Aerobic L-ascorbate metabolism and associated oxidative stress in Escherichia coli

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The anaerobic utilization of L-ascorbate by gene products of the ula regulon in Escherichia coli has been widely documented. Under aerobic conditions, we have shown that this metabolism is only functional in the presence of casein acid hydrolysate. Transcriptional fusions and proteomic analysis indicated that both the ula regulon and the yiaK-S operon are required for the aerobic utilization of this compound. The aerobic dissimilation of L-ascorbate shares the function of three paralogous proteins, UlaD/YiaQ, UlaE/YiaR and UlaF/YiaS, which encode a decarboxylase, a 3-epimerase and a 4-epimerase, respectively. In contrast, L-ascorbate enters the cells through the ula-encoded phosphotransferase transport system, but it is not carried by the yiaMNO-encoded ABC transporter. Proteomic analysis also indicated enhanced expression of the alkyl hydroperoxide reductase encoded by the ahpC gene, suggesting a response to oxidative stress generated during the aerobic metabolism of L-ascorbate. Control of ahpC expression by the OxyR global regulator in response to L-ascorbate concentration is consistent with the formation of hydrogen peroxide under our experimental conditions. The presence of certain amino acids such as proline, threonine or glutamine in the culture medium allowed aerobic L-ascorbate utilization by Escherichia coli cells. This effect could be explained by the ability of these amino acids to allow yiaK-S operon induction by L-ascorbate, thus increasing the metabolic flux of L-ascorbate dissimilation. Alternatively, these amino acids may slow the rate of L-ascorbate oxidation.

INTRODUCTION

Several studies have reported the ability of a number of enteric bacteria to both ferment and oxidize L-ascorbate (Esselen & Fuller, 1939; Volk & Larsen, 1962; Young & James, 1942). Under anaerobic conditions, dissimilation of L-ascorbate by Escherichia coli has been extensively documented (Campos et al., 2002; Yew & Gerlt, 2002; Zhang et al., 2003) and demonstrated to be a function of proteins encoded by the ula regulon (Fig. 1a). This system is formed by two divergently transcribed operons (Campos et al., 2004): the ulaG operon, believed to encode L-ascorbate-6-P lactonase (Yew & Gerlt, 2002), and the ulaA-F operon, encoding the three components of the L-ascorbate phosphotransferase transport system (UlaABC) (Zhang et al., 2003) as well as three catabolic enzymes (UlaDEF) (Yew & Gerlt, 2002). The three gene products UlaABC (formerly designated SgaTBA) are involved in the uptake and phosphorylation of L-ascorbate (Zhang et al., 2003). Intracellular L-ascorbate-6-P could be transformed by L-ascorbate-6-P lactonase to 3-keto-L-gulonate-6-P. It has been proposed that this compound is decarboxylated by UlaD to L-xylulose-5-P, which is then converted to D-xylulose-5-P by the sequential action of UlaE (3-epimerase activity) and UlaF (4-epimerase activity) (Yew & Gerlt, 2002). Thus the function of the gene products of the ula system is the transport of L-ascorbate and its transformation into D-xylulose-5-P (Fig. 1c) (Yew & Gerlt, 2002), which is subsequently metabolized by the pentose phosphate pathway. The ula regulon is under the control of the UlaR repressor (Campos et al., 2004), which belongs to the DeoR repressor family. These types of regulators normally recognize a phosphorylated sugar as an effector molecule. In the E. coli genome three paralogous proteins to UlaDEF were identified. These proteins are encoded by genes yiaQRS belonging to the yiaK-S operon (Fig. 1b), located at centisome 80.7, for which no specific role has been established so far (Campos et al., 2002).

Under aerobic conditions, metabolism of L-ascorbate is hindered by the special reactivity and toxicity of this compound in the presence of oxygen. In this context the observation that aerobic utilization of L-ascorbate in E. coli requires the presence of nutrient broth (Young & James, 1942) is of interest. L-Ascorbate is an extremely effective antioxidant, thanks to its ability to donate additional...
Electrons, which react with and scavenge a wide range of reactive oxygen species (ROS), such as singlet oxygen, superoxide anions and hydroxyl radicals (Blokhina et al., 2003). In this process ascorbate is converted into an ascorbyl radical, which is more stable than ROS, and thereby diminishes any potential cell damage resulting from ROS-induced oxidative activity on macromolecules (Moslen, 1992). In spite of such antioxidant reactivity, it is well known that in the presence of redox-active iron and oxygen, L-ascorbate acts as iron-reducing agent, triggering the Fenton reaction, which yields ROS from hydrogen peroxide and causes significant damaging oxidative stress (Dekker & Dickinson, 1940; Imlay, 2003; Meneghini, 1997; Winterbourn, 1995). Under these conditions L-ascorbate enhances the stress caused by the natural and unavoidable by-products of aerobic life. To face the toxicity effects of excessive intracellular iron levels, complex regulatory networks have evolved in E. coli cells to keep only 1% of the iron free or in a loosely bound state (Semsey et al., 2006).

The cellular response to oxidative stress can be mediated by the expression of a set of enzymes primarily regulated by the soxRS and oxyR regulons that detoxify oxygen-free radicals (Demple, 1991). It is widely believed that the soxRS regulon is activated by the superoxide anion and the oxyR regulon by H2O2 (Aslund et al., 1999; Pomposiello & Demple, 2001). To detoxify both the superoxide anion and H2O2, E. coli has developed superoxide dismutase and catalase, respectively. In addition, alkyl hydroperoxide reductase, encoded by aphC, acts as a primary scavenger of endogenous H2O2 (Seaver & Imlay, 2001; Storz et al., 1990). This gene is regulated by the oxidative stress sensor OxyR.

In this study, we describe the metabolic system involved in the aerobic dissimilation of L-ascorbate in E. coli, and how E. coli cells overcome the oxidative stress caused by this substrate in the presence of ubiquitous iron atoms.
**H₂O₂ determination.** H₂O₂ was detected using the Amplex Red Assay (Yan et al., 2005). Cuvettes containing 0.5 ml of the working solution [50 μl 10 mM Amplex Red, 100 μl horseradish peroxidase (10 U ml⁻¹) and 4.85 ml PBS] and either 0.5 ml of various H₂O₂ calibration standards or 0.5 ml of samples were incubated 30 min at room temperature. Fluorescence was then measured with a spectrophotometer using excitation at 530 nm and fluorescence detection at 585 nm. Background fluorescence, determined for a control reaction lacking H₂O₂, was subtracted from each value.

**Enzyme activities.** β-Galactosidase activity was assayed by hydrolysis of O-nitrophenyl-β-D-galactopyranoside and expressed in Miller units (Miller, 1992). The data reported are a representative set of at least three separate experiments performed in duplicate.

**DNA manipulation.** Bacterial genomic DNA was obtained as described by Silhavy et al. (1984). Plasmid DNA was routinely obtained by using either the Wizard Plus SV Midiprep DNA purification system or the boiling method (Holmes & Quigley, 1981). DNA manipulations were performed as described by Sambrook & Russell (2001). DNA sequencing was done by using the M13 primer to ensure that no mutation was introduced. Single-copy fusions on the chromosomal DNA were amplified by PCR using specific primer sets (which yielded no PCR products, thus revealing the absence of DNA contamination). The reactions were performed with Taq DNA polymerase under standard conditions.

**Reverse transcriptase PCR (RT-PCR) analysis.** Total RNA was obtained using an SV total RNA isolation kit (Promega) from cultures of strain ECL1 grown aerobically on 0.4 % CAA in the absence or presence of 10 mM l-ascorbate, followed by treatment with RNase-free DNase I (Ambion) and inactivation according to the manufacturer’s protocol. RNA was quantified spectrophotometrically. Quality was assessed via RNA analysis of 1 μg RNA samples with specific primer sets (which yielded no PCR products, thus revealing the absence of DNA contamination). The reactions were performed with 1 μg total RNA at 37 °C for 30 min with 200 U M-MLV-reverse transcriptase (Life Technologies). Subsequent PCRs were carried out with *ahpC* primers ahp-fw (5′-AGATACCGAAGGCGCTGAG-GCTTTC-3′) and ahp-c-rv (5′-ATTCCTACGATGTGGAATGTTACGGA-GTACGG-3′) for 25 cycles. The rRNA 16S gene (5′-GGTTACGG-3′) and 16S rDNA fragment from the 5′ upstream region of *ahpC* was obtained by PCR using primers ahp-C1 (5′-CCGGATCTACTATCATGATAG-GTAAGAGCTTAG-3′) and ahp-C2 (5′-CCGGATCTACTATCATGATAG-GTAAGAGCTTAG-3′) and a 300 bp DNA fragment from the 5′ upstream region of *dps* gene was obtained using primers DPS-1 (5′-CCGGATCTACTATCATGATAG-GTAAGAGCTTAG-3′) and DPS-2 (5′-CCGGATCTACTATCATGATAG-GTAAGAGCTTAG-3′). The PCR product was digested with BamHI and EcoRI (restriction sites for these endonucleases are underlined in the primer sequences) and cloned into plasmid pRS550 or pRS551. The pRS plasmids carry a cryptic lac operon and genes that confer resistance to both kanamycin and ampicillin. Recombinant plasmids were selected, after transformation of strain XLI-Blue, as blue colonies on LB plates containing X-Gal, ampicillin and kanamycin. Plasmid DNA was then sequenced by using the M13 primer to ensure that no mutation was introduced. Single-copy fusions on the *E. coli* chromosome were obtained by the method of Elliott (1992) using strain TE2680. The transformants were selected for kanamycin resistance and screened for sensitivity to ampicillin and chloramphenicol. P1 vir lysates were made to transduce the fusions into the desired genetic background.

**In vivo l-ascorbate transport assays.** Cells were collected, washed twice, suspended in phosphate buffer (pH 7.0) at a final cell density of 0.5 mg dry wt ml⁻¹ and maintained at 25 °C. The rate of l-ascorbate uptake was assayed with 0.6 ml cell suspension in 1.5 ml microcentrifuge tubes with 50 μM ascorbate (0.1 mCi mmol⁻¹). The radioactive substrate l-[1-³⁵S]Gclascorbate (13 mCi mmol⁻¹) was purchased from Amersham. Samples of 100 μl were taken at different intervals and filtered through 0.45 μm pore size cellulose nitrate filters. The filters were washed in 4 ml phosphate buffer, placed in plastic vials and counted in the presence of Emulsifier-safe (Packard).

**DNA binding studies.** The DIG gel shift kit for 3′-end labelling of DNA fragments (Roche Applied Science) was used for protein–DNA binding assays. The fragments obtained by PCR were labelled at the 3′ end with terminal transferase and digoxigenin-dUTP and used in gel shift reactions, according to the manufacturer’s instructions (Roche). Protein samples consisted of crude extracts obtained as described by Nunoshiba et al. (1992). A 5 % native polyacrylamide gel containing 5 % glycerol in 0.5 × TBE (1 × TBE is 0.089 M Tris-borate, pH 8, 0.002 M EDTA) was prepared and used for electrophoresis of the gel shift reactions. Following electrophoretic separation, the oligonucleotide–protein complex was transferred by contact-blotting to nylon membranes. Chemiluminescent detection of DIG-labelled DNA–protein complexes on the nylon membranes was obtained with X-ray film.

**Preparation of bacterial proteins and analysis by 2-D electrophoresis.** Once harvested, cells were washed twice with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) and then transferred to a lysis buffer that contained 8 M urea, 2 % CHAPS, 1.2 % DeStreak, 0.5 % immobilized pH gradient and 80 mM DTT. After sonic disruption on ice, the cell extract was obtained and the protein concentration was determined by the RC DC Protein Assay (Bio-Rad) following the manufacturer’s instructions.

Cell extract (300 μg) of strain ECL1 grown aerobically in 0.4 % CAA or 0.4 % CAA plus 10 mM l-ascorbate to stationary phase were used to generate proteomes for comparative analysis. Proteins were dissolved in 450 μl rehydration buffer (7 M urea, 2 M thiourea, 80 mM DTT, 2 % CHAPS, 0.5 % immobilized pH gradient buffer, bromophenol blue). Isoelectric focusing was performed using an immobilized pH 3–10 gradient strip. Second dimension SDS-PAGE was performed in a 12.5 % acrylamide gel and gels were fixed overnight followed by Coomassie blue staining.

**In-gel digestion with trypsin and identification of proteins by peptide mass mapping.** Gels were scanned and the relevant protein spots were excised from the 2-D gels and digested with trypsin (sequencing grade modified; Promega) using the automatic Investigator ProGest robot from Genomic Solutions. Proteins excised from the 2-D gels were analysed by MALDI-TOF-MS (4700 Proteomics Analyser; Applied Biosystems) or ESI-MS-MS (Q-TOF Global; Micromass-Waters) by the Serveis Científico-technics, University of Barcelona, Spain. Some of the digested peptide samples were analysed by online liquid chromatography tandem mass spectrometry (Cap-LC-nano-ESI-Q-TOF; CapLC; Micromass-Waters). In those cases, samples were dissolved in 10 % formic acid solution and 4 μl was injected into a reverse-phase capillary C₁₈ column (75 μm i.d., 15 cm long; PepMap column; LC Packings) for chromatographic separation. The eluted peptides were ionized via coated nano-ES needles (PicoTip; New Objective). A capillary voltage of 1800–2200 V was applied together with a cone voltage of 80 V. The collision in the CID (collision-induced dissociation) was 20–35 eV.
RESULTS

Aerobic growth in l-ascorbate

Growth of *E. coli* (strain ECL1) on l-ascorbate was tested in solid medium containing 5 or 10 mM l-ascorbate as a carbon source, or in liquid medium containing l-ascorbate at concentrations ranging from 0.05 to 5 mM. Although no colonies were obtained on solid medium, an increase in OD<sub>600</sub> up to 0.3 after 12 h was detected in liquid medium containing 1 mM l-ascorbate. Higher concentrations of l-ascorbate in liquid cultures diminished the growth yield, and when the concentration was over 2 mM, the culture acquired a brownish colour and cell lysis was observed. This phenotypic behaviour was blunted by the addition of CAA. The growth of *E. coli* in mineral medium containing 0.4 % CAA plus 5 or 10 mM l-ascorbate is shown in Fig. 2. The culture was started with an inoculum that yielded an initial cell density of 5 × 10<sup>7</sup> c.f.u. ml<sup>-1</sup> and displayed two different phases of growth. In the second phase, the generation time and yield clearly increased with the L-ascorbate concentration. Utilization of L-ascorbate was confirmed by the disappearance of this compound from the medium, as indicated by its absence in the supernatant of a centrifuged culture after reaching the stationary phase. Concentrations of CAA below 0.3 % did not allow L-ascorbate utilization, and cell lysis was observed after several hours of incubation. The additional growth due to L-ascorbate utilization was not observed when glycerol, L-rhamnose or glucose were used as carbon source instead of CAA or when l-ascorbate was added to the CAA cultures at the end of the exponential phase (data not shown). Toxicity of l-ascorbate was evident by the inhibition of growth when this compound was added at 10 mM during the exponential phase of a culture growing in 2.5 mM glycerol (not shown).

Formation of H<sub>2</sub>O<sub>2</sub>

Formation of H<sub>2</sub>O<sub>2</sub>, the oxidant proposed to be responsible for l-ascorbate toxicity, was checked under our culture conditions. In cell-free supernatants of CAA cultures without l-ascorbate the concentration of H<sub>2</sub>O<sub>2</sub> was negligible (0.27 μM) after 2 h incubation. This concentration increased gradually with the concentration of l-ascorbate present in the cultures, yielding 3.2, 6.1 or 7.4 μM H<sub>2</sub>O<sub>2</sub> with 1, 5 or 10 mM l-ascorbate, respectively.

Proteomic analysis of cells induced by l-ascorbate in the presence of oxygen

To search for proteins likely to be involved in l-ascorbate utilization, we employed a proteomic approach. To this end, cell extracts of strain ECL1 grown to stationary phase in 0.4 % CAA or 0.4 % CAA plus 10 mM l-ascorbate were analysed by 2-D gel electrophoresis and the protein profiles were compared after Coomassie blue staining. In the presence of l-ascorbate, the expression of several proteins increased (Fig. 3). On the basis of their peptide masses, the following proteins were identified: alkyl hydroperoxide reductase, C22 subunit involved in the detoxification of hydroperoxides, encoded by *ahpC*; 3-keto-L-gulonate 6-phosphate decarboxylase, previously known as 3-hexulose-6-phosphate synthase, encoded by *yiaQ* (also known as *sgbH*); transaldolase B, encoded by *talB*; phosphoglyceromutase 1, encoded by *gpmA*; l-lactate dehydrogenase encoded by *ltkD* and dihydrolipoamide acyltransferase (E2), encoded by *aceF*, a component of the pyruvate/2-oxoglutarate dehydrogenase complex (see numbered spots in Fig. 3b).

Aerobic l-ascorbate utilization requires expression of the yiaK-S operon and ula regulon

The *yiaK-S* operon (Fig. 1b), of unknown function, has been revealed by proteomic analysis as one of the systems expressed in cells grown aerobically in CAA in the presence of l-ascorbate. The expression of this operon was analysed by monitoring growth and β-galactosidase activity in strain ECL1 carrying the transcriptional fusion ϕ(*yiaK-lacZ*). In this experiment, cells were grown aerobically in 0.4 % CAA in the absence or presence of 10 mM l-ascorbate. The expression of ϕ(*yiaK-lacZ*) was induced by the presence of l-ascorbate, increased through exponential phase to a maximum during transition, and decreased slightly as the culture reached stationary phase (Fig. 4a). Thus regulation of *yiaK-S* operon expression in *E. coli* occurs under aerobic conditions at the transcriptional level in a growth-phase-dependent manner.

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Fig. 2. Growth curves of the wild-type strain ECL1 and the ulaA mutant strain in the absence or presence of L-ascorbate. Cells of the wild-type strain ECL1 (closed symbols) and the ulaA mutant strain (open symbols) were grown in CAA at 0.4 % in the absence (circle) or in the presence of 5 mM (square) or 10 mM (triangle) l-ascorbate. Cells were grown at 37 °C.
In addition, we also checked the induction of yiaK-S by oxidative stress by adding H₂O₂ to cultures without L-ascorbate. Several spots of proteins whose expression was upregulated in the crude extracts obtained from CAA plus L-ascorbate cultures are ringed in black. The numbered proteins in (b) are: 1, alkyl hydroperoxide reductase, C22 subunit involved in the detoxification of hydroperoxides, encoded by ahpC; 2, 3-keto-L-gulonate 6-phosphate decarboxylase, previously named 3-hexulose-6-phosphate synthase, encoded by yiaQ which is also known as sgbH; 3, transaldolase B, encoded by talB; 4, phosphoglyceromutase 1, encoded by gpmA; 5, L-lactate dehydrogenase encoded by lctD; 6, dihydrolipoamide acyltransferase (E2) encoded by aceF, a component of the pyruvate/2-oxoglutarate dehydrogenase complex.

The expression of the yiaK-S operon and ula regulon in cultures of CAA plus L-ascorbate suggested the coordinated contribution of both systems to the aerobic dissimilation of L-ascorbate. This was confirmed by the observation that neither yiaK::Tn5 nor ulaA::Tn10 mutants grew efficiently when inoculated at the same c.f.u. into a standard L-ascorbate utilization test medium containing 0.4% CAA plus 5 or 10 mM L-ascorbate. Results obtained with the ulaA mutant showed no growth when the CAA culture was supplemented with 10 mM L-ascorbate (Fig. 2). Cell lysis appeared after a few hours incubation under these conditions. Slow growth was observed when the culture was supplemented with 5 mM L-ascorbate and displayed no additional yield when compared to a culture in CAA with no L-ascorbate added. The coordinated contribution of ula and yiaK-S systems was further demonstrated by an experiment in which an ulaD::cat mutant, with no metabolic function of the ula operon but keeping the

**Fig. 3.** Coomassie-blue-stained 2-D polyacrylamide gels of crude extract proteins of strain ECL1 grown in 0.4% CAA in the absence (a) or presence (b) of 10 mM L-ascorbate. Several spots of proteins whose expression was upregulated in the crude extracts obtained from CAA plus L-ascorbate cultures are ringed in black. The numbered proteins in (b) are: 1, alkyl hydroperoxide reductase, C22 subunit involved in the detoxification of hydroperoxides, encoded by ahpC; 2, 3-keto-L-gulonate 6-phosphate decarboxylase, previously named 3-hexulose-6-phosphate synthase, encoded by yiaQ which is also known as sgbH; 3, transaldolase B, encoded by talB; 4, phosphoglyceromutase 1, encoded by gpmA; 5, L-lactate dehydrogenase encoded by lctD; 6, dihydrolipoamide acyltransferase (E2) encoded by aceF, a component of the pyruvate/2-oxoglutarate dehydrogenase complex.
UlaABC transport function and the YiaK-S metabolic function, grew in CAA plus L-ascorbate with the same yield as wild-type strain ECL1.

Effect of amino acids on yiaK-S expression

In this context the results obtained when using single amino acids instead of CAA to counteract L-ascorbate toxicity became relevant. This study was approached by growing E. coli strain ECL1 ψ(yiaK-lacZ) in mineral medium with 5 mM L-ascorbate and specific amino acids previously shown to support growth. In these cultures, growth and β-galactosidase activity were monitored. Cells grown in proline, threonine, glutamate, glutamine or aspartate, but not those grown in serine or cysteine, displayed both high β-galactosidase activity and an additional growth yield when L-ascorbate was present (not shown). This led us to check whether the lack of L-ascorbate utilization in cells growing in glycerol, glucose or L-rhamnose was a consequence of the low expression of ψ(yiaK-lacZ). β-Galactosidase analysis in these cells showed a negligible level of activity, reinforcing the idea that a certain level of yiaK-S expression is required for the aerobic assimilation of L-ascorbate.

L-Ascorbate transport

We also studied the entry of L-ascorbate into cells growing under aerobic conditions. Based on the results of the 2-D separation and the data obtained from the transcriptional fusion analysis, it is clear that the yiaK-S operon is induced in the presence of L-ascorbate. Since the yiaMNO-encoded transport system is unable to transport this compound, another transporter for L-ascorbate should be functionally active.

Uptake of L-[1-14C]ascorbate by ECL1 cells grown in the presence or absence of L-ascorbate revealed that transport of the radioactive substrate was reduced by 78%
when the cells were grown in the absence of this compound (Fig. 6). This suggests the contribution of an l-ascorbate-inducible transporter. A transport assay was also performed with ECL1 *ulaA::Tn10*. In this case, to overcome l-ascorbate toxicity, cells were grown in 0.4% CAA and at the end of the exponential phase l-ascorbate was added to 10 mM final concentration. After 3 h incubation to induce the *ula* system, cells were processed for the transport assay. Results obtained with mutant cells indicated a reduced uptake, matching the levels presented by ECL1 cells grown in CAA (Fig. 6).

These results indicate that the *ulaABC*-encoded transport system contributes to the uptake of l-ascorbate, while another as yet unidentified transporter, of constitutive expression, must account for the remaining l-ascorbate transport activity.

**Expression of ϕ(ahpC-lacZ) in CAA plus l-ascorbate**

The increased expression of alkyl hydroperoxide reductase detected in the proteome of cells grown in the presence of l-ascorbate suggested a response to the oxidative stress caused by this carbon source. We therefore examined the expression of the *ahpC*, which encodes alkyl hydroperoxide reductase. A preliminary estimation of the amount of *ahpC* transcript was analysed by RT-PCR. A substantially increased amount of transcript was detected in cells grown in the presence of l-ascorbate. The experiment was normalized by RT-PCR amplification of the *rrsB* gene encoding rRNA 16S (not shown). To measure the transcriptional activity of the *ahpC* promoter, a transcriptional fusion to *lacZ* was constructed and transferred into strain ECL1. These cells, containing ϕ(*ahpC-lacZ*), were grown in 0.4% CAA in the absence or presence of 5 mM l-ascorbate. Cell density (OD₆₀₀) and β-galactosidase activity were subsequently monitored throughout the growth of these cultures. Expression of ϕ(*ahpC-lacZ*) in the presence of 10 mM l-ascorbate exhibited a transient increase, reaching a maximum after 1 h incubation and returning to basal level after 4 h (Fig. 7a). These data imply that in the presence of l-ascorbate, *ahpC* expression in *E. coli* is mainly regulated at the transcriptional level during early exponential phase.

**Ascorbate activation of ahpC is dependent on OxyR**

Two lines of evidence confirm that the increased expression of *ahpC* observed in the presence of l-ascorbate is controlled by the activator OxyR. First, we performed

![Fig. 6. Time-course of uptake of l-[1-¹⁴C]ascorbate. Uptake of l-ascorbate was studied in whole cells of strain ECL1 grown in 0.4% CAA in the absence (open circles) or in the presence (closed circles) of 10 mM l-ascorbate. Uptake with mutant strain ECL1 *ulaA::Tn10* grown in the presence of l-ascorbate (closed triangles) is also plotted.](http://mic.sgmjournals.org)

![Fig. 7. Expression profiles of ϕ(*ahpC-lacZ*) (a) and ϕ(*dps-lacZ*) (b) transcriptional fusions. β-Galactosidase activity, expressed as Miller units, of cells of strain ECL1 carrying these fusions grown aerobically in 0.4% CAA in the absence of l-ascorbate (open circles) or in the presence 10 mM l-ascorbate (closed circles) were plotted against time.](http://mic.sgmjournals.org)
electrophoretic mobility shift assays (EMSA) with two probes of the $ahpC$ gene promoter: P140, containing the consensus for OxyR recognition; and P130, containing the consensus for MetJ recognition (Fig. 8a). Binding reactions were performed with extracts of strain ECL1 grown in 0.4% CAA in the absence or presence of L-ascorbate at 1, 5 or 10 mM. As a positive control we used extracts obtained from CAA cultures of strain ECL1 grown in the presence of 5 μM H₂O₂. A retarded band appeared only in the case of probe P140 when L-ascorbate or H₂O₂ was present in the growth medium. No retarded band appeared with probe P130, or in the absence of L-ascorbate. The retarded complex species was clearly more abundant for those extracts obtained from cells grown with 5 or 10 mM than with 1 mM L-ascorbate.

A second line of evidence stemmed from the phenotype of a $\Delta$oxyR mutant. These mutant cells (strain JTG101) did not grow in glucose, glycerol or even CAA in the presence of 10 mM L-ascorbate. Under these conditions no induction of $\phi$($ahpC$-lacZ) in the genetic background of this OxyR-deficient strain was observed. In these experiments, due to the toxicity of L-ascorbate to the OxyR mutant, cells were first grown to stationary phase in CAA and then incubated for 3 h in the presence of 10 mM L-ascorbate. Furthermore, gel shift experiments using an OxyR mutant as a source of protein showed no bands with any of the probes tested (not shown). These results suggest an OxyR dependence in the L-ascorbate activation of $ahpC$.

If OxyR mediates the high expression of $ahpC$ observed in cell cultures grown in the presence of l-ascorbate, other genes regulated by OxyR such as $dps$ (Altuvia et al., 1994) must be activated under these conditions. To this end, a transcriptional fusion of the promoter region of this gene was constructed as described in Methods, and β-galactosidase activity was analysed in cultures grown in CAA in the presence or absence of 10 mM l-ascorbate. Results presented in Fig. 7(b) indicate that expression of this gene was also activated under these conditions. The involvement of OxyR was confirmed by EMSA using a $dps$ promoter probe containing only the OxyR binding site. As a negative control, an $lldP$ promoter probe was used. The presence of bands when using a $dps$ probe and cells extracts of cells grown in the presence of l-ascorbate (Fig. 8b) reinforce the idea that OxyR mediates the high-level expression of $ahpC$ in the presence of l-ascorbate. The formation of H₂O₂ in our cultures, as indicated above, guarantees the required activation of OxyR through oxidation of this transcriptional factor.

**DISCUSSION**

An explanation for the inability of E. coli to metabolize L-ascorbate as sole carbon source in the presence of oxygen has been based on the observation that the addition of CAA allows the dissimilation of this compound. In addition to the metabolic intermediate supplementation, which results in the formation of intermediate products that can be used as carbon sources, the presence of oxygen in the growth medium is essential for the activation of the OxyR transcription factor. OxyR is a member of the HapR family of regulators, which are activated by oxidative stress and play a key role in the expression of genes involved in the detoxification of oxidants and the regulation of the respiratory chain. In the presence of L-ascorbate, OxyR activation is facilitated by the formation of H₂O₂, which acts as a redox mediator, converting OxyR to its oxidized form that can bind to its target DNA sequences. The formation of this complex results in the activation of the $ahpC$ gene, encoding a peroxidase that is involved in the detoxification of hydrogen peroxide. The induction of $ahpC$ in the presence of L-ascorbate is therefore a result of the oxidative stress induced by the compound, which is sensed by OxyR and leads to the transcriptional activation of the gene.

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**Fig. 8.** Gel shift experiments with the $ahpC$ and $dps$ promoter regions. Diagram of the promoter regions with fragments used as probes (solid lines) are indicated in the upper part. Experiments were carried out with DIG-labelled fragment P130, P140 or P135 mixed with 5 μg protein extracts of strain ECL1 for 20 min at 30 °C and then subjected to gel electrophoresis. (a) Cells were grown in 0.4% CAA (lanes 2 and 8), 0.4% CAA in the presence of 1 (lane 3), 5 (lane 4) or 10 mM (lanes 5 and 9) L-ascorbate. As a control 5 μM H₂O₂ (lane 6) was added to the 0.4% CAA. Free probe was applied to lanes 1 and 7. (b) Cells were grown in 0.4% CAA (lane 2), 0.4% CAA in the presence of 5 mM L-ascorbate (lane 3) or 5 μM H₂O₂ (lane 4). Free probe was applied to lane 1.
from the dissipation of the given amino acids, some of them function as ROS scavengers. Amino acids such as histidine or tryptophan, among others, are free-radical scavengers (Moslen, 1992; Stadtman, 1993). Recently, assessment of the potential of proline to scavenge free radicals was made in a couple of in vitro assays (Kaul et al., 2006). This led us to investigate whether aerobic growth of E. coli on L-ascorbate was hampered by the generation of oxidative stress in the culture. It has been reported that L-ascorbate reacts with oxygen in the presence of metal ions to produce dehydroascorbate and H$_2$O$_2$ (Richter & Loewen, 1981). This reaction takes place mainly outside the cell. Our results indeed show formation of H$_2$O$_2$ under our culture conditions. Extracellular H$_2$O$_2$ enters the cells where it can form oxygen free radicals by the Fenton reaction, which takes place when a reducing agent like L-ascorbate is in the presence of ferrous ions (wardman & Candeias, 1996). In the cytoplasm, as a consequence of oxidative stress, iron is released from the macromolecules with which it is complexed (Kärkönen & Fry, 2006), thus increasing the normally low proportion of free iron and contributing to oxidative damage.

The response of the cell to oxidative stress follows a typical mechanism involving H$_2$O$_2$ detoxification and ROS scavenging. The enhanced expression of ahpC observed in the proteomic experiment, confirmed by RT-PCR and transcriptional fusion experiments, is indeed an efficient way for E. coli cells to cope with the H$_2$O$_2$ formed internally or found in the culture medium (Seaver & Imlay, 2001). As has been reported, AhpC is a more efficient scavenger of H$_2$O$_2$ than catalase when H$_2$O$_2$ is at a low concentration and thus remains the primary scavenger of endogenous H$_2$O$_2$. Regulation of ahpC by the global regulator OxyR has been widely documented (Schellhorn & Hassan, 1988). The increasing intensity of the retardation band corresponding to the complex OxyR-DNA when L-ascorbate is increased from 1 to 5 or 10 mM in the culture suggests increased H$_2$O$_2$ formation as well as increased oxidation of OxyR with its concomitant activation. This activation is responsible for the increase in ahpC expression (Aslund et al., 1999). This idea was reinforced by the fact that a retarded complex was also observed when the same experiments were performed with a dps probe containing only the OxyR binding site.

A deeper comprehension of the metabolic strategy that allows the surmounting of problematic L-ascorbate metabolism in the presence of oxygen has come from the discovery of some genetic products of the yiaK-S operon to this metabolism. Indeed, the finding in the proteomic analysis of enhanced expression for a protein encoded by a gene belonging to the yiaK-S operon suggests that this system participates in the aerobic metabolism of L-ascorbate. We have also shown that the ula regulon exhibits increased expression under our conditions, consistent with the description that L-ascorbate enters the cell through the ula-encoded phosphotransferase system. Thomas et al. (2006) have shown that the ABC transporter encoded by yiaMNO does not recognize this compound as a substrate. The transport of L-ascorbate through the ulaA-encoded system is further demonstrated by the substantial reduction of L-ascorbate uptake in the ulaA::Tn10 mutant. Since some nucleobase transporters can recognize L-ascorbate in different organisms (De Koning & Diallinas, 2000) the basal uptake level observed in ulaA-deficient cells may be carried out by them. Consequently, the proposed metabolic pathway for L-ascorbate dissimilation requires genes of the ula regulon and the yiaK-S operon. This pathway probably shares the genes encoding the sets of paralogous proteins UlaD/YiaQ, UlaE/YiaR and UlaF/YiaS displaying decarboxylase, 3-epimerase and 4-epimerase activity, respectively. Despite the high similarity between YiaR and UlaE, it has not been possible to assign the 3-epimerase function to YiaR by measuring the corresponding activity in vitro (Ibañez et al., 2006b; Yew & Gerlt, 2002).

The need to overcome L-ascorbate-generated oxidative stress for E. coli to grow with this compound as sole carbon source requires a certain metabolic flux. This is most probably only accomplished when both the ula regulon and the yiaK-S operon are activated. Recruitment of paralogous proteins to quickly assimilate this compound is an interesting and complex phenomenon, particularly in evolutionary terms. The finding that assimilation of L-ascorbate only takes place in strains constitutively expressing the yiaK-S operon or under conditions in which this operon is induced, reinforces the idea that expression of this operon is needed for L-ascorbate metabolism in the presence of oxygen. Why the yiaK-S operon is induced by L-ascorbate in the presence of certain amino acids like proline, threonine or glutamine, and not in the presence of serine or cysteine, remains to be elucidated.

We conclude that the effects of these amino acids to counteract L-ascorbate toxicity are due to their ability to allow yiaK-S operon induction by L-ascorbate, in this way reaching an efficient metabolic flux compatible with L-ascorbate assimilation in the presence of oxygen. Alternatively, these amino acids may act to slow the rate of L-ascorbate oxidation.

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