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

GISELA STORZ, FREDRIC S. JACOBSON,[†] LOUIS A. TARTAGLIA, ROBIN W. MORGAN,[‡]
LINDA A. SILVEIRA, AND BRUCE N. AMES*

Department of Biochemistry, University of California, Berkeley, California 94720

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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* (*oxyR1*) 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 HT105/1 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. Susskind (unpublished data) was used to generate the random pool of Tn5 insertions. Phage P22 carrying a random pool of Tn10 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 2×10^9 cells per ml and then diluted in 10 ml of VBC (22) salts to 1×10^8 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

* Corresponding author.

[†] Present address: Genentech, Inc., South San Francisco, CA 94080.

[‡] Present address: Department of Animal Science and Agricultural Biochemistry, University of Delaware, Newark, DE 19717-1303.

TABLE 1. Bacterial strains

Strain ^a	Genotype
TA4130.....	<i>zii-614::Tn10 oxyRA2</i>
TA4173.....	<i>zac-119::Tn5</i>
TA4174.....	<i>zac-120::Tn10</i>
TA4175.....	<i>entΔ30</i>
TA4190.....	<i>ahp::Tn10</i>
TA4191.....	<i>ahp-1 zii-614::Tn10 oxyRA2</i>
TA4266.....	<i>ahp-1</i>
TA4267.....	<i>ahp-2 zii-614::Tn10</i>
TA4281.....	<i>ahp-2 oxyRA2</i>
TA4314.....	<i>ahpΔ4</i>
TA4315 ^b	<i>ahpΔ5</i>
TA4316.....	<i>entΔ31</i>
TA4317.....	<i>ahpΔ4(pAQ9)</i>
TA4318 ^b	<i>ahpΔ5(pAQ9)</i>
TA4319.....	<i>ahp-1 zii-614::Tn10</i>
TA4320.....	<i>ahp-2 zii-614::Tn10 oxyRA2</i>
TA4321 ^b	<i>ahpΔ5(pAQ10)</i>
TA4322.....	<i>ahpΔ4(pAQ10)</i>
TA4334 ^b	<i>zbe-279::Tn10</i>
TA4460 ^b	<i>RK4936 (oxyR⁺)(pAQ9)</i>
TA4461 ^b	<i>TA4112 (oxyRA3)(pAQ9)</i>
TA4462 ^b	<i>SC122 (rpoH⁺)(pAQ9)</i>
TA4463 ^b	<i>K165 (rpoH165)(pAQ9)</i>

^a All strains are *S. typhimurium* unless otherwise indicated.

^b *E. coli* strain.

plated on hydrogen peroxide (40 to 880 μ M on NB plates and 40 to 150 μ M on minimal-glucose plates), cumene hydroperoxide (100 to 400 μ M on NB plates and 100 μ M on minimal-glucose plates), and menadione (60 to 190 μ M on NB plates and 20 μ M 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 μ l) of solutions containing 3% hydrogen peroxide dissolved in water or 3% cumene hydroperoxide dissolved in dimethyl sulfoxide were applied to 0.25-in. (1 in. = 2.54 cm) paper disks (BBL Microbiology Systems, Cockeysville, Md.), and the disks 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- μ l 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 *sieA* gene of P22 and a deletion (Δ Ap68tpfr-251) that removes both *att* and *int* (Susskind, unpublished data). The mixture was plated in top agar onto Luria broth plates containing 75 μ g 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 μ M cumene hydroperoxide and 10 mM EGTA (ethylene glycol-bis(β -aminoethyl ether)-*N,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 *oxyRA2* 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 (*leu-485*) cloned into the unique *Bam*HI site of pBR328 (19). A 0.1-ml volume of an overnight culture of TA4314 was mixed with 5 μ l containing P22 (8×10^8 PFU/ml) carrying the library per ml and top spread on NB plates containing 20 μ M chloramphenicol and 150 μ M 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 lacΔU169 galK2 ara-14 xyl-15 mtl-1 str-31 tsx? sup? fur-1::Tn5*) are cloned into an *Eco*RI 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 *entA* recipient AN193 (*F'* *proC leu trp purE thi lacY rpsL galK ara mtl xyl azi tonA supE44 entA*) in the presence of 0.1 ml of BN402 (*F'* *thr-1 thi-1 his-4 argE3 lacΔU169 galK2 ara-14 xyl-15 mtl-1 str-31 tsx? sup?*) carrying the helper plasmid pRS2013 (7) in microtiter wells. 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 BN402), 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-bipyridyl 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 μ g 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 μ g 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 μ g of F52a protein (9a) by electrophoresis and cut it out of the polyacrylamide gels. The mashed 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 μ g 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 μ l of Laemmli buffer (11) for TA4317 and TA4321 and 50 μ l of buffer for all of the other strains. Samples (10 μ l) were electrophoresed on 12% polyacrylamide gels. The proteins were then transferred to nitrocellulose filters by electroblotting, and the filter was probed with either 5 μ l (1:2,000

dilution) of F52a antibody or 2 μ l (1:5,000 dilution) of C22 antibody. Bound antibody was visualized by the reaction catalyzed by alkaline phosphatase conjugated to goat anti-rabbit antibody (2, 10).

(iii) **Immunoprecipitation.** Cells were labeled with 200 μ Ci of L-[³⁵S]methionine per ml during steady-state growth or heat shock as described previously (4, 14). TA4460 and TA4461 were labeled in VBC-glucose containing 0.3 mM methionine, 5 mM glutamate, 0.6 mM arginine, 0.3 mM aspartate, and 35 μ M ampicillin, and TA4462 and TA4463 were labeled in VBC-glucose containing 2 mM proline, 0.3 mM isoleucine, 0.3 mM leucine, 0.3 mM valine, 0.6 mM arginine, 0.1 mM tryptophan, 0.05 mM thiamine, and 35 μ M ampicillin. A 0.5-ml volume of labeled cells was mixed with 1.5 ml of an overnight culture of unlabeled *ahp* deletion-carrying strains and lysed in 0.1 ml of phosphate-buffered saline (200 mM NaCl, 12.5 mM sodium phosphate, pH 7.4) with 2% sodium dodecyl sulfate by six freeze-thaw cycles. A 0.9-ml volume of phosphate-buffered saline with 1% Triton X-100 was added to the lysed cells, and cell debris was precipitated after addition of IgG-sorb (The Enzyme Center, Inc., Malden, Mass.) The supernatant was then incubated overnight with 5 μ l of F52a antibody (1:200 dilution) at 4°C. A 30- μ l volume of protein A-Sepharose was added to precipitate the bound antibody complex. The precipitated complexes were washed with phosphate-buffered saline–0.1% sodium dodecyl sulfate–1% Triton X-100 (twice), 2 M urea–0.2 M NaCl–1% Triton X-100–0.1 M Tris chloride (pH 7.5) (twice), 0.5 M NaCl–20 mM Tris chloride (pH 7.5)–1% Triton X-100 (once), and 10 mM Tris chloride (pH 7.5)–50 mM NaCl (twice). The protein was eluted from the complexes by being boiled in Laemmli buffer, and then it was electrophoresed on 12% polyacrylamide gels which were subsequently treated with Amplify (Amersham Corp., Arlington Heights, Ill.) and exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Alkyl hydroperoxide-resistant mutants. To learn more about bacterial defenses against oxidative damage, we isolated mutants resistant to different oxidants in *S. typhimurium*. Two classes of mutants isolated in this manner overexpress proteins F52a and C22 (as seen on two-dimensional gels), shown to constitute alkyl hydroperoxide reductase activity.

The first class of *ahp* was isolated when wild-type *S. typhimurium* LT2 was screened for mutants resistant to cumene hydroperoxide, a representative mutagenic alkyl hydroperoxide. LT2 was plated on minimal-glucose plates containing 200 μ M cumene hydroperoxide, the MIC of cumene hydroperoxide for the wild-type strain. Colonies that grew on this concentration of cumene hydroperoxide were tested for resistance to cumene hydroperoxide by disk inhibition assays. One of the four cumene hydroperoxide-resistant mutants isolated, TA4266 (*ahp-1*), was treated with L-[³⁵S]methionine, and the labeled proteins were examined on two-dimensional gels (4, 14). Compared with the wild type, the only change in the protein pattern was an increase in the intensities of the F52a and C22 protein spots (data not shown). The increased syntheses of the F52a and C22 proteins suggested that the resistance to cumene hydroperoxide was due to elevated levels of alkyl hydroperoxide reductase activity. Consistent with this result, we found that the mutant cells had steady-state levels of alkyl hydroperoxide reductase activity (4, 9a) threefold higher than that of wild-type cells.

The *oxyR1* mutant constitutively overexpresses the F52a and C22 proteins, and *oxyR* deletion strains fail to induce these proteins upon treatment with 60 μ M 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-1*-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 μ M 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 2. Sensitivities of *ahp-1*- and *ahp-2* strains to killing by oxidants^a

<i>S. typhimurium</i> strain (genotype)	Zone of inhibition (mm) by:	
	Hydrogen peroxide (3%)	Cumene hydroperoxide (3%)
TT2385 (<i>oxyR+</i>)	26	27
TA4130 (<i>oxyRΔ2</i>)	46	40
TA4319 (<i>oxyR+ ahp-1</i>)	27	19
TA4191 (<i>oxyRΔ2 ahp-1</i>)	46	38
TA4267 (<i>oxyR+ ahp-2</i>)	27	24
TA4320 (<i>oxyRΔ2 ahp-2</i>)	44	27

^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.

FIG. 1. Map position of *ahp* in *S. typhimurium*. The map positions of *zac120::Tn10*, *zac119::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.

containing a range of cumene hydroperoxide (10 to 100 μ M), 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, *zac119::Tn5* (TA4173), 88% linked to the *ahp-2* mutation by P22 transduction. *zac119::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 (*zac119::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 (*zac120::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 *zac119::Tn5* and *zac120::Tn10* to *ent-7* (TA2443) (17). The 59% linkage of *zac119::Tn5* and the 75% linkage of *zac120::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 *zac120::Tn10* (data not shown). By analogy to *E. coli* mutants, the fact that the *S. typhimurium*

entΔ30 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

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

Strain (genotype)	Zone of inhibition (mm) by:	
	Hydrogen peroxide (3%)	Cumene hydroperoxide (3%)
<i>S. typhimurium</i>		
LT2	20	24
TA4314 (<i>ahpΔ4</i>)	22	37
TA4317 (<i>ahpΔ4</i>)(pAQ9)	29	19
TA4322 (<i>ahpΔ4</i>)(pAQ10)	23	20
<i>E. coli</i>		
K-12	17	23
TA4315 (<i>ahpΔ5</i>)	18	37
TA4321 (<i>ahpΔ5</i>)(pAQ10)	22	20
TA4318 (<i>ahpΔ5</i>)(pAQ9)	17	23

^a 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.

FIG. 2. Immunoblots of strains carrying *ahp* deletions and clones. Extracts were prepared from overnight cultures of the indicated strains and blotted and visualized as described in Materials and Methods. The top lanes show the presence or absence of the F52a protein in *S. typhimurium* and *E. coli* strains carrying *ahp* deletions and clones. The bottom lanes show the presence or absence of the C22 protein.

and *oxyR1* cells, *ahpΔ4* cells had low, uninducible levels of alkyl hydroperoxide reductase activity. The residual activity could be due to nonspecific reduction by glutathione reductase or free sulfhydryls 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*) carry-

FIG. 3. Restriction map of pAQ9. The heavy line corresponds to the 8.5-kilobase chromosomal fragment cloned into the *Bam*HI site of pBR328. The approximate positions of the *ahpF* and *ahpC* genes as determined by subsequent subcloning and sequence analysis are indicated (L. Tartaglia, G. Storz, M. Brodsky, and B. Ames, unpublished data).

ing 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

FIG. 4. Heat shock induction of F52a. Total cellular proteins were labeled with L-[³⁵S]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* (*ahp*Δ4) and *E. coli* (*ahp*Δ5) deletion backgrounds. (B) Effects of an *oxyR* deletion and an *rpoH* mutation on heat shock induction of the *S. typhimurium*(pAQ9) 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.

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* (*ahp*Δ4) and *E. coli* (*ahp*Δ5) 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-[³⁵S]methionine. Antibodies to the F52a protein were then used to immunoprecipitate the labeled F52a protein (Fig. 4A). Densitometer scans of the autoradiograph corresponding to 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 *rpoH*165 mutant strains (16) and *E. coli* *oxyR*⁺ and *oxyR*Δ3 (4) strains. The autoradiograph corresponding to the gels of the immunoprecipitated protein

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 *rpoH*165 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 significance 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-1* and *ahp-2* mutants suggest that they are both regulatory mutants. Since *ahp-1* 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

a *cis*-acting heat shock element which is not present at the *E. coli ahp* 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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