An Alkyl Hydroperoxide Reductase Induced by Oxidative Stress in *Salmonella typhimurium* and *Escherichia coli*: Genetic Characterization and Cloning of *ahp*

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The *ahp* genes encoding the two proteins (F52a and C22) that make up an alkyl hydroperoxide reductase were mapped and cloned from *Salmonella typhimurium* and *Escherichia coli*. Two classes of oxidant-resistant *ahp* mutants which overexpress the two proteins were isolated. *ahp-1* was isolated in a wild-type background and is dependent on *oxyR*, a positive regulator of defenses against oxidative stress. *ahp-2* was isolated in an *oxyR* deletion background and is *oxyR* independent. Transposons linked to *ahp-1* and *ahp-2* or inserted in *ahp* mapped the genes to 13 min on the *S. typhimurium* chromosome, 59% linked to *ent*. Deletions of *ahp* obtained in both *S. typhimurium* and *E. coli* resulted in hypersensitivity to killing by cumene hydroperoxide (an alkyl hydroperoxide) and elimination of the proteins F52a and C22 from two-dimensional gels and immunoblots. *ahp* clones isolated from both *S. typhimurium* and *E. coli* complemented the cumene hydroperoxide sensitivity of the *ahp* deletion strains and restored expression of the F52a and C22 proteins. A cis-acting element required for *oxyR*-dependent, *rpoH*-independent heat shock induction of the F52a protein was present at the *S. typhimurium* but not the *E. coli* *ahp* locus.

When *Salmonella typhimurium* and *Escherichia coli* cells are pretreated with low doses of hydrogen peroxide, they become transiently resistant to lethal doses of hydrogen peroxide (4, 5). Coincident with this increased resistance is the induction of at least 30 proteins as seen by two-dimensional gel electrophoresis (4, 14). The synthesis of some of the hydrogen peroxide-inducible proteins is also elevated by heat shock and treatment with nalidixic acid or ethanol, although each of these stresses also induces a group of unique proteins (14, 21). We have identified and characterized the *oxyR* gene that regulates the expression of nine of the hydrogen peroxide-inducible proteins and have isolated mutants in *S. typhimurium* (*oxyRI*) and *E. coli* (*oxyR2*) that are resistant to hydrogen peroxide and constitutively overexpress the nine proteins (4).

Some of the stress proteins that are overexpressed in the *oxyR1* mutant have been identified, including catalase, manganese superoxide dismutase, and glutathione reductase (4). We found that two of the proteins overexpressed by the *oxyR1*- and the *oxyR2* mutants, designated F52a and C22 on two-dimensional gels, make up a novel alkyl hydroperoxide reductase activity (4, 9a). The proteins were purified to homogeneity and characterized. The F52a flavoprotein, together with the smaller C22 protein, reduces lipid hydroperoxides and other alkyl hydroperoxides directly to their corresponding alcohols by using either NADH or NADPH as an electron donor (9a).

We are interested in studying the role of the alkyl hydroperoxide reductase in defending against oxidative stress, since hydroperoxides have been shown to be mutagenic in bacteria (12). The expression of the F52a and C22 proteins is also of interest, especially because the F52a protein is induced by both heat shock and oxidative stress in *S. typhimurium*. Heat shock induction is dependent on *oxyR* and is not seen in *E. coli* (14). This paper describes the isolation of two *S. typhimurium* mutants that constitutively overexpress only the F52a and C22 proteins and show increased resistance to alkyl hydroperoxides. These mutants allowed us to delete, map, and clone the genes encoding the alkyl hydroperoxide reductase activity and allowed us to study heat shock induction of the F52a protein in both *S. typhimurium* and *E. coli*.

MATERIALS AND METHODS

Bacterial strains and bacteriophage stocks. The bacterial strains constructed for this study are listed in Table 1. P22 Ht105/I int-201 (1) was used in transductions with *S. typhimurium*, and phage P1 was used in transductions with *E. coli*. A tail-dependent Tn5 P22 vector of M. Suskind (unpublished data) was used to generate the random pool of Tn5 insertions. Phage P22 carrying a random pool of Tn5 insertions generated by D. Speiser (unpublished data) was used to obtain zac-120::Tn10 and *ahp*::Tn10.

Bacterial methods. (i) Mutagenesis with diethylsulfate. *S. typhimurium* TA4108 (4) was grown overnight in nutrient broth (NB) to 2 × 10⁹ cells per ml and then diluted in 10 ml of VBC (22) salts to 1 × 10⁶ cells per ml. Diethylsulfate (0.1 ml) was added to the 10-ml culture, and the tube was vortexed vigorously for 20 s. After the culture was left at room temperature for 20 min, 1.0 ml of the mutagenized culture was withdrawn from the top of the tube and used to inoculate 10 ml of NB. This culture was grown for 6 h at 37°C, and then 0.1-ml samples were plated on NB plates or VBC plates containing 2% glucose (minimal glucose) and supplemented with 0.5 mM L-arginine (L-arginine is required by *oxyRΔ2* strains). To isolate suppressors of *oxyRΔ2*, unmutagenized and mutagenized cells (strain TA4108) were...
plated on hydrogen peroxide (40 to 880 µM on NB plates and 40 to 150 µM on minimal-glucose plates), cumene hydroperoxide (100 to 400 µM on NB plates and 100 µM on minimal-glucose plates), and menadione (60 to 190 µM on NB plates and 20 µM on minimal-glucose plates).

(ii) Disk inhibition assays. Cells were grown overnight in NB, Luria broth, or Luria broth with the appropriate antibiotic at 37°C before testing. Culture samples (0.1 ml) were then added to 2 ml of soft agar and plated on minimal-glucose plates (supplemented with 0.5 mM L-arginine for the strains in Table 2). Samples (10 µl) of solutions containing 3% hydrogen peroxide dissolved in water or 3% cumene hydroperoxide dissolved in cumene sulfoxide were applied to 0.25-in. (1 in. = 2.54 cm) paper discs (BBL Microbiology Systems, Cockeysville, Md.), and the discs were placed in the center of the agar. The diameter of the zone of killing was measured after 24 h at 37°C.

(iii) Isolation of a Tn5 insertion linked to ahp. A 0.1-µl volume of an overnight culture of strain TA4281 was mixed with 10⁶ PFU of a tail-dependent P22 bacteriophage containing a Tn5 insertion in the siaE gene of P22 and a deletion (ΔAp68tpfr-251) that removes both att and sin (Susskind, unpublished data). The mixture was plated in top agar onto Luria broth plates containing 75 µg of kanamycin per ml and grown overnight at 37°C. We pooled 7,500 kanamycin-resistant colonies in Luria broth-kanamycin, P22 HT105 int-201 was grown on the pool of kanamycin-resistant colonies. After addition of chloroform, the resultant P22 lysate was used to transduce strain TA4108 (4) to kanamycin resistance at a multiplicity of infection of 0.5, such that roughly 500 transductants per plate were obtained. After 24 h at 37°C, the plates were replica plated onto NB-kanamycin plates containing 100 to 400 µM cumene hydroperoxide and 10 mM EGTA (ethylene glycol-bis(β-aminoethylether)-N,N',N'-tetraacetic acid) (to prevent multiple additional infections). Colonies that grew on the cumene hydroperoxide-containing plates after 24 h were purified and tested for ahpR2 and ahp-2 phenotypes.

Cloning methods. (i) pAQ9. The S. typhimurium library packaged in phage P22 was obtained from C. Miller (unpublished data). The library contains 8- to 12-kilobase fragments derived from a partial Sau3A digest of strain TN1379 (lev-485) cloned into the unique BamHI site of pBR328 (19). A 0.1-ml volume of an overnight culture of TA4314 was mixed with 5 µl containing 22 (8 × 10⁶ PFU/ml) carrying the library per ml and top spread on NB plates containing 20 µM chloramphenicol and 150 µM cumene hydroperoxide. Cumene hydroperoxide-resistant transductants were picked after 24 h at 37°C. pAQ9 was transformed into strain TA4315 after being cycled through E. coli DH1 (restriction minus, modification plus).

(ii) pAQ10. The E. coli cosmid library was obtained from A. Bagg (Ph.D. thesis, University of California, Berkeley, 1987). In this library, 22- to 35-kilobase fragments of BN4020 (F' thr-1 thi-1 his-4 argE3 lacA1U169 galK2 ara-14 xyl-15 mtl-1 str-31 tsx+ supF fur-1::Tn5) are cloned into an EcoRI site of cosmid vector pLAFR (8). A 0.1-ml volume of the DH1 strain carrying the cosmid library was mated with 0.1 ml of the enteric recipient AN193 (F' proC leu trp purE thi lacY rpsL galK ara mtl xyl aro ind supE44 entA) in the presence of 0.1 ml of BN4020 (F' thr-1 thi-1 his-4 argE3 lacA1U169 galK2 ara-14 xyl-15 mtl-1 str-31 tsx+ supF) carrying the helper plasmid pRS2013 (7). After incubation for 2 h at 37°C, the cells were plated on minimal glucose containing proline, tryptophan, leucine, and dihydroxybenzoic acid (which allows growth of the recipient but not BN4020), streptomycin (which selects against the donor strain), and tetracycline (which selects against cells without a cosmid). The patches of streptomycin- and tetracycline-resistant cells which grew were then purified on NB-bipiridyl plates to test for an EntA + phenotype. Cosmids conferring EntA + were mated with TA4315 by using the same selections and helper strain and transduced into TA4314 after being cycled through S. typhimurium SL4213 (restriction minus, modification plus).

All plasmid DNA was isolated and restriction mapped as described by Maniatis et al. (13).

Immunological techniques. (i) Production of antibodies. We mixed 300 µg of purified C22 protein (9a) in 0.5 ml of H₂O 1:1 with Freund complete adjuvant, sonicated it to form an emulsion, and injected it into a female New Zealand White rabbit. The rabbit was boosted after 15 days with another 300 µg of C22 protein mixed 1:1 with Freund incomplete adjuvant. At 15 days after the boost, serum was obtained which reacted only with the C22 protein and one smaller protein (present as a minor contaminant in the purified C22 fraction) on immunoblots of whole cells. We further purified 300 µg of F52a protein (9a) by electrophoresis and cut it out of the polyacrylamide gels. The washed polyacrylamide gel was suspended in 0.5 ml of H₂O, mixed 1:1 with Freund complete adjuvant, sonicated to form an emulsion, and injected into a female New Zealand White rabbit. The rabbit was boosted twice at 15-day intervals with 300 µg of electrophoretically purified F52a protein cut out of gels and mixed 1:1 with Freund incomplete adjuvant. The serum obtained 15 days after the second boost, which reacted predominantly with the F52a protein and weakly with two other proteins on immunoblots of whole cells, was used.

(ii) Immunoblots. Equal volumes (0.3 ml) of overnight cultures were centrifuged, and the pellets were suspended in 200 µl of Laemmli buffer (11) for TA4317 and TA4321 and 50 µl of buffer for all of the other strains. Samples (10 µl) were electrophoresed on 12% polyacrylamide gels. The proteins were then transferred to nitrocellulose filters by electrophoretic transfer, and the filter was blocked with either 5 µl (1:2,000) or Tween 20.
genetic characterization and cloning of ahp

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The oxyR1 mutation constitutively overexpresses the F52a and C22 proteins, and oxyR deletion strains fail to induce these proteins upon treatment with 60 μM hydrogen peroxide. We tested the effects of a deletion of oxyR on ahp-1 by transducing oxyRΔ2 linked to zii-614::Tn10 into an ahp-l-carrying strain. The resultant oxyRΔ2 ahp-1 strain (TA4191) was not resistant to cumene hydroperoxide when compared with oxyRΔ2 ahp+ strain (TA4130) as determined by disk inhibition assays (Table 2) and did not overexpress the F52a and C22 proteins when examined on two-dimensional gels (data not shown), indicating that the Ahp-1 phenotype is oxyR dependent.

A second class of ahp mutations was isolated in an oxyR deletion background (TA4108) when we screened for mutations which suppressed the oxyRΔ2 sensitivity to oxidants. The oxyR deletion strain was plated onto a range of inhibitory concentrations of hydrogen peroxide, cumene hydroperoxide, and menadione. The latter compound causes redox cycling and can lead to generation of oxygen radicals. Of the 100 colonies that arose spontaneously (the selecting agents are mutagens [12]) and after diethylsulfate mutagenesis, 30 showed increased resistance to cumene hydroperoxide as shown by zones of inhibition. Although we screened for resistance to several different oxidants, only mutants that overexpressed alkyl hydroperoxide reductase activity were obtained. The zones of inhibition for one representative spontaneous mutant that carried oxyRΔ2 and ahp-2, isolated on 150 μM hydrogen peroxide on a minimal-glucose plate, are given in Table 2. Compared with oxyRΔ2 parent strain TA4130, TA4320 (oxyRΔ2 ahp-2) was greatly resistant to cumene hydroperoxide and slightly resistant to hydrogen peroxide. This mutant, therefore, represents a second class of ahp mutation, since it is necessarily independent of oxyR. A wild-type oxyR strain carrying ahp-2 (TA4267) was only slightly more resistant to cumene hydroperoxide than the wild-type oxyR+ ahp+ strain (TT2385; 4). The ahp-2 mutant also overexpressed the F52a and C22 proteins, as seen on two-dimensional gels (data not shown), and had alkyl hydroperoxide reductase activity fivefold higher than that of wild-type cells. ahp-1 and ahp-2 are linked and map to 13 min. The ahp-1 and ahp-2 mutations were mapped and shown to be linked to each other through the isolation of a Tn5 insertion and a Tn10 insertion linked to the ahp mutations. A random S. typhimurium Tn5 pool was transduced into TA4281 (oxyRΔ2 ahp-2). Phage P22 grown on TA4281 (oxyRΔ2 ahp-2) carrying the random Tn5 insertions were used to transduce TA4108 (oxyRΔ2) to kanamycin resistance. The kanamycin-resistant transductants were then replica plated onto plates

<table>
<thead>
<tr>
<th>TABLE 2. Sensitivities of ahp-1- and ahp-2 strains to killing by oxidantsa</th>
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<tbody>
<tr>
<td><strong>S. typhimurium</strong> strain (genotype)</td>
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<tr>
<td>--------------------------------------</td>
</tr>
<tr>
<td>TT2385 (oxyR+)</td>
</tr>
<tr>
<td>TA4130 (oxyRΔ2)</td>
</tr>
<tr>
<td>TA4319 (oxyR+ ahp-1)</td>
</tr>
<tr>
<td>TA4191 (oxyRΔ2 ahp-1)</td>
</tr>
<tr>
<td>TA4267 (oxyR+ ahp-2)</td>
</tr>
<tr>
<td>TA4520 (oxyRΔ2 ahp-2)</td>
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</tbody>
</table>

a The cultures were grown overnight in NB, and zones of inhibition were determined as described in Materials and Methods. The values are means of three determinations.
containing a range of cumene hydroperoxide (10 to 100 μM), which allowed TA4281 (oxyRΔ2 ahp-2) but not TA4108 (oxyRΔ2) to grow. Colonies that were both kanamycin and cumene hydroperoxide resistant were screened for cotransduction of kanamycin and cumene hydroperoxide resistance indicative of a Tn5 linked to the ahp-2 mutation. In this manner, we isolated one Tn5 insertion, zac-119::Tn5 (TA4173), 88% linked to the ahp-2 mutation by P22 transduction. Zac-119::Tn5 was also 73% linked to ahp-1, indicating that the two ahp mutations are linked (Fig. 1).

To facilitate deletion mutagenesis of the ahp locus (see below), we isolated a Tn10 insertion to replace the Tn5 insertion. TA4173 (zac-119::Tn5) was transduced to tetracycline resistance with phage grown on a Tn10 pool. These transductants were replica plated onto kanamycin. The colonies that were kanamycin sensitive yet tetracycline resistant were tested for linkage of tetracycline resistance to the ahp genes. One Tn10 insertion isolated in this fashion in strain TA4174 (zac-120::Tn10) was 64% linked to ahp-1 and 67% linked to ahp-2 (Fig. 1).

By using positive selection for tetracycline sensitivity (3) to isolate Tn10-mediated deletions in TA4174, two classes of deletions were isolated. One class of deletion mutants was auxotrophic and required dihydroxybenzoic acid to grow on minimal-glucose medium. Dihydroxybenzoic acid is a precursor of enterobactin, a siderophore required to chelate extracellular iron. The products of entA, entB, and entC convert chorismate to dihydroxybenzoic acid, and E. coli strains with mutations in any of these genes require dihydroxybenzoic acid to grow on low-iron medium (6). The auxotrophic requirement for dihydroxybenzoic acid, therefore, indicated a deletion in the ent locus at 13 min and prompted us to test the linkage of zac-119::Tn5 and zac-120::Tn10 to ent-7 (TA2443) (17). The 59% linkage of zac-119::Tn5 and the 75% linkage of zac-120::Tn10 to ent-7 allowed us to map the ahp genes to 13 min on the S. typhimurium chromosome (Fig. 1). This map position agrees with a position of 5 to 25 min determined by Hfr mapping experiments (4) with zac-120::Tn10 (data not shown). By analogy to E. coli mutants, the fact that the S. typhimurium ent30 deletion mutant was still able to utilize dihydroxybenzoic acid suggested that the entDEFG genes (which convert dihydroxybenzoic acid to enterobactin) had not been deleted and defined the extent of the deletion (Fig. 1).

The phenotype of the second class of deletion mutants suggested that they carried deletions of the genes encoding alkyl hydroperoxide reductase activity. The mutants were hypersensitive to cumene hydroperoxide, as shown by the zones of inhibition for one representative mutant, TA4314 (ahpΔ4) (Table 3). Labeled cell extracts of strain TA4314 (ahpΔ4) no longer had protein spots in the F52a and C22 positions on two-dimensional gels (data not shown). The absence of the F52a and C22 proteins in this strain was further confirmed by the absence of the proteins on Western blots (immunoblots) probed with antibodies to the purified F52a and C22 proteins (Fig. 2). Compared with wild-type

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Map position of ahp in S. typhimurium. The map positions of zac-120::Tn10, zac-119::Tn5, and ahp::Tn10 relative to ahp-1, ahp-2, ent-7, and lip-1 were determined by P22 transductions. For all transductions, the Tn10 and Tn5 insertions were moved into the mutant backgrounds and the ability of each insertion to move in the wild-type phenotype was scored for more than 50 transductants. The extents of the ahpΔ4 and entΔ30 deletions are indicated by the heavy lines. The broken line corresponds to the E. coli map.

**TABLE 3.** Sensitivities of strains carrying ahp deletions and clones to killing by oxidants

<table>
<thead>
<tr>
<th>Strain (genotype)</th>
<th>Hydrogen peroxide (3%)</th>
<th>Cumene hydroperoxide (3%)</th>
</tr>
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<tbody>
<tr>
<td>S. typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT2</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>TA4314 (ahpΔ4)</td>
<td>22</td>
<td>37</td>
</tr>
<tr>
<td>TA4317 (ahpΔ4)(pAQ9)</td>
<td>29</td>
<td>19</td>
</tr>
<tr>
<td>TA4322 (ahpΔ4)(pAQ10)</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-12</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>TA4315 (ahpΔ5)</td>
<td>18</td>
<td>37</td>
</tr>
<tr>
<td>TA4321 (ahpΔ5)(pAQ10)</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>TA4318 (ahpΔ5)(pAQ9)</td>
<td>17</td>
<td>23</td>
</tr>
</tbody>
</table>

* The strains were grown in Luria broth (or Luria broth with antibiotic for strains carrying pAQ9 or pAQ10), and zones of inhibition were determined as described in Materials and Methods. The zone sizes are means of three determinations. pAQ9 carries the S. typhimurium ahp genes, and pAQ10 carries the E. coli ahp genes.
and oxyR1 cells, ahpΔ4 cells had low, undetectable levels of alkyl hydroperoxide reductase activity. The residual activity could be due to nonspecific reduction by glutathione reductase or free sulphydryls present in the cells.

Finally, a Tn10 insertion was isolated in the ahp locus by transducing a Tn10 pool into the ent-7 mutant (TA2443) and screening tetracycline-resistant Ent+ transductants for cumene hydroperoxide sensitivity. TA4190 (ahp::Tn10) carries a Tn10 insertion that results in cumene hydroperoxide sensitivity and elimination of the F52a and C22 proteins on two-dimensional gels. ahp::Tn10 is 59% linked to ent-7 and 1% linked to a lip-1 mutation (SA320; Salmonella Genetic Stock Center) at 14 min (18), as determined by P22 transduction crosses (Fig. 1).

The analogous cumene hydroperoxide-sensitive and dihydroxybenzoic acid-requiring deletion mutants were obtained in E. coli by selecting for Tn10-mediated deletions in E. coli K-12 carrying zbe-279::Tn10 (TA4334), a Tn10 insertion that cotransduces with rna at 14 min (S. Kushner, unpublished data). The zones of inhibition for one cumene hydroperoxide-sensitive strain, TA4315 (ahpΔ5), are given in Table 3. Antibodies to the S. typhimurium F52a and C22 proteins did not react with any proteins on Western blots of extracts of a strain carrying ahpΔ5, but they did bind proteins in the K-12 parent strain (Fig. 2). The linkage of zbe-279::Tn10 in E. coli to both ahp and ent, as indicated by the phenotype caused by the Tn10-mediated deletions, showed that ahp maps to similar positions in S. typhimurium and E. coli.

**Cloning ahp.** The ahp genes were isolated from an S. typhimurium library contained in pBR328 (Miller, unpublished data) by complementation of the cumene hydroperoxide sensitivity of the ahp deletion. Strain TA4314 (ahpΔ4) was infected with P22 carrying the library and plated on NB-chloramphenicol plates containing 150 μM cumene hydroperoxide, a concentration of cumene hydroperoxide which allowed the growth of strain LT2 but not that of strain TA4314 (ahpΔ4). Six colonies that grew on this selection were tested, and four were found to confer greater-than-wild-type resistance to cumene hydroperoxide to TA4190 (ahpΔ4). The zones of inhibition for TA4317 (ahpΔ4) carrying one representative clone, pAQ9, are given in Table 3. We also found that pAQ9 could complement the cumene hydroperoxide sensitivity of E. coli TA4318 (ahpΔ5) (Table 3) and restore the F52a and C22 proteins in both TA4317 (ahpΔ4) and TA4318 (ahpΔ5), as seen on Western blots (Fig. 2). Interestingly, pAQ9, while conferring greater resistance to cumene hydroperoxide, caused increased sensitivity to hydrogen peroxide, especially in the S. typhimurium ahp deletion strain.

The restriction map of pAQ9 is given in Fig. 3. The clone is contained on an 8.5-kilobase fragment. We subsequently subcloned and sequenced portions of pAQ9 and found sequences corresponding to the amino acid sequence of the N-terminal 25 amino acids of both the F52a and C22 proteins (9a; L. Tartaglia, G. Storz, M. Brodsky, and B. Ames, unpublished data), showing that pAQ9 carries the structural genes for the alkyl hydroperoxide reductase.

We used the knowledge that ahp is linked to the enterobactin (ent) biosynthesis operon to clone ahp in E. coli. We screened a pLAFR cosmid library (Bagg, Ph.D. thesis) for a cosmid which complemented a point mutation in entA. Cosmids in E. coli DH1 were mated with an EntA+ recipient in the presence of a helper plasmid. The mated cells were then screened for complementation of the EntA+ phenotype by streaking on NB-bipyridyl plates, selective plates that chelate iron and prevent growth of EntA- mutants. Of the 800 cosmids screened in this way, 13 could confer the ability to grow on the NB-bipyridyl plates, and 11 of these 13 cosmids were mated into the ahp deletion strain and screened for ability to complement the cumene hydroperoxide sensitivity of the strain. Only one of the cosmids did not complement the sensitivity, two partially restored resistance, and the other eight fully complemented the cumene hydroperoxide sensitivity, as shown by the zones of inhibition. Table 3 gives the zones of inhibition for one representative cosmid clone, pAQ10, in E. coli TA4321 (ahpΔ5) and S. typhimurium TA4322 (ahpΔ4). pAQ10 also restored
is shown in Fig. 4B. As with S. typhimurium, the F52a protein was not induced in the E. coli oxyR deletion strain. However, unlike most E. coli heat shock proteins, the F52a protein was induced equally in the rpoH+ wild-type and rpoH mutant strains. Two-dimensional gels confirmed that other E. coli heat shock proteins were not induced by the 28-

to-42°C temperature shift in the rpoH165 mutant strain (data not shown).

**DISCUSSION**

We have shown that the locus (ahp) encoding an alkyl hydroperoxide reductase in S. typhimurium and E. coli plays an important role in protecting the bacterial cells against mutagenic alkyl hydroperoxides. Mutations at *ahp*, which caused increased expression of the alkyl hydroperoxide reductase, conferred increased resistance to killing by alkyl hydroperoxides, such as cumene hydroperoxide. The finding that all suppressors of the hydrogen peroxide and cumene hydroperoxide sensitivity of an oxyR deletion strain map to the *ahp* locus further emphasizes the importance of these genes in protecting against oxidative stress. Deletions of the *ahp* genes which eliminated the F52a and C22 proteins on two-dimensional gels and Western blots resulted in hypersensitivity to cumene hydroperoxide. Clones which restored the expression of the F52a and C22 proteins restored resistance to cumene hydroperoxide. We have also recently found that the high frequency of spontaneous mutagenesis in oxyR deletion strains can be suppressed by a multicopy clone of the *S. typhimurium ahp* genes, which causes overexpression of alkyl hydroperoxide reductase activity (20).

Furthermore, Greenberg and Demple have found that mutations that suppress the hydrogen peroxide sensitivity and high rate of mutagenesis of the *E. coli* strain carrying the oxyR deletion cause overexpression of alkyl hydroperoxide reductase activity (9).

The phenotypes of the *ahp-l* and *ahp-2* mutants suggest that they are both regulatory mutants. Since *ahp-l* is dependent on oxyR, it may be a site of oxyR action, possibly within an OxyR-binding site. *ahp-2* may be a more general promoter mutation. We are now mapping the promoters and cis-acting, oxyR-dependent regulatory sequences of the *ahp* genes.

The finding that both the *S. typhimurium* and *E. coli* clones can complement the *ahp* deletions in both organisms indicates that the two activities and functions of the *S. typhimurium* and *E. coli* enzymes are very similar. Interestingly, while the *ahp* deletion strains carrying the *ahp* clones were more resistant to cumene hydroperoxide than were the wild-type strains, the deletion strains with the clones were more sensitive to hydrogen peroxide than were the wild-type strains. This was especially true for the *ahp* deletion strains carrying the *ahp* clones of the same species. Possibly, hydrogen peroxide induction of multiple copies of the *ahp* genes leads to vast overproduction of alkyl hydroperoxide reductase proteins, which in turn may cause general cell stress. Alternatively, multiple copies of the *ahp* genes may cause the oxyR regulator to be titrated away from other oxyR-regulated genes, which confer greater resistance to hydrogen peroxide, such as the katG gene encoding catalase.

As we have noted previously, regulation of *S. typhimurium* and *E. coli* *ahp* genes is distinct (14). The current finding that the *S. typhimurium* F52a protein can be induced by heat shock in both *S. typhimurium* and *E. coli* strains while the *E. coli* F52a protein is not induced in either background indicates that the *S. typhimurium ahp* locus has the F52a and C22 proteins in both deletion-carrying strains, as seen on Western blots (Fig. 2). The positions of the *E. coli* *ahp* genes were not mapped on the cosmid clone.

**Heat shock induction of the F52a protein.** As described previously, the F52a component of the alkyl hydroperoxide reductase is induced by heat shock in *S. typhimurium* but not in *E. coli* in an oxyR-dependent manner (14). The differing heat shock induction of the F52a protein in *S. typhimurium (ahpΔ4)* and *E. coli (ahpΔ5)* was studied with the two *ahp* clones *S. typhimurium*(pAQ9) and *E. coli*(pAQ10). The *ahp* deletion strains carrying one of the two *ahp* clones were shifted from 28 to 42°C and pulse-labeled with L-

\[^{35}S\]methionine. Antibodies to the F52a protein were then used to immunoprecipitate the labeled F52a protein (Fig. 4A). Densitometer scans of the autoradiograph corresponding to the gels of the immunoprecipitated protein showed that the *S. typhimurium* F52a protein was induced at least twofold by heat shock in *S. typhimurium* and *E. coli*. In contrast, the expression of the *E. coli* F52a protein was not induced in either species and even decreased in the *S. typhimurium* background.

The induction of most *E. coli* heat shock proteins is under the control of rpoH, which acts as an alternate sigma factor to reprogram RNA polymerase to recognize the promoters of genes induced by heat shock (recently reviewed in reference 15). We previously observed that heat shock induction of the F52a protein in *S. typhimurium* was dependent on oxyR (14). To determine whether heat shock induction of the *S. typhimurium* F52a protein also required rpoH in addition to oxyR, we tested the heat shock induction of the F52a protein in *E. coli* rpoH+ and rpoH165 mutant strains (16) and *E. coli* oxyR*+* and oxyRAΔ3 (4) strains. The autoradiograph corresponding to the gels of the immunoprecipitated protein was monitored with a densitometer. The results are shown in Fig. 4B. As with *S. typhimurium*, the F52a protein was not induced in the *E. coli* oxyR deletion strain. However, unlike most *E. coli* heat shock proteins, the F52a protein was induced equally in the rpoH+ wild-type and rpoH mutant strains. Two-dimensional gels confirmed that other *E. coli* heat shock proteins were not induced by the 28-

to-42°C temperature shift in the rpoH165 mutant strain (data not shown).

**FIG. 4.** Heat shock induction of F52a. Total cellular proteins were labeled with L-

\[^{35}S\]methionine during log-phase growth with and without heat shock. The cells were then lysed, and the F52a protein was precipitated as described in Materials and Methods. (A) Effect of heat shock induction on the *S. typhimurium*(pAQ9) and *E. coli*(pAQ10) clones in *S. typhimurium (ahpΔ4)* and *E. coli (ahpΔ5)* deletion backgrounds. (B) Effect of an oxyR deletion and an rpoH mutation on heat shock induction of the *S. typhimurium* F52a clone. The predominant band in each case corresponds to the F52a protein; the minor bands are due to cross-reaction with F52a degradation products or proteins which copurified with the F52a protein.
a cis-acting heat shock element which is not present at the E. coli oxyR locus. Interestingly, the heat shock induction is oxyR dependent but rpoH independent. This finding raises several new questions. Why is heat shock induction of the F52a protein rpoH independent while heat shock induction of most other bacterial proteins requires rpoH? How does oxyR act to induce the F52a protein after heat shock? Using the cloned ahp genes from S. typhimurium, we are now determining the sequences required for heat shock induction of the F52a protein and the manner of oxyR regulation.

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