, 40% methanol) for 15 min, destained in sterile deionized water until protein spots appeared clearly, and subsequently dried at room temperature between sheets of blotting paper (Whatmann). The spots of interest were excised from the membranes, and automated Edman degradation was performed directly on the membrane fragments using an Applied Biosystems 477A Pulsed Liquid Phase sequencer (Foster City, CA) with an online PTH 120A Analyzer.

Measurement of rates of synthesis of individual proteins

At indicated times, a portion (1 ml) of a culture was removed and placed in a flask containing [^{3}H]leucine (5 mCi/mmol, 100 μCi/ml). Incorporation was allowed to proceed for 5 min, after which non-radioactive leucine (2.4 mM) was added for a chase. To this sample was added a portion of a culture of the same strain grown in [^{35}S]methionine labeling medium as described (Nyström and Neidhardt, 1992). The mixed sample was then prepared as previously described (VanBogelen and Neidhardt, 1990) to produce extracts for resolution of the cellular proteins on two-dimensional gels. An autoradiogram was prepared to permit visualization of labeled proteins. Protein spots chosen for quantitative assay were sampled from the dried gel with a syringe needle and treated as described (Pedersen *et al.*, 1976) to permit measurement of their ^{3}H and ^{35}S content by scintillation counting. The differential rate of synthesis of a sampled protein was defined as the ^{3}H/^{35}S ratio of the sampled spot divided by the same isotope ratio of unfractionated mixed extracts.

Flow cytometry

Bacteria isolated from the exponential growth phase and at different times of glucose starvation were run on a flow cytometer (Skantron) to determine cell size distributions and DNA content per bacterial cell. Cells were fixed in 70% ethanol–10 mM Tris (pH 7.4) and stored at 4°C until staining. All samples were stained just before being applied to the flow cytometer. Stored cells were centrifuged and resuspended in 10 mM Tris (pH 7.4) containing 10 mM $MgCl_2$. DNA staining was performed by mixing equal parts of the resuspended cells with mithramycin (200 μg/ml) and ethidium bromide (40 μg/ml) in the same buffer.

Microcalorimetry

The heat production rate (dQ/dt) was measured with a heat conduction-type multichannel microcalorimeter (Bioactivity Monitor LKB 2277; Thermometric AB, Järfälla, Sweden) equipped with flow-through cells, as described by Larsson *et al.* (1991). The microcalorimeter was operated at 30.0°C at a measuring range of 1000 μW. The cultures to be analyzed were pumped at a rate of 80 ml/h from the growth vessel by a peristaltic pump as previously described (Ölz *et al.*, 1993). External and internal calibrations were performed as described by Ölz *et al.* (1993).

Respirometry

The respirometric measurements were performed in a Cyclobios oxygraph (A.Paar KG, Graz, Austria; Haller *et al.*, 1994). Samples (2×2 ml) were withdrawn from the culture connected to the calorimeter and the oxygen consumption rate (mmol O_2/l×h) was determined simultaneously with the heat production rate both at 30°C.

Glucose determination

Samples (1 ml) were centrifuged for 2 min at 15 000 *g* and the supernatants were frozen immediately. Concentrations were analyzed by using enzyme combination kits (Biochemica Test Combination; Boehringer Mannheim GmbH, Mannheim, Germany).

Calculations of heat production accompanying respiration

The experimental set-up in these experiments results in a heat production of 468.9 kJ/mol O_2 consumed (von Stockar *et al.*, 1993). Consequently, the heat production rate accompanying respiration was calculated as: $J_Q = J_{O2} \times 468.9$, where J_Q is the rate of heat production (kJ/h) and J_{O2} is the oxygen consumption rate (mol O_2/h).

Reproducibility

Each experiment was repeated at least twice to confirm reproducibility. Representative results are presented in the figures and tables of this paper.

Acknowledgements

Nan H.Albertson and Anne Farewell are gratefully acknowledged for valuable suggestions on the manuscript. We are grateful to Knud Rasmussen for running the flow cytometer and to Søren Molin for letting T.N. invade his laboratory. Strains were kindly provided by Danielé Touati. This work was supported by the Swedish Natural Science Research Council, NFR, to T.N.

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Received on February 23, 1996; revised on March 15, 1996