Bacterial Ohr and OsmC paralogues define two protein families with distinct functions and patterns of expression

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**INTRODUCTION**

Organic peroxides are highly toxic and can damage cellular macromolecules, including proteins, lipids and DNA. Furthermore, these compounds participate in free-radical reactions that generate more reactive organic radicals which thereby increases their toxicity (Halliwell & Gutteridge, 1984). In bacteria, organic peroxides are generated as by-products of aerobic metabolism (Gonzales-Flecha & Demple, 1997). In addition, pathogenic bacteria are exposed to reactive oxygen species, including organic peroxides, generated by the host as a part of the active defence response (Baker & Orlandi, 1995; Levine et al., 1994). Thus, detoxification of organic peroxides is important for bacterial survival and proliferation in the host.

Bacteria have evolved complex systems to protect themselves from organic-peroxide toxicity. Alkyl hydroperoxide reductase (Ahp) is the best-characterized bacterial enzyme involved in the metabolism of organic peroxides (Poole, 1996; Niiura et al., 1995). This enzyme consists of two subunits: catalytic subunit C (AhpC) and reductase subunit F (AhpF). AhpC reduces organic peroxides to the corresponding alcohols (Poole & Ellis, 1996). AhpC belongs to a large family of peroxidases (the AhpC family) found in organisms ranging from bacteria to man (Chae et al., 1994a). Some organisms express multiple AhpC/thiol-specific antioxidant paralogues, presumably with distinct functions (regulation or cellular localization) (Baillon et al., 1999; Bsat et al., 1996; Hillas et al., 2000).

In the bacterial phytopathogen Xanthomonas campestris pv. phaseoli, the defence against organic-peroxide toxicity is complex (Loprasert et al., 1996). In addition to AhpC, there is a recently characterized novel...
organic hydroperoxide resistance gene, obr (Loprasert et al., 1997; Mongkolsuk et al., 1998a). X. campesistris obr mutants are sensitive to organic peroxides, but not to other oxidants (Mongkolsuk et al., 1998a). In addition, obr has a unique pattern of oxidant-induced expression; only organic peroxides induce high levels of expression (Mongkolsuk et al., 1998a). This unusual pattern of induction distinguishes obr from other known oxidative stress genes. Analysis of Ohr primary structure shows that it has homology to proteins with unknown functions from both Gram-positive and Gram-negative bacteria, and that it has moderate homology to an osmotically inducible protein (OsmC) from Escherichia coli (Gutiérrez & Devedjian, 1991).

On the basis of sequence analysis of Ohr and OsmC homologues, we propose that these two proteins define two protein subfamilies. In this report, we focus on two organisms with one member of each subfamily: Pseudomonas aeruginosa and Deinococcus radiodurans. Genetic analyses in P. aeruginosa, and expression studies in both organisms, support the hypothesis that these proteins are functionally, as well as structurally, distinct.

METHODS

Bacterial strains, growth conditions and oxidant killing. Xanthomonas strains, D. radiodurans and P. aeruginosa PAO1 were grown in Silva–Buddenhagen medium (0.5% sucrose, 0.5% yeast extract, 0.5% peptone, 0.1% glutamic acid; pH 7.0) at 28 °C, TGY medium (0.1% glucose, 0.8% tryptone, 0.4% yeast extract; pH 7.2) at 32 °C and Luria–Bertani (LB) medium at 37 °C, respectively. Bacterial growth was monitored spectrophotometrically at OD600.

Quantitative determinations of plating efficiency in the presence of various oxidants of Pseudomonas strains were performed as described previously (Hassett et al., 2000; Ochsner et al., 2000). Essentially, cells from exponential phase cultures were serially diluted and plated on LB agar containing various concentrations of tert-butyl hydroperoxide (tBOOH). The numbers of colonies at the different oxidant concentrations were counted after 24 h incubation at 37 °C. Percentage survival is defined as the percentage ratio between the c.f.u. growing on plates containing tBOOH and those growing on plates without tBOOH.

Alignment and phylogenetic analysis. Protein sequences related to Ohr and OsmC were retrieved from public sequence databases using the CLUSTAL W program (Altschul et al., 1997). These amino acid sequences were aligned using the program CLUSTAL W, version 1.7 (Thompson et al., 1994). A phylogenetic tree was constructed by the neighbour-joining method, using the TREE program from the phylogenetic analysis page of D. L. Robertson, E. Beaudoin & J. M. Claverie (http://igs-server.cnrs-mrs.fr/ansr/phylogenetics). The results were drawn using the program PHYLODENDRON, version 0.8d (D. G. Gilbert, Department of Biology, University of Indiana, USA; http://ubio.bio.indiana.edu).

Stress-induced expression of ohr and osmC. Exponential phase cultures (OD600 = 0.4) were divided into flasks and oxidants or other chemicals were added. The following concentrations of chemicals were used: 250 µM H2O2, 200 µM cumene hydroperoxide, 200 µM tBOOH, 100 µM menadione, 2% (w/v) sodium chloride and 4% (v/v) ethanol for P. aeruginosa; 250 µM H2O2, 100 µM tBOOH, 4% (w/v) sodium chloride and 4% (v/v) ethanol for D. radiodurans. Treated and untreated cultures were harvested after 20 min incubation at appropriate temperatures.

Cloning of P. aeruginosa ohr and osmC. Full-length P. aeruginosa obr and osmC genes were cloned using PCR. Primers 5′-ohrP (5′-TCGAGAGGTGACTCTC-3′), 3′ohrP (5′-AGTGGGAAGTTGCAGAC-3′), 5′osmCP (5′-CGAGGGAGGGATGC-3′) and 3′osmCP (5′-AGCGTTCGCCGCAGTTGTC-3′) were designed using sequence data obtained from the genome sequence of P. aeruginosa (Stover et al., 2000). A primer pair, P. aeruginosa genomic DNA, the PCR reaction mix and 2 U Pfu polymerase were mixed and used to amplify either obr or osmC genes, under the following conditions: denaturation at 96 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min. The 450 bp obr and 470 bp osmC PCR-generated fragments were cloned into pBRIIMCS-4 and pBBIIMCS-5, respectively (Kovach et al., 1994), giving two recombinant plasmids, pBBRohrP and pBBRosmCP. The nucleotide sequences of both genes were determined using a BigDye terminator cycle sequencing kit on an automated DNA sequencer (ABI 310).

Construction of ohr and osmC mutants in P. aeruginosa. Mutants were constructed by insertional inactivation of obr and osmC genes. Essentially, pBBRohrP was digested with SfiI and SacII. The ends of the 340 bp fragment containing the coding region of obr were gap-filled by DNA polymerase, and the blunt-ended fragment was cloned into Smal-digested pKnoCK-GM (Alexeyev, 1999) to give pKnoCK-ohr. Similarly, pBBRosmCP was digested with Sall and BstEl. The ends of the 240 bp DNA fragment containing part of the osmC coding region were gap-filled by DNA polymerase, and cloned into Smal-digested pKnoCK-Ap (Alexeyev, 1999) to give pKnoCK-omscP. The sequences of the cloned DNA in both recombinant plasmids were determined using an automated DNA sequencer (ABI 310). pKnoCK-ohrP and pKnoCK-omscP were conjugated into P. aeruginosa PAO1 as described previously (Hassett et al., 2000). Gentamicin-resistant and carbenicillin-resistant colonies will arise from homologous recombination of the insert plasmid with either obr or osmC genes on the chromosome, depending on the fragment on the plasmid. Insertion of the plasmid into the chromosome is expected to inactivate the gene. Transconjugants containing pKnoCK-ohr and pKnoCK-omsc were selected with gentamicin (15 µg ml−1) and carbenicillin (200 µg ml−1), respectively. Putative mutants were screened by PCR using a universal sequence primer for a site located in pKnoCK vectors and either the 3′ohr or the 3′osmC primer. The expected insertions resulting in inactivation of obr and osmC were confirmed by Southern analysis of genomic DNA extracted from the mutants and were probed with gene-specific probes (data not shown).

Northern analysis of ohr and osmC homologues. Total RNA was extracted from P. aeruginosa and D. radiodurans by using the hot acid phenol method performed as described previously (Mongkolsuk et al., 1997). RNA samples were separated by electrophoresis in formaldehyde agarose gels and were then transferred by capillary action to pieces of nylon membrane. Total RNA (10 µg) was loaded into each well. Probes were prepared, and RNA hybridization and membrane washing were performed as described previously (Mongkolsuk et al., 1997). P. aeruginosa obr and osmC probes of 300 bp and 375 bp, respectively, were made from MluI-digested pKnoCK-ohrP and SfiI–HindIII-digested pKnoCK-omscP. The DNA fragments were separated on an agarose gel, extracted and then purified prior to being radioactive labelled using a
random prime DNA-labelling kit. *D. radiodurans ohr* and *osmC* probes were made using PCR. Primers corresponding to coding regions of either *ohr* (5′-GCTTGTTACTCAGCCGACA-3′) or *osmC* (5′-GCTTGTTACTCAGCCGACA-3′) were designed using the *D. radiodurans* genome sequence (White et al., 1999). PCR was performed with *D. radiodurans* genomic DNA and the gene-specific primers in the PCR reactions noted above, using the following conditions: denaturation at 96 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min for 35 cycles.

The strongly nucleophilic regions in thiol-specific antioxidant proteins such as AhpC (Chae et al., 1994b) and in the peroxide-scavenging protein ovothiol (Turner et al., 1988) have been shown to be the catalytic sites for the breakdown of peroxides. This suggests that the C-125 residue in members of the Ohr family could participate in peroxide reduction. This idea is being investigated. The amino acid sequences were used to construct a phylogenetic tree (Fig. 2); it clearly shows that there are two separate groups of proteins, defined here as the Ohr and OsmC subfamilies.

Several bacteria produce either Ohr or OsmC. For example, *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Vibrio cholerae* and *Xylella fastidiosa* have only the Ohr homologue, whereas *E. coli* has only an OsmC homologue. In *Bacillus subtilis*, *Mycoplasma genitalium* and *Sinorhizobium meliloti*, proteins described as ‘OsmC homologues’ (Volker et al., 1998) clearly belong to the Ohr subfamily (Fig. 2).

Interestingly, *Mycoplasma genitalium* has no known proteins, other than the Ohr homologue, involved in peroxide detoxification (Fraser et al., 1995). This suggests that in some bacteria Ohr might have a crucial role (or roles) in protecting against peroxide toxicity. Neither Ohr nor OsmC homologues were found in the genomes of several bacteria such as *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Neisseria meningitidis* and *Rickettsia prowazekii*. Overall, members of the Ohr family appear to be more widely distributed among diverse bacteria than members of the OsmC family.

Unexpectedly, several bacteria have homologues from both subfamilies. Several Gram-negative bacteria (*P. aeruginosa*, *Pseudomonas putida*) and a Gram-positive bacterium (*D. radiodurans*) have one member each from the OsmC and Ohr subfamilies. Other Gram-positive bacteria, such as *B. subtilis* and *Streptomyces coelicolor*, have one member of the OsmC family and two or more members of the Ohr family. Multiple Ohr homologues have not been identified in genomes from Gram-negative bacteria. At present, the functions of the multiple Ohr homologues are unknown but are the subject of further investigation.

**Ohr and OsmC homologues have different physiological roles**

The separation of Ohr and OsmC homologues into two subfamilies raises an important question: do these two subfamilies have distinct or overlapping functions? Bacteria such as *P. aeruginosa*, having one member each from the *ohr* and *osmC* subfamilies, offer an attractive model system for investigating this question. Using insertional inactivation, we generated mutants of the *P. aeruginosa ohr* and *osmC* genes. The *P. aeruginosa ohr* mutant, but not the *osmC* mutant, has a much reduced (more than 100 times lower) plating efficiency on agar containing 500 µM tBOOH when compared with the
Fig. 1. Multiple amino acid sequence alignment of Ohr and OsmC homologues. Various homologues of Ohr and OsmC were aligned by using the CLUSTAL W program (Thompson et al., 1994). The origins of these proteins are as follows: OhXp is from Xanthomonas campestris pv. phaseoli (AF036166); OhSp is from Shewanella putrefaciens (TIGR24 spuret6401)*; OhVc is from Vibrio cholerae (AE003853); OhLp is from Legionella pneumophila (CUGCC446 lneumo W/G.011079-R)*; OhCc is from Caulobacter crescentus (TIGR Carcesntens1257a)*; OhXf is from Xylella fastidiosa (AE003849); OhPs is from Pseudomonas aeruginosa (PAGP 287 contig1)*; OhAc is from Acinetobacter (Y09102); OhSc1, OhSc2, OhSc3 and OsSc are from Streptomyces coelicolor (AL133423.1, AL163672.1, AL031515.1 and AL031515.1, respectively); OhDr and OsDr are from Deinococcus radiodurans (AE000205 and AE000513); OhSm is from Sinorhizobium meliloti (Stanford 382 smelil 423032c12)*; OhBj is from Bradyrhizobium japonicum (AAFF78793.1); YklAAbb, YkbABBs and YmaDBs are from Bacillus subtilis (AJ002571 and Z99113); OhMg is from Mycoplasma genitalium (U39732); OhMp is from Mycoplasma pneumoniae (MPAE000018); OhEf is from Enterococcus faecalis (TIGR1351 gef6391)*; OsPs is from P. aeruginosa (PAGP287 contig1)*;
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Fig. 2. An unrooted phylogenetic tree of Ohr and OsmC homologues. The tree was obtained by a neighbour-joining method by TREE phylogenetic analysis and was displayed using the PHYLODENDRON program (see Methods). Bar, 0.1 changes per site. Protein designations are the same as those in Fig. 1.

Fig. 3. Plating efficiencies of P. aeruginosa ohr and osmC mutants in the presence of oxidants. P. aeruginosa PAO1 (○), an ohr mutant (●), an osmC mutant (□), an ohr mutant harbouring pBBRohrP (▲) and an ohr mutant harbouring pBBRosmCP (■) were grown to exponential phase, serially diluted and plated on plates containing various concentrations of tBOOH. The experiments were performed independently four times, and the error bars represent standard error of the mean.

OsBb is from Bordetella bronchiseptica (Sanger518 bbronchi contig2522)*; OsEc is from Escherichia coli (X57433); OsTf is from Thiobacillus ferrooxidans (TIGR tferroxidans4156)*. Asterisks indicate data from unfinished genome sequences. The conserved regions found in either Ohr homologues (Oh regions) or OsmC homologues (Os regions) are shown by regions of black shading with white lettering. Grey shading with black lettering indicates identical amino acid residues found in both Ohr and OsmC (15 out of 26 sequences). ▼. Highly conserved C residues. The numbers at the ends of each line on the right-hand side refer to the numbers of amino acid residues.

parent strain (Fig. 3). No changes in the plating efficiency in the presence of H$_2$O$_2$ or menadione for either mutant were observed (data not shown). Mutations in oxidative stress genes can lead to decreased aerobic growth rate and plating efficiency (Mongkolsuk et al., 1998b; Hassett et al., 2000). However, both mutants had the same growth rate as the parent strain in rich medium, and no deficiency in aerobic plating was detected (data not shown).

The ohr phenotype can be complemented by a plasmid containing the ohr gene (pBBRohrP), but not by a plasmid carrying the osmC gene (pBBRosmCP) (Fig. 3). The ohr mutant carrying pBBRohrP showed a 60-fold increase in plating efficiency in the presence of tBOOH when compared with the mutant harbouring the vector alone (Fig. 3), though this level was slightly lower than the tBOOH-resistance level attained by the parent strain. In the parental strain, ohr is expressed at high levels after exposure to tBOOH (Fig. 4); however, ohr expression in a moderate-copy-number expression vector (pBBR1MCS-4) might not be high enough to confer full protection against tBOOH toxicity in the ohr mutant. pBBRohrP did not raise tBOOH resistance in either the parental strain or the osmC mutant. These findings are similar to those presented in a previous
**ohr and osmC homologues have different expression patterns**

*P. aeruginosa* and *D. radiodurans* bacteria, each of which has one gene from each subfamily. In both organisms, *ohr* was strongly induced by low concentrations of organic peroxides (cumene hydroperoxide and tBOOH) (Fig. 4) but not by other oxidants such as menadione (not shown for *D. radiodurans*) or *H*₂*O*₂. Neither osmotic stress (a high salt concentration) nor ethanol induced expression of the *ohr* homologues. In contrast, expression of *osmC* homologues in both bacteria was induced by ethanol, while salt stress induced *osmC* expression only in *P. aeruginosa*; none of the oxidants tested induced the gene expression (Fig. 4). Thus, the patterns of *ohr* and *osmC* expression in *P. aeruginosa* and *D. radiodurans* are consistent with the known regulation of *X. campestris pv. phaseoli* *ohr* (Mongkolsuk et al., 1998a) and *E. coli osmC* (Gutierrez & Devedjian, 1991). The *ohr* and *osmC* mRNAs in both bacterial species were each approximately 0.7 kb in length, indicating that these genes are transcribed as monocistronic mRNAs. Expression of both genes is different: *ohr* and *osmC* are induced by organic peroxide and osmotic stress, respectively. At present, well-characterized regulators of stress-induced gene expression such as OxyR, SoxRS and RpoS cannot account for the *ohr* and *osmC* patterns of expression, implying that these genes are regulated by novel regulators.

It was noticeable that basal levels of *ohr* and *osmC* from *P. aeruginosa* and *D. radiodurans* varied greatly, ranging from barely detectable amounts in the former to moderately high levels in the latter. In addition, the degree of induction varied significantly between these bacteria: *D. radiodurans* showed a lower magnitude of induction than *P. aeruginosa*. It remains to be seen if these differences in basal level expression and degree of induction are related to the ability of each bacterium to cope with organic peroxide stress or are simply indicative of the differences between Gram-negative and Gram-positive bacteria. It is remarkable that the patterns of stress-induced expression of *ohr* and *osmC* homologues are highly conserved in a diverse range of bacteria. This suggests that both genes might have important functions.

**Concluding remarks**

Members of the Ohr family are widely distributed in both Gram-negative and Gram-positive bacteria. Analysis of primary structure, the physiological characterization of mutants and expression patterns show that Ohr and OsmC proteins belong to different, but related, subfamilies. We have shown, in *P. aeruginosa* and *X. campestris pv. phaseoli* (Mongkolsuk et al., 1998a), that mutations in *ohr* increase susceptibility to organic peroxides. This phenotype, coupled with the specific induction of *ohr* by organic peroxides, suggests that *ohr* represents a novel organic peroxide protection system. Recent results from Ochsner et al. (2001) confirm our finding that mutation in *P. aeruginosa* *ohr* results in response to osmotic and oxidative stresses in *P. aeruginosa* and *D. radiodurans* bacteria, each of which has one gene from each subfamily. In both organisms, *ohr* was strongly induced by low concentrations of organic peroxides (cumene hydroperoxide and tBOOH) (Fig. 4) but not by other oxidants such as menadione (not shown for *D. radiodurans*) or *H*₂*O*₂. Neither osmotic stress (a high salt concentration) nor ethanol induced expression of the *ohr* homologues. In contrast, expression of *osmC* homologues in both bacteria was induced by ethanol, while salt stress induced *osmC* expression only in *P. aeruginosa*; none of the oxidants tested induced the gene expression (Fig. 4). Thus, the patterns of *ohr* and *osmC* expression in *P. aeruginosa* and *D. radiodurans* are consistent with the known regulation of *X. campestris pv. phaseoli* *ohr* (Mongkolsuk et al., 1998a) and *E. coli osmC* (Gutierrez & Devedjian, 1991). The *ohr* and *osmC* mRNAs in both bacterial species were each approximately 0.7 kb in length, indicating that these genes are transcribed as monocistronic mRNAs. Expression of both genes is different: *ohr* and *osmC* are induced by organic peroxide and osmotic stress, respectively. At present, well-characterized regulators of stress-induced gene expression such as OxyR, SoxRS and RpoS cannot account for the *ohr* and *osmC* patterns of expression, implying that these genes are regulated by novel regulators.

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increased organic-peroxide sensitivity. More studies are needed to discover the physiological function of OsmC. The osmotically inducible expression of the gene suggests that it could have some kind of role in the bacterial osmotic-stress response. Recently, Conter et al. (2001) reported contradictory results that E. coli osmC mutants showed increased sensitivity to tBOOH but not to cumene hydroperoxide.

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