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

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Xanthomonas campestris Ohr (a protein involved in organic peroxide protection) and Escherichia coli OsmC (an osmotically inducible protein of unknown function) are related proteins. Database searches and phylogenetic analyses reveal that Ohr and OsmC homologues cluster into two related subfamilies of proteins widely distributed in both Gram-negative and Gram-positive bacteria. To determine if these two subfamilies are functionally distinct, ohr and osmC in Pseudomonas aeruginosa (a bacterium with one representative from each subfamily) were analysed. Only ohr mutants are hypersensitive to organic peroxide, and this phenotype can be restored by complementation with ohr but not osmC. In addition, expression of ohr was highly induced only by organic peroxides, and not by other oxidants or stresses. In contrast, osmC was induced by ethanol and osmotic stress. A similar pattern of regulation was observed for Ohr and OsmC homologues in the Gram-positive bacterium Deinococcus radiodurans, though uninduced expression was much higher and induction lower in this species. These data clearly support the conclusion that Ohr and OsmC define two functionally distinct subfamilies with distinct patterns of regulation.

Keywords: Pseudomonas aeruginosa, Deinococcus radiodurans, organic peroxide resistance, osmotic stress

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; Niimura 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, ohr (Loprasert et al., 1997; Mongkolsuk et al., 1998a). X. campesiris ohr mutants are sensitive to organic peroxides, but not to other oxidants (Mongkolsuk et al., 1998a). In addition, ohr 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 ohr 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 (Gutierrez & 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 OD

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 (OD

Alignment and phylogenetic analysis. Protein sequences related to Ohr and OsmC were retrieved from public sequence databases using the BLAST 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 (OD

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 ohr and osmC probes of 300 bp and 375 bp, respectively, were made from MluI-digested pKnoch-ohrP and SfiI–HindIII-digested pKnoch-osmCP. The DNA fragments were separated on an agarose gel, extracted and then purified prior to being radioactively labelled using a
random prime DNA-labelling kit. D. radiodurans obr and osmC probes were made using PCR. Primers corresponding to coding regions of either obr (5'-ohrD, 5'-TGCGGGCCAG-GGAATAG-3', and 3'ohrD, 5'-TGTCCTTATTCGCCGG-AC-3') or osmC (5'-osmCD, 5'-CAGCGACACACTGGGC-3', and 3'osmCD, 5'-GTTTGGCCAGACTCAGGC-3') were designed using the D. radiodurans genomic 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 445 bp obr and 470 bp osmC PCR-generated fragments were gel-purified, and radioactively labelled probes were made using the random primer DNA-labelling kit.

**RESULTS AND DISCUSSION**

**Analysis of Ohr and OsmC homologues**

Analysis of Ohr homologues from various bacteria suggested that there were two groups of related proteins (Koonin et al., 2000; Mongkolsum et al., 1998a; Volker et al., 1998). Some homologues, such as Ohr from *Acinetobacter calcoaceticus* and *P. aeruginosa*, have high levels of identity (50% or more), while others, such as *E. coli* OsmC, have moderate levels of identity (around 20%) when compared with the Ohr from *X. campestris* pv. *phaseoli* (Mongkolsum et al., 1998a). Since the physiological role of these homologues is unknown, it is not yet clear whether these structural distinctions are of functional significance. To address this question, we have performed a phylogenetic analysis of Ohr homologues and initiated molecular genetic studies in two model systems containing homologues from both the Ohr and the OsmC subfamilies.

*X. campestris* pv. *phaseoli* Ohr and *E. coli* OsmC amino acid sequences were used to search the GenBank and bacterial genome databases for related proteins. Homologues of both proteins are widely distributed in both Gram-negative and Gram-positive bacteria, but no homologues were detected in eukaryotes. Amino acid alignments generated using CLUSTAL W (Thompson et al., 1994) suggest that the Ohr/OsmC family can be divided into two subfamilies, each being defined by sequence motifs conserved only among Ohr (designated Oh regions) or only among OsmC (designated Os regions) homologues (Fig. 1). At present, we do not know the biological significance of these different motifs. A notable feature of the primary structure of Ohr and OsmC family members is the two highly conserved cysteine residues. C residues have been shown to be the active site of AhpC, an enzyme that metabolizes organic peroxide (Chae et al., 1994b). The amino acid sequences around the second C residue are conserved within members of the Ohr and OsmC families but are very diverse between the two families. The conserved amino acid region around C-125 of the Ohr family members contains the sequence motif VCPY (Fig. 1). This region is not present in members of the OsmC family. The VCPY motif places the cysteine residue in an environment of abnormally strong nucleophilicity that makes it highly susceptible to reactive oxygen species (Lim et al., 1994). 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 obr and osmC subfamilies, offer an attractive model system for investigating this question. Using insertional inactivation, we generated mutants of the *P. aeruginosa* obr and osmC genes. The *P. aeruginosa* obr 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 spntrf4c9)*; OhVc is from Vibrio cholerae (AE003853); OhLp is from Legionella pneumophila (CUCGC446 1pneumo W.G.011079-R)*; OhCc is from Caulobacter crescentus (TIGR Carucsntus12574)*; OhEf is from Xylella fastidiosa (AE003849); OhPs is from Pseudomonas aeruginosa (PAGP 287 contig1)*; OhAc is from Actinobacillus (Y90102); OhSc1, OhSc2, OhSc3 and OhSc4 are from Streptomyces coelicolor (AL133424.1, AL163672.1, AL031515.1 and AL031515.1, respectively); OhDr is from Deinococcus radiodurans (AE000205 and AE000513); OhMg is from Sinorhizobium meliloti (Stanford 382 smelilot 4230832.12); OhAs is from Bradyrhizobium japonicum (AAF78797.1); YkLAbs, YkzABs and YmaDBs are from Bacillus subtilis (AJ002571 and Z99113); OhMg is from Mycoplasma genitalis (U39732); OhMp is from Mycoplasma pneumoniae (MPA800018); OhEf is from Enterococcus faecalis (TIGR1351 gef6391)*; OhPs 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.
report on *X. campestris pv. phaseoli*, in which the wild-type strain harbouring an *ohr* expression vector did not display increased resistance to tBOOH (Mongkolsuk et al., 1998a).

We have tested several parameters, including aerobic growth, response to osmotic stress, and ethanol resistance, in the *P. aeruginosa* osmC mutant and found no significant changes in these parameters in comparison with the parent strain (data not shown). A similar analysis of *E. coli* osmC mutants failed to detect any physiological alterations (Gutierrez & Devedjian, 1991). The distinct phenotypes of the *ohr* and osmC mutants, and the inability of the osmC gene to complement the *ohr* mutant, support the idea that these genes play different roles in the cell.

**ohr and osmC homologues have different expression patterns**

*X. campestris pv. phaseoli* *ohr* has a unique expression pattern in that its expression is induced only by organic peroxide, and not by menadione or H$_2$O$_2$ (Mongkolsuk et al., 1998a). In contrast, *E. coli* osmC is under both growth-phase (RpoS) and osmotic-stress regulation (Bouvier et al., 1998; Gordia & Gutierrez, 1996). This suggests that members of the *ohr* and osmC subfamilies may have different patterns of stress-inducible expression.

We used Northern blotting experiments to determine the expression patterns of *ohr* and osmC homologues 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.

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...
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.

**ACKNOWLEDGEMENTS**

We thank P. Bennett for editing the manuscript. The research was supported by a grant from Chulabhorn Research Institute to the Laboratory of Biotechnology, by grants to S. M. from the Thai Research Fund (BRG/10/2543), by a career-development award (RCF 01-40-005) from the NSTDA (to S. M.), and by grant NSF 9983634 to J. D. H.

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Received 29 December 2000; revised 16 March 2001; accepted 2 April 2001.