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Novel Antioxidant Role of Alcohol Dehydrogenase E from *Escherichia coli**

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Alcohol dehydrogenase E (AdhE) is an Fe-enzyme that, under anaerobic conditions, is involved in dissimilation of glucose. The enzyme is also present under aerobic conditions, its amount is about one-third and its activity is only one-tenth of the values observed under anaerobic conditions. Nevertheless, its function in the presence of oxygen remained ignored. The data presented in this paper led us to propose that the enzyme has a protective role against oxidative stress. Our results indicated that cells deleted in *adhE* gene could not grow aerobically in minimal media, were extremely sensitive to oxidative stress and showed division defects. In addition, compared with wild type, mutant cells displayed increased levels of internal peroxides (even higher than those found in a Δ*katG* strain) and increased protein carbonyl content. This pleiotropic phenotype disappeared when the *adhE* gene was reintroduced into the defective strain. The purified enzyme was highly reactive with hydrogen peroxide (with a K_i of 5 μM), causing inactivation due to a metal-catalyzed oxidation reaction. It is possible to prevent this reactivity to hydrogen peroxide by zinc, which can replace the iron atom at the catalytic site of AdhE. This can also be achieved by addition of $ZnSO_4$ to cell cultures. In such conditions, addition of hydrogen peroxide resulted in reduced cell viability compared with that obtained without the Zn treatment. We therefore propose that AdhE acts as a H_2O_2 scavenger in *Escherichia coli* cells grown under aerobic conditions.

Under anaerobic conditions *Escherichia coli* carries out mixed-acid fermentation of sugars. One of the major products is ethanol, which is synthesized from acetyl coenzyme A by two consecutive Fe^{2+} and NADH-dependent reductions, catalyzed by alcohol dehydrogenase E (AdhE)[1] (1). This enzyme, encoded by the a*dhE* gene (2), belongs to the group III Fe-activated dehydrogenases and shares a high degree of structural homology with other microbial alcohol dehydrogenases (3). AdhE is also known as pyruvate-formate lyase-deactivase because it converts the active radical form of pyruvate-formate lyase into the non-radical form (4, 5). AdhE is abundantly synthesized (about 3×10^4 copies per cell) during anaerobic growth on glucose and forms helical structures, called spirosomes, which are around 0.22 μm long and contain 40–60 AdhE molecules (6). This structure has also been detected in *Entamoeba hystolitica* (7), *Salmonella typhimurium* (8), *Yersinia enterolytica* (9), *Lactobacillus brevis*, and *Lactobacillus reuteri* (10, 11).

When *E. coli* cells are shifted from anaerobic to aerobic conditions, transcription of the *adhE* gene is reduced and maintained within 10% of the range found under anaerobiosis (12–15). Translation is also regulated and requires RNase III (15, 16). AdhE has been identified as one of the major targets when *E. coli* cells were submitted to hydrogen peroxide stress (17). In fact, under aerobic conditions, AdhE has no assigned function, accounts for about 1% of total protein, and is inactivated by metal-catalyzed oxidation (18). This apparent wastefulness prompted us to investigate why this enzyme has been maintained under aerobic conditions. Evidences about the relationship between AdhE and protection against oxidative stress are provided.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—The merodiploid strain *E. coli* ECL4000 (MC4100 adhE$^+$ Φ(*adhE-lacZ*)) was used as the wild type parental strain (18, 19). Isogenic strains were used in all experiments. A previously obtained null *adhE::kan* strain (ECL4002) was also used (20). An *E. coli* strain *katG::tet* (PEL1) was constructed by P1 transduction (21) from UM202 (22). The DHB4 strain was a kind gift from J. Beckwith and was also used as a wild type strain (23). *S. typhimurium* LT2 was from ATCC (catalog number E23564) and *Serratia marcescens* was from Colección Española de Cultivos Tipo (Valencia, Spain, catalog number 846).

Cells were grown at 30 °C either in Luria broth (LB) medium (0.5% yeast extract, 1% NaCl, and 0.5% tryptone) or minimal medium (64 mM K_2HPO_4, 34 mM NaH_2PO_4, 20 mM $(NH_4)_2SO_4$, 0.1 mM $MgSO_4$, 10 μM $CaCl_2$, and 1 μM $FeSO_4$, pH 7.4) supplemented with one of the following carbon and energy sources: glucose (10 mM), glucuronate (10 mM), gluconate (10 mM), acetate (30 mM), succinate (15 mM), fucose (10 mM), manitol (10 mM), glycerol (20 mM), or casein acid hydrolysate (0.1% w/v). Solid media contained 1.5% Bacto-agar (Difco) in LB. Aerobic cultures of 10 ml were grown in 100-ml flasks shaken at 250 rpm. Anaerobic cultures were grown in 100-ml flasks filled to the brim. When appropriate, antibiotics (Sigma) were added at the following concentrations: 100 μg/ml kamamycine and 25 μg/ml tetracycline. To study ECL4002 auxotrophies amino acids were added to minimal medium-glucose at 100 μg/ml each and cells were aerobically cultured.

Sensitivity to Stress Conditions—Exponentially growing cells (A_{600}: 0.3) were treated with the stressing compound, which was directly added to the growth medium at the concentrations and periods indicated for each experiment. Untreated cultures were incubated in parallel over the same periods. Sensitivity to the treatment was determined by serially diluted (1/10) bacterial suspension with phosphate-buffered saline and then plated by triplicates on LB agar.

Enzymatic Activity Assays—Cells were disrupted by sonication in an ice bath, and the centrifuged extracts were assayed for the following enzyme activities, as described: ethanol dehydrogenase (15), aconitase (24), malate dehydrogenase (25), catalase, and superoxide dismutase

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[1] The abbreviations used are: AdhE, alcohol dehydrogenase E; AhpCF, alquil hydroperoxide reductase; H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; LB, Luria broth; DNP, dinitrophenyl.

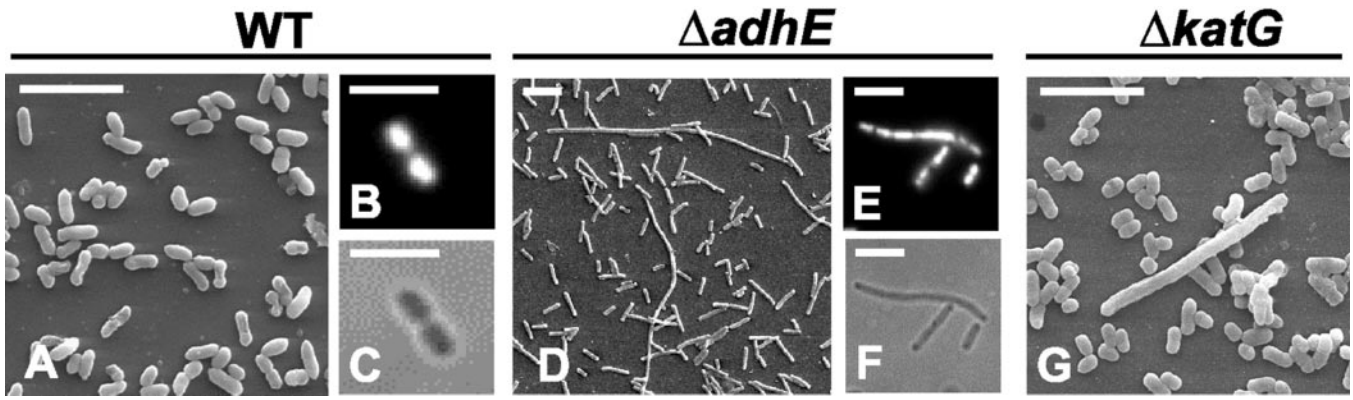

FIG. 1. **In the absence of AdhE, morphologic defects are associated with deficient septum formation.** Scanning electron microscopy from ECL4000 (*WT*) (*A*) and ECL4002 (*DadhE*) (*D*) cells grown in LB, and PEL1 (Δ*katG*) (*G*) cells grown in minimal medium-glucose supplemented with casein acid hydrolysate. *Bars*, 5 μm. Diaminophenylindole fluorescence from ECL4000 (*B*) and ECL4002 (*E*) cells and the corresponding Nomarski pictures (*C* and *F*). *Bars*, 2 μM.

(26). Protein concentration was determined by the Bradford method, using bovine serum albumin as standard.

Western Blot Analysis—Oxidized proteins in cell extracts were revealed immunochemically by their carbonyl content (27) after derivatization with dinitrophenylhydrazine (17). The anti-dinitrophenyl (DNP) antibody (Dako) was used at 1:5,000 dilution. Anti-AdhE Western blot was performed using the primary antibody at 1:2,000 dilution. In both cases, the secondary antibody was a goat anti-rabbit conjugated with alkaline phosphatase (Tropix) used at 1:25,000 dilution.

Measurement of Intracellular Oxidation Level—The oxidant-sensitive probe H_2DCFDA was used to measure the intracellular peroxide levels (28). Cells growing aerobically in LB (A_{600}: 0.3) were washed with 10 mM potassium phosphate buffer, pH 7.0, and incubated for 30 min in the same buffer with 10 μM H_2DCFDA (dissolved in dimethyl sulfoxide). Cells were washed, resuspended in the same buffer, and disrupted by sonication. Cell extracts (100 μl) were mixed in 1 ml phosphate buffer and fluorescence intensity was measured using a Shimadzu RF 5000 spectrofluorimeter (excitation, 490 nm; emission, 519 nm). Emission values were normalized by protein concentration.

In Vitro Inactivation Assays of AdhE—Purified AdhE (2.5 μM) in 50 mM Tris-HCl, 160 mM NaCl, pH 8.5, was incubated for 4 min at room temperature with different concentrations of either KO_2 (superoxide donor) plus catalase (50 units) or H_2O_2 and ethanol dehydrogenase was assayed. Potassium superoxide (KO_2) was maintained in dimethyl sulfoxide due to its instability in aqueous solutions. KO_2 concentration was monitored spectrophotometrically ($e_{260} = 2086\ M^{-1}\ cm^{-1}$) (29).

Other Methods—Total cellular iron was determined under reducing conditions (30), with bathophenanthroline sulfonate as chelator (31). Quantification of AdhE expression was carried out by Laurell rocket immunoelectrophoresis (32), according to a calibration curve (not shown) obtained with AdhE purified from strain ECL4000 as described previously (18). For DNA staining, cells were resuspended for 10 min in 70% (v/v) ethanol, washed and incubated with 1 μg/ml diaminophenylindole in phosphate-buffered saline for 10 min, and then rinsed with the same buffer. Fluorescence was viewed using a Nikon fluorescence microscope and images were taken using a Sony camera system. To obtain scanning electron microscopy images, exponentially growing cells were washed in 0.1 M phosphate potassium buffer, pH 7.0, and fixed for 30 min in phosphate-buffered glutaraldehyde (2.5% v/v). Cell suspension was transferred to silanized glass. After washing with potassium phosphate, cells were postfixed with osmium tetraoxide (1% v/v) for 30 min, followed by 10 min washes with 30, 50, 70, and 90% acetone. Cells were then washed 3 times with 100% acetone (30 min each) and dried. After carbon evaporation, gold was added to the cells (Balzers ScD 050 Sputer Coater), and samples were observed using a scanning electronic microscope (DMS 940 A Zeiss).

RESULTS

Growth Defects of ECL4002 Strain—When *E. coli* grows anaerobically on glucose, full expression of the *adhE* gene gives about 30,000 molecules/cell. In contrast, the amount of AdhE protein present under aerobic conditions, as measured by both Western blot and immunoelectrophoresis (see "Experimental Procedures"), was only 30% of that present under anaerobic conditions (9.6 ± 0.7 ng AdhE/μg total protein *versus* 28.1 ± 3.7 ng AdhE/μg total protein). Consequently, when oxygen was present in the culture, there were ~10,000 molecules of AdhE per cell, of which 70% were inactive. It thus seemed inefficient to synthesize such amounts of a 96-kDa protein that apparently had no physiological role. An explanation for this apparent paradox was found during the study of the Δ*adhE* strain (ECL4002).

ECL4002 cells were unable to grow aerobically in minimal medium on any of the carbon sources tested (glucose, succinate, fucose, acetate, glucuronate, gluconate, manitol, and glycerol). Aerobic metabolism of these sugars did not require any AdhE activity. This mutant strain grew under aerobic conditions in LB medium, but with a duplication time of 60 min (whereas wild type cells duplicated in 35 min in this medium). Addition of 0.1% casein acid hydrolysate to the minimal medium-glucose allowed growth but duplication time increased progressively over time. Analysis of amino acids needed for growth revealed that Δ*adhE* cells presented auxotrophies for Arg, His, Leu, Pro, and Thr, and growth without Lys was extremely limited.

As expected, the Δ*adhE* strain was unable to grow anaerobically in minimal medium plus glucose, but it did grow when glucuronate was the carbon source (33). The control lysogen ECL4063 (*adhE::kan λatt::adhE*$^+$) grew as the wild type strain in all the media and showed aerobic and anaerobic levels of AdhE activity indistinguishable from those of the wild type strain ECL4000 (18)

The growth defects of Δ*adhE* cells were also associated with changes in size and shape. When grown in LB medium, these mutant cells presented a filamentous appearance in comparison with wild type cells. Although, as known, wild type cells displayed a homogeneous size of 1–2 μm long (Fig. 1*A*), Δ*adhE* cells were, on average, 4–5 μm long, but cells 20–50 μm long could also be observed (Fig. 1*D*). DNA stain with the fluorochrome diaminophenylindole revealed that in these filamentous cells, replication and segregation of DNA was apparently normal (Fig. 1*E*). The filamentous phenotype was probably due to a problem in septum formation. Indeed, transmission electron microscopy revealed that there was no septum in the long Δ*adhE* cells (not shown). The same phenotype was also observed in DHB4 cells used as a parallel control.

AdhE Deletion Causes an Oxidative Stress Phenotype—During our study of Δ*adhE* cells, we observed that they showed less viability after freeze-thawing and lysozyme treatment than ECL4000 cells, which could indicate that their cell membrane was defective. This phenomenon and the previously described growth defects led us to hypothesize an internal oxidative stress problem in cells deficient in AdhE. To analyze this possibility, wild type and Δ*adhE* cells were tested for viability

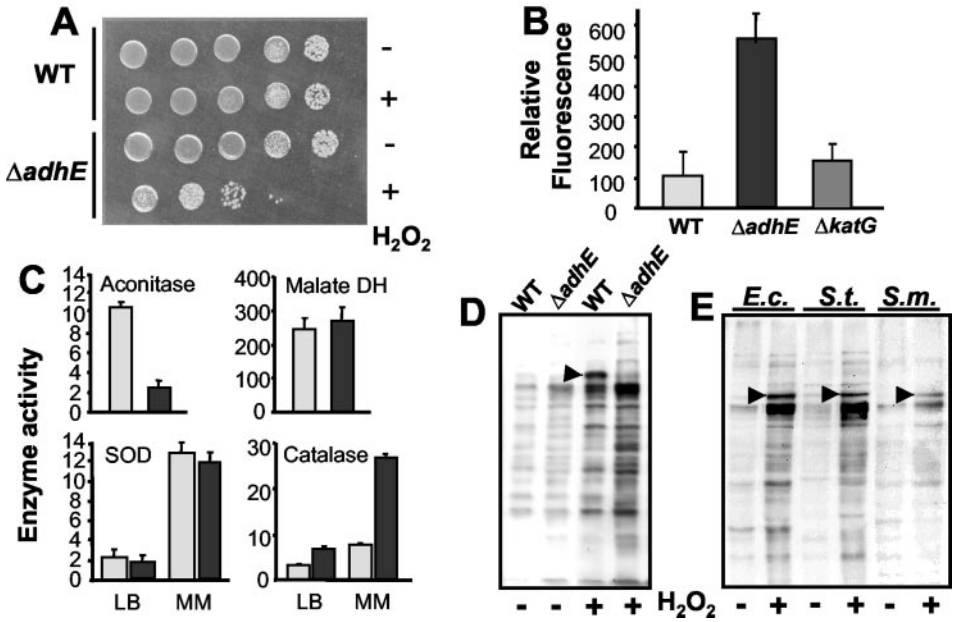

FIG. 2. **Cells without AdhE displayed a phenotype characteristic of endogenous oxidative stress.** ECL4000 (*WT*) and ECL4002 (Δ*AdhE*) cells growing in LB cells were tested for sensitivity to 2 mM H_2O_2 treatment (60 min). Serial dilutions were plated on LB plates (*A*). ECL4000, ECL4002, and PEL1 (Δ*katG*) cells grown in LB were loaded with 10 μM H_2DCFDA. Intracellular peroxides were measured as arbitrary units of fluorescence intensity (*B*). Enzyme activities were determined in ECL4000 (*shaded bars*) and ECL4002 (*closed bars*) cells growing exponentially in LB or minimal medium-glucose supplemented with casein acid hydrolysate. Units were expressed as nmols·min^{-1}·mg^{-1} in the case of aconitase and malate dehydrogenase and as μmols·min^{-1}·mg^{-1} in the case of superoxide dismutase and catalase (*C*). Protein oxidative damage was detected with anti-DNP Western blot from ECL4000 and ECL4002 cells treated with 10 mM H_2O_2 for 30 min. Untreated cells were used as a control (*D*). Anti-DNP Western blot from wild type cells of *E. coli*, *S. typhimurium*, and *S. marcescens* treated with 10 mM H_2O_2 for 30 min. Untreated cells were used as controls (*E*). *Arrowheads* in *D* and *E* indicate the band corresponding to AdhE.

after treatment with 2 mM H_2O_2 for 60 min. The mutant strain had a decreased viability (around two orders of magnitude) both in aerobic (Fig. 2*A*) and anaerobic conditions (not shown) compared with the wild type cells. No differences in viability between the two strains were observed when cells were treated with paraquat, a superoxide generator (not shown).

The fluorescent probe H_2DCFDA was used to measure intracellular peroxide levels (Fig. 2*B*). Basal levels of endogenously generated peroxides were 5 times greater in Δ*adhE* cells compared with wild type cells. When treated with 10 mM H_2O_2 for 60 min, peroxides in the mutant strain were only slightly higher than those found in wild type cells (not shown).

Several enzyme activities known to be affected by reactive oxygen species were measured in cells grown aerobically in LB (Fig. 2*C*). Aconitase, an enzyme with an Fe/S cluster, has been reported to be very sensitive to oxidative stress (31, 34, 35). In Δ*adhE* cells, aconitase activity was reduced 5-fold with respect to wild type strain. As a control, malate dehydrogenase, which has been reported to be resistant to oxidative stress (31, 35, 36), showed no differences between wild type and Δ*adhE* cells. Catalase (HPI) and Mn-superoxide dismutase are enzymes regulated by the transcriptional regulators OxyR (activated in response to H_2O_2) and SoxRS (activated in response to superoxide), respectively. Levels of catalase activity in Δ*adhE* cells grown either in LB medium or in glucose-minimal medium supplemented with casein acid hydrolysate, were, respectively, two and four times greater with respect to those displayed in wild type cells. However, there was no difference in superoxide dismutase activity between both strains, either when grown in LB or in glucose-minimal medium. This indicated that H_2O_2, but not $O_2^{\cdot-}$ levels, were higher than normal in the Δ*adhE* cells (Fig. 2*C*).

The endogenous oxidative stress measured with H_2DCFDA could have been due to a defective iron metabolism, resulting in iron accumulation within the cell, as reported in several *E. coli* mutant strains (37). To investigate this possibility, total iron was measured using batophenanthroline sulfonate (see "Experimental Procedures"). Results demonstrated that there were no significant differences between wild type and Δ*adhE* strains grown in LB with respect to total cell iron (3.3 nmol Fe/6 × 10^8 cells).

One of the markers under oxidative stress conditions is the formation of carbonyl groups on proteins due to modification of some amino acid side chains (27). Anti-DNP Western blot experiments revealed that in Δ*adhE* cells, grown aerobically in LB and stressed with 10 mM H_2O_2 for 60 min, showed increased levels of protein damage as compared with stressed wild type cells (Fig. 2*D*). As shown, even in non-stressed cells, protein carbonylation was slightly higher in Δ*adhE* cells than in wild type cells.

Many microorganisms, including human pathogens, also synthesize a multifunctional alcohol dehydrogenase. These enzymes are members of the "iron-dependent" dehydrogenase family and share a high sequence homology to AdhE from *E. coli*. To verify whether these homologues were also major targets under H_2O_2 stress conditions, wild type cells from other two enterobactericeae, *S. typhimurium* and *S. marcescens*, were submitted to 10 mM H_2O_2 for 60 min, and protein oxidation was analyzed by anti-DNP Western blot and compared with *E. coli* (Fig. 2*E*). As expected, a band corresponding to AdhE became strongly carbonylated in both *S. typhimurium* and *S. marcescens*, which means that homologues of AdhE exhibit the same reactivity to H_2O_2.

*Comparison Between Δ*adhE* and Δ*katG* Cells*—From the above results one can hypothesize that strain ECL4002 was deficient at detoxifying endogenously generated peroxides. In this context, to compare the role of AdhE with that of catalase, a *katG* defective mutant was constructed (see "Experimental Procedures"). This gene encoded for the exponential phase catalase, HPI. Several parameters were measured to compare Δ*katG* cells with Δ*adhE* cells. Cultures of Δ*katG* cells were grown aerobically in LB with a duplication time of 45 min (as compared with 60 min for Δ*adhE* and 35 min for wild type

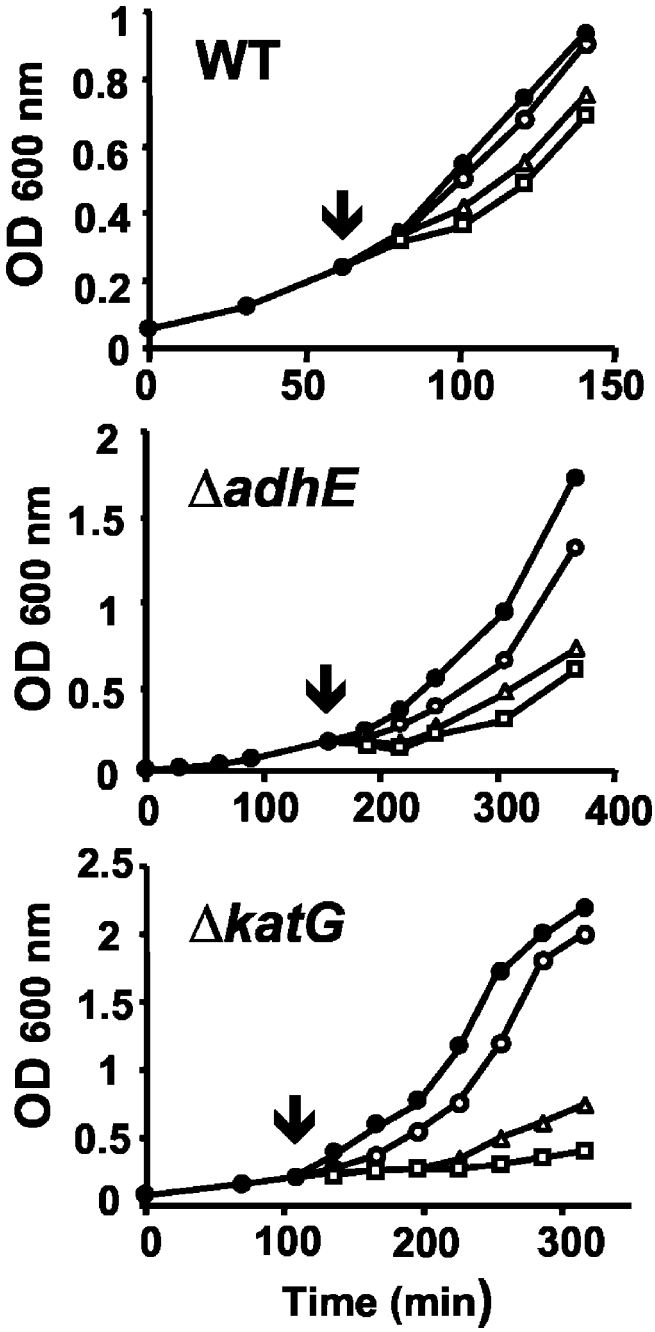

FIG. 3. **AdhE plays an important role in protection against low levels of H_2O_2.** Growth of ECL4000 (*WT*), ECL4002 (Δ*AdhE*), and PEL1 (Δ*katG*) cells in LB was monitored. Cell cultures were divided and, at the indicated time (*arrow*): 0 (●), 0.1 (○), 0.5 (△), or 1 (□) mM H_2O_2 added. Results are the average of three independent experiments with a variation of less than 15%.

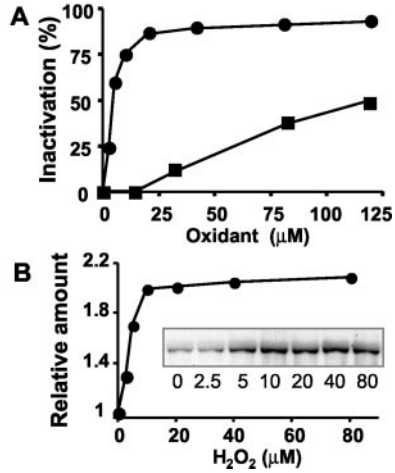

FIG. 4. **AdhE reacts with H_2O_2 with high affinity.** Purified AdhE (2.5 μM) was incubated for 4 min with the indicated concentrations of H_2O_2 (●) or KO_2 plus 50 units of catalase (■), and ethanol dehydrogenase activity was immediately assayed (*A*). Samples of AdhE treated with H_2O_2 as described above, were submitted to anti-DNP Western blot to measure carbonyl formation and densitometric analysis of the Western blot was performed. One arbitrary unit corresponds to the signal intensity given by the unstressed sample (*B*).

strain). The HPI-deficient cells grew on minimal medium with glucose as a carbon source under aerobic conditions, although only at the permissive temperature of 30 °C (Δ*adhE* strain did not grow in this medium at any temperature). When observed under the electron microscope, a minor fraction (fewer than 0.1%) of the cells were longer than normal when grown in this minimal medium (Fig. 1*G*). No apparent defects were observed when grown in LB (not shown).

Peroxide levels measured in Δ*katG* cells growing under aerobic conditions in LB (Fig. 2*B*) were found slightly increased compared with wild type cells, although smaller than those found in Δ*adhE* cells. It is known that cells submitted to a non-lethal oxidative stress by H_2O_2 transiently arrest their growth. After a lag period cells recover from the stress and growth is restored. This lag time was measured in wild type, Δ*katG* and Δ*adhE* cultures, grown aerobically in LB and stressed with various concentrations of H_2O_2 (Fig. 3). Wild type cells presented only slight growth delay at the highest dose of H_2O_2 used (1 mM) and almost no effect at lower concentrations. On the contrary, both Δ*adhE* and Δ*katG* cells arrested their growth after stress. As expected, at 0.5 and 1 mM H_2O_2, HPI was more important than AdhE, because Δ*katG* cells arrested growth longer than Δ*adhE* cells. However, at 0.1 mM H_2O_2, growth of Δ*adhE* cells was almost as equally affected as Δ*katG* cells. In addition, Δ*adhE* strain had a duplication time higher than Δ*katG* strain. These results indicated that AdhE was more important than HPI for maintaining basal levels of H_2O_2.

AdhE is Highly Reactive to H_2O_2—The reactivity between AdhE and H_2O_2 was observed *in vivo* measuring the rapid inactivation of ethanol dehydrogenase activity following the addition of 0.5 or 1 mM H_2O_2 for 5 min to ECL4000 cultures. An inactivation of 55 and 80% was, respectively, obtained. Under these conditions, aconitase was only inactivated 25 and 40%, respectively. AdhE inactivation by H_2O_2 was irreversible because addition of 10 μg/ml chloramfenicol to ECL4000 cells simultaneous to H_2O_2 treatment prevented the recovery of ethanol dehydrogenase activity. When no antibiotic was added, AdhE activity increased in parallel with the duplication of cells (not shown).

Using pure preparations of AdhE, the plot of H_2O_2 concentration *versus* enzyme inactivation gave an inactivation constant (K_i) for H_2O_2 of 5 μM (Fig. 4*A*). However, when KO_2 was used as a superoxide donor (in the presence of catalase to remove spontaneously formed H_2O_2), AdhE reactivity was clearly lower (K_i for superoxide was 120 μM) (Fig. 4*A*). As expected, inactivation of AdhE by H_2O_2 resulted in carbonyl formation (Fig. 4*B*). Taken together, these results were consistent with the idea that AdhE plays an important role in combating H_2O_2 stress, but not superoxide stress.

Zn Inactivation of AdhE Generates Cells Highly Sensitive to Oxidative Stress—Assuming the aerobic role of AdhE as an H_2O_2 scavenger, the addition of any inhibitor of its scavenging activity should result in cells highly sensitive to oxidative stress, resembling Δ*adhE* cells. In previous studies (38, 39) on ADHII, a dehydrogenase from *Zymomonas mobilis*, which is also a member of the iron-activated dehydrogenase family, our

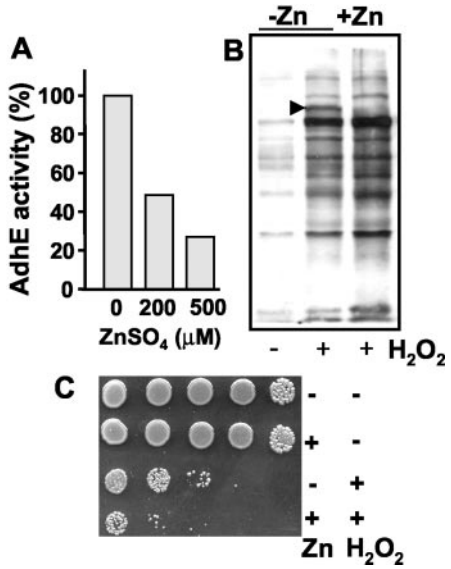

FIG. 5. **Inactivation of AdhE by Zn decreases the resistance of wild type cells to H_2O_2 stress.** ECL4000 cells growing in LB were treated with the indicated concentrations of $ZnSO_4$ for 20 min. Cell extracts were obtained, and ethanol dehydrogenase activity was measured (*A*). Anti-DNP Western blot from ECL4000 cells grown in LB treated in both the absence and presence of 500 μM $ZnSO_4$ for 20 min prior to 10 mM H_2O_2 stress (60 min). Unstressed cells were used as a control (*B*). ECL4000 cells growing in LB were pretreated in the absence or the presence of 500 μM $ZnSO_4$ for 20 min prior to 10 mM H_2O_2 stress (60 min). Cell viability was assayed by plating serial dilutions on LB plates. Unstressed cells were used as a control (*C*).

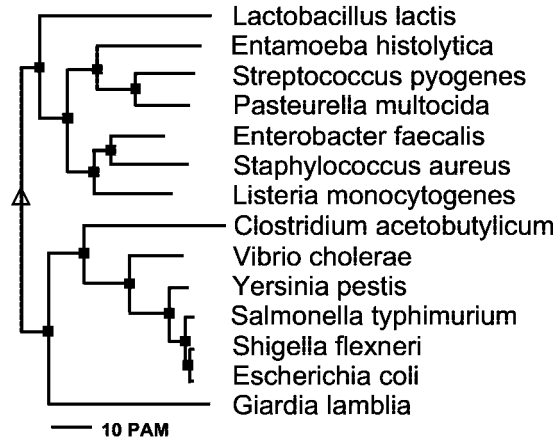

FIG. 6. **Phenogram of representative AdhE homologs.** The Multialin program (54) was used to obtain the phenogram from sequences available from Swiss-Prot (www.expasy.org), The Wellcome Trust Sanger Institute (www.sanger.ac.uk), and The Institute for Genomic Research (www.tigr.org).

group demonstrated that the Fe-ion was easily exchanged by Zn, which results in alcohol dehydrogenase inactivation and inability to carry out Fenton reaction. As expected, when $ZnSO_4$ was added to ECL4000 cultures, AdhE was inactivated in a dose-dependent manner (Fig. 5*A*). In addition, carbonyl groups formation in AdhE under oxidative stress were undetectable when zinc was present (Fig. 5*B*). Fig. 5*C* shows that the effect of Zn treatment prior to H_2O_2 stress reduced cell viability (around 1 order of magnitude), demonstrating the importance of Fe-AdhE as an H_2O_2 scavenger.

DISCUSSION

AdhE displays its activity under anaerobic or microoxic conditions; in aerobiosis, transcription is down-regulated and the enzyme is inactivated by metal-catalyzed oxidation. To date, no role has been assigned to this enzyme when oxygen is present. Our results indicated that cells lacking AdhE showed markers of a stress situation with a phenotype resembling that of a cell deficient in antioxidant defense systems. This conclusion was based on the following observations. (i) The increased H_2O_2 concentrations inside the cell, measured with H_2DCFDA. (ii) Inactivation of aconitase, an enzyme highly sensitive to oxidative stress (31, 34, 35, 40), which in this situation, liberates iron ions present in its Fe/S cluster after oxidation (41). This, in turn, may further increase production of reactive oxygen species. (iii) Increased levels of catalase activity, which are probably the result of OxyR activation. This transcription factor becomes activated when H_2O_2 concentration is 10^{-5} M or higher (42). (iv) Filamentous morphology may indicate the activation of the SOS system (43), due to damage to DNA produced by H_2O_2 (44). SOS activation blocks septum formation, producing cells longer than normal (45). (v) Auxotrophies observed in Δ*adhE* could not be explained solely on the basis of a higher susceptibility to inactivation of enzymes implicated in the amino acid biosynthesis. This could also have been due to membrane permeation produced by reactive oxygen species as described in Δ*sod* cells (46).

The results clearly demonstrated that AdhE was able to react with H_2O_2, both *in vivo* and *in vitro*, producing an irreversible inactivation of the enzyme. This was indicated by the lack of recovery when chloramfenicol and H_2O_2 were simultaneously added to the culture. This scavenging power would imply an equimolar reaction between H_2O_2 and AdhE. Although a reducing cycle (with Fe^{3+} being recycled to Fe^{2+}, perhaps by NAD(P)H^+ or superoxide) would make the system more efficient by allowing to scavenge more than 1 molecule of H_2O_2 per molecule of AdhE, several indirect results seemed to rule out this possibility. First, AdhE has lower binding affinity to ferrous ion than to ferric ion.[2] And second, propanediol oxidoreductase, an enzyme with the same iron-binding signature is inactivated by metal-catalyzed oxidation due to the modification of a highly conserved histidine residue, which is essential for iron binding (39, 47).

AdhE is important for controlling H_2O_2 homeostasis under normal aerobic conditions because reactive oxygen species produced in the electron transport chain are of the same order of magnitude as K_i for H_2O_2 (5 μM). A similar role has been proposed for alquil hydroperoxide reductase (AhpCF) (48). Although it would be difficult to establish which is more important, in contrast to Δ*adhE* strain, Δ*ahpCF* mutants grow aerobically in LB as well as a wild type strain (48). AhpCF is regulated by OxyR (AdhE is not), which means that at intermediate H_2O_2 concentrations (10^{-5}–10^{-4} M), the amount of AhpCF would rise considerably and, consequently, will perform its physiological role. At high concentrations, catalases are the predominant enzyme against H_2O_2 as demonstrated in the case of our HPI-deficient cells and by other authors (48, 49). HPI has a K_m of 5.9 mM (50), and HPII (reported as important only in stationary phase, when it is induced by RpoS) (48, 51) has similar kinetic parameters (52). One can hypothesize that these sets of enzymes could constitute a three-step barrier against H_2O_2, with AdhE being the most important at lowest H_2O_2 concentrations. An additional advantage, such as the fast adaptability to an anoxic situation, can be deduced from the maintenance of such an expensive system as AdhE under aerobic conditions. In this context, it must be remembered that 30% of AdhE molecules remain active and can start immediately working as a dehydrogenase.

The observation that these mutant cells exhibited lower resistance to freeze-thawing and to lysozyme treatment (not

[2] P. Echave, J. Tamarit, E. Cabiscol, and J. Ros, unpublished results.

shown) led us to believe that Δ*adhE* cells were very fragile as a result of the continuous attacks from H_2O_2 generated inside the cell (the membrane was probably affected). The decreased viability observed by a strong stress (2–10 mM H_2O_2), would be the result of this fragility and not strictly due to the lack of AdhE scavenging activity, because in this situation HPI is by far the most important enzyme.

There is an increasing list of microorganisms with orthologues to AdhE sharing a 60–90% sequence homology (Fig. 6). These include human pathogens, which give this enzyme clinical importance. It seems reasonable to assume that all these enzymes should act in the same way that AdhE did in *E. coli*. The results obtained in *S. typhimurium* and *S. marcescens* are in agreement with this assumption. Additionally, in a systematic study of genes involved in survival to macrophage attack in *S. typhimurium*, it was observed that *adhE* mutant cells were more sensitive than wild type cells (53). The role of these orthologues as H_2O_2 scavengers provide clues to explain why even microorganisms that neither produce alcohols nor use them as carbon sources (*Listeria, Staphylococcus*, and *Streptococcus*) have maintained this enzyme.

It would be especially interesting to be able to inactivate these AdhEs in bacterial pathogens making them more susceptible to the immune system. From previous studies (38), we know that Zn can replace Fe easily in these dehydrogenases *in vitro*. As we have demonstrated in this study, treatment of *E. coli* cells with $ZnSO_4$ inactivated AdhE, and this Zn-AdhE was not able to perform the Fenton reaction. More interestingly, these Zn-treated *E. coli* cells became more susceptible to oxidative stress. It is possible to speculate on the possible role of Zn treatment in infections involving such pathogen microorganisms. Developing specific inhibitors to AdhE could be of great interest in the near future.

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Novel Antioxidant Role of Alcohol Dehydrogenase E from *Escherichia coli*

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