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/thiol-specific antioxidant 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; Bsai *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

Abbreviations: Ahp, alkyl hydroperoxide reductase; tBOOH, *tert*-butyl hydroperoxide.

organic hydroperoxide resistance gene, *ohr* (Loprasert *et al.*, 1997; Mongkolsuk *et al.*, 1998a). *X. campestris 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 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 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/anrs/phylogenetics>). The results were drawn using the program PHYLODENDRON, version 0.8d (D. G. Gilbert, Department of Biology, University of Indiana, USA; <http://iubio.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 ohr* and *osmC* genes were cloned using PCR. Primers 5'ohrP (5'-TCAGACAGGTGACTCTC-3'), 3'ohrP (5'-AGTCGGAAGCTTCAGAC-3'), 5'osmCP (5'-CGACGCGAGCGGATGTC-3') and 3'osmCP (5'-AGCGTTCCGCTCAGCCG 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 *ohr* 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 *ohr* and 470 bp *osmC* PCR-generated fragments were cloned into pBBR1MCS-4 and pBBR1MCS-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 *ohr* and *osmC* genes. Essentially, pBBRohrP was digested with *Sfi*I and *Sac*II. The ends of the 340 bp fragment containing the coding region of *ohr* were gap-filled by DNA polymerase, and the blunt-ended fragment was cloned into *Sma*I-digested pKnock-GM (Alexeyev, 1999) to give pKnock-ohr. Similarly, pBBRosmCP was digested with *Sall* and *Bst*EII. The ends of the 240 bp DNA fragment containing part of the *osmC* coding region were gap-filled by DNA polymerase, and cloned into *Sma*I-digested pKnock-Ap (Alexeyev, 1999) to give pKnock-osmCP. The sequences of the cloned DNA in both recombinant plasmids were determined using an automated DNA sequencer (ABI 310). pKnock-ohrP and pKnock-osmCP 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 in-coming recombinant plasmid with either *ohr* 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-osmC 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 *ohr* 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 ohr* and *osmC* probes of 300 bp and 375 bp, respectively, were made from *Mlu*I-digested pKnock-ohrP and *Sfi*I-*Hind*III-digested pKnock-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* *ohr* and *osmC* probes were made using PCR. Primers corresponding to coding regions of either *ohr* (5'-ohrD, 5'-TGCGGGCGA-GGGAATAG-3', and 3'-ohrD, 5'-GTGTCTTATTTCGCGG-AC-3') or *osmC* (5'-osmCD, 5'-CAGCGAGCACACTGGGC-3', and 3'-osmCD, 5'-GCTTGAGCGACTCAGCC-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 445 bp *ohr* 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; Mongkolsuk *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* (Mongkolsuk *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 environ-

ment 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 *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

OhXp	:	-----MASPEKVLVTAHATATGCR-EGRAVSSDKALDAKLISTR--ELGGAGGDGTNPEOLFAAGYAACFIGAMKAVAA	:	71
OhSp	:	-----MKTLYETRATASACR-QQVSTDDLLNVALAYPKAMETGGSGCL-ATNPEOLFAAGYAACFSNALHVAK	:	68
OhVc	:	-----MSTIVQTSATASACR-NGVVSTEDKLELNLSYFK--EMGSGT-ATNPEOLFAAGYAACFSNALHVAR	:	66
OhLp	:	-----MKALYTTIAKAHGCGR-NGHVETDGLLKLDLAMPR--ELGGEGG-ATNPEOLFAAGYAACFESAIRHVA	:	66
OhCc	:	-----MTTLYTTRATVVGCR-EGHARTEDGLLDVQLSMFK--SLGGKET-GTNPEOLFAAGYAACFQSMGHVAR	:	66
OhXf	:	-----MNSLEKVLVTAIVTATGCR-DGSVVSSDNVLNVKLSVQ--GLGGPGSGGTNPEOLFAAGYACFEGALKFVA	:	71
OhPs	:	-----MQT-IKALYTATATATGCR-DGRAVSSDGLVDVQLSTR--ELGGQGAATNPEOLFAAGYACFEGALKFVAG	:	70
OhAc	:	-----MSLEQVVVTAHAKATGCR-DGRATSSDNILDVOLTVFK--EMGGMGG-GTNPEOLFAAGYACFEGALKFVAT	:	69
OhSc1	:	-----MPIQQSEVLVTAIVATAENGR-DGRVATDDGRLDVVNVPFK--EMGGNGA-GTNPEOLFAAGYACFEGALKFVAT	:	71
OhSc2	:	-----MDALYTAAATATHCR-DGRAVSSDGLVDLALGIFV--EMGGNGQ-GTNPEOLFAAGYACFEGALKFVGR	:	66
OhDr	:	-----MANVYTAEATATGCR-AGTTRSSDRLNLDLSVPA--EMGGDGGGTNPEOLFAAGYACFEGALKFVSR	:	67
OhSm	:	-----MTTHDEVLYTGKTHTTDCR-EGFARSDDAQLDVLRSF--GGVKC-VTNPEOLFAAGYACFEGALKFVSR	:	67
OhBj	:	-----MTQAALLYTARTHTGGGRQDGMSSDGLDVLRSF--GGAGI-GTNPEOLFAAGYACFEGALKFVSR	:	68
OhSc3	:	-----MTVHG--GAASH--CRASGHARSADGLDLRMPA--ELGGDGR-GTNPEOLFAAGYACFEGALKFVSR	:	64
YklABs	:	-----MSQPLFTATVSAVGGCR-EGKVISSDRLVLDVAMP--TPRAKKLEKATNPEOLFAAGYACFEGALKFVSR	:	69
YkzABs	:	-----MALETAKVATARGCR-AGHITSDDGLDVLDFIVMPN-AK--KEGQTGTNPEOLFAAGYACFEGALKFVSR	:	65
OhMg	:	MLFNIFTKILSILINMALIKTVAQTETGR-EGSVKTLTG--FQTKLSFPK--PDLVQVTENNPEOLFAAGYACFEGALKFVSR	:	80
OhMp	:	-----MAVYKTTAASACR-EGVVQTVDG--FTQSLAFK--PCATHQDKNNPEOLFAAGYACFEGALKFVSR	:	65
OhEf	:	-----MKKIHETTTIINTGCR-AGEVHSPD--KSFYAVAS--PCVKKENTNPEOLFAAGYACFEGALKFVSR	:	64
OsPs	:	---MSIHSSGGVDMKKTASAVVQGLKDK--GTLSTESGALKDNPYGENTREFEAPG---TNPEELIGAHAAGCFSMALSMMLG	:	77
OsBb	:	-----MKKTASAVVQGLKDK--GTLSTESGALKDNPYGENTREFEAPG---TNPEELIGAHAAGCFSMALSMMLG	:	67
OsEc	:	-----MTIHKKGQAHWEDIKRKG--GTVSTESGVLNQQPYGENTREFEAPG---TNPEELIGAHAAGCFSMALSMMLG	:	69
OsDr	:	-----MADIARKASAHWEDIKRSGN--GTVSTESGVLNQQPYGENTREFEAPG---TNPEELIGAHAAGCFSMALSMMLG	:	70
OsTf	:	-----MPIREAHTSMSGSLQGV--GTVVLTSSGIGSYEVSFKRAESAEG--ATSPBELIAAHTSCYANQFSAALLG	:	70
OsSc	:	-----MPTSAHTVWENLLEGN--GVVTFSSGIGEPQVSWPFR--AEQANG-KTSPBELIAAHTSCYANQFSAALLG	:	70
YmaDBs	:	-----MADHHFYLKANWPGNR-NDVGTTESGNLITSISIK--EMDGPGE-GTNPEMLLGAATCYIITLAAMME	:	67
OhXp	:	QD-KLKLPGEVSDIDSSVGIGQI-PG-GFGIVVELRIAVPG--MKAEQLTLVDKAAHQVCFYSNATRGNIDVITLTA-----	:	142
OhSp	:	IA-KVAIT-EAPVSAVVGIGAN-ETGGFALTVALEVSLD---LPQADAQALVRHAAHQVCFYSNATRGNIDVITLTA-----	:	143
OhVc	:	EA-KVALK-EAPVTATVGIGPN-GQGGFALSVALAAHIA---LEDEQARQLVTVAAHQVCFYSNATRGNIDVITLTA-----	:	140
OhLp	:	VQ-KISLE-DVMSSTSEVSLYAT-PEKGKFLGVALHAHIT--LNQNEAALVAAHQVCFYSNATRGNIDVITLTA-----	:	139
OhCc	:	TQ-KIALA-GSTVTGQVSL-AT-AEGERLEVALEVETQG--LSQADAEALVAAHQVCFYSNATRGNIDVITLTA-----	:	138
OhXf	:	KE-KVDLPAEPRVEGRVIGIGI-PG-GFGLVVELRIAVPG--MERSMLQTLVDKAAHQVCFYSNATRGNIDVITLTA-----	:	143
OhPs	:	QR-KQTLPADASITGVIGIGI-PG-GFGLVVELRIAVPG--MERSMLQTLVDKAAHQVCFYSNATRGNIDVITLTA-----	:	142
OhAc	:	RD-KFNIPKDAYVEGDVGIGPI-PN-GFGEIVELHVLHPG--MDREAKKLVDAAHQVCFYSNATRGNIDVITLTA-----	:	143
OhSc1	:	QE-GADIS-GSTVTAKVGIGKN-DD-GFGIIVEISAEIPT--VDAATARSLEKAAHQVCFYSNATRGNIDVITLTA-----	:	139
OhSc2	:	AA-KVDVS-DAAVTAVVGIGKQ-GE-GFALAVTLRVLELPEG--LDEETGRKLEVAHQVCFYSNATRGNIDVITLTA-----	:	137
OhDr	:	RQ-KIDVPADSTITARVGLQKA-GL-APALDVELEGHFP--LSREQAEGLMHAAHQVCFYSNATRGNIDVITLTA-----	:	139
OhSm	:	KH-RLTLPAETAVIDAEDDLAKS--DGGELLQDRLAVSLPG--MDADLARSLEKAAHQVCFYSNATRGNIDVITLTA-----	:	138
OhBj	:	KR-KITLPRKCAIDAIDLLD--EGAYSRLARLNVSLPG--LDREVARDIVDEAHQVCFYSNATRGNIDVITLTA-----	:	139
OhSc3	:	QE-ALD-PAASVSVATVAFGRDPEDGGYLLRVLDVVRWPG--VDPATADELIGKADALCFYARMARGTPTTITLAS-----	:	137
YklABs	:	TE-RVKVE--TEVTANVSLKDEADQGYKLGVLQVKGEG--VSASEALEALVKKAAHQVCFYSNATRGNIDVITLTA-----	:	141
YkzABs	:	EQ-NIED--SEIEGQVSLMKDESDDGFKIGVTLVNTKD--MDREKAELVNAHEFCFYSNATRGNIDVITLTA-----	:	136
OhMg	:	QH-QFSFSPKPPVSVKVELHQE--NGLSHIKAGVELTNS--NDQEVGKKLIQKAAHEMCFFSRLIRNENFLGITLNGIKL--	:	155
OhMp	:	QH-QLQIATQPIVGVSVELHDQ--DGLSHIKAGVELAIT--VDQTTAQTVITANHAMCFFSRLIKPENFLGITLNGAKL--	:	140
OhEf	:	TD-QEKVEGKSTVTARVSLFQG--EDGSGVGAELVHIDG--VDQAKAEELVKKAAHEI-CFYSNATRGNIDVITLTA-----	:	135
OsPs	:	EASLTAERIETRAEVLTDKQSDG--FAITAVHLVLRARVPG--ADAQTFEQIANKAAKAGCFVSKVLN--AKISLDASLDG---	:	151
OsBb	:	EASLKADEIETRAEVLTDKQSDG--FAITAVHLVLRARVPG--ATKDSFADAAKAAKAGCFVSKVLN--ARISMDATLKG---	:	141
OsEc	:	EASFTPTSIDTTADVSLDKVDAG--FAITKIALKSEVAVPG--IDASTFDGIIQKAAKAGCFVSKVLN--AEITLD-----	:	138
OsDr	:	EHSHEIKALDTDATCEMVKDGPG--FKINNHMLRVRAQLTG--SDQADFEAHVKDAEKCPFLSRIMQGNVEVTHEALEG---	:	146
OsTf	:	AAGGVDIHLEVGAKVHLEPDPDGGFFHPRIDETVDGSAQG--VAQABEHKIAEEAKRSCFVSKVLSG--TQIELIVV-----	:	143
OsSc	:	GASTPPTKLTTSDATVTFQPG--EG--IKGIHITVEGTVP--LDNDAFVAAEDAKKNCFVSKVLSG--TTITLSAKLA-----	:	141
YmaDBs	:	RSLEKEDLQMESEGINVNTKGVFTYKIIHRPSVVLKHDASQDVALAHKLCKKLESSCMISRAIQENVEIQLQEASVKLGGE	:	150

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 sputre6401)*; OhVc is from *Vibrio cholerae* (AE003853); OhLp is from *Legionella pneumophila* (CUCGC446 lpneumo WG.011079-R)*; OhCc is from *Caulobacter crescentus* (TIGR C.crescentus12574)*; 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* (AE002025 and AE000513); OhSm is from *Sinorhizobium meliloti* (Stanford 382 smelil 423032c12)*; OhBj is from *Bradyrhizobium japonicum* (AAF78793.1); YklABs, YkzABs 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)*;

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 PHYLONDENDRON 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.

parent strain (Fig. 3). No changes in the plating efficiency in the presence of H_2O_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

OsBb is from *Bordetella bronchiseptica* (Sanger518 bbronchi contig2522)*; OsEc is from *Escherichia coli* (X57433); OsTf is from *Thiobacillus ferrooxidans* (TIGR tferrooxidans4156)*. 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.

Fig. 4. Expression analysis of *ohr* and *osmC* in response to stresses in *D. radiodurans* and *P. aeruginosa*. *P. aeruginosa* and *D. radiodurans* cultures were grown and were either left untreated (U) or treated with oxidants (C, cumene hydroperoxide; H, H_2O_2 ; M, menadione; T, tBOOH) or subjected to osmotic stress (S, salt) and ethanol stress (Et) as described in Methods. RNA isolation, gel electrophoresis, blotting and hybridization were as described in Methods. Northern blots were probed with radioactively labelled *P. aeruginosa ohr* or *osmC* or *D. radiodurans ohr* or *osmC* probes.

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_2O_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_2O_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 increase sensitivity to tBOOH but not to cumene hydroperoxide.

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