AhpC Is Required for Optimal Production of Enterobactin by Escherichia coli

Li Ma and Shelley M. Payne

Section of Microbiology and Molecular Genetics and Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas, USA

Escherichia coli alkyl hydroperoxide reductase subunit C (AhpC) is a peroxiredoxin that detoxifies peroxides. Here we show an additional role for AhpC in cellular iron metabolism of E. coli. Deletion of ahpC resulted in reduced growth and reduced accumulation of iron by cells grown in low-iron media. Liquid chromatography–mass spectroscopy (LC-MS) analysis of culture supernatants showed that the ahpC mutant secreted much less enterobactin, the siderophore that chelates and transports ferric iron under iron-limiting conditions, than wild-type E. coli did. The ahpC mutant produced less 2,3-dihydroxybenzoate, the intermediate in the enterobactin biosynthesis pathway, and providing 2,3-dihydroxybenzoate restored wild-type growth of the ahpC mutant. These data indicated that the defect was in an early step in enterobactin biosynthesis. Providing additional copies of entC, which functions in the first dedicated step of enterobactin biosynthesis, but not of other enterobactin biosynthesis genes, suppressed the mutant phenotype. Additionally, providing either shikimate or a mixture of para-aminobenzoate, tryptophan, tyrosine, and phenylalanine, which, like enterobactin, are synthesized from the precursor chorismate, also suppressed the mutant phenotype. These data suggested that AhpC affected the activity of EntC or the availability of the chorismate substrate.

The ability to acquire sufficient iron from the environment is essential for growth of most bacteria, including Escherichia coli. An efficient mechanism of iron acquisition by E. coli under iron-limiting conditions involves the synthesis and secretion of the siderophore enterobactin, a cyclic trimer of 2,3-dihydroxybenzoyl serine (35, 36, 39). This low-molecular-weight, high-affinity chelator sequesters ferric iron and facilitates its transport to the cytoplasm of the cell (35, 36, 39).

There are six genes, entA to -F, encoding enzymes for enterobactin biosynthesis from the precursor chorismate (7, 49, 50). In the initial stage of enterobactin biosynthesis, the enzymes EntC, EntB, and EntA convert chorismate to 2,3-dihydroxybenzoate (DHB) (27, 28, 44). The remaining enzymes, EntD to -F, together with the bifunctional enzyme EntB, convert DHB to enterobactin, which is secreted by the cells (Fig. 1) (10, 17, 50). Extracellular enterobactin binds ferric iron, and the complex is transported back into the cells via a ligand-specific transport system encoded by fepABCDG (4, 23). E. coli also has the ability to transport xeno siderophores, including the fungal siderophore ferrichrome (Fc) (5).

The expression of genes encoding iron transport proteins is regulated by the transcriptional repressor Fur (20, 29). Under iron-rich conditions, iron in the cytoplasm binds Fur, and Fe-Fur binds at the promoter regions of iron-responsive genes, repressing their transcription (29). Under iron-limiting conditions, there is insufficient iron available in the cytoplasm to bind Fur, so Fur no longer binds the promoters and transcription is derepressed (29).

In a screen for mutants defective in iron acquisition, we noted a mutant deleted for genes in both the glutathione and thioredoxin reductase systems (gor and trxB) that a mutant deleted for genes in both the glutathione and thioredoxin reductase systems similarly to the wild type in rich medium, the gor mutant grew very poorly and rapidly accumulates suppressor mutations (40). The suppressor mutations were mapped to a gene encoding alkyl hydroperoxide reductase subunit C (AhpC) (40). One frequent suppressor mutation, designated ahpC*2, has a triplet expansion at position 38 in ahpC (40), and this mutation was present in the gor trxB mutant we tested.

AhpC is a member of the alkyl hydroperoxide reductase system, which in E. coli consists of two proteins: a peroxiredoxin (AhpC) and a flavoprotein disulfide reductase (AhpF). AhpC converts peroxides to alcohol and water, and AhpF recycles the oxidized AhpC (22, 48). AhpC is a homodimer and belongs to the 2-Cys peroxiredoxin family, with C46 of one subunit attacking the peroxide substrate, being oxidized to a cysteine sulfenic acid and resolved by C165 of the other subunit (11, 22). Furthermore, the oligomeric states of AhpC correlate with the oxidation–reduction states of AhpC. The reduced form of AhpC is exclusively dimeric. Disulfide bond formation results in a loss of stability of the decamer and in dissociation to the dimeric form (53).

Here we show that mutations in ahpC affect cellular iron metabolism of E. coli and that it is the ahpC* mutation in the gor trxB ahpC* mutant that is responsible for reduced growth under low-iron conditions. The participation of AhpC is in an early step in enterobactin biosynthesis, demonstrating a previously uncharacterized role for AhpC.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids are listed in Table 1, and primer sequences are listed in Table 2. Strains BW25113ahpF and BW25113trxB were constructed by bacteriophage P1 transduction of the ahpF and trxB deletion mutations from Keio collection mutants (2) into E. coli strain BW25113. To verify that this strain does

Received 25 August 2012 Accepted 26 September 2012 Published ahead of print 5 October 2012

Address correspondence to Shelley M. Payne, smpayne@austin.utexas.edu.

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doi:10.1128/JB.01574-12
EntDEF, together with the bifunctional enzyme EntB, converts DHB to enterobactin biosynthesis in E. coli. There are six genes, entA to -F, encoding enzymes for enterobactin biosynthesis in E. coli. EntC, the isochorismate synthase, converts chorismate to isochorismate; EntB, the isochorismatase, converts isochorismate to 2,3-dihydro-2,3-dihydroxybenzoate; and EntA, the 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, converts 2,3-dihydro-2,3-dihydroxybenzoate to DHB. EntA, the 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, converts 2,3-dihydro-2,3-dihydroxybenzoate to DHB. EntDEF, together with the bifunctional enzyme EntB, converts DHB to enterobactin (50). (Modified from reference 54.)

**FIG 1** Enterobactin biosynthesis branch of the shikimic acid pathway of E. coli. In E. coli, enterobactin is synthesized from chorismate, which is derived from the shikimic acid pathway (9). There are six genes, entA to -F, encoding enzymes for enterobactin biosynthesis in E. coli. EntC, the isochorismate synthase, converts chorismate to isochorismate; EntB, the isochorismatase, converts isochorismate to 2,3-dihydro-2,3-dihydroxybenzoate; and EntA, the 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, converts 2,3-dihydro-2,3-dihydroxybenzoate to DHB. EntDEF, together with the bifunctional enzyme EntB, converts DHB to enterobactin (50). (Modified from reference 54.)

not carry the rpoS mutation found in some related strains, the rpoS gene was analyzed by DNA sequencing and confirmed to be the wild-type allele. Strain BW25113entF was derived by bacteriophage P1 transduction of the entF mutation from ARM110 (33) into BW25113. Strains ML100 and ML300 were derived by introducing the ahpC deletion from the ahpC mutant of the Keio collection into BW25113 and BW25113entF by allelic exchange, as follows. The ΔahpC::kan cassette was amplified from the ahpC mutant by use of primers P1 and P2 and Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The product was digested with XbaI and cloned into pHM5 digested with XbaI and XhoI. pEnt1 (entCEBA) and pEnt2 (entC) were constructed by amplifying the genes from E. coli strain BW25113 with Platinum Pfx DNA polymerase, using primers P5 and P6. Each PCR fragment was digested with XbaI and Xhol and inserted into pWSK29 digested with XbaI and Xhol. pEnt1 (entCEBA) and pEnt2 (entC) were constructed by amplifying the genes from E. coli strain BW25113 with Platinum Pfx DNA polymerase, using primers P7 and P8 for entCEBA and primers P9 and P10 for entC. Each PCR fragment was cloned into the EcoRV site of pWSK29. All constructs were confirmed by DNA sequencing.

Site-directed mutagenesis was used to replace each of the two cysteine residues in AhpC with serine. The plasmid pAhpC16S was constructed by splice overlap extension PCR as described previously (21), using Platinum Pfx DNA polymerase and primers P5, P6, P11, and P12. The plasmid pAhpC16S was constructed using primers P13 and P14 and Phusion Hotstart thermostable proofreading DNA polymerase (NEB, Ipswich, MA), following the QuikChange site-directed mutagenesis protocol (Agilent Technologies, Santa Clara, CA). All constructs were confirmed by DNA sequencing.

**Iron metabolism assays.** 
(i) **Bioassay.** For ent-negative strains, 4.8 × 10⁴ CFU/ml of bacterial cells were added to molten LB agar containing 0.2 mg/ml EDDA and allowed to solidify. Iron sources (5 μl) were spotted onto the surface of the agar, and the diameter of the zone of growth around each iron source was measured after 24 h of incubation at 37°C. Iron sources used were 10 μl purified enterobactin (generously provided by K. N. Raymond, University of California, Berkeley, CA), 10⁷ cells/ml BW25113 as a source of enterobactin and related catechols, 0.8 mM ferriochrome (Fc) (Sigma, St. Louis, MO), and 25 mM FeSO₄.

(ii) **Sensitivity assays.** To measure sensitivity to iron chelation by EDDA, overnight cultures were diluted 1:600 in 5 ml LB broth containing EDDA at concentrations ranging from 0 to 2.5 mg/ml and grown with aeration at 37°C for 8 h, at which time the OD₆₅₀ was measured.

To measure sensitivity to cumene hydroperoxide, 100-μl overnight cultures of each strain were mixed with 3 ml of LB top agar and poured onto LB plates. Ten microliters of 5% cumene hydroperoxide was spotted on a sterile paper disc that was placed in the center of the plate. After 24 h of incubation at 37°C, the diameters of the killing zones of bacteria were measured.

**Detection of catechols and enterobactin.** To quantitatively measure extracellular catechol concentrations, overnight cultures in LB broth were centrifuged, and the cells were resuspended in the same volume of saline and then diluted 1:500 in T medium. The cultures were grown with aeration at 37°C for 24 h. Catechols were assayed by a modification of the method of Arnow (1), as follows. The culture was centrifuged, and 0.5 ml of the supernatant was collected and acidified by adding 250 μl of concentrated HCl, followed by extraction with a 1/8 volume of ethyl acetate, twice. The extracts were dried using a SpeedVac system and analyzed not para-aminobenzoic acid (50 μg/ml). The ferric iron chelator ethylenediamine-N,N’-bis(2-hydroxyphenylacetic acid) (EDDA or EDDHA) (15), deferred by the method of Rogers (42), was added where indicated.

Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 12.5 μg/ml. For growth assays, cultures were inoculated to an initial optical density at 650 nm (OD₆₅₀) of 0.01 and grown at 37°C with aeration. Growth was monitored by measuring the OD₆₅₀ over 16 h or by diluting and plating cells to determine the number of CFU.

**Plasmid construction.** To construct pAhpC and pAhpC*, the ahpC and ahpC* genes of strains BW25113 and SMG96, respectively, were amplified with Platinum Pfx DNA polymerase, using primers P5 and P6. Each PCR fragment was digested with XbaI and Xhol and inserted into pWSK29 digested with XbaI and Xhol. pEnt1 (entCEBA) and pEnt2 (entC) were constructed by amplifying the genes from E. coli strain BW25113 with Platinum Pfx DNA polymerase, using primers P7 and P8 for entCEBA and primers P9 and P10 for entC. Each PCR fragment was cloned into the EcoRV site of pWSK29. All constructs were confirmed by DNA sequencing.

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Direct detection of enterobactin in the supernatant was performed by a modification of the method of Furrer et al. (16), as follows. Fifty milliliters of the supernatant was collected and acidified by adding 250 μl of concentrated HCl, followed by extraction with a 1/8 volume of ethyl acetate, twice. The extracts were dried using a SpeedVac system and analyzed...
by liquid chromatography–mass spectrometry (LC–MS) at the University of Texas at Austin Protein and Metabolite Analysis Facility. For LC–MS, the flow rate was 0.15 ml/min and fractions were monitored at a wavelength of 220 nm.

**Measurement of total cellular iron content.** Overnight LB broth cultures were centrifuged, resuspended in an equal volume of saline, diluted 1:500 in 25 ml LB broth or T medium, and grown with aeration at 37°C for 12 h (LB broth) or 24 h (T medium). A total of 4 × 10⁹ cells from each sample were collected, washed once with saline, and lysed by adding an equal volume of concentrated HNO₃. The homogenized sample was analyzed in an Agilent 7500ce inductively coupled plasma mass spectrometer (ICP–MS) at the University of Texas at Austin School of Geological Sciences.

**In vitro competition assay.** Overnight cultures of the wild-type (BW25113) and ahpc mutant (ML100) strains were centrifuged and resuspended in saline. Equal numbers of cells (1.25 × 10⁹ CFU) of each strain were mixed in 50 ml T medium and grown with aeration at 37°C for 18 h. Each strain (2.5 × 10⁸ CFU) was also grown individually in T medium under the same conditions. Growth was monitored by diluting and plating cells in triplicate on LB agar every 2 h. The growth of the ahpc mutant in the mixture was determined by patching the colonies from LB agar to LB agar containing kanamycin, and the growth of the wild type was determined by subtraction of the number of Kanr colonies from the total.

**β-Galactosidase assay.** Cells carrying lacZ transcriptional fusions were grown with aeration at 37°C for 3 h in LB broth containing EDDA and 50 µg/ml ampicillin. β-Galactosidase was measured as described by Miller (34).

**SDS-PAGE and Western immunoblotting.** After growing overnight at 37°C with aeration, 1 × 10⁹ bacterial cells were collected by centrifugation, resuspended in 75 µl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and boiled for 5 min. Five microliters of each sample was loaded onto a 12% polyacrylamide gel. Following electrophoresis, the gels were either stained with Coomasie blue or electroblotted for 1.5 h at 45 V onto Hybond ECL nitrocellulose (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). Western immunoblots were incubated with an anti-AhpC rabbit polyclonal primary antibody (generously provided by L. Poole, Wake Forest University School of Medicine) and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA) secondary antibody (diluted 1:10,000). The signal was detected by developing the blot with an enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL).

## RESULTS

Deletion of ahpc CLeads to increased sensitivity to iron chelation. Analysis of a trxB gor mutant (SMG96) in the EDDA sensitivity assay indicated that it had increased sensitivity to iron chelation by EDDA compared to its parent strain (Fig. 2A). SMG96 not only carries a suppressor mutation in ahpc, which encodes an alkyl hydroperoxidase. This suppressor mutation, designated ahpc*, has a
codon expansion at position 38 that gives the protein reductase activity but eliminates the peroxidase function (39). Therefore, to determine the genetic basis for the reduced fitness of SMG96 under iron-limiting conditions, single deletions in gor, trxB, and ahpC were constructed in strain BW25113, and the resulting mutants were tested for EDDA sensitivity.

Deletion of ahpC, but not gor or trxB, resulted in an increased EDDA sensitivity compared to that of the wild type (Fig. 2B). Wild-type resistance to iron chelation was restored by providing the ahpC gene carried on a plasmid (Fig. 2C). However, the ahpC* gene, which suppressed the gor trxB mutant’s reductase phenotype, did not suppress the increased EDDA sensitivity of the ahpC mutant (Fig. 2C). The ahpC mutant carrying pAhpC* was tested by Western blot analysis to confirm that the protein was produced. The results showed that the amount of AhpC* protein produced by that strain was comparable to the level of AhpC detected in the wild-type strain (Fig. 3B). These data indicate that the functional activity of AhpC is required for wild-type resistance to iron chelation and that deletion of ahpC or mutation of ahpC to ahpC* results in reduced growth in the presence of an iron chelator.

To confirm that the increased EDDA sensitivity of the ahpC mutant was due to iron starvation, the wild type and the ahpC mutant were tested in the EDDA sensitivity assay, with or without added iron. In the presence of added iron, the ahpC mutant grew to the same level as the wild type (Fig. 2D), confirming that the reduced growth of the ahpC mutant in the presence of EDDA was due to iron limitation. The ahpC mutant also showed reduced growth in a low-iron minimal medium (T medium). Similarly, the ahpC mutant grew more slowly and plateaued at a lower cell density than the wild type in T medium, and the difference was eliminated by addition of iron (Fig. 4).

**Internal iron levels are lower in the ahpC mutant.** To determine whether the reduced growth in low-iron medium indicated that the ahpC mutant was more iron starved than the wild type, expression of a reporter plasmid was used as a measure of the internal iron level. The reporter plasmid (32) has a transcriptional fusion of the ryhB promoter, which is iron regulated via Fur, with lacZ. Thus, transcription of lacZ increases as intracellular iron levels decrease. In the presence of EDDA, the β-galactosidase activities were significantly higher in the ahpC mutant than in the wild type (Fig. 5), suggesting that the ahpC mutant had a lower level of internal iron available to Fur.

To directly measure the total cellular iron levels in the wild type and the ahpC mutant, inductively coupled plasma mass spectrometry was used to measure the iron contents of wild-type and ahpC mutant cells under both iron-limiting and iron-rich conditions. The results showed that under iron-limiting conditions, the ahpC mutant had a total cellular iron content of 8 × 10⁻⁶ µg/10⁹ cells, which was reduced relative to the content of 17 × 10⁻⁶ µg/10⁹ cells measured in the wild type. The total cellular iron contents of the wild type and the ahpC mutant were essentially the same under...
grown in T medium, with or without added Fe(III) ions. The wild-type (BW25113) and the ahpC mutant (ML100) strains were grown to stationary phase were separated in a 12% SDS-PAGE gel and visualized by Coomassie blue staining. AhpC is indicated by an arrow. (B) The wild-type strain (WT) carrying the empty vector (BW25113/pWSK29), the ahpC mutant carrying the empty vector (ML100/pWSK29), and the ahpC mutant carrying plasmids containing wild-type ahpC (pAhpC), ahpC* (pAhpC*), ahpC^C46S (pAhpC^C46S), or ahpC^C165S (pAhpC^C165S) were analyzed by Western immunoblotting with anti-AhpC rabbit polyclonal primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody.

AhpC affects enterobactin production but not enterobactin uptake and utilization. Enterobactin-mediated iron acquisition is a major pathway for E. coli to acquire ferric iron under iron-limiting conditions. To determine whether AhpC affected enterobactin production, the extracellular concentration of enterobactin was estimated by the Arnow assay for catechols (1). There was a significant difference in the amounts of extracellular catechols detected between the wild type (35 ± 6 Arnow units) and the ahpC mutant (12 ± 5 Arnow units) when the strains were grown in T medium. The Arnow assay detects total catechols, including enterobactin and some of its precursors and breakdown products. Therefore, LC-MS was used to determine whether there was a difference in the amount of enterobactin produced in the cultures. Supernatants of the wild-type strain showed a peak at 29 min, the expected retention time for enterobactin, whereas the ahpC mutant lacked a corresponding peak (Fig. 6A). The fraction containing the peak for the wild-type strain was further analyzed by MS. The m/e ratio matched that of enterobactin (Fig. 6B). These data indicate that AhpC is required for the uptake of enterobactin biosynthesis or secretion and that most of the catechols detected by the Arnow assay are not enterobactin.

To determine if AhpC participates in enterobactin transport or utilization in addition to enterobactin biosynthesis, a competition assay was used. If the defect of the ahpC mutant was in transport or utilization of enterobactin, the ahpC mutant would be expected to be outcompeted by the wild type, even though both strains would have equal access to the pool of secreted siderophores. To test this, equal numbers of the wild type and the ahpC mutant were grown separately or inoculated together in T medium, and their growth was monitored. The ahpC mutant showed reduced growth compared to that of the wild type when the strains were grown separately (Fig. 7A). However, when the wild-type and ahpC strains were inoculated together, they grew equally well (Fig. 7B). It should be noted that in the mixture, each strain was inoculated at one-half the number of cells used for inoculation of that strain alone, so the total number of cells per ml was the same in each culture. The mixture reached a final density comparable to that of the wild type grown alone, with each strain contributing approximately half the total cell number. This indicates that AhpC is not required for enterobactin transport or utilization.

The ahpC mutant was also tested for the ability to use iron sources other than ferri-enterobactin. This was assessed in a bioassay in which the strains were seeded at low density into agar containing an iron chelator and then siderophores or iron was spotted onto the surface of the agar. Both the parent strain and the ahpC mutant contained an entF mutation to eliminate endogenous enterobactin biosynthesis. The bioassay results showed no
AhpC participates in 2,3-dihydroxybenzoic acid production.

Enterobactin biosynthesis in E. coli can be divided into two main stages: the early steps lead to the synthesis of DHB, and later reactions produce enterobactin from DHB and serine (50) (Fig. 1). The amount of DHB produced can be estimated by the Arnow assay using an entF mutant, which is blocked in the conversion of DHB to enterobactin. The results of the Arnow assay showed a significant reduction in catechols secreted by the ahpC entF double mutant compared to the entF strain in T medium (100 ± 29 Arnow units and 183 ± 66 Arnow units, respectively) (P < 0.05 by Student’s t test), indicating that deletion of ahpC led to a reduction in the production of the catechol DHB. The intermediate product DHB was also tested for the ability to suppress the increased EDDA sensitivity of the ahpC mutant. Supplying the ahpC mutant with DHB enabled the mutant to grow to the same level as the wild-type strain under iron-limiting conditions (Fig. 8A). These results suggested that AhpC participates in the early stage of enterobactin biosynthesis. This was further supported by the observation that introducing a plasmid carrying the entABC genes was able to suppress the increased EDDA sensitivity of the ahpC mutant, while a plasmid carrying entF did not (Fig. 8B). Thus, increasing the copy number of genes involved in the early stage of enterobactin biosynthesis suppressed the deleterious effects of the ahpC mutant on growth in low-iron medium.

To determine which gene or genes of the entABC cluster were needed to suppress the ahpC mutant phenotype, plasmids carrying only the entC gene, encoding the isochorismate synthase syntheses of chorismate to isochorismate (28), or entAB, encoding the enzymes for making DHB from isochorismate (27, 44), were constructed and tested in the EDDA sensitivity assay. A plasmid carrying only the entC gene was able to suppress the increased EDDA sensitivity of the ahpC mutant, while a plasmid carrying entAB failed to do so (Fig. 8B). Thus, enhanced expression of entC was sufficient to suppress the effect of the ahpC mutation. This suggests that AhpC participates in enterobactin biosynthesis via EntC, which is the first enzyme in the enterobactin biosynthesis pathway.

Providing extra copies of entC suppressed the increased EDDA sensitivity of the ahpC mutant grown in rich medium containing EDDA. However, the ahpC mutant carrying entC on a plasmid still showed reduced growth compared to that of the wild type in minimal medium (Fig. 9A). Because the substrate for EntC is chorismate, which is also the precursor for other aromatic compounds in the cell (9), it appeared likely that increased competition for chorismate due to synthesis of aromatic amino acids and vitamins in minimal medium could reduce the amount of chorismate available to EntC. To determine whether the reduced synthesis of enterobactin could be alleviated by reducing competition for chorismate in minimal medium, a mixture of the aromatic amino acids and para-aminobenzoate, all of which are synthesized from cho-

### Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diam of growth zone around iron source (mm)</th>
<th>E. coli&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ent&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Fe&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Fe&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>entF mutant (BW25113entF)</td>
<td>32 ± 1</td>
<td>20 ± 1</td>
<td>22 ± 2</td>
<td>15 ± 1</td>
<td></td>
</tr>
<tr>
<td>ahpC entF mutant (ML300)</td>
<td>31 ± 2</td>
<td>20 ± 1</td>
<td>22 ± 2</td>
<td>13 ± 3</td>
<td></td>
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<sup>a</sup> Data are averages ± 1 standard deviation for at least three independent experiments.

<sup>b</sup> Five microplates containing 10<sup>9</sup> cells/ml BW25113 as a source of enterobactin and related catechols.

<sup>c</sup> Five microplates of 10 µM purified enterobactin.

<sup>d</sup> Five microplates of 0.8 mM ferrichrome.

<sup>e</sup> Five microplates of 25 mM FeSO<sub>4</sub>.
FIG 8 DHB and entC suppressed the increased EDDA sensitivity of the \( \text{ahpC} \) mutant. The strains were grown in LB broth containing EDDA for 8 h, and the OD\(_{650}\) was measured. Data presented are averages for at least three independent experiments, and error bars represent 1 standard deviation. *, \( P < 0.01 \) compared to the wild type, determined by Student’s \( t \) test. (A) The wild-type (BW25113) and \( \text{ahpC} \) mutant (ML100) strains were grown in medium containing EDDA, with or without 1 mM DHB. (B) The wild-type strain (BW25113) carrying the empty vector (pWSK29) and the \( \text{ahpC} \) mutant (ML100) carrying the empty vector (pWSK29), \( \text{entCEBA} \) (pEnt1), \( \text{entF} \) (pS4700), \( \text{entC} \) (pEnt2), or \( \text{entEBA} \) (pCP410) were grown in LB broth containing appropriate antibiotics and with or without EDDA.

rismate, was added to T medium. Adding these compounds to the minimal medium allowed the \( \text{ahpC} \) mutant, with (Fig. 9B) or without (data not shown) \( \text{entC} \) on a plasmid, to grow to the wild-type level. However, adding the amino acids individually did not support full growth of the mutant (data not shown).

Addition of the amino acids and \( \text{para} \)-aminobenzoate increased the amounts of extracellular catechols in both the wild-type and \( \text{ahpC} \) strains (Table 4). The presence of the \( \text{entC} \) plasmid also increased the amounts of extracellular catechols produced in both the wild-type and mutant strains, and there was an additive effect when there was both supplementation and the presence of \( \text{entC} \) on a plasmid (Table 4). In each of these cases, the amount of catechol secreted by the \( \text{ahpC} \) mutant was comparable to that for the wild type. Thus, the reduced siderophore production by the \( \text{ahpC} \) mutant in minimal medium is suppressed when it has more copies of \( \text{entC} \) or when competition of EntC for its substrate is reduced. Additionally, providing the \( \text{ahpC} \) mutant with the chorismate precursor shikimate restored wild-type growth to the \( \text{ahpC} \) mutant in the EDDA sensitivity assay (Fig. 10).

Taken together, these data indicate that AhpC may participate in DHB synthesis through increasing the availability of chorismate or increasing the efficiency with which EntC uses chorismate, roles that were not anticipated given its characterization as a peroxidase.

C46 and C165 are both required for the role of AhpC in cellular iron metabolism. AhpC belongs to the 2-Cys peroxiredoxin family and has cysteines at positions 46 and 165. It has been shown that a C46S mutation of AhpC abolishes its peroxidase activity \textit{in vitro}, while a C165S mutant maintains wild-type activity (11). In contrast, the ability of AhpC* to generate reduced glutathiones depends on C165 but not C46; the C165S mutation in AhpC* abolishes its ability to suppress the loss of Gor and TrxB, but the C46S mutation has no effect on suppression (11, 55). To determine whether either of these cysteine residues is important for AhpC to play a role in cellular iron metabolism, plasmids encoding AhpC\textit{C46S} and AhpC\textit{C165S} were constructed. Western blot analysis confirmed that the proteins were produced at the same level as wild-type AhpC expressed from a plasmid (Fig. 3).

The plasmids containing the cysteine mutations were tested for the ability to complement the \( \text{ahpC} \) deletion for growth under iron-limiting conditions, and the results showed that mutation of either C46 or C165 in AhpC resulted in an increased EDDA-sensitive phenotype (Fig. 11). This indicated that both cysteine residues are important for AhpC’s role in cellular iron metabolism. Analysis of the peroxide sensitivity of strains carrying the cysteine mu-

![FIG 9](https://jb.asm.org/10.1128/JB.01099-07)

**FIG 9** Supplying a mixture of aromatic amino acids and \( \text{para} \)-aminobenzoate suppressed the growth defect of the \( \text{ahpC} \) mutant in T medium. The wild-type (BW25113) and \( \text{ahpC} \) (ML100) strains carrying \( \text{entC} \) (pEnt2) were grown in T medium (A) or T medium supplemented with a mixture of aromatic amino acids and \( \text{para} \)-aminobenzoate (B), and growth was monitored by measuring the OD\(_{650}\) over 14 h. Data presented are averages for at least three independent experiments, and error bars represent 1 standard deviation. *, \( P < 0.01 \) compared to the wild type, determined by Student’s \( t \) test.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No additions</th>
<th>With supplements</th>
<th>With pEntC</th>
<th>With supplements and pEntC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (BW25113)</td>
<td>31 ± 9</td>
<td>151 ± 34</td>
<td>125 ± 40</td>
<td>245 ± 25</td>
</tr>
<tr>
<td>( \text{ahpC} ) mutant (ML100)</td>
<td>16 ± 3(^a)</td>
<td>221 ± 12</td>
<td>138 ± 9</td>
<td>254 ± 20</td>
</tr>
</tbody>
</table>

\(^a\) Strains carrying the empty vector or the \( \text{entC} \) plasmid pEnt2 (pEntC) were grown in T medium or T medium supplemented with a mixture of Trp, Tyr, Phe, and \( \text{para} \)-aminobenzoate (supplements). Data are expressed in Arnow units and are averages ± 1 standard deviation for at least three independent experiments.

\(^b\) \( P < 0.05 \) compared to the wild type, determined by Student’s \( t \) test.
FIG 10 Shikimate suppressed the increased EDDA sensitivity of the *ahpC* mutant. Strains used were the wild-type (BW25113) and *ahpC* (ML100) strains. The strains were grown in LB broth containing EDDA, with or without 200 μM shikimate, for 8 h, and the OD_{600} was measured. Data presented are means for at least three independent experiments, and error bars represent 1 standard deviation. *, *P* < 0.01 compared to the wild type, determined by Student’s *t* test.

tations confirmed that the C164S mutant had peroxidase activity and was resistant to cumene hydroperoxide, while the C46S mutant was as sensitive as the *ahpC* deletion mutant to the compound (Fig. 12). The fact that both cysteines are required for participation of AhpC in cellular iron metabolism suggests that the peroxidase activity of AhpC is not sufficient for AhpC to participate in cellular iron metabolism.

**DISCUSSION**

In *E. coli*, it has been shown that AhpC detoxifies peroxides, is produced at high levels, and exhibits broad substrate specificity (26, 37). The expression level of AhpC is the same during aerobic and anaerobic growth at physiological pH (3). It has also been shown that the peroxidase catalytic efficiency of AhpC is not as great as that of glutathione peroxidase (37, 53). The abundance and broad specificity of AhpC could be evolutionary adaptations to compensate for its moderate peroxidase activity. However, it is also possible that AhpC participates in other cellular events that require such abundance or broad substrate specificity. It has been shown that *ahpC* can undergo mutation to *ahpC C46S*, which encodes a protein that gains a new function to generate reduced glutathione in the absence of the Gor or TrxB cellular reductase (40). In

Streptococcus agalactiae, AhpC has been shown to serve as an intracellular chaperone for heme to optimize its trafficking and transfer to cellular targets such as catalases (25). In mammalian cells, peroxiredoxins are reported to control cytokine-induced peroxide levels that mediate signal transduction (12). These findings imply that AhpC may have diverse functionalities or be involved in multiple cellular events in *E. coli*.

The data presented in this work provide evidence that AhpC participates in cellular iron metabolism of *E. coli*. The role for AhpC in cellular iron metabolism is indicated by its reduced growth in iron-limiting medium. Under low-iron conditions, the *ahpC* mutant had a lower internal iron level and a reduction of total cellular iron content. These phenotypes correlated with a reduced production of the siderophore enterobactin in the *ahpC* mutant. The reduced secretion of DHB by the *entF ahpC* mutant and the ability to suppress the growth defect in the *ahpC* mutant by either the addition of exogenous DHB or the introduction of a plasmid carrying the *entC* gene indicated that the defect was in an early step in enterobactin biosynthesis. A mixture of aromatic amino acids and *para*-aminobenzoate, all of which were synthesized from chorismate, suppressed both the growth defect and the catechol production of the *ahpC* mutant in minimal medium. This suggests that AhpC either facilitates the delivery of the substrate chorismate to the enterobactin biosynthesis pathway or helps maintain an optimal concentration of chorismate inside *E. coli* cells.

It is possible that AhpC is required to maintain chorismate availability to the enterobactin biosynthesis pathway. Chorismate is used in the biosynthesis of many aromatic compounds, including the aromatic amino acids and folate, so multiple enzymes must compete for chorismate (9). Based on their *K_m* values for chorismate, these enzymes can be divided into three groups: the strong competitor group, with *K_m* values between 1 and 10 μM; the medium competitor group, with *K_m* values between 10 and 100 μM; and the weak competitor group, with *K_m* values of >100 μM. With a *K_m* value of 14 μM, EntC falls into the medium competitor group (28). If the deletion of *ahpC* leads to a reduction of the chorismate pool or a less efficient shuttling of chorismate to EntC, EntC might be at a disadvantage in competing for chorismate compared with some of the strong competitors, such as the aminodeoxychorismate synthase of the Trp biosynthesis pathway, given the relatively high *K_m* value of EntC for chorismate. This
competitive disadvantage would lead to a lowered production of DHBA and enterobactin, causing reduced growth under iron-limiting conditions, a smaller intracellular ferrous iron pool, and a lowered total cellular iron content in the ahpC mutant. Providing the ahpC mutant with extra copies of entC might help EntC to compete for the limited chorismate pool. Additionally, providing the ahpC mutant with exogenous DHBA or iron would bypass the requirement for the precursor chorismate, and supplementing shikimate or the mixture of aromatic amino acids and para-aminobenzoate would increase the available chorismate concentration for the enterobactin biosynthesis pathway.

The peroxidase activity of AhpC may contribute to the efficiency of enterobactin production if EntC or the chorismate pool is very sensitive to peroxide. A recent report (18) showed that under peroxide stress, a small RNA (sRNA), RybA, downregulates aromatic amino acid biosynthesis, and the authors of that report suggest that this may increase the availability of chorismate to other biosynthetic pathways. Waters et al. (52) observed that this gene encodes a small protein, which they designated MntSt. They noted an increase in entC expression in an MntSt mutant, which overexpressed the MntSt sRNA, when the cells were grown with a high manganese concentration. These data indicate a link between peroxide stress, the chorismate pool, and enterobactin gene expression. However, in the absence of other factors, these observations would predict that peroxide stress such as that induced by the ahpC mutation would increase the availability of chorismate for EntC and also increase enterobactin biosynthesis. Thus, AhpC would appear to have an effect in addition to its role in reducing peroxide in the cell.

Additional evidence that the peroxidase activity of AhpC is not solely responsible for AhpC’s participation in cellular iron metabolism is seen in the requirement for both cysteine residues. The C146S mutant had peroxidase activity and was resistant to cumene hydroperoxide, but it failed to support wild-type growth under iron-limiting conditions. The fact that ahpC* on a plasmid was not able to suppress the ahpC mutant phenotype suggests that the ability to generate reduced glutathione is not important for AhpC’s participation in cellular iron metabolism. Thus, AhpC’s participation in cellular iron metabolism is a new role of AhpC. This undesignated role of AhpC provides, for the first time, an example of a peroxidase participating in a cellular process other than detoxifying peroxides in E. coli, which expands the versatility of peroxidoxins in prokaryotes.

Although increasing the copy number of entC in the cell compensated for the loss of ahpC, it is unlikely that AhpC enhances the enzymatic activity of EntC or is involved in the recycling of an EntC cofactor. The crystal structure of EntC (46) with bound isochorismate indicates that the catalytic center contains a magnesium ion and the isochorismate product, but no cofactor, such as flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN), has been found to participate in EntC’s catalytic reaction (28, 46). There is no indication that EntC forms any inter- or intramolecular disulfide bonds or catalyzes any oxidation-reduction reaction (28, 46). It has been shown that the function of the chorismate synthase in E. coli requires reduced FMN, but the NADPH:FMN oxidoreductase that provides the cofactor has not been identified (30). Since AhpC catalyzes oxidation-reduction reactions, and as both of its cysteine residues are important for its participation in cellular iron metabolism of E. coli, AhpC may participate in the reduction of FMN for chorismate synthesis, either directly or indirectly. This participation would enable AhpC to influence cellular chorismate levels, which would offset multiple pathways, including enterobactin biosynthesis. However, further studies are needed to elucidate these possibilities.

ACKNOWLEDGMENTS

This work was supported by grants AI091957 and AI16935 from the National Institutes of Health.

We thank George Georgiou for providing strains DHBA and SMG96, Leslie B. Poole for providing the anti-AhpC antibody, the National BioResource Project (NIG, Japan) for providing E. coli Keio mutant strains, Nathan Miller for performing ICP-MS, and Marvin Mercado for performing LC-MS. Alexandra Mey provided assistance with the real-time PCR analysis and review of the manuscript. We thank Elizabeth Wyckoff and other members of the laboratory for critical reviews of the manuscript and for helpful discussions.

REFERENCES

Goldberg JB, Ohman DE. 1997. Enterobactin biosynthesis in Escherichia coli: isochorismate lyase (EntB) is a bifunctional enzyme that is phosphopantetheinylated by EntD and then acylated by EntE using ATP and 2,3-dihydroxybenzoate. Biochemistry 36:8495–8503.


