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The unique glutathione reductase from *Xanthomonas campestris*: Gene expression and enzyme characterization ☆

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Abstract

The glutathione reductase gene, *gor*, was cloned from the plant pathogen *Xanthomonas campestris* pv. *phaseoli.* Its gene expression and enzyme characteristics were found to be different from those of previously studied homologues. Northern blot hybridization, promoter-*lacZ* fusion, and enzyme assay experiments revealed that its expression, unlike in *Escherichia coli*, is OxyR-independent and constitutive upon oxidative stress conditions. The deduced amino acid sequence shows a unique NADPH binding motif where the most highly conserved arginine residue, which is critical for NADPH binding, is replaced by glutamine. Interestingly, a search of the available Gor amino acid sequences from various sources, including other *Xanthomonas* species, revealed that this replacement is specific to the genus *Xanthomonas*. Recombinant Gor enzyme was purified and characterized, and was found to have a novel ability to use both, NADPH and NADH, as electron donor. A *gor* knockout mutant was constructed and shown to have increased expression of the organic peroxide-inducible regulator gene, *ohrR*.

Keywords: Glutathione reductase; NADPH binding motif; Organic hydroperoxide-responsive promoter; Oxidative stress

Glutathione is a major cellular free thiol-containing compound that is present in animals, plants, fungi, and a large number of prokaryotic species. Glutathione is synthesized by glutathione synthetase and functions as an important cellular antioxidant that can react with a variety of compounds containing electrophilic centers. Apart from its function as an antioxidant, glutathione is also responsible for the maintenance of the intracellular thiol redox status and thus contributes to the function of many biological processes within the cell [1,2]. For most of its functions glutathione must be in the reduced form. Glutathione reductase (Gor) is the enzyme that reduces the oxidized form of glutathione, glutathione disulfide (GSSG), to reduced glutathione (GSH). In *Escherichia coli*, high steady-state levels of glutathione maintain a strong reducing environment in the cell [3]. Glutathione can react with H_2O_2, O_2^-, or $HOO^\cdot$ to form stable glutathione radicals that will then dimerize to form glutathione disulfide. Finally, glutathione reductase can then transfer an electron to glutathione disulfide, to re-form reduced glutathione [4]. Typically, reduction of GSSG to GSH is catalyzed by Gor, which in most cases exhibits a marked preference for NADPH over NADH as the electron donor. One of the most important functions of glutathione is to reduce disulfide bridges in proteins caused by oxidative stress. Although formation of the disulfide bonds is easily reversible, their presence can drastically alter protein function.

Glutathione reductase is a member of an important class of flavoprotein enzymes, the disulfide oxidoreductases, containing two active-site electron acceptors: FAD and a redox-active disulfide. The other members of this class include lipoamide dehydrogenase [5],

☆ *Abbreviations:* *t*-BOOH, *tert*-butyl hydroperoxide; NEM, *N*-ethylmaleimide.

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mercury reductase [6], trypanothione reductase [7], and thioredoxin reductase [8]. These proteins share extensive amino acid sequence similarities, in particular, sequences surrounding the redox-active cysteine residues, implying that they have arisen by divergent evolution from a common ancestor [9]. Glutathione reductase is the most important enzyme in maintaining a high intracellular ratio of reduced:oxidized glutathione (approximately 500:1) [10]. Gor is involved in redox cycles that are important in maintaining the anti-oxidative capacity of cells engaged in a wide variety of functions in which reactive oxygen species may be produced and is considered to be a key enzyme involved in maintaining the redox status of the cell during oxidative stress.

Xanthomonas belongs to an important family of plant bacterial pathogens. The bacterial enzymes and genes involved in the oxidative stress response and in regulating cellular redox status are likely to play important roles in disease development. Therefore, study of the glutathione reductase in this phytopathogen would certainly yield crucial information relating to pathogenesis and how *Xanthomonas* adapts to the host plant environment during infection.

This work reports that *Xanthomonas campestris* glutathione reductase has an atypical NADPH binding motif, in which the most highly conserved arginine residue, that is critical for NADPH binding, is replaced by glutamine. This unique change is specific only to Gor from the *Xanthomonas* genus. Furthermore, recombinant *Xanthomonas* Gor was found to have the ability to utilize both NADPH and NADH as electron donors. A *gor*-disrupted *Xanthomonas* mutant displayed increased expression of the organic peroxide-responsive regulator gene (*ohrR*).

Materials and methods

Bacterial cultures and media. Xanthomonas campestris pv. *phaseoli* was grown aerobically at 28 °C in SB medium as previously described [11,12]. All *E. coli* strains were grown aerobically in Luria–Bertani (LB) broth at 37 °C.

Nucleic acid extraction and analysis, cloning, and nucleotide sequencing. Genomic DNA extraction from *X. campestris* was performed according to the method of Mongkolsuk et al. [13]. Total RNA was isolated by hot-phenol method [13]. Molecular cloning, gel electrophoresis, and nucleic acid hybridizations were performed as previously described [14]. Nucleotide sequences were determined using an automated sequencer, model 310 (Applied Biosystems). *E. coli* and *Xanthomonas* were genetically transformed by a chemical method [14] and by electroporation [13], respectively.

In vitro transcription–translation analysis. Plasmid pGR1800 was used as a template for the expression of cloned gene products using a coupled in vitro transcription–translation *E. coli* S-30 extract system (Promega). A ^{14}C-methylated protein molecular weight standard (Amersham) was used as a standard marker.

Construction of chromosomal gor promoter::cat transcriptional fusion strains. The 540-bp *gor* promoter fragment was generated by PCR amplification using pZL-G1 as the template and primers corresponding to the 5′ region starting 340-bp upstream of the translation start site (5′ CGCGAGCGCCTGCGCATCGG 3′) and 3′ region (5′ CGCT GGCCAACTCGATCTTGC 3′). A *Bam*HI–*Hin*cII *gor* promoter fragment was ligated into *Bam*HI–*Eco*ICRI digested pUC18*Sfi*I *cat*, subsequently the *Sfi*I fragment containing the *gor* promoter and *cat* reporter was excised and ligated into the minitransposon pUT-Tn5 [15] to create pUT-Pgor which was then conjugally transferred into *Xanthomonas* and a stable kanamycin-resistant transconjugant was selected and named *X. campestris* strain TnPgor.

Amplification and sequencing of the conserved region of Gor in Xanthomonas species. Two oligonucleotide primers (5′ CACATCGT GATCGCCACCGG 3′ and 5′ GCCGCAATCGCCACCGGTGT 3′) corresponding to the conserved amino acid regions HIVIATG and TPVAIAA were synthesized and used to PCR amplify *gor* gene-internal fragments from *Xanthomonas vessicatoria*, *Xanthomonas translucens*, and *Xanthomonas hyacinthi* chromosomal DNA. The 560-bp fragments were cloned into pDrive (Qiagen) and their DNA sequence was determined.

High-level production and purification of Gor. High levels of *gor* expression for Gor purification were achieved using a His-tagged gene fusion expression vector system (Qiagen) in *E. coli*. Oligonucleotide primers corresponding to the 5′ (5′ CGGCATGCATGAGT GCGCGTTA 3′) and 3′ (5′ CGAAGCTTCGCAACCAACCAT 3′) non-coding regions of the *Xanthomonas gor* locus were used to amplify *gor* from pZL-G1. The resulting 1400-bp PCR product was then digested with *Sph*I and *Hin*dIII, gel purified, and cloned into pQE30 vector (Qiagen). A clone that expressed high levels of the fusion protein was obtained and named pQEG. A 200-ml culture of *E. coli* harboring pQEG was grown at 37 °C to an optical density at 600 nm of 0.6 and induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. The following purification steps were all done at 4 °C. The cells were subsequently pelleted, and the pellet was resuspended in sonication buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl). The suspension was then sonicated for a total of 10 min, with periodic cooling intervals. His-tagged Gor fusion protein was purified using nickle affinity columns according to the manufacturer's recommendations. The purified fusion protein was eluted with 100 mM imidazole in sonication buffer and the homogeneity of the eluted protein fractions was judged by SDS–PAGE. The eluted fractions containing the pure protein were pooled and dialyzed overnight against 20 mM Tris–HCl, pH 7.0, to remove imidazole.

Molecular weight determination of Gor. Protein concentration was measured by the dye binding method [16]. Determination of the molecular weight under denaturing conditions in the presence of SDS was performed as previously described [17]. For molecular weight determination under non-denaturing conditions, the addition of reducing agent (mercaptoethanol) to the protein sample and sample heating were omitted. The native molecular weight of recombinant His-tagged Gor was determined by gel filtration chromatography on a FPLC Akta Purifier (Pharmacia) using a Superdex 75 HR10/30 column (Pharmacia).

Gor enzyme assay. Gor activity was measured by monitoring the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) to thiobis(2-nitrobenzoic acid) by GSH which is produced by Gor according to a previously described method [18].

Disruption of gor gene. A *gor* insertion mutant was created by single recombination of plasmid, pBX170, into the chromosomal copy of *gor*. Specifically, a 1800-bp *Sph*I–*Hin*dIII fragment from pZL-G1 was gel purified and cloned into similarly digested pUC18 resulting in pGR1800. A 800-bp fragment was deleted from pGR1800 by digestion with *Bst*EII, *Hin*dIII, and gap-filled with Klenow polymerase, and ligated to form pBX1000. pBX1000 was further deleted by removing a 830-bp *Xba*I fragment followed by religation to form pBX170. Therefore, pBX170 contains a 170-bp gene-internal *gor* fragment in pUC18. Plasmid pBX170 was then electroporated into *X. campestris* and ampicillin resistant/*gor*-disrupted mutants were selected.

The correct integration of pBX170 into *gor* was verified by Southern blot hybridization (data not shown).

ohrR promoter assay. A previously described mini-Tn5 pP1lacZ construct, in which the *ohrR* promoter has been placed in front of a promoterless *lacZ* gene, was used as an indicator to measure the cellular redox status [19]. β-Galactosidase activity assays were carried out as previously reported [20].

Nucleotide sequence accession number. The nucleotide sequence of the *X. campestris* pv. *phaseoli gor* gene has been deposited in GenBank under Accession No. AY742859.

Results and discussion

Cloning of the X. campestris pv. phaseoli gor gene and its expression

Analysis of multiple amino acid sequence alignments of many Gor proteins revealed the presence of two conserved regions, VGCVPKK and GYIAVE [21], which were suitable for the application of reverse genetics and PCR gene cloning techniques. Degenerate oligonucleotide primers corresponding to the conserved regions were synthesized, taking into account the fact that *Xanthomonas* frequently uses G or C in the last position of codons. One primer corresponding to amino acid region VGCVPKK (5′ GTXGGXTGYGTXCCXAA ZAA 3′) and the second primer corresponding to amino acid region GYIAVE (5′ YTCXACXGCZATZTAXCC IXC 3′) (where X represents G and C, Y represents C and T, Z represents A and G, and I represents inosine) were used to amplify a 400-bp gene-internal portion of the *X. campestris* pv. *phaseoli gor* gene, which was cloned, sequenced, and used as a probe to screen an *X. campestris* pv. *phaseoli* genomic library constructed in a ZipLox vector (BRL Life Technology). A number of positively hybridizing clones were isolated, and plaques were purified. One positive clone, pZL-G1, was completely sequenced. Analysis of the nucleotide sequence revealed the presence of an open reading frame with a predicted amino acid sequence that shared high homology with Gor from a number of different sources. The *gor* gene was then subcloned into pGR1800, and in vitro transcribed and translated using the *E. coli* S-30 system (Promega). A 50-kDa protein band was detected (Fig. 1A) that corresponded to the calculated molecular mass of Gor verifying that the cloned *gor* could be in vitro translated to yield a full-length protein. Next, we examined the transcription pattern of *gor* in *X. campestris* using Northern blot hybridization experiments. The results, shown in Fig. 1B, revealed that *X. campestris* pv. *phaseoli gor* is transcribed as a 1.5-kb monocistronic mRNA. The level of *gor* mRNA was unaltered when cells were exposed to the oxidative stress inducing agents; diamide, paraquat, *N*-ethylmaleimide (NEM), cadmium, and nickel (Fig. 1B). *gor* promoter activity was also monitored in exponential phase cells of the *X. campestris* strain TnPgor, a strain that contains a

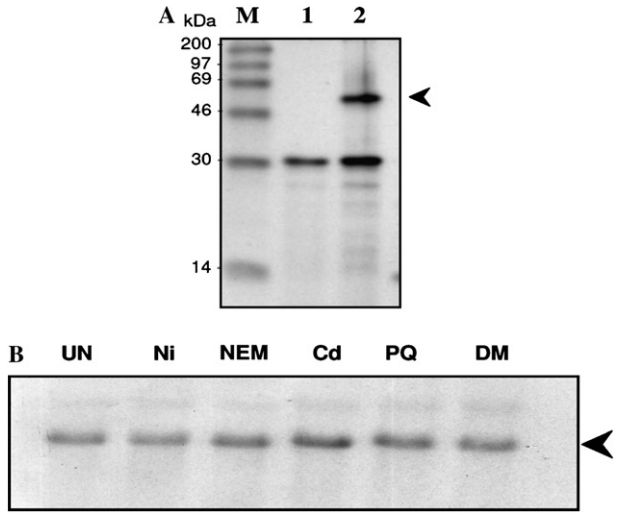

Fig. 1. In vitro translation products (A) and constitutive expression (B) of *gor*. (A) In vitro transcription-translation of pGR1800-encoded proteins with *E. coli* S-30 extracts. Lane M, protein molecular mass markers; lane 1, pUC18; and lane 2, pGR1800. The arrow indicates in vitro translation products of *gor*. The second band at around 30 kDa is the product of the ampicillin resistance gene. (B) Northern blot of total RNA isolated from *X. campestris* uninduced (UN) or induced with 0.2 mM Ni ($NiCl_2$), NEM (*N*-ethylmaleimide), Cd ($CdCl_2$), or PQ (paraquat), and 2 mM DM (diamide). The membrane was probed with a radioactively labeled *gor* DNA fragment. Ten micrograms of total RNA was loaded in each lane. The arrow indicates the 1.5-kb mRNA of *gor*.

chromosomal *gor::cat* transcriptional fusion, that had been exposed to 2 mM of either menadione, H_2O_2, *tert*-butyl hydroperoxide (*t*-BOOH), cumene, or paraquat for 30 min. Consistent with the mRNA analysis, no significant change in *gor* promoter activity was observed in the presence of any of the oxidants tested (data not shown). Moreover, exposure of cells to 10 μM paraquat for up to 24 h resulted in no increase in *gor* promoter activity (data not shown). This is in contrast to the situation in the yeast, *Schizosaccharomyces pombe*, where *gor* expression has been shown to increase upon exposure to oxidants such as: organic hydroperoxide, diamide, and the superoxide generator, menadione [22].

The constitutive expression of *X. campestris gor* raised the question of whether *gor* is in the OxyR regulon as is the case in *E. coli* [23]. To answer this question Gor enzyme and promoter activities were measured in *X. campestris* wild type, an *X. campestris oxyR* knockout mutant, and an *X. campestris oxyR5* strain that has spontaneous mutations at G197 and L301 of OxyR that render it constitutively active [24,25]. Both Gor enzyme activity and *gor* promoter activity were not significantly different in the three strains (data not shown) indicating that *X. campestris gor* expression differs from that of *E. coli gor* in that it is not regulated by OxyR. Similar OxyR-independent expression of *gor* has thus far only been observed in the photosynthetic bacterium

Rhodobacter capsulatus, where *gor* expression was found not to be induced by H_2O_2 [26].

Enzyme kinetic study and the coenzyme binding motif analysis of Gor

A His-tagged Gor protein fusion was constructed as described in Materials and methods. His-tagged *Xanthomonas* Gor was expressed at high level in *E. coli* harboring pQEG and purified using nickle affinity column chromatography. The purity of each eluted protein fraction was determined by SDS–PAGE (Fig. 2). Both SDS- and non-denaturing PAGE indicated that the recombinant *Xanthomonas* Gor enzyme ran as a single band of approximately 50 kDa. This was confirmed using gel filtration column chromatography by FPLC which indicated that the enzyme was active as a monomer of 50 kDa in size (data not shown). This is atypical of the known Gor from various sources which are generally dimeric enzymes [27]. The only monomeric Gor reported to date is from the photosynthetic alga *Chlamydomonas reinhardtii* [28].

The kinetic parameters of the recombinant *Xanthomonas* Gor catalyzed reduction of oxidized glutathione were determined (Table 1). Interestingly, the K_m for NADH of *Xanthomonas* Gor was 55.5 μM which is approximately 3.5- and 36-fold lower than those of human erythrocyte [29] and *E. coli* [30] Gor, respectively. Surprisingly, *Xanthomonas* Gor utilized both NADH and NADPH with nearly equal affinity (K_m of 52.6 μM for NADPH versus 55.5 μM for NADH) (Table 1). This was unusual given that the Gor enzymes that have been studied in detail either use NADPH exclusively or show only a very low affinity for NADH [27]. Only Gor from *Chromatium vinosum* has thus far been reported to preferentially utilize NADH (K_m of 60 μM for NADH versus a K_m of 3000 μM for NADPH) [31]. The deduced amino acid sequence of *Xanthomonas* Gor was compared with other Gor sequences from various sources including bacteria, plant, and human using the Clustal W program [32], in order to identify sequence differences that might explain the enzymes' unique NADH/NADPH specificity (data not shown). *Xanthomonas* Gor showed a high degree of sequence identity with Gor sequences from *E. coli* (45%), *Haemophilus influenzae* (45%), human (44%), and *Pseudomonas aeruginosa* (40%). All active-site amino acid residues, as well as those involved in FAD binding and GSSG binding, were conserved among the different Gor homologs [27]. Most Gor homologs contained the highly conserved NADPH binding site sequence ($GxGYIAx_{18}Rx_5R$) where the first arginine residue (R200) in the Rx_5R motif is virtually 100% conserved. However, *X. campestris* Gor was found to have a unique NADPH binding site sequence ($GxGYIAx_{18}Qx_5E$) in which the highly conserved arginine residues are replaced by glutamine (Q200) and glutamic acid (E206) (Fig. 3). While this unique NADH/NADPH binding sequence is likely the reason for *Xanthomonas* Gor's ability to utilize both electron donors, the mechanism by which this is made possible remains unknown. A previous study of human glutathione reductase found that NADH also binds to Gor but with less affinity than NADPH, (i.e., a 60-fold higher K_m than that for NADPH) due to its lack of a 2′-phosphate group [30] that can interact with the positively charged residues R218 and R224 [30]. In *E. coli* Gor, replacement of R218 and R224 with M and L, respectively, substantially decreased the enzyme's affinity for NADPH and resulted in a catalytically less favorable configuration for bound NADPH [30]. In the NADH-dependent enzymes, like dihydrolipoamide dehydrogenase, conserved E residues replace the R residues in equivalent positions of the NADH binding motif where they were suggested to be involved in binding the 2′-OH group of the ribose moiety of NADH [30]. Therefore, E206 in *Xanthomonas* Gor may facilitate NADH ribose group binding thus allowing the enzyme to use NADH as a cofactor. Rationalizing how the Qx_5E motif facilitates NADPH binding is more difficult since Q200 is an uncharged residue and E206 is negatively charged, so both do not favor binding of the negatively charged phosphate group of NADPH.

A comparison of a total of 86 deduced Gor amino acid sequences, that included those identified from 209 completed microbial genomes as well as all the Gor protein sequences deposited in the SwissProt database, revealed that the $Q200x_5E206$ NADH/NADPH binding motif was present only in Gor from two *Xanthomonas* species, *X. campestris* pv. *campestris* and *X. axonopodis*

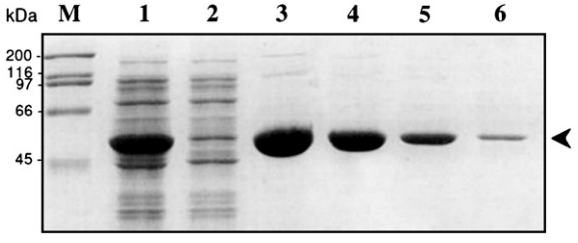

Fig. 2. SDS–PAGE of Gor at different stages of purification. Lane M, molecular mass standard; lane 1, crude extract; lane 2, after nickle affinity column; and lanes 3–6, eluted fractions. Each lane was loaded with 5 μl protein. Arrow indicates Gor protein bands.

Table 1
Kinetic parameters of glutathione reductase from *Xanthomonas*

Parameter	NADPH	NADH
K_m (μM)	52.6	55.5
k_{cat} (min^{-1})	2250	1950
k_{cat}/K_m (min^{-1} μM^{-1})	42.8	35.1
V_{max} (U/ml)	39.2	39.2
Specific activity (U/mg)	45	39

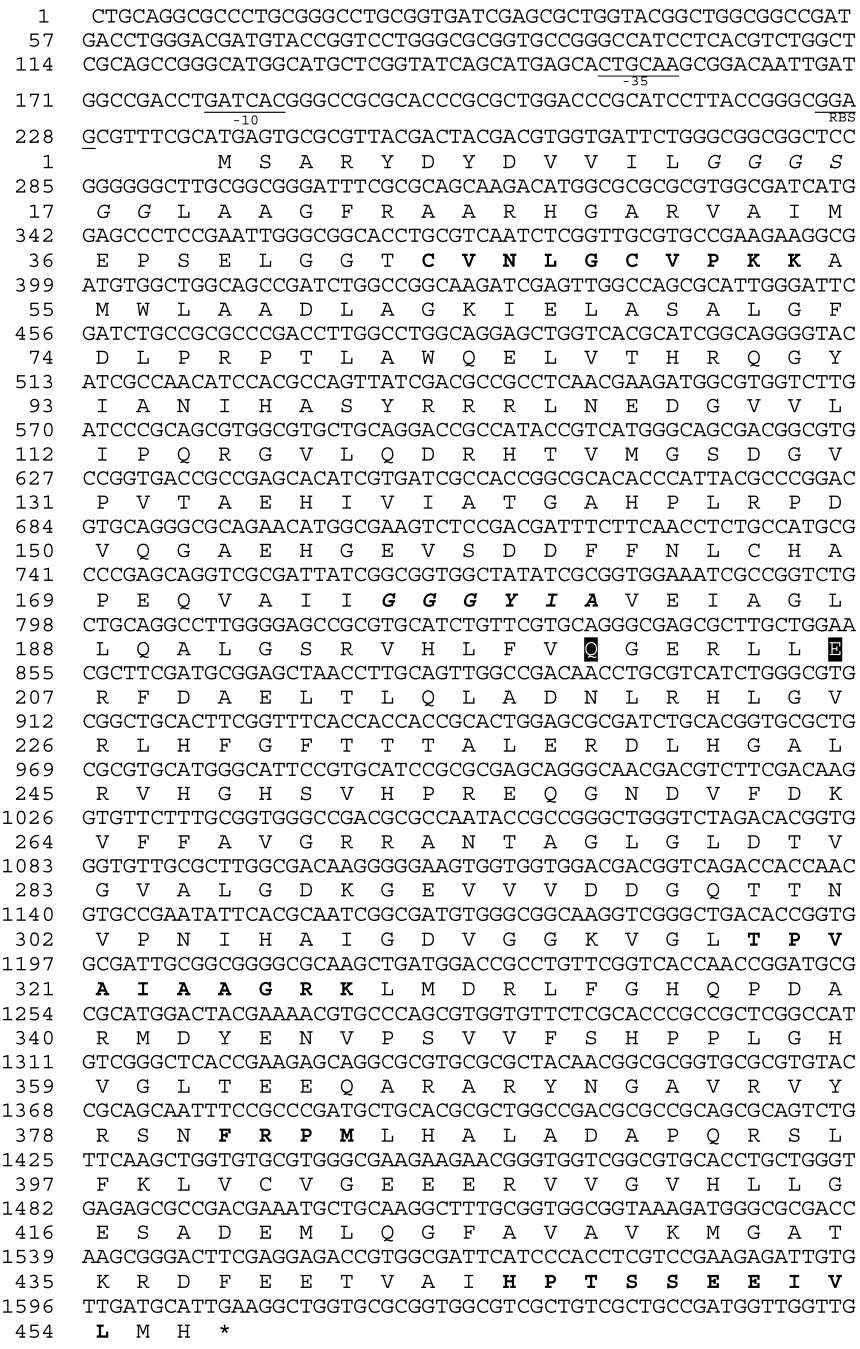

Fig. 3. Nucleotide sequence and predicted amino acid sequence of *Xanthomonas gor*. The putative −35, −10 promoter regions, and ribosome binding site (RBS) are underlined. Regions of residues important for GSSG binding are shown in bold letters. Residues involved in NADPH binding are in italic and bold. Q and E residues that replace the most conserved R at the NADPH binding sites are marked by white letters on a black background.

pv. *citri*, while all other Gor sequences contained the highly conserved NADPH binding motif ($GxGYIAx_{18}Rx_5R$) in which R200 was absolutely conserved among Gor from all sources except *Xanthomonas*. In order to determine if the Qx_5E sequence motif was shared between other members of the genus, *Xanthomonas* DNA fragments spanning the Qx_5E region within *gor* in *X. vessicatoria*, *X. translucens*, and *X. hyacinthi* were amplified by PCR, cloned, and sequenced. The sequences from all three *Xanthomonas* species contained the Qx_5E binding motif indicating that the Gor NADH/NADPH binding specificity is common to members of the genus. *X. campestris* Gor also differed from Gor of other organisms in respect to its specific activity, that was comparatively low relative to the specific activities of Gor isolated from other sources [30,33,34]. Presumably, the relatively low specific activity of *Xanthomonas* Gor may be compensated for by

the enzyme's unique ability to utilize both NADH and NADPH.

Increased expression of an organic peroxide-inducible regulator gene (ohrR) in gor mutants

In order to define the physiological role of *Xanthomonas* atypical Gor, the expression of the well-characterized organic peroxide-inducible *ohrR* promoter system [19,35–37] was used as an indicator of the cellular redox state in *Xanthomonas* wild type and *gor* mutant strains. The organic hydroperoxide resistance protein (Ohr) was first identified in *X. campestris* [12] and its expression is regulated by a novel transcription repressor, OhrR (Fig. 4A) [37]. Expression of the *ohrR-ohr* operon is highly induced by organic peroxide through the oxidation of a highly conserved cysteine residue that prevents the protein from binding to its target promoter region [19,37]. Thus, expression of the *ohrR-ohr* operon is a sensitive indicator of oxidative stress that is induced either by exposure to organic oxidants in the external environment or those generated as a result of internal cellular processes. The question of whether *Xanthomonas* atypical Gor affects the cells' ability to respond to oxidative stress was investigated through the use of a highly sensitive *ohrR* promoter-*lacZ* fusion system. A mini-Tn5 pP1lacZ construct was transferred to both wild type and *gor*-disrupted mutant strains of *X. campestris* pv. *phaseoli* and their response towards organic peroxide exposure was determined and compared (Fig. 4B). In the absence of peroxide, *ohrR* promoter in *Xanthomonas* lacking Gor exhibited marginally higher β-galactosidase activity (Fig. 4B, uninduced) when compared to the wild type level indicating that the absence of Gor enzyme causes the intracellular environment to become more

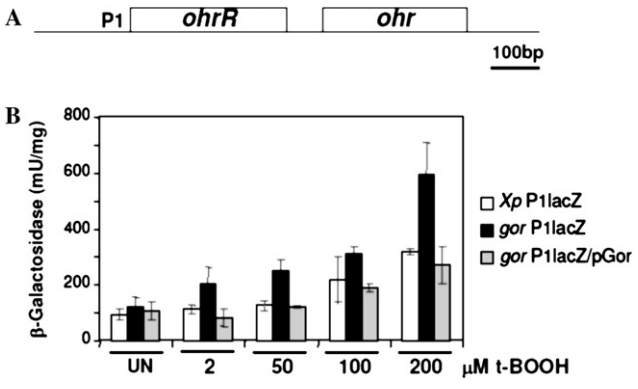

Fig. 4. Diagram of the genetic organization of *ohrR* and *ohr* (A). Expression of the *ohrR* promoter in *Xanthomonas* wild type and *gor* mutants when exposed to organic hydroperoxide (B). β-Galactosidase activities in crude extracts of an *ohrR-lacZ* fusion in parental (*Xp*P1lacZ) and *gor* mutant (*gor* P1lacZ), as well as the complemented strain (*gor* P1lacZ/pGor) when uninduced (UN) or induced with 2, 50, 100, or 200 μM *t*-BOOH. Each value shown is the mean of three separate experiments and error bars indicate standard error of the mean.

oxidized and the OhrR mediated derepression of the *ohrR* promoter. The situation became more pronounced when both strains were exposed to higher concentrations of organic peroxide (*t*-BOOH). The *ohrR* promoter in *gor* mutants responded more strongly to all concentrations of *t*-BOOH (Fig. 4B, 25–100 μM *t*-BOOH). Complementation of the *gor* mutant *gor* P1lacZ with a plasmid-borne *gor* in strain *gor* P1lacZ/pGor reduced *ohrR* promoter activity to the level in the wild type background. The result demonstrated that *Xanthomonas* Gor indeed plays a key anti-oxidative stress role in maintaining the reduced cellular redox state.

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