**Organic Hydroperoxide Resistance Gene Encodes a Thiol-dependent Peroxidase**

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**ohr** (organic hydroperoxide resistance gene) is present in several species of bacteria, and its deletion renders cells specifically sensitive to organic peroxides. The goal of this work was to determine the biochemical function of Ohr from *Xylella fastidiosa*. All of the Ohr homologues possess two cysteine residues, one of them located in a VCP motif, which is also present in all of the proteins from the peroxiredoxin family. Therefore, we have investigated whether Ohr possesses thiol-dependent peroxidase activity. The *ohr* gene from *X. fastidiosa* was expressed in *Escherichia coli*, and the recombinant Ohr decomposed hydroperoxides in a dithiothreitol-dependent manner. Ohr was about twenty times more efficient to remove organic hydroperoxides than to remove H₂O₂. This result is consistent with the organic peroxyxidase activity of Ohr strains. The dependence of Ohr on thiol compounds was ascertained by glutamine synthetase protection assays. Approximately two thiol equivalents were consumed per peroxide removed indicating that Ohr catalyzes the following reaction: 2RSH + ROOH → RSSR + ROH + H₂O. Pretreatment of Ohr with N-ethyl maleimide and substitution of cysteine residues by serines inhibited this peroxidase activity indicating that both of the Ohr cysteines are important to the decomposition of peroxides. C12SS still had a residual enzymatic activity indicating that Cys-61 is directly involved in peroxide removal. Monothiol compounds do not support the peroxidase activity of Ohr as well as thioredoxin from *Saccharomyces cerevisiae* and from *Spirulina*. Interestingly, dithiothreitol and dithiodiglycoic acid, which possess two sulphydryl groups, do support the peroxidase activity of Ohr. Taken together our results unequivocally demonstrated that Ohr is a thiol-dependent peroxidase.

The infection of both plants and animals induces a defense response that results in an oxidative burst with the increased generation of ROS1 (1). Lipid hydroperoxides can be generated from the attack of ROS to the bacterial membrane. Organic hydroperoxides can also be formed during metabolism of certain drugs or during oxidation of n-alkanes (2). These peroxides can then react with metals or with metalloproteins leading to the production of secondary free radicals (3, 4), which may be related to the fact that organic peroxides possess bactericidal activity (5).

The alkyl hydroperoxide reductase (AhpR) is frequently considered the main enzyme responsible for the conversion of organic peroxides to the corresponding alcohols in bacteria (6, 7). This enzyme comprises two subunits, AhpF and AhpC. AhpC is a thiol-dependent peroxidase that belongs to the peroxiredoxin family (8). A cysteine residue of AhpC is oxidized to sulfenic acid (R-SOH) by peroxides. NADH reduces the sulfenic acid back to its sulphydryl (R-SH) form in a reaction catalyzed by AhpF. AhpF is a flavo-enzyme that shares homology with thioredoxin reductase (9).

Recently a gene was isolated in *Xanthomonas campestris pv. phaseoli* because its deletion rendered cells highly sensitive to killing by organic peroxides but not to H₂O₂ or superoxide generators (10). Therefore, it was named *organic hydroperoxide resistance (ohr)* gene. *ohr* gene expression was highly induced by t-bOOH, weakly induced by H₂O₂, and not induced at all by superoxide (10). Recently, homologues of this gene were also characterized in other bacteria such as *Bacillus subtilis* and *Pseudomonas aeruginosa* (11, 12) among others. Interestingly, Ohr, but not AhpR, appears to play a significant role in CuOOH resistance in *B. subtilis* (12). In *Enterococcus faecalis*, ohr deletion rendered the cells more sensitive to t-bOOH and also to ethanol (13). Sequence analysis has shown that *ohr* homologues are widely spread among different bacteria genera, many of them pathogenic (14). Ohr also shares similarities with OsmC, which is involved in bacterial defense against osmotic stress (14).

All the Ohr and OsmC homologues have two cysteine residues located in motifs that are also very conserved. One of the cysteine residues is part of a VCP motif that is also found in peroxiredoxins. Therefore, it was postulated that Ohr could decompose peroxides directly, similarly to AhpC, a peroxiredoxin found in bacteria (14). In fact, AhpC complement *ohr* deletion in *Escherichia coli* and in *X. campestris* (10). In *P. aeruginosa*, the deletion of *ohr* rendered the cells more sensitive to organic peroxide than *ahpC* deletion, and the double mutant *ohr*, *ahpC* is more sensitive than the single mutants (11). Finally, media from mutants for Ohr contain higher levels of organic peroxides than the correspondent wild-type cells (11, 15).

Despite the suggestions that Ohr might directly detoxify organic hydroperoxides, it was not possible to rule out the possibility that Ohr is involved in other processes such as the transport of organic molecules (10) or in yet undefined signal-
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ing pathways that lead to activation of secondary molecules that would then inactivate organic peroxides. Here, for the first time, the biochemical activity of Ohr was elucidated: Ohr from Xylella fastidiosa possesses a thiol-dependent peroxidase activity, which is probably responsible for the hypersensitivity of Δohr mutants to treatment with organic hydroperoxides.

MATERIALS AND METHODS

Materials—All the reagents were purchased with the highest degree of purity. DHLA was purchased in the reduced form from Sigma (T8620). DHLA is a yellow oil, and its stock solution was prepared by dilution to 50 mM concentration in 20 mM phosphate buffer, pH = 7.5 and 150 mM NaCl for 30 min. DHLA concentration was ascertained by the use of the Ellman’s reagent as described below.

Nucleic Acid Extraction, Cloning, and Nucleotide Sequencing—The ohr gene was PCR-amplified from the cosmide XF-07F02 that was used in the X. fastidiosa sequencing genome project (16). The following forward 5'-CCGGAAGTTCCATATAGTACCTAGGAA (Xf01) and reverse 5’-GGGCTGATCTTCTCATTGGACATCCGACATCG (Xf02) primers were used. The underlined bases represent the NdeI and BamHI sites, respectively. The PCR product was cloned into the pGEM-T easy vector (Promega) resulting in the pGEMohr plasmid. An E. coli DH5-α strain was transformed with pGEMohr, and white colonies were selected from LB-ampicillin-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) medium. A second extraction was performed using the Rapid Plasmid Miniprep System Concert kit (Invitrogen). The plasmid pGEMohr was used to generate the two individually ohr mutant proteins, C61S and C125S, in X. fastidiosa and E. coli, in which cysteines Cys-61 and Cys-125 were replaced by serines through PCR megaprimer methods (17, 18). In the case of the C61S construct, PCR was performed first with the mutagenic primer Xf0C1 forward 5'-CATCCTTGGACATCCGACATCG-3' and Xf02 reverse, where the bold letters denote the mutation performed. A single band of 277 bp was eluted out from the agarose gel and used as a primer (megaprimer) along with the primer Xf01 to the second PCR step to amplify the rest of the Ohr gene. In the case of the C125S construct, replacement was performed in one-step PCR using the primers Xf0C1 and a large terminal mutagenic primer Xf0C2 5’-GGGCTGATCTTCTCATTGGACATCCGACATCG-3’, which, while reducing the typing represents the codon mutation and the underlined bases represents the BamHI restriction site. The final mutated PCR products were ligated in pGEM/T easy vector to produce PGEM/C61S and PGEM/C125S, independently.

Protein Expression and Purification—E. coli B215(DE3) cells were transformed with the same constructs. The resulting strains were used for expression and purification of Ohr, C61S, and C125S.

Protein Expression and Purification—E. coli B215(DE3) strains transformed with pET15b, pGEMohr, pGEMohr/C61S, and pGEMohr/C125S were first digested with NdeI and BamHI. The fragments generated by NdeI/BamHI digestion of plasmids derived from pGEMs were extracted from agarose gel by the Rapid Gel Extraction Concentration kit (Invitrogen) and were individually ligated to the digested pET-15b expression vector. The resulting pET15b, pET15b/C61S, and pET15b/C125S plasmids were transformed into competent E. coli BL21(DE3) in an Applied Biosystems ABI Prism 377 96 to 100-μl reaction mixtures. No peroxide consumption was detected in the absence of thiols. H2O2 concentration in stock solutions was checked by its absorbance (ε240 nm = 43.6 M-1 cm-1).

Determination of Sulphydryl Groups Concentration—The amount of thiol groups remaining in solution was determined by the Ellman’s reagent (DTNB), using the ε412 nm = 13,600 M-1 cm-1 for 2-nitro-5-thiobenzoic acid (TNB) (19). As described above, reactions were stopped at different intervals by addition of 20 μl of HCl (1 M) into 100-μl reaction mixtures. Samples were neutralized by dilution (1:10) in a solution containing Hepes (1 x, pH 7.4) and DTNB (5 mM). Absorbance at 412 nm was immediately recorded.

Glutamine Synthetase Protection Assay—Antioxidant activities of Ohr, OhrC61S, OhrC125S, and cTPx were measured by their ability to protect glutamine synthetase from oxidative inactivation. Several H2O2-removing enzymes such as GSH peroxidase, catalase, and cTPx were used as positive controls (20). The procedure used to determine glutamine synthetase activity was the same described by Kim et al. (20).

Ohr Inactivation by NEM Treatment—Recombinant Ohr was digested with thrombin (0.01 units/μl) using the thrombin cleavage capture kit from Promega. The digestion was carried out for 16 h at 25 °C. The concentration of the recombinant protein without histidine tag was determined spectrophotometrically, using the same extinction coefficient ε280 nm = 3960 M-1 cm-1 determined above because the histidine tag does not contain optically active residues.

Sulfenic Acid Formation—Determination of sulfenic acid (R-SOH) in wild-type as well as in mutant proteins was performed by the TNB anion method described by Ellis and Poole (21). In summary, TNB was prepared by incubation of an almost equimolar mixture of DTNB and DTT (1 DTNB:0.9 SH). Proteins preincubated or not with peroxides were treated with a 20-fold excess of TNB. As described before (21), TNB reacts with sulfenic acids in a 1:1 stoichiometry, generating a molar extinction coefficient between TNB and a cysteine residue. Excess of TNB was removed by PD-10 desalting column (Amersham Biosciences). Mixed disulfides were then treated with 10-fold excess of DTT, and the amount TNB released (which was equal to the amount sulfenic acid formed) was determined spectrophotometrically.

RESULTS

Genetic and Biochemical Analysis of Ohr—Ohr from X. fastidiosa possesses a very high degree of similarity with proteins from various bacteria such as X. campesiris pv. phaseoli and P. aeruginosa (14). In general, the ohr gene is present in a single copy, but in some cases such as B. subtilis, Menozobium loti, and Ralstonia solanacearum two copies of ohr are present (12, 22, 23). In Streptomyces coelicolor, three copies of ohr appear to be present (24). A blastp analysis on the X. fastidiosa genome using the tools available at the site aeg.lib.ic.unicamp.br/Idf/ detected only one copy of ohr in this bacteria located between coordinates 1,742,868 and 1,743,299 with 432 nucleotides. The predicted amino acid sequence possesses 143 residues and a molecular mass equivalent to 14.9 kDa. Among other characteristics Ohr proteins have two conserved cysteine residues that are at positions 61 and 125 in the homologue from X. fastidiosa. Based on sequence homology two domains of Ohr can be defined: domain 1, which contains cysteine 61 and a high number of hydrophobic residues, and domain 2, which has cysteine 125 in a hydrophilic environment (Fig. 1B). To investigate whether Ohr possesses thiol-dependent peroxidase activity, ohr gene from X. fastidiosa was expressed in
A. The sequences represented in this scheme were the consensus obtained from the Clustal W method from the MegAlign 5.01 software (DNAstar Inc.) including the Ohr proteins from: Deinococcus radiodurans (Ohr Dr = GI:15808857); Caulobacter crescentus (Ohr Cc GI: 13422184); Mesorhizobium loti (Ohr Ml = GI:14024371); Ohr M2 = GI:13473574); Vibrio cholerae (Ohr Vc = GI:15601759); Mycoplasma genitalium (Ohr Mg = GI:1723186); Mycoplasma pneumoniae (Ohr Mp = GI:13508407); B. subtilis (Ohr Bc1or Ykna = GI:16078381, Ohr Bc2 = GI:16078379); Staphylococcus aureus ssp. aureus Mu50 (Ohr Sa = GI:15923818); Lactococcus lactis ssp. lactis (Ohr L1 = GI:15672574); Listeria monocytogenes EGD-e (Ohr L1 = GI:16804238); Listeria innocua (Ohr Li = GI:16801366); S. coelicolor (Ohr Sc1 = GI:6562797, Ohr Sc2 = GI:7546676, Ohr Sc3 = GI:9885209); Agrobacterium tumefaciens (Ohr At GI:15888188); Sinorhizobium meliloti (Ohr Sm1 = GI:16283744, Ohr Sm2 = GI:15964715); Bradyrhizobium japonicum (Ohr Bj = GI:8709920); Acinetobacter calcoaceticus (Ohr Ac = GI:7531260); Pseudomonas aeruginosa (Ohr Pa = GI:15598046); Ralstonia solanacearum (Ohr Rs1 = GI:17549328, Ohr Rs2 = GI:17548547); Xanthomonas campestris pv. phaseoli (Ohr Xc = GI:7531169); Xylella fastidiosa (Ohr Xf = GI:15838425). The residues that match the consensus. The histogram shows the strength of the residues belonging to the two domains. B, hydrophilicity plot is displayed in the y-axis, whereas hydrophilicity values are displayed in the y-axis. According to this method, hydrophilic residues have positive values, whereas hydrophobic residues have negative values. The arrows show the positions of cysteine in Ohr sequence.

E. coli and purified by nickel affinity chromatography (see “Materials and Methods”). A histidine tag recombinant Ohr was obtained with high degree of purity as ascertained by SDS-PAGE (Fig. 2). Two bands corresponding to Ohr were observed, both of which migrate closely to 17 kDa, as expected for a monomer (Fig. 2A). Removal of histidine tag by thrombin proteolysis also resulted in two bands both of which migrated at ~15 kDa (data not shown). The abundance of each was dependent on the oxidative condition to which Ohr was exposed (Fig. 2) and was independent of the presence of the histidine tag (data not shown). Bands corresponding to dimers did not appear even after treatments of Ohr with H2O2 or organic hydroperoxide at various concentrations (Fig. 2B) indicating that no disulfide bond was formed between two Ohr molecules. Since the two bands observed in Fig. 2 have approximately the same monomer size, they should represent different configurations of Ohr monomers. The lower band (band a) should correspond to an oxidized state of Ohr, which is generated after H2O2 treatment or at mild concentrations of t-b-OOH (Fig. 2B). On the other side, the upper band (band b) may represent a mixture of reduced and oxidized states of Ohr. This is because Ohr treatment with increasing concentrations of DTT provoked the oxidized state of Ohr (Fig. 2A) as well as Ohr treatment with high organic peroxide concentrations (Fig. 2B). The meanings of these two bands are further discussed below, after the description of results with Ohr site-specific mutants. In any case, it is important to emphasize that different monomeric configurations were also observed before for cTPxI, a thiol-dependent peroxidase from Saccharomyces cerevisiae (27).

Thiol-dependent Peroxidase Activity of Ohr—Recombinant Ohr decomposed peroxides only if DTT was also present in the reaction mixture (Fig. 3). Because the substrates were present in a 350–4100-excess over the protein, it is reasonable to think that Ohr was acting catalytically. In the conditions described in Fig. 3, the specific activity of Ohr was 20.0, 17.0, and 1.3 μM/min/ng when, respectively, t-b-OOH, Cu-OOH, and H2O2 were considered the substrates. This indicates that Ohr was 10–20 times more efficient in the removal of organic peroxides than in the removal of H2O2. These results are consistent with the high susceptibility of ohr mutants to organic peroxides but not to H2O2 (Ref. 14 and references cited herein). In any case, Ohr did decompose H2O2 when DTT was present in the

FIG. 1. Domains of Ohr proteins that contain cysteine residues. A, the sequences represented in this scheme were the consensus obtained by the Clustal W method from the MegAlign 5.01 software (DNAstar Inc.) including the Ohr proteins from: Deinococcus radiodurans (Ohr Dr = GI:15808857); Caulobacter crescentus (Ohr Cc GI: 13422184); Mesorhizobium loti (Ohr Ml = GI:14024371); Ohr M2 = GI:13473574); Vibrio cholerae (Ohr Vc = GI:15601759); Mycoplasma genitalium (Ohr Mg = GI:1723186); Mycoplasma pneumoniae (Ohr Mp = GI:13508407); B. subtilis (Ohr Bc1or Ykna = GI:16078381, Ohr Bc2 = GI:16078379); Staphylococcus aureus ssp. aureus Mu50 (Ohr Sa = GI:15923818); Lactococcus lactis ssp. lactis (Ohr L1 = GI:15672574); Listeria monocytogenes EGD-e (Ohr L1 = GI:16804238); Listeria innocua (Ohr Li = GI:16801366); S. coelicolor (Ohr Sc1 = GI:6562797, Ohr Sc2 = GI:7546676, Ohr Sc3 = GI:9885209); Agrobacterium tumefaciens (Ohr At GI:15888188); Sinorhizobium meliloti (Ohr Sm1 = GI:16283744, Ohr Sm2 = GI:15964715); Bradyrhizobium japonicum (Ohr Bj = GI:8709920); Acinetobacter calcoaceticus (Ohr Ac = GI:7531260); Pseudomonas aeruginosa (Ohr Pa = GI:15598046); Ralstonia solanacearum (Ohr Rs1 = GI:17549328, Ohr Rs2 = GI:17548547); Xanthomonas campestris pv. phaseoli (Ohr Xc = GI:7531169); Xylella fastidiosa (Ohr Xf = GI:15838425). The letters in blue represent Ohr treated with the following concentrations of DTT: 0 μM, 0.5 μM, 1 μM, and 10 μM, respectively. A, lane 1, molecular mass standard (Benchmark™ Protein Ladder, Invitrogen); lane 2, untreated Ohr; lanes 3–6 represent Ohr treated with the following concentrations of DTT: 0 μM, 1 μM, 10 μM, and 100 μM, respectively. B, lane 1, molecular mass standard (Benchmark™ Protein Ladder, Invitrogen); lanes 2–5 represent Ohr treated with the following concentrations of H2O2: 60 μM, 0.5 mM, 1 mM, and 10 mM, respectively; lanes 6–9 represent Ohr treated with the following concentrations of t-b-OOH: 60 μM, 0.5 mM, 1 mM, and 10 mM, respectively. Letters a and b denote the lower and upper band, respectively.

FIG. 2. SDS-PAGE analysis of purified recombinant Ohr from X. fastidiosa. Recombinant Ohr was expressed in E. coli and purified as described under “Material and Methods.” Treatments were carried out for 1 h at room temperature. A, lane 1, molecular mass standard (Benchmark™ Protein Ladder, Invitrogen); lane 2, untreated Ohr; lanes 3–6 represent Ohr treated with the following concentrations of DTT: 0 μM, 1 μM, 10 μM, and 100 μM, respectively. B, lane 1, molecular mass standard (Benchmark™ Protein Ladder, Invitrogen); lanes 2–5 represent Ohr treated with the following concentrations of H2O2: 60 μM, 0.5 mM, 1 mM, and 10 mM, respectively; lanes 6–9 represent Ohr treated with the following concentrations of t-b-OOH: 60 μM, 0.5 mM, 1 mM, and 10 mM, respectively. Letters a and b denote the lower and upper band, respectively.
reaction mixture. This ability of Ohr to decompose peroxides was dependent on the integrity of its cysteine residues because pretreatment of Ohr with NEM inhibited its peroxidase activity (Fig. 3C). Therefore, it is reasonable to think that Cys-61, Cys-125, or both are directly involved in the decomposition of peroxides by Ohr.

Ohr also possesses antioxidant property as demonstrated by its capacity to protect glutamine synthetase from inactivation by thiol-containing oxidative system composed of DTT/Fe³⁺/H₂O₂ or composed of DHLA/Fe³⁺/H₂O₂ (Fig. 4A). Probably, this protection was due to the thiol-dependent peroxidase activity of Ohr because other thiol-dependent peroxidase such as GSH peroxidase and cTPxI also protect glutamine synthetase from inactivation (20). In fact, the glutamine synthetase protection assay has been used to investigate whether proteins possess peroxide-removing activity (28, 29). Ohr did not protect glutamine synthetase against the ascorbate/Fe³⁺/H₂O₂ system, indicating that this protein is a thiol-specific antioxidant protein (Fig. 4A).

The protective activity of Ohr was dependent on the concentration of protein and is comparable to the cTPxI activity (Fig. 4B). Removal of histidine tag by thrombin treatment did not alter the enzymatic characteristics of Ohr nor its ability to protect glutamine synthetase (data not shown).

When H₂O₂ was used as substrate, the specific activity of Ohr (Fig. 3) was similar to cTPxI (data not shown). These results are consistent with the glutamine synthetase assays (Fig. 4B) where these two proteins were also equally protective. The oxidative inactivation of glutamine synthetase is dependent on H₂O₂ formation by metal catalyzed oxidation systems

**Fig. 3. Thiol-dependent peroxidase activity of Ohr.** Peroxide concentration was determined at different periods by FOX assay as described under “Material and Methods.” The reactions were carried out in Hepes buffer 50 mM, pH 7.4, in the presence of azide (1 mM) and DTPA (0.1 mM). The concentration of peroxides at time zero was 500 μM. Reactions were initiated by the addition of DTT (0.5 mM). A represents the kinetics of H₂O₂ decomposition in the presence of Ohr (100 ng/μl). B and C represent the kinetics of Cu-OOH and t-BOOH decomposition, respectively, in the presence of Ohr (10 ng/μl). ● represents the reaction mixture without Ohr (DTT + peroxide); ▲ represents the full system (DTT + peroxide + Ohr). Reaction mixtures without DTT did not show any decomposition of peroxides even in the presence of Ohr. The symbol ■ in C represents Ohr whose cysteine residues were previously alkylated with NEM.

**Fig. 4. Ohr protected glutamine synthetase from oxidative inactivation.** Glutamine synthetase (GS) protection assay was performed as described under “Material and Methods.” All reaction mixtures contain: Fe³⁺ = 1 μM; glutamine synthetase 1 mg/ml; azide = 1 mM in Hepes buffer 50 mM, pH 7.4. A, the symbols represent: ● (DTT 10 mM addition); ▲ (DTT 10 mM + Ohr 100 ng/μl addition); ◀ (ascorbate 10 mM addition); ■ (ascorbate 10 mM + Ohr 100 ng/μl addition); △ (DHLA 10 mM addition); ○ (DHLA 10 mM + Ohr 100 ng/μl addition). B, reactions were carried out for 15 min. The symbols represent: ▲ = Ohr and ■ = cTPxI at the concentrations described in the x-axis.
and by its posterior conversion to hydroxyl radical through the Fenton reaction (30). Therefore protection of glutamine synthetase from inactivation probably occurs through the removal of \( \text{H}_2\text{O}_2 \) by antioxidant enzymes (31).

Analysis of Cysteine Replacements on Ohr Activity—The role of Ohr cysteines in peroxide reduction was strongly suggested since treatment of Ohr with NEM lead to protein inactivation (Fig. 3). To specifically investigate the roles of Cys-61 and Cys-125 on Ohr catalysis these two residues were individually replaced by serine generating, respectively, C61S and C125S. Initially, the capacity of the mutant proteins to decompose hydroperoxides was investigated. C61S and C125S had no detectable peroxidase activity when \( \text{t-bOOH} \) was used as a substrate (Fig. 5A). When \( \text{H}_2\text{O}_2 \) was the substrate, C125S had a residual activity, whereas C61S did not decompose any peroxide (Fig. 5B). These results indicated that both cysteine residues are important for catalysis. Replacement of either Cys-61 or Cys-125 also provoked great decreases in the ability of Ohr to protect glutamine synthetase from oxidative inactivation (Fig. 5C). C61S only showed some protective effect at very high doses, which might be attributable to nonspecific activity. Interestingly, replacement of two of the cysteines of human peroxiredoxin V (prx V), which forms a stable intramolecular disulfide intermediate during its catalytic cycle, produced similar effects (32). Therefore, the results described in Fig. 5 represented an initial suggestion that an intramolecular disulfide is also a reaction intermediate of wild-type Ohr. Further evidences are presented below.

The migration of Ohr mutants was also analyzed by SDS-PAGE (Fig. 6) under both reducing and non-reducing conditions to understand the meaning of the two monomeric bands observed in Fig. 2. As standards for mutant proteins, wild-type Ohr was treated with DTT (100 mM), \( \text{t-bOOH} \) (60 \( \mu \text{M} \)) and \( \text{H}_2\text{O}_2 \) (60 \( \mu \text{M} \)), respectively; lanes 5–7 represent C125S treated with DTT (100 mM), \( \text{t-bOOH} \) (60 \( \mu \text{M} \)), and \( \text{H}_2\text{O}_2 \) (60 \( \mu \text{M} \)), respectively; lane 1, molecular mass standard (BenchMark™ Protein Ladder, Invitrogen); lanes 2–4 represent wild-type Ohr treated with DTT (100 mM), \( \text{t-bOOH} \) (60 \( \mu \text{M} \)) and \( \text{H}_2\text{O}_2 \) (60 \( \mu \text{M} \)), respectively; lanes 5–7 represent C125S treated with DTT (100 mM), \( \text{t-bOOH} \) (60 \( \mu \text{M} \)), and \( \text{H}_2\text{O}_2 \) (60 \( \mu \text{M} \)), respectively. Letters a, b, c, and d refer to the bands described under “Results.”
substitution of band c after C125S treatment with organic peroxides (data not shown). The meaning of band c is unknown and could be attributed to proteolysis or to generation of cross-links in the dimer.

Since band d should correspond to a dimer of two C125S proteins bound through their Cys-61, it appears that this sulf-hydryl group is relatively reactive toward peroxides. As expected for a dimer exposed to reducing condition, when C125S was treated with DTT in denaturing conditions, only a monomeric band (band b) was detected (Fig. 6A, lane 5).

On the contrary, C61S migrated preferentially as a monomer (Fig. 6B). Only after treatment of cells with very high peroxide concentrations could a dimer be observed (data not shown), indicating that Cys-125 is not very oxidizable by peroxides.

The possible formation of stable sulfenic acid intermediates (R-SOH) in Ohr, C125S, and C61S was also analyzed. Using the compound TNB, we could only clearly detect sulfenic acid intermediates in C125S protein (Fig. 7). This result further indicated that Cys-61 but not Cys-125 is very reactive.

Thiol Substrate Specificity—The possibility that other thiol compounds besides DTT support the peroxidase activity of Ohr was also analyzed. No decomposition of t-hydroperoxide by Ohr was detected when DTT was replaced by monothiols such as GSH, 2-mercaptoethanol (Fig. 8A), and cysteine (data not shown). It is important to note that even when GSH was added at a concentration 10-fold higher than DTT no peroxidase activity could be observed (Fig. 8A). Therefore, Ohr does not possess GSH peroxidase activity.

To check if thioredoxin could be the biological substrate for Ohr, thioredoxin was added to the reaction mixture containing DTT, t-hydroperoxide, and Ohr. In the case of cTPxI, addition of thioredoxin to the reaction mixture increased the specific activity of this protein (28). The addition of thioredoxin from Spirulina or from S. cerevisiae did not increase significantly the ability of Ohr to decompose t-hydroperoxide, taking into account the decomposition of peroxides by thioredoxin itself (Fig. 8B). The peroxidase activity of Ohr may be specific for thioredoxin from X. fastidiosa. Alternatively, other thiol compound than thioredoxin can be the reducing agent of Ohr.

Interestingly, Ohr was also capable of decomposing peroxides (Fig. 8A) and protecting glutamine synthetase from inactivation (Fig. 4A) if DHLA was present in the reaction mixture. Like DTT, DHLA is also a dithiol compound. Enzymes required for DHLA biosynthesis are present in X. fastidiosa (XF1289, XF1270) in an operon configuration indicating that this thiol compound should be present in this bacteria. The possibility that DHLA is the in vivo reducing power of Ohr is discussed below.

Because the ability of Ohr to decompose peroxides is dependent on the presence of DTT, the stoichiometry of the reaction catalyzed by this protein was investigated as described before for cTPxI (31). The data described in Table I indicated that the ratio of thiol consumption per peroxide consumption is around 2, which is consistent with the same reaction catalyzed by proteins belonging to the peroxiredoxin family: 2RSSR + ROOH → RSSR + ROH + H2O. Therefore, Ohr is a thiol-dependent peroxidase.

**DISCUSSION**

The present report attribute for the first time a biochemical function for a protein belonging to the Ohr/OsmC family. Taken together, our results demonstrate unequivocally that Ohr from X. fastidiosa possesses thiol-dependent peroxidase activity. This biochemical activity is consistent with the increased sensitivity to organic peroxides observed for several bacterial species in which this gene is deleted (10–13, 15).

Ohr possesses a very high specific activity for organic peroxides in comparison with peroxiredoxins. In our hands, the specific activity of Ohr is approximately 10–20 times higher than the specific activity of cTPxI when organic peroxides were used as substrates (data not shown). AhpC is the other thiol-dependent peroxidase present in X. fastidiosa and in several other bacteria. AhpC belongs to the peroxiredoxin family like cTPxI, and therefore they are expected to behave similarly. Results showing that mutation of ohr renders cells more sen-
sitive to organic peroxide killing than ahpC deletion (11, 12) lead us to speculate that this probably occurs because Ohr has higher specific activity toward organic peroxides than peroxiredoxins, but this suggestion awaits experimental confirmation.

The relationship between Ohr and AhpCF proteins has been studied in other bacteria. AhpC acts in concert with AhpF (a thioredoxin reductase homologue) to reduce peroxides to the corresponding alcohols at the expense of NADH (9). It is well known that the expression of ahpC and ahpF are regulated by OxyR, a transcriptional regulator that is activated by H₂O₂ (33). This should also occur in X. fastidiosa because ahpC, ahpF, and oxyR genes are contiguous and therefore probably belong to the same operon (aeg.lbi.ic.unicamp.br/xf/). On the other hand, the expression of ohr genes in B. subtilis and X. campestris is not regulated by OxyR but by OhrR (12, 34). OhrR is a member of the MarR family of transcriptional repressors. No OhrR homologue was found in a search through the site of X. fastidiosa genome suggesting that ohr is regulated by a different mechanism in this microorganism.

X. fastidiosa contains several peroxide-removing enzymes as analyzed by the bioinformatic tools available at aeg.lbi.ic.unicamp.br/xf/and using the sequencing data generated by the genome project supported by FAPESP (16). Besides ahpC (XF1530), two other genes codify for proteins that contain AhpC/TSA domains as defined by the pFAM analysis. Additionally, one catalase and one GSH peroxidase homologue are also present. Each one of these peroxide-removing enzymes may utilize different substrates or may act during specific stress conditions.

Ohr is also capable of decomposing H₂O₂ although with a lower efficiency compared with the removal of organic peroxides (Fig. 3). Probably Ohr does not play an important role in the defense of X. fastidiosa against this oxidant. In other related bacteria, ohr mutants are not hypersensitive to H₂O₂ (10, 11, 15). Moreover, in X. campestris pv. phaseoli and in B. subtilis ohr expression is not regulated by OxyR, which is activated by H₂O₂ (12, 34). Catalases appear to be the primary defense of Xanthomonas against exogenous H₂O₂ (35, 36). X. fastidiosa possesses at least one catalase (XF2232), similar to HPI, (katG) from E. coli, which is OxyR-regulated (33) and should be a key component of the antioxidant defense against exogenous H₂O₂. On the other side, several studies indicate that AhpR should be an important component in the removal of H₂O₂ endogenously generated in bacteria (37–39).

The reduction of peroxides by Ohr requires at least one of its cysteine residues because NEM pretreatment abolishes its per-oxidase activity (Fig. 3C). Ohr has two cysteine residues that are present in all of its homologues (Fig. 1A) and therefore potentially could be involved in peroxide reduction. Substitution of Cys-61 of Cys-125 by serine dramatically reduces the ability of Ohr to decompose peroxides (Fig. 5), indicating that both cysteine residues are important for the catalytic activity. However, it is important to note that C125S, but not C61S, still has a residual peroxidase activity, suggesting an essential role for Cys-61. In fact, Cys-61, but not Cys 125, was easily oxidized by any of the peroxides (Fig. 6), and it appears that Cys-61 is directly involved on peroxide reduction, whereas Cys-125 is the resolving cysteine. Our data fit very well in the scheme described in Fig. 9. At low concentrations of organic peroxides, Cys-61 could be oxidized to sulfenic acid, which should be rapidly converted to the intramolecular disulfide intermediate (electrophoretic band a). In support with this model, we could only detect sulfenic acid intermediate in C125S protein (Fig. 7). Cys-61-SOH should be more stable in C125S than in wild-type Ohr because the mutant protein lacks Cys-125 to react with Cys-61-SOH. At high levels of organic peroxides, sulfenic acid of Cys-61 should react first with another peroxide molecule and with Cys-125 sulfhydryl group, leading to the formation of a cysteine sulfonic acid (R-SO₂H). Further reaction of Cys-61-SO₂H with another organic peroxide molecule can provoke the formation of Cys-61 sulfonyl acid (R-SO₃H). Both Cys-61-SO₂H and Cys-61-SO₃H should correspond to the electrophoretic band b observed in Fig. 2 when Ohr was exposed to high concentrations of organic peroxides.

Disulfide intermediates are stable compounds among other factors because they can not be overoxidized as sulfenic acids can be (40). Therefore, it is tempting to speculate that Cys-125 prevents Ohr inactivation by avoiding Cys-61 overoxidation to sulfinic or sulfonic acids. It is well described that overoxidation of peroxiredoxin provoked their inactivation (41). In the case of the mutant protein C125S, in addition to overoxidation of Cys-61, dimer formation (Fig. 6A) could represent another pathway of protein oxidation.
Bond b was never observed when Ohr was treated with H$_2$O$_2$ (Fig. 2), indicating that this peroxide has lower capacity than organic peroxides to oxidize Cys-61 to sulfenic or sulfonyl acids. In fact, H$_2$O$_2$ had lower ability than organic peroxides to induce dimer formation in the mutant protein C125S (data not shown). Cys-61 is located in a very hydrophobic environment (Fig. 1D), which is probably ideal to accommodate an organic peroxide but not H$_2$O$_2$. This hypothesis would explain the higher specific activity of Ohr toward organic peroxides in comparison with H$_2$O$_2$ (Fig. 3).

The biological-reducing substrate of Ohr is still unknown. GSH should be present in X. fastidiosa because this bacteria contains homologues for the two genes (gsh1 and gsh2) involved in its biosynthesis (aeg bụi.unicamp.br/xfl). However, this thiol was not capable of reducing peroxides in the presence of Ohr, even when it was present in a concentration ten times higher than DTT concentration (Fig. 8A). Thioredoxin from either Spirulina (data not shown) nor from S. cerevisiae (Fig. 8B) increased the rate of peroxide removal by Ohr. We can not exclude, however, the possibility that thioredoxin or glutaredoxin systems of X. fastidiosa specifically reduces Ohr.

In addition to DTT, DHLA supported the peroxidase activity of Ohr (Figs. 4A and 8A). Therefore, Ohr utilized only dithiols, but not monothiols, as substrate. Dithiols such as DTT and DHLA have very negative redox potentials, which indicate that these compounds have very high reducing power. The redox potentials for dithiols are in the range from $-0.31$ to $-0.33$, whereas for monothiols such as GSH and cysteine the redox potentials are in the range of $-0.24$ to $-0.25$ (42). Probably the intramolecular disulfide bond of Ohr is very stable, and only very strong reducing agents are able to convert them to the reduced form. Another possibility is related to possible structural constrains of the Ohr active site. According to our results, the active site of this protein is very hydrophobic and may not be capable of accommodating two monothiols but can interact with only one dithiol molecule. This is because in the case of dithiols only one molecule would be enough to reduce Ohr back to the dihthiol configuration according to Reaction 1.

\[
\text{Ohr}(-SS)+R-SH\rightarrow \text{Ohr}(-[Cys-61])SSR-SH+\text{Ohr}(-SH)+RSSR
\]

**REACTION 1**

In the case of monothiols, two molecules would be required to fully reduce Ohr. First, a mixed disulfide between Ohr and the reducing substrate is formed (Reaction 2), which would then be reduced to the Ohr dihthiol configuration by another monothiol molecule and the release of a disulfide compound (Reaction 3).

\[
\text{Ohr}-SS+R-SH\rightarrow \text{Ohr}-SS
\]

**REACTION 2**

\[
\text{Ohr}-SS+R-SH\rightarrow \text{Ohr}-SH+RSSR
\]

**REACTION 3**

The possibility that DHLA is the biological substrate of Ohr is supported by the fact that its biosynthetic pathway is present in X. fastidiosa (XF 1269, XF1270). Interestingly, Bryk et al. (43) characterized a peroxidase system dependent on lipoic acid in Mycobacterium tuberculosis. Bryk et al. (43) demonstrated that lipoic acid utilized came from a thiol linked through an amide linkage dihydrolipoamide succinyltransferase, a component of $\alpha$-keto acid oxidases, and was reduced by NADH in a reaction catalyzed by dihydrolipoamide dehydrogenase. Both dihydrolipoamide succinyltransferase (XF1549) and dihydrolipoamide dehydrogenase (XF1548) enzymes are also present in X. fastidiosa (aeg.ılıc.unicamp.br/xfl). Several reducing systems from X. fastidiosa are in the process to be expressed to find the reducing substrate of Ohr. In any case, our data suggest that Ohr may be a dihydrolipoic acid peroxidase.

Contrary to peroxiredoxins, GSH peroxidase, and catalases, Ohr belongs to a family of proteins that are present only in bacteria, most of them pathogenic to plants or mammals. Thus Ohr may be promising as a target for drug development in agriculture and medicine, considering the fact that plant and animal defenses against pathogens involves oxidative burst (1).
Ohr Is a Thiol-dependent Peroxidase

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