Complementation of the *dpsA* and *dpsA oxyR* mutants

The *dpsA* structural gene and its ribosome-binding site (500 bp) were amplified by PCR and cloned into pGEM-T (Promega) to generate pGEM-D. The primers were D303 (5′AAGGAGTTTCGAGGATGG3′) and D304 (5′TCAC-CGAGCAGCGAACG3′). *dpsA* was then removed from pGEM-D and cloned into the *ApaI-SpeI* site of pBBR-Sp (specinomycin resistant), which was created by replacing *cat* (chloramphenicol resistance gene) of pBBR-Cm with the Sp^r gene from pKRP13 (Reece and Phillips 1995), to create pDps, which was then mobilized into the *dpsA* mutant DR18 by conjugation. In order to complement the *dpsA oxyR* mutant DR17, pUT-*oxyR-ery* (erythromycin resistant) was mobilized into the chromosome of DR17 which harbors pDps, creating DR17R/pDps (*dpsA oxyR* TnR/pDps).

Growth on oxidant agar plates

Cultures of the desired strains grown overnight in M9 low glucose medium were adjusted to OD_{600}=1.0 and serially diluted. Ten microliters of each dilution was spotted onto LB agar containing 150 µM *tert*-butyl hydroperoxide (*t*-BOOH) and the extent of growth was observed after 24 h of incubation at 37°C.

Survival in oxidant medium

Overnight cultures in LB medium were subcultured (starting OD_{600}=1.0) into fresh modified M9 (0.2% casamino acids, 0.4% glucose) with and without 150 µM *t*-BOOH and the optical density was measured after 7 h of incubation at 37°C with shaking. The relative growth was calculated by comparing the optical density of treated cultures with comparable untreated cultures.

Growth inhibition zone assays

To test the susceptibility of *E. coli* strains to organic hydroperoxides, disk inhibition assays were done as previously described (Mongkolsuk et al. 1998). Briefly, bacterial cells from an exponential-phase culture (10^8 cells) were added to 3 ml of warm top LB agar. The mixture was then overlayed onto an LB agar plate. When the agar had set, 6-mm paper discs containing 6 µl of 250 µM *t*-BOOH were placed on the cell lawn. Zones of growth inhibition were measured after a 24-h incubation.

Reduction of organic hydroperoxide assay

The reduction of organic hydroperoxide in the growth medium was measured at different times by a reaction using chromogen xylenol orange, ammonium ferrous sulfate, and sulfuric acid as previously described (Shea and Mulks 2002).

Results and discussion

Regulation of *dpsA* expression by OxyR

We have previously shown that *dpsA* is co-transcribed with *katG* upon exposure to oxidative stress (Loprasert et al. 2003b) (Fig. 1A). To test whether the global peroxide sensor OxyR is a regulator of *dpsA* expression, the relative amounts of *dpsA* mRNA in *oxyR*, *katG*, and *oxyR katG* double mutant strains were determined by Northern blot analysis. A lack of OxyR in *oxyR* (R957) and *oxyR katG* (RG27) mutants abolished the induction of *dpsA* following treatment with the superoxide generator menadione (Fig. 1B lane M of *oxyR* and *oxyR katG*). Transcripts of *katG–dpsA* (3.5 kb) and *dpsA* (0.6 kb) were not induced in OxyR-deficient strains when cells were exposed to oxidant (menadione). In the *katG* mutant, a *katG–dpsA* transcript was not detected while *dpsA* mRNA was highly induced, indicating that OxyR can activate *dpsA* expression from the *dpsA* promoter (Fig. 1B lane M of *katG*). It is worthwhile noting that, in the wild-type following oxidant treatment, a transcript of approximately 3.5 kb (*katG–dpsA*) was highly induced while the 0.6-kb mRNA of *dpsA* showed no increase. *dpsA* transcripts were apparent only when *katG* was disrupted, suggesting that OxyR may preferably activate *dpsA* via the *katG* promoter instead of the downstream *dpsA* promoter. Arrangement of *katG* and *dpsA* in an operon would certainly benefit cells by allowing a prompt increase in expression of both genes in response to oxidative stress. While KatG detoxifies the peroxide threat, DpsA would simultaneously protect DNA from peroxide-induced damage.

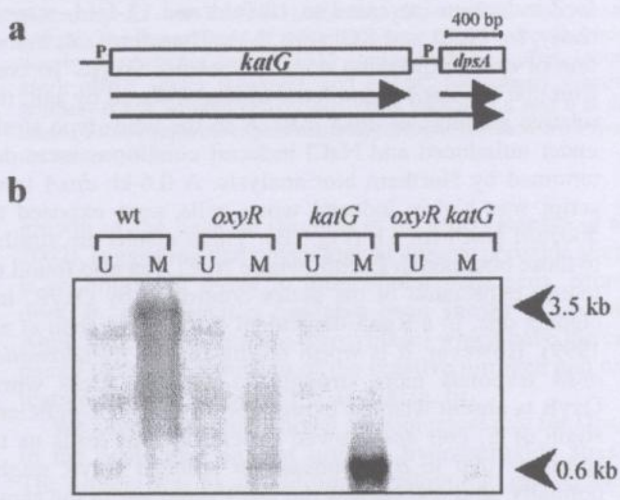

Fig. 1 Gene organization and transcriptional regulation of *katG dpsA* operon expression in response to oxidative stress. **a** The genetic organization of *katG* and *dpsA*. *Arrows* indicate the direction and extent of transcription, *P* promoter regions. **b** Northern analysis of *dpsA* mRNA prepared from *Burkholderia pseudomallei* P844 cells (wt), *oxyR* (R957), *katG* (G221), and *oxyR katG* (RG27) mutants under uninduced (U) and menadione-induced (M) conditions. *Arrowheads* indicate hybridizing mRNAs and their sizes (kb) are shown

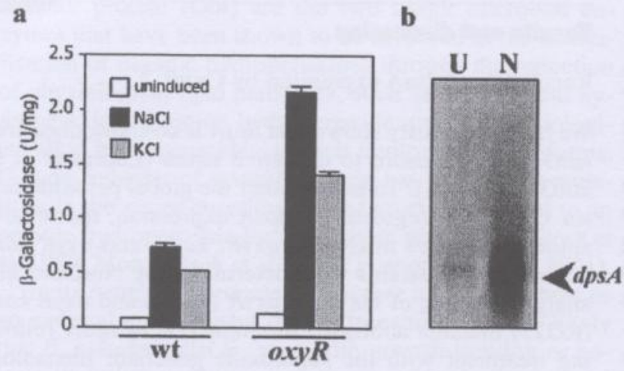

Fig. 2 Regulation of the *dpsA* promoter in response to osmotic stress. **a** β-Galactosidase activities in crude extracts of *dpsA-lacZ* fusion parent P844D (wt), and *oxyR* mutant R957D (*oxyR*) prepared from uninduced cells and cells induced with NaCl and KCl. Each value shown is the mean of three separate experiments: *error bars* standard error from the mean. **b** Northern analysis of *dpsA* mRNA prepared from *B. pseudomallei* P844 cells under uninduced (U) and NaCl-induced (N) conditions

Inducible transcription of *dpsA* by osmotic stress

To determine whether *dpsA* could be induced by oxidative and osmotic stresses, cells were treated with 0.5–10 mM H_2O_2, 500 mM NaCl or 500 mM KCl and *dpsA-lacZ* expression was monitored. Under these conditions, H_2O_2 did not cause any significant induction of the *dpsA* promoter (data not shown). Expression of *dpsA-lacZ* in wild-type (P844D) cells was induced eightfold and sixfold by NaCl and KCl, respectively, compared to an uninduced control. In the *oxyR*-disrupted mutant R957D, the level of *dpsA-lacZ* induction increased to 18-fold and 12-fold, respectively, for NaCl and KCl (Fig. 2A). Therefore, salt induction of *dpsA* expression does not require OxyR. To confirm that the *dpsA* promoter is indeed induced by salt, the relative amounts of *dpsA* mRNA in the wild- type strain under uninduced and NaCl-induced conditions were determined by Northern blot analysis. A 0.6-kb *dpsA* transcript was highly induced when cells were exposed to 500 mM NaCl for 1 h (Fig. 2B). These results are similar to those obtained in *E. coli*, where NaCl was also found to induce expression of the genes controlled by OxyR, including *dps*, in a RpoS-dependent manner (Michan et al. 1999). However, it is worth noting that *B. pseudomallei dpsA* responds more strongly to osmotic stress when OxyR is absent whereas expression in an OxyR-deficient strain of *E. coli dps* showed no effect. This leads us to speculate that in *B. pseudomallei* reduced OxyR might normally bind and repress the *dpsA* promoter in the same manner that it has previously been shown to bind to, and repress expression of, the *katG* promoter in uninduced *B. pseudomallei* (Loprasert et al. 2003b). Therefore, a lack of OxyR would certainly facilitate the RpoS dependence of the *dpsA* promoter by RNA polymerase. Expression of *dps* was also shown to be induced by general stress, e.g. heat shock, exposure to high salt or ethanol, and after glucose starvation in *B. subtilis* (Antelmann et al. 1997).

t-BOOH sensitivity of *dpsA* mutants

The physiological role of DpsA in *B. pseudomallei* was determined by testing the sensitivity of the various mutants to organic hydroperoxide stress. DpsA-deficient mutants exhibited hypersensitivity to *t*-BOOH. The *dpsA* mutant D18 did not grow well on 150 µM *t*-BOOH-containing agar while the growth of *dpsA oxyR* double mutant DR1, was even poorer. In both strains, growth was restored to the wild-type level after complementation with the *dpsA*-containing plasmid pDps (Fig. 3A). The ability of the wild-type and mutant strains to grow in M9 minimal liquid medium containing 150 µM *t*-BOOH was also studied (Fig. 3B) and the results are in good agreement with those determined on agar plates. In the wild-type strain P844, overexpression of DpsA from pDps (strain P844/pDps) increased relative growth following *t*-BOOH exposure (75% compared to 55% for wild-type). In strain D18, which lacks a functional *dpsA*, the relative growth following *t*-BOOH exposure was reduced to 36%. When either DpsA or AhpC was overexpressed in the *dpsA* mu-

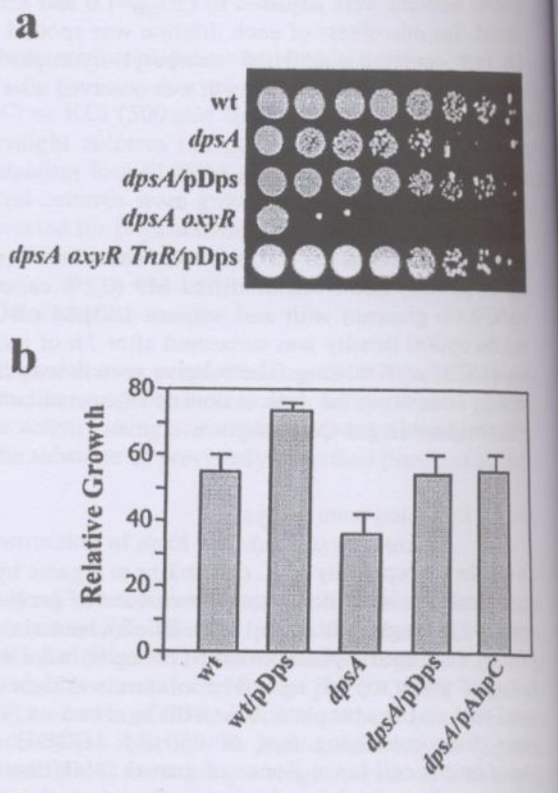

Fig. 3 Sensitivity of *B. pseudomallei* to *t*-BOOH. **a** Growth on *t*-BOOH-containing agar plate of serially diluted *B. pseudomallei* P844 (wt), *dpsA* mutant (*dpsA*), *dpsA* mutant complemented with *dpsA* on plasmid pDps (*dpsA*/pDps), *dpsA oxyR* mutant (*dpsA oxyR*), and the *dpsA oxyR* mutant complemented strain (*dpsA oxyR TnR*/pDps). **b** Relative growth of various strains in *t*-BOOH-containing M9 medium. *B. pseudomallei* P844 (wt), overexpressed DpsA (wt/pDps), *dpsA* mutant (*dpsA)*, complemented *dpsA* mutant (*dpsA*/pDps), and *dpsA* mutant with AhpC plasmid pAhpC (*dpsA*/pAhpC). Each value shown is the mean of three separate experiments; *error bars* standard error from the mean

tant strains D18/pDps and D18/pAhpC the relative rate of growth was restored to the wild-type level. This restoration of *t*-BOOH resistance in the *dpsA* strain expressing AhpC was expected since AhpC reduces and detoxifies *t*-BOOH (Storz et al. 1989). Dps-deficient *E. coli* mutants have been shown to be hypersensitive to H_2O_2 (Almiron et al. 1992), *N*-ethylmaleimide (NEM) (Ferguson et al. 1998), and acid stress (Choi et al. 2000). *B. subtilis mrgA* mutants are sensitive to H_2O_2 (Chen and Helmann 1995). To our knowledge, this is the first report demonstrating that Dps protects cells from organic hydroperoxide toxicity. We have previously found that a *B. pseudomallei katG* mutant shows increased sensitivity to H_2O_2, menadione, NEM, and sodium hypochlorite (Loprasert et al. 2003b). In order to rule out the possibility that KatG expression might be reduced in the *dpsA* mutant strain, the sensitivity of this strain to each of the aforementioned oxidants was measured. It was found that the *dpsA* mutant had the same sensitivity, as determined by growth inhibition zone assays, to H_2O_2 (0.5 M), menadione (100 mM), NEM (0.1 M), and sodium hypochlorite (0.6%) as the wild-type (data not shown).

Protection of *E. coli* against *t*-BOOH by *B. pseudomallei* DpsA

To test whether the protective property of DpsA to organic hydroperoxide is specific to *B. pseudomallei*, *dpsA* was overexpressed in the organic-oxidant-sensitive *E. coli* strain TA4315 (Storz et al. 1989) which lacks functional *ahpC*. Growth inhibition studies clearly demonstrated that both *B. pseudomallei* DpsA and AhpC could protect *E. coli* against *t*-BOOH toxicity, suggesting that this is a common property of DpsA (Fig. 4A). Since *B. pseudomallei* AhpC also conferred protection to *t*-BOOH, we were interested in finding out whether both AhpC- and DpsA-mediated protection involve the same or different mechanisms. It has been well documented that AhpC is a reductase that catalyzes the reduction of alkyl hydroperoxide to alcohol (Storz et al. 1989). The levels of *t*-BOOH in the culture medium during growth of *E. coli* strain TA4315 expressing either *B. pseudomallei* AhpC or DpsA from plasmid were therefore determined. As anticipated, the AhpC-expressing strain (TA4315/pAhpC) completely reduced the *t*-BOOH in the culture medium within 20 min, whereas the DpsA-expressing strain (TA4315/pDps) showed no significant reduction of *t*-BOOH levels relative to strain TA4315 carrying plasmid vector pBBR (Fig. 4B). This indicates that the mechanisms of protection employed by DpsA and AhpC during organic hydroperoxide exposure are distinct. It is likely that the binding of DpsA to DNA acts as a physical barrier to organic-oxidant-induced DNA damage in a manner analogous to that observed for hydrogen peroxide protection (Wolf et al. 1999). By contrast, AhpC enzymatically detoxifies the organic hydroperoxides. Analysis of *ahpC* expression in *Legionella pneumophila* and *Salmonella typhimurium* showed that *ahpC* levels increased several-fold during intracellular growth of the bacteria (Francis et al. 1997; Rankin et al. 2002), suggesting that physiological organic peroxide stress is an important threat to intracellular pathogens. Moreover, *B. pseudomallei* has also been shown to contain AhpC and KatG (catalase–peroxidase) which have the capacity to relieve a portion of the reactive nitrogen and oxidative stresses (Loprasert et al. 2003a, b).

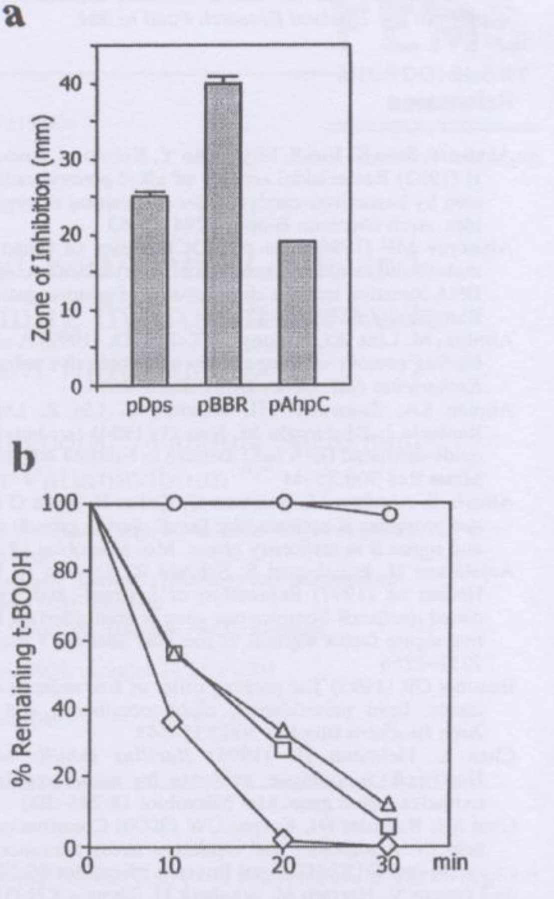

Fig. 4 Growth inhibitory zone and *t*-BOOH degradation assays. **A** Determination of the levels of resistance to *t*-BOOH killing displayed by *E. coli* TA4315 harboring pDps, pBBR vector, and pAhpC. **B** Measurement of the remaining *t*-BOOH after degradation by *Escherichia coli* TA4315 strains harboring pDps (*triangles*), pBBR vector (*squares*), and pAhpC (*diamonds*). Control (circles) is LB without cells. Each value shown in **a** and **b** is the mean of three separate experiments; *error bars* standard error from the mean.

We have uncovered a novel physiological role for DpsA in the protection against organic hydroperoxide stress. The protein acts as an additional system that can be used by *B. pseudomallei* to guard against attack by host immune systems. This study demonstrates that DpsA is a key component of the stress protection system important for the survival of the infectious pathogen *B. pseudomallei*.

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Novel Roles of *ohrR-ohr* in *Xanthomonas* Sensing, Metabolism, and Physiological Adaptive Response to Lipid Hydroperoxide

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Lipid hydroperoxides are highly toxic to biological systems. Here, the *Xanthomonas campestris* pv. phaseoli sensing and protective systems against linoleic hydroperoxide (LOOH) were investigated by examining the phenotypes, biochemical and regulatory characteristics of various *Xanthomonas* mutants in known peroxide resistance pathways. Analysis of LOOH resistance levels indicates that both alkyl hydroperoxide reductase (AhpC) and organic hydroperoxide resistance enzyme (Ohr) have important and nonredundant roles in the process. Nonetheless, inactivation of *ohr* leads to a marked reduction in LOOH resistance levels. The regulatory characteristics of an *ohr* mutant add further support to its primary role in LOOH protection. Northern analysis shows that LOOH had differential effects on induction of *ahpC* and *ohr* expression with the latter being more sensitive to the inducer. Analysis of the *ahpC* and *ohr* promoters confirmed that the LOOH-dependent induction of these promoters is mediated by the transcription regulators OxyR and OhrR, respectively. Using the in vivo promoter assays and the in vitro gel mobility shift assay, we show that LOOH directly oxidized OhrR at the sensing residue Cys-22 leading to its inactivation. In addition, physiological analysis shows that pretreatment of *X. campestris* pv. phaseoli with a sublethal dose of LOOH induced high levels of resistance to subsequent exposure to lethal concentrations of LOOH. This novel LOOH-induced adaptive response requires a functional *ohrR-ohr* operon. These data illustrate an important novel physiological role for the *ohrR-ohr* system in sensing and inactivating lipid hydroperoxides.

During normal growth *Xanthomonas* spp. are exposed to harmful reactive oxygen species (ROS) including H_2O_2, organic peroxide, and superoxide anions generated from other soil organisms and as a part of active plant defense responses. Lipid hydroperoxides are important components of the ROS produced during the plant defense response (8), and are both highly reactive and toxic to bacterial cells. Plant lipoxygenases catalyze the formation of fatty acid hydroperoxides through the reaction of fatty acid precursors such as linoleic or linolenic acids with molecular oxygen (3, 8). The expression of these enzymes has been shown to be induced in response to microbial invasion and has been linked to the plant microbial defense response (10). Consequently, in order to survive and proliferate during infection, invading bacteria must detoxify lipid hydroperoxides.

To date, very little is known regarding how bacteria protect themselves from fatty acid hydroperoxides. The best-characterized bacterial system for the detoxification of organic hydroperoxides is the alkyl hydroperoxide reductase (AhpC). AhpC catalyzes the reduction of organic peroxides to their corresponding alcohols (24). In many bacteria, inactivation of *ahpC* results in increased sensitivity to organic peroxides and pleiotropic alterations in the oxidative stress response (2, 20, 27, 31, 32). A second system for organic hydroperoxide protection, designated *ohr*, has been discovered in *Xanthomonas* (19). *ohr* confers resistance to organic hydroperoxides, and inactivation of the gene leads to increased sensitivity to organic peroxides (19). *ohr* homologues are widely distributed in both gram-positive and gram-negative bacteria (1, 6, 12, 22, 25, 28). The structure and biochemical mechanism of Ohr have been elucidated (12). Ohr is a thiol peroxidase that catalyses the reduction of an organic hydroperoxide to its corresponding organic alcohol (4). AhpC and Ohr appear to have similar biochemical properties and possibly overlapping physiological functions. The genes are independently regulated. *ahpC* is regulated by OxyR (14, 32), whereas *ohr* is controlled by the transcription repressor OhrR (16). In *Xanthomonas*, AhpC and Ohr were shown to have slightly different organic peroxide substrate preferences (30). Recently, the thiol peroxidases, bactoferritin comigratory protein (BCP), and glutathione peroxidases (Gpx-like) have been reported to contribute to the protection of bacteria from organic peroxide (7, 9). However, the corresponding genes are either found only in a few bacteria and are not well characterized or they have highly specialized physiological roles. Thus, their general role in the protection of bacteria from organic peroxide has yet to be elucidated.

Here, we examined the physiological and biochemical roles of AhpC and Ohr in the protection against lipid hydroperoxide toxicity. The results of the study demonstrate the importance of the *ohrR-ohr* system in the ability to tolerate lipid hydroperoxides and revealed a novel bacterial adaptive response to lipid hydroperoxide exposure. (Parts of this work are from the dis-

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sertation of C.K. submitted for the Ph.D. degree from Mahidol University, Bangkok, Thailand.)

Different sensitivity to LOOH in *ahpC* and *ohr* mutants. Many organic hydroperoxide-metabolizing systems have been studied in bacteria; however, these studies have not addressed the integral roles of gene regulation and bacterial physiology in these defense systems (7, 9, 12, 17, 24, 27, 30). Thus, a growth inhibition zone assay (19) was used to measure the sensitivity to LOOH (prepared as described by Evans et al. [5]) of wild-type *Xanthomonas campestris* pv. phaseoli and various *Xanthomonas* strains. Wild-type *X. campestris* pv. phaseoli was highly resistant to LOOH and exhibited no zone of growth inhibition when exposed to 50 mM LOOH. However, mutants in which the *ahpC* (17) and *ohr* (19) genes were inactivated gave zones of growth inhibition of 6 and 12 mm, respectively. In the double mutant, a zone of inhibition of 16.5 mm was observed. At present, the mechanism responsible for uptake of LOOH is not known. At high concentrations of LOOH diffusion is thought to contribute to the uptake process while at low concentrations of LOOH, the energy-dependent fatty acid uptake system could be involved (21).

We extended these studies to determine the ability of *ahpC1*, *ohr*, and *ahpC ohr* mutants to metabolize LOOH using the Fox assay as described by Ochsner et al. (22) and Shea and Mulks (28). Exponential-phase cultures (optical density at 600 nm [OD_{600}] of 0.5) of the parental strain, *ahpC1*, *ohr*, and *ahpC1 ohr* mutants were incubated with 200 μM LOOH, and the amount of LOOH remaining after a 30-min incubation was determined. The results mirrored the resistance studies in that both *ahpC1* and *ohr* single mutants displayed a decreased ability to metabolize LOOH, with the *ohr* mutant showing the higher degree of impairment, while an *ahpC1 ohr* double mutant was less able to metabolize LOOH than either of the single mutants (data not shown). The ability of the *ahpC1 ohr* double mutant to metabolize LOOH could be restored to levels that were equal to or greater than that of wild type by the overexpression of plasmid-borne *ahpC* and *ohr*, respectively (data not shown), indicating that both enzymes could use LOOH as a substrate. The data suggest that the two systems act through independent pathways with *ohr* being the major protective system and *ahpC* playing a secondary backup role in protecting *Xanthomonas* from LOOH. A possible explanation for this observation could be due to a difference in the cellular locations of the two enzymes. Ohr is structurally related to OsmC, a putative thiol peroxidase that is localized in the periplasmic space (12, 13), and initial studies in our laboratory have shown that Ohr is found in both the periplasm and the cytoplasm (S. Mongkolsuk et al., unpublished observation). By contrast, AhpC is likely to be a cytoplasmic protein (24). Thus, periplasmic Ohr could detoxify LOOH before it entered the cytoplasm, thereby limiting damage to intracellular macromolecules.

LOOH induced the expression of *ahpC* and *ohr*. The LOOH-dependent regulation of *ahpC* and *ohr* is of particular interest, due to the fact that the genes are regulated by different global peroxide-sensing transcriptional regulatory systems and display different patterns of oxidant-induced expression (14, 29). Thus, the effect of treatment with LOOH or the synthetic organic hydroperoxide, *tert*-butyl hydroperoxide (tBOOH), on the expression of these genes in *X. campestris* pv. phaseoli was investigated using Northern blot hybridization analysis. It was found that LOOH was a strong inducer of *ohr* expression. *ohr* was induced by exposure to 10 μM LOOH whereas a similar treatment with 10 μM tBOOH did not induce expression of the gene (Fig. 1A). As inducing concentrations of LOOH increased, there was a parallel increase in the magnitude of induction of *ohr* expression that reached a maximum level of 80-fold (as determined by densitometry), relative to the level in uninduced cells, following treatment with 100 μM LOOH (Fig. 1A). *ohr* expression was also induced by tBOOH, but to a lesser degree (Fig. 1A). Treatment with 100 μM tBOOH induced *ohr* expression by less than 10-fold. When *ahpC* expression was examined, the situation was reversed. As was the case with *ohr*, both peroxides were able to induce *ahpC* expression.

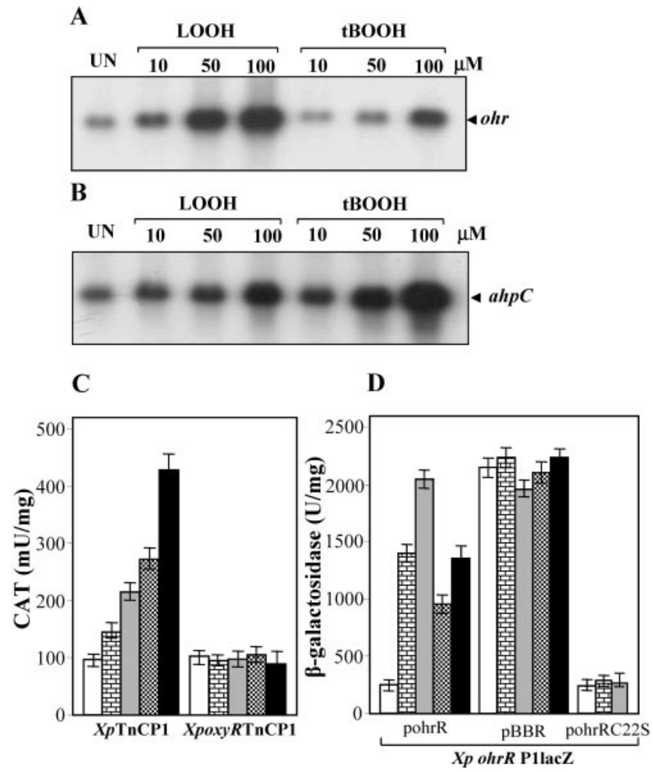

FIG. 1. Induction of *ahpC* and *ohr* expression in *X. campestris* pv. phaseoli by LOOH and tBOOH. Total RNA (10 μg) prepared from *X. campestris* pv. phaseoli cultures induced with 10, 50, 100 μM LOOH; 10, 50, 100 μM tBOOH; and uninduced cells (UN) were loaded into each lane and hybridized with either ^{32}P-labeled *ohr* (A) or *ahpC* (B) probes as previously described (30). The numbers above each lane in both panels A and B indicate the concentrations of peroxide added to the cultures. (C) Induction of the *ahpC* promoter fused to *cat* was monitored by determination of chloramphenicol acetyltransferase (CAT) activity (26) in *X. campestris* pv. phaseoli TnCP1 (*Xp* TnCP1) and an *oxyR* mutant containing TnCP1 (*Xp oxyR* TnCP1). (D) Induction of the *ohrR* P1 promoter fused to *lacZ* was monitored by determining β-galactosidase activity (15) in an *ohrR* mutant containing a P1 *lacZ* fusion (*Xp ohrR* P1lacZ) harboring pohrR, pBBR1MCS-5 (11) (pBBR), and pohrRC22S. For experiments in both panels C and D, exponential-phase cultures were untreated (open bars) or treated with LOOH (100 μM, brick bars, and 200 μM, gray bars), or tBOOH (100 μM, checkered bars, and 200 μM, black bars) for 30 min. Crude lysate preparation and enzymatic assays were performed as previously described (23). The CAT- or β-galactosidase-specific activities from induced cultures and uninduced cultures are shown.

However, tBOOH was the more effective of the two. Treatment with 100 μM tBOOH produced an 80-fold induction in *ahpC* expression levels compared to a 30-fold increase in the *ahpC* levels following treatment with 100 μM LOOH (Fig. 1B). The data clearly showed that the regulation of *ohr* responded more sensitively to the complex organic hydroperoxide, LOOH, than to the simple organic hydroperoxide molecule, tBOOH. By contrast, induction of *ahpC* expression was more sensitive to tBOOH than to LOOH treatments.

We extended these observations by determining the effect of LOOH and tBOOH on the transcription of *ahpC* and *ohrR* by monitoring the promoter activities of these genes using strains containing transcriptional fusions of the *ahpC* promoter with chloramphenicol acetyltransferase (*cat*) (*Xp* TnCP1 [14]) and the *ohrR* P1 promoter with β-galactosidase (*Xp ohrR* P1lacZ [23]) that were constructed by insertion of the reporter gene cassette within the chromosomal copy of *ahpC* or *ohrR*. The results reinforced those of the Northern blot analyses in demonstrating that the *ahpC* promoter was more efficiently induced by tBOOH. Treatment of *Xp* TnCP1 with 200 μM tBOOH resulted in a 4.5-fold increase in *ahpC* promoter activity, relative to an uninduced culture, compared with only a 2.2-fold increase in the presence of 200 μM LOOH (Fig. 1C). Furthermore, induction of the *ahpC* promoter by either organic peroxide depended on the presence of functional OxyR since no induction of the *ahpC* promoter was observed in an *oxyR* mutant background (*Xp oxyR* TnCP1) (Fig. 1C). Analysis of the hydroperoxide dependent induction of *ohrR* P1 promoter activity was complicated by the fact that the *lacZ* reporter gene insertion in this strain inactivates *ohrR*, encoding the *ohr* repressor (23). Thus, it was necessary to first complement this strain with a plasmid-borne copy of *ohrR* (pohrR) (18). As expected, LOOH was more efficient at inducing *ohrR* P1 promoter activity than tBOOH. Treatment of the strain containing the *ohrR* P1*lacZ* fusion (*Xp ohrR* P1lacZ harboring pohrR) with 100 and 200 μM LOOH induced P1 promoter activity by 6.8- and 9.7-fold, respectively, while treatment with the same concentrations of tBOOH resulted in respective increases in P1 promoter activity of 4.5- and 6.4-fold (Fig. 1D). The induction of the P1 promoter was found to be dependent on the presence of functional OhrR since the uncomplemented *ohrR* mutant strain (pBBR) did not show hydroperoxide-specific induction of the P1 promoter (Fig. 1D). The in vivo promoter fusion data supported the Northern blot results and confirmed that the observed increases in the levels of *ahpC* and *ohr* mRNA, in response to organic hydroperoxide treatments, were due to increased rates of *ahpC* and *ohr* transcription. Furthermore, the data show that in the presence of LOOH and tBOOH, the *ahpC* and *ohrR* promoters are induced by separate peroxide sensing regulatory systems. It appears that both OxyR and OhrR can sense changes in lipid hydroperoxide levels with the latter being more sensitive to the presence of the more complex hydroperoxide, LOOH, while OxyR is more sensitive to the simple organic hydroperoxide molecule, tBOOH.

In *Xanthomonas* the mechanism of organic hydroperoxide-dependent derepression of *ohr* transcription is thought to proceed via the oxidation of the highly conserved peroxide sensing cysteine residue, Cys-22, of OhrR (23). In order to test whether Cys-22 is required for LOOH inactivation of OhrR, a plasmid carrying a copy of the mutant *ohrR* (pohrRC22S), in which

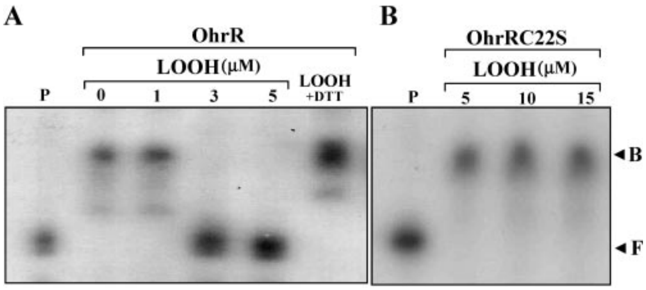

FIG. 2. The effect of LOOH and DTT on OhrR binding to the *ohrR-ohr* promoter. The results of DNA mobility shift experiments, testing oxidation of OhrR by LOOH. The DNA binding reaction contained ^{32}P-labeled P1, the *ohrR-ohr* promoter fragment (18), 3 fmol of purified OhrR or purified OhrRC22S (23). The binding reactions and electrophoresis were performed as previously described (23). In panel A, the binding reactions containing the P1 promoter fragment and OhrR were treated with either various concentrations of LOOH or 5 μM LOOH followed by 10 mM DTT treatment (LOOH+DTT) as previously described (23). In panel B the binding of P1 fragment to 3 fmol of purified OhrR C22S before 5, 10, and 15 μM LOOH were added to the binding reactions. The numbers above each lane indicate the concentration of LOOH added. P indicates free probe. The positions of bound (B) and free (F) probes are indicated.

Cys-22 has been changed to serine (C22S), was transformed into *Xp ohrR* P1lacZ and the ability of LOOH to induce the P1 promoter in this strain was evaluated. The results showed that LOOH-dependent induction of the P1 promoter was abolished in *Xp ohrR* P1lacZ harboring pohrRC22S (Fig. 1D). This indicates that residue Cys-22 of OhrR is essential for LOOH-dependent derepression of the P1 promoter. This favors the idea that in vivo, LOOH or its metabolites mediate the oxidation of residue Cys-22 thus inactivating OhrR.

LOOH oxidizes and inactivates OhrR binding to the promoter. In vivo experiments suggested that LOOH or its metabolites probably oxidized OhrR at Cys-22, but the experiment could not provide a definitive answer regarding the mechanism of LOOH sensing. Thus, gel mobility shift experiments were performed to further characterize the LOOH-sensing mechanism of OhrR. First purified OhrR and OhrRC22S (23) were incubated with a radioactively labeled 170-bp P1 promoter fragment in the presence and absence of LOOH. In the absence of LOOH, OhrR strongly bound to the P1 promoter fragment as shown by the slower-migrating P1 promoter fragment OhrR complex (Fig. 2A). Addition of 3 μM LOOH to the binding reaction completely negated OhrR binding to the P1 promoter fragment. The concentration of LOOH required to completely inhibit the binding of OhrR to the P1 promoter was 100-fold lower than that previously determined for tBOOH (18). Next, we tested whether the inactivation of OhrR by LOOH was due to direct oxidation of the protein by assessing whether the process could be reversed by treatment with a reducing agent (dithiothreitol [DTT]) and determining the effect of LOOH on a nonsensing mutant protein OhrRC22S. The results show that 10 mM DTT reversed the inhibitory effects of LOOH on the binding of OhrR to the P1 promoter (Fig. 2A). In addition, the mutant OhrRC22S had no binding defect as shown by its ability to efficiently bind to the promoter fragment at a similar concentration as wild-type OhrR (Fig. 2B). However, treatment of OhrRC22S with in-

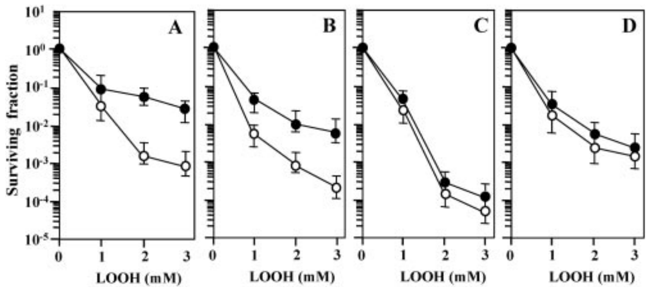

FIG. 3. Induced adaptive protection responses to LOOH in *X. campestris* pv. phaseoli, *ohr* and *ohrR* mutants. The results of LOOH-induced adaptive protection response experiments testing the effect of a 30-min preexposure to 50 μM LOOH on the survival of *X. campestris* pv. phaseoli exponential-phase cultures to subsequent exposure to 0, 1, 2 or 3 mM LOOH for 30 min. Plots of the surviving fraction of cells in cultures that did (●) or did not (○) receive a pretreatment are shown. (A) *Xp*, *X. campestris* pv. phaseoli; (B) *ahpC1* mutant; (C) *ohr* mutant; (D) *ohrR* mutant. Each value presented is the mean and standard deviation of four replicates.

creasing concentrations of LOOH had no effect on the mutant protein's ability to bind to the P1 promoter (Fig. 2B). The results support the idea that LOOH inactivated OhrR through the direct oxidation of the sensing Cys-22 residue. The available in vitro DNA binding data from this and previous studies (18) support the in vivo promoter analyses in showing that OhrR is 80-fold more responsive to the complex hydroperoxide, LOOH, than to the simple hydroperoxide, tBOOH. This favors the idea that OhrR may have evolved to preferentially sense complex organic hydroperoxides such as lipid hydroperoxides via oxidation of the highly conserved peroxide sensing residue Cys-22. The in vivo and in vitro regulatory characteristics of the *ohrR-ohr* operon support its role as the major system for the sensing of and protection from lipid hydroperoxides such as LOOH.

The novel physiological LOOH adaptive response required functional *ohrR* and *ohr*. The adaptive response is an important strategy for microbial survival under stressful conditions; however, an adaptive response to lipid peroxide has not been reported previously. Experiments were done to test if *Xanthomonas* has the capacity to mount an adaptive response to LOOH and whether *ahpC* and *ohr* are involved in the process. *Xanthomonas* cultures that had been pretreated with 50 μM LOOH were exposed to lethal concentrations (1, 2, 3 mM) of LOOH for 30 min and the fraction of surviving cells was determined. The results in Fig. 3A show that LOOH induced cells were 50-fold more resistant to LOOH killing than uninduced cells. This is the first demonstration of a bacterial adaptive response to a lipid hydroperoxide. Similar experiments were then performed using *ohr* and *ahpC* mutants in order to determine the roles of *ohr* and *ahpC* in the LOOH adaptive response. Pretreatment of an *ahpC1* mutant with LOOH induced high-level resistance to subsequent LOOH killing (Fig. 3B). By contrast, a similar preexposure of the *ohr* mutant to LOOH failed to induce increased protection, relative to uninduced cells, against subsequent LOOH killing treatments (Fig. 3C). Clearly, *ohr*, but not *ahpC*, is required for the LOOH adaptive response. We extended the investigation by determining whether proper regulation of *ohr* or simply the presence of functional *ohr* was required for the LOOH adaptive response. In the previous section, we showed that the transcription repressor, OhrR, was involved in LOOH-dependent induction of *ohr*. We therefore tested whether OhrR was also the regulator involved in the LOOH adaptive response. The LOOH adaptive response experiment was repeated using the *ohrR* mutant. As expected, pretreatment of the *ohrR* mutant with LOOH did not induce adaptive protection against subsequent LOOH killing (Fig. 3D) indicating that proper regulation of the operon is required for the LOOH-induced adaptive response. Loss of the induced adaptive protection in *ohrR and ohr*, mutants, but not in *ahpC1* mutant is consistent with the data from the physiological, and gene regulation analyses indicating that the *ohrR-ohr* system plays the major role in protecting *Xanthomonas campestris* pv. phaseoli from LOOH.

An important physiological question is whether *Xanthomonas* is likely to be exposed to LOOH in its natural environment. *Xanthomonas* spp. are important bacterial phytopathogens. During plant microbe interactions, bacteria are likely to be exposed to lipid hydroperoxide produced by plants as part of an active defense response against microbial invasion. It has been shown that increased lipoxygenase, an enzyme involved in lipid hydroperoxide synthesis, is associated with the plant defense response and fatty acid precursors such as linoleic or linolenic acids are abundant in plants (3, 8). Thus, *Xanthomonas* is likely to encounter LOOH during its interaction with host plants. Interestingly, *ohr* homologues have been found in all genomes of bacterial plant pathogens thus far sequenced (Mongkolsuk et al., unpublished observation). This conservation of *ohr* implies its important physiological role in the protection against lipid hydroperoxide exposure during plant-microbe interactions.

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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 $HOO^{\bullet}$ 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–*Hinc*II *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

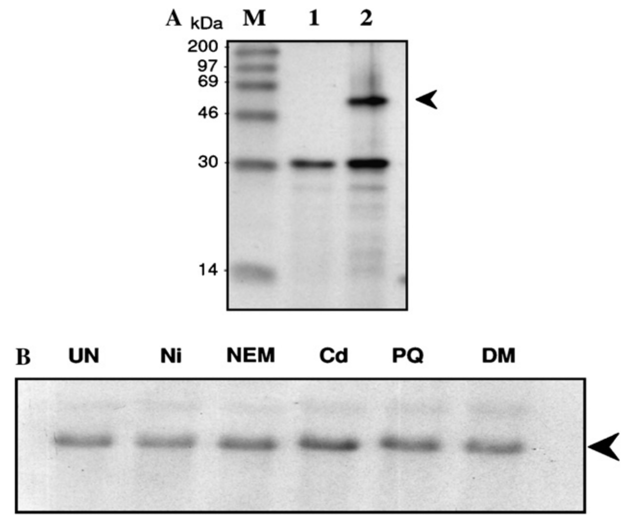

Fig. 1. In vitro translation products (A) and constitutive expression (B) of *gor*. (A) In vitro transcription-translation of pGR1800-encoded proteins with *E. coli* S-30 extracts. Lane M, protein molecular mass markers; lane 1, pUC18; and lane 2, pGR1800. The arrow indicates in vitro translation products 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*

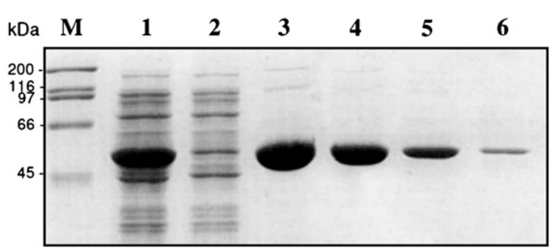

Fig. 2. SDS–PAGE of Gor at different stages of purification. Lane M, molecular mass standard; lane 1, crude extract; lane 2, after nickle affinity column; and lanes 3–6, eluted fractions. Each lane was loaded with 5 μl protein. Arrow indicates Gor protein bands.

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

```
   1   CTGCAGGCGCCCTGCGGGCCTGCGGTGATCGAGCGCTGGTACGGCTGGCGGCCGAT
  57  GACCTGGGACGATGTACCGGTCCTGGGCGCGGTGCCGGGCCATCCTCACGTCTGGCT
 114  CGCAGCCGGGCATGGCATGCTCGGTATCAGCATGAGCACTGCAAGCGGACAATTGAT
                                              -35
 171  GGCCGACCTGATCACGGGCCGCGCACCCGCGCTGGACCCGCATCCTTACCGGGCGGA
                  -10                                       RBS
 228  GCGTTTCGCATGAGTGCGCGTTACGACTACGACGTGGTGATTCTGGGCGGCGGCTCC
   1              M  S  A  R  Y  D  Y  D  V  V  I  L  G  G  G  S
 285  GGGGGGCTTGCGGCGGGATTTCGCGCAGCAAGACATGGCGCGCGCGTGGCGATCATG
  17   G  G  L  A  A  G  F  R  A  A  R  H  G  A  R  V  A  I  M
 342  GAGCCCTCCGAATTGGGCGGCACCTGCGTCAATCTCGGTTGCGTGCCGAAGAAGGCG
  36   E  P  S  E  L  G  G  T  C  V  N  L  G  C  V  P  K  K  A
 399  ATGTGGCTGGCAGCCGATCTGGCCGGCAAGATCGAGTTGGCCAGCGCATTGGGATTC
  55   M  W  L  A  A  D  L  A  G  K  I  E  L  A  S  A  L  G  F
 456  GATCTGCCGCGCCCGACCTTGGCCTGGCAGGAGCTGGTCACGCATCGGCAGGGGTAC
  74   D  L  P  R  P  T  L  A  W  Q  E  L  V  T  H  R  Q  G  Y
 513  ATCGCCAACATCCACGCCAGTTATCGACGCCGCCTCAACGAAGATGGCGTGGTCTTG
  93   I  A  N  I  H  A  S  Y  R  R  R  L  N  E  D  G  V  V  L
 570  ATCCCGCAGCGTGGCGTGCTGCAGGACCGCCATACCGTCATGGGCAGCGACGGCGTG
 112   I  P  Q  R  G  V  L  Q  D  R  H  T  V  M  G  S  D  G  V
 627  CCGGTGACCGCCGAGCACATCGTGATCGCCACCGGCGCACACCCATTACGCCCGGAC
 131   P  V  T  A  E  H  I  V  I  A  T  G  A  H  P  L  R  P  D
 684  GTGCAGGGCGCAGAACATGGCGAAGTCTCCGACGATTTCTTCAACCTCTGCCATGCG
 150   V  Q  G  A  E  H  G  E  V  S  D  D  F  F  N  L  C  H  A
 741  CCCGAGCAGGTCGCGATTATCGGCGGTGGCTATATCGCGGTGGAAATCGCCGGTCTG
 169   P  E  Q  V  A  I  I  G  G  G  Y  I  A  V  E  I  A  G  L
 798  CTGCAGGCCTTGGGGAGCCGCGTGCATCTGTTCGTGCAGGGCGAGCGCTTGCTGGAA
 188   L  Q  A  L  G  S  R  V  H  L  F  V  Q  G  E  R  L  L  E
 855  CGCTTCGATGCGGAGCTAACCTTGCAGTTGGCCGACAACCTGCGTCATCTGGGCGTG
 207   R  F  D  A  E  L  T  L  Q  L  A  D  N  L  R  H  L  G  V
 912  CGGCTGCACTTCGGTTTCACCACCACCGCACTGGAGCGCGATCTGCACGGTGCGCTG
 226   R  L  H  F  G  F  T  T  T  A  L  E  R  D  L  H  G  A  L
 969  CGCGTGCATGGGCATTCCGTGCATCCGCGCGAGCAGGGCAACGACGTCTTCGACAAG
 245   R  V  H  G  H  S  V  H  P  R  E  Q  G  N  D  V  F  D  K
1026  GTGTTCTTTGCGGTGGGCCGACGCGCCAATACCGCCGGGCTGGGTCTAGACACGGTG
 264   V  F  F  A  V  G  R  R  A  N  T  A  G  L  G  L  D  T  V
1083  GGTGTTGCGCTTGGCGACAAGGGGGAAGTGGTGGTGGACGACGGTCAGACCACCAAC
 283   G  V  A  L  G  D  K  G  E  V  V  V  D  D  G  Q  T  T  N
1140  GTGCCGAATATTCACGCAATCGGCGATGTGGGCGGCAAGGTCGGGCTGACACCGGTG
 302   V  P  N  I  H  A  I  G  D  V  G  G  K  V  G  L  T  P  V
1197  GCGATTGCGGCGGGGCGCAAGCTGATGGACCGCCTGTTCGGTCACCAACCGGATGCG
 321   A  I  A  A  G  R  K  L  M  D  R  L  F  G  H  Q  P  D  A
1254  CGCATGGACTACGAAAACGTGCCCAGCGTGGTGTTCTCGCACCCGCCGCTCGGCCAT
 340   R  M  D  Y  E  N  V  P  S  V  V  F  S  H  P  P  L  G  H
1311  GTCGGGCTCACCGAAGAGCAGGCGCGTGCGCGCTACAACGGCGCGGTGCGCGTGTAC
 359   V  G  L  T  E  E  Q  A  R  A  R  Y  N  G  A  V  R  V  Y
1368  CGCAGCAATTTCCGCCCGATGCTGCACGCGCTGGCCGACGCGCCGCAGCGCAGTCTG
 378   R  S  N  F  R  P  M  L  H  A  L  A  D  A  P  Q  R  S  L
1425  TTCAAGCTGGTGTGCGTGGGCGAAGAAGAACGGGTGGTCGGCGTGCACCTGCTGGGT
 397   F  K  L  V  C  V  G  E  E  E  R  V  V  G  V  H  L  L  G
1482  GAGAGCGCCGACGAAATGCTGCAAGGCTTTGCGGTGGCGGTAAAGATGGGCGCGACC
 416   E  S  A  D  E  M  L  Q  G  F  A  V  A  V  K  M  G  A  T
1539  AAGCGGGACTTCGAGGAGACCGTGGCGATTCATCCCACCTCGTCCGAAGAGATTGTG
 435   K  R  D  F  E  E  T  V  A  I  H  P  T  S  S  E  E  I  V
1596  TTGATGCATTGAAGGCTGGTGCGCGGTGGCGTCGCTGTCGCTGCCGATGGTTGGTTG
 454   L  M  H  *
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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 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.

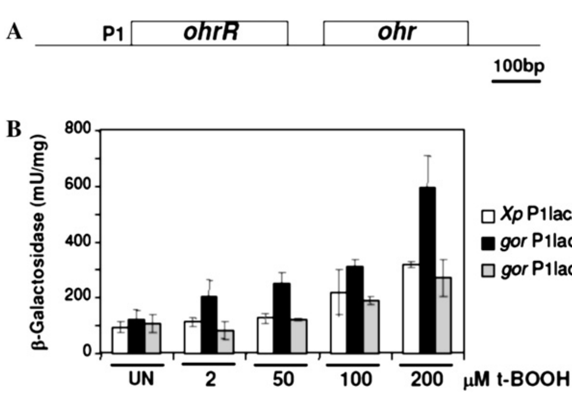

Fig. 4. Diagram of the genetic organization of *ohrR* and *ohr* (A). Expression of the *ohrR* promoter in *Xanthomonas* wild type and *gor* mutants when exposed to organic hydroperoxide (B). β-Galactosidase activities in crude extracts of an *ohrR-lacZ* fusion in parental (*Xp*P1lacZ) and *gor* mutant (*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.

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Brief note

Protection of *Xanthomonas* against arsenic toxicity involves the peroxide-sensing transcription regulator OxyR

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Abstract

Arsenic has been shown to mediate its toxicity through induced generation of reactive oxygen species. Here, we examined the role of oxidative stress-inducible genes (*katA*, *ahpC* and *ohr*) and their regulators (*oxyR* and *ohrR*) in the response to arsenic treatment in a plant pathogenic bacterium, *Xanthomonas campestris* pv. phaseoli (*Xp*). Overproduction of peroxide-scavenging enzymes (KatA, AhpCF and Ohr) did not enhance arsenic tolerance in wild-type *Xp*. Furthermore, inactivation of *katA*, *ahpC*, *ohr*, and *ohrR* genes had no effect on the level of arsenic resistance. By contrast, an *oxyR* mutant (*Xp oxyR*) showed increased sensitivity to both pentavalent arsenate and, to a greater extent, trivalent arsenite. The resistance of cells to arsenite treatment was significantly affected by the level of iron. Cells were 10-fold more sensitive to arsenite killing in the presence of excess iron, while removal of iron by an iron chelator (2,2′-dipyridyl) protected *Xanthomonas* from arsenite toxicity. The arsenite-sensitive phenotype of *Xp oxyR* could be complemented by the expression of functional OxyR from a plasmid vector, but not by the expression of other known OxyR-regulated peroxide-scavenging enzymes such as KatA and AhpCF, Ohr and OhrR. The data suggested that as yet unidentified, OxyR-regulated gene(s) are involved in conferring arsenic resistance in *Xp*. To our knowledge, this is the first report showing that the peroxide-sensing regulator OxyR is involved in arsenic resistance.

Keywords: Arsenic resistance; OxyR; KatA; AhpCF; Ohr; OhrR

1. Introduction

Arsenic is a toxic metal that is found in both natural environments and in sites contaminated by fungicides, pesticides and herbicides. Arsenic mainly exists in two oxidation states, arsenite As(III) and arsenate As(V). Arsenite is more toxic than arsenate and readily reacts with the thiol and nitrogen groups of proteins, thus disrupting their function. In addition, the production of reactive oxygen species (ROS) associated with arsenic toxicity has been reported [5,13]. Exposure to arsenic may exert an effect on soil and plant-pathogenic bacteria. Plants induce oxidative stress in infecting bacteria by generating ROS such as H_2O_2, superoxides, and organic peroxides, to inhibit microbial invasion [2]. In response to certain peroxides, bacteria have evolved enzymatic oxidant-scavenging systems, including catalase (KatA) and alkyl hydroperoxide reductase (AhpCF), to defend against host-derived oxidative killing. The genes encoding these enzymes are regulated by OxyR, a global regulator of the peroxide stress regulon [14]. Ohr is an additional protective enzyme against organic peroxide toxicity [9]. The regulation of Ohr expression is independent of OxyR and is controlled by the negative regulator OhrR [16].

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Table 1
Strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Reference or source
Xanthomonas campestris pv. phaseoli strains		
Xp	Wild type	Laboratory collection
Xp HR	Spontaneous multiple peroxide resistant mutant	[3]
Xp oxyR	Gen^r, *oxyR* mutant	[10]
Xp katA	Amp^r, *katA* mutant	Laboratory collection, unpublished
Xp ahpC	Kan^r, *ahpC* mutant	[12]
Xp ohr	Tet^r, *ohr* mutant	[9]
Xp ohrR	Tet^r, *ohrR* mutant	[16]
Plasmids		
pOxyR	Amp^r, *oxyR* coding region cloned into pBBR1MCS-4	[7]
pOxyRC199S	Amp^r, pOxyR mutated to convert Cys199 to Ser	[7]
pOxyR5	Amp^r, OxyR locked in the oxidized form	[11]
pKatA	Kan^r, *katA* coding region cloned into pUFR047	[8]
pAhpCF	Amp^r, *ahpCF* coding region cloned into pUFR047	[6]
pOhr	Amp^r, *ohr* coding region cloned into pUFR047	[9]
pOhrR	Amp^r, *ohrR* coding region cloned into pBBR1MCS-4	[16]

On the molecular level, the regulation of oxidant-responsive regulons during arsenic stress is poorly understood. Here we examined the role of oxidative stress-inducible genes and their products in response to arsenic exposure in the plant pathogenic bacterium *Xanthomonas campestris* pv. phaseoli. We found that arsenite resistance is dependent on functional OxyR, suggesting that OxyR plays an important role in the defense against arsenite exposure in *Xanthomonas*.

2. Materials and methods

2.1. Bacterial strains, plasmids, media, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. Cells were grown aerobically at 28 °C in Silva–Buddenhagen (SB) medium containing the appropriate antibiotics. SB medium contains 0.5% yeast extract, 0.5% peptone, 0.1% glutamic acid, and 0.5% sucrose (pH 7.0). Ampicillin (200 $\mu g\,ml^{-1}$), gentamicin (15 $\mu g\,ml^{-1}$), kanamycin (15 $\mu g\,ml^{-1}$), and tetracycline (15 $\mu g\,ml^{-1}$) were added as required.

2.2. Arsenic resistance of Xanthomonas strains using inhibition zone assay

Cells from an overnight culture (10^8) were subcultured into 10 ml of SB medium and grown at 28 °C with shaking for 4 h. Exponential phase cells (10^8) were added to 10 ml of prewarmed (50 °C) top agar (0.7% SB agar) and layered onto SB agar plates containing 40 ml of medium (14-cm-diameter petri dishes). After the top agar solidified, sterile 6-mm-filter paper disks containing 5 µl of a solution containing varying concentrations of either trivalent sodium arsenite ($NaAsO_2$) or pentavalent sodium arsenate (Na_2AsO_4) were placed on the surface. Plates were incubated at 28 °C for 24 h and the diameters of the inhibition zones were measured. All assays were performed in triplicate.

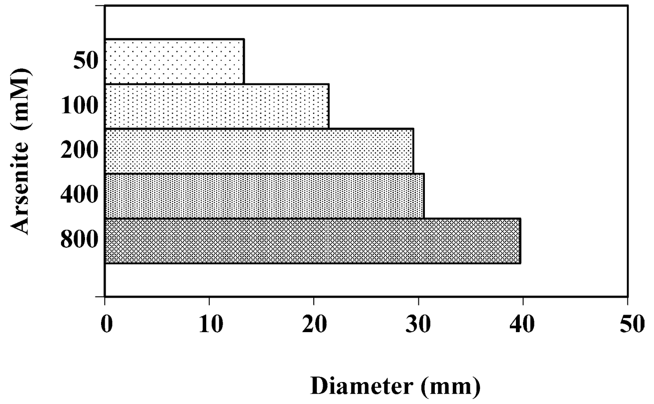
2.3. Survival assay

Exponential phase cells (10^8) were treated with 150 mM $NaAsO_2$ at room temperature. After 30-min treatment, samples were washed once with fresh SB medium before appropriate dilutions were plated on SB agar plates containing 0.1% pyruvate. Cells that survived were counted after incubation at 28 °C for 48 h. The percent survival is defined as the number of colony forming units (CFUs) obtained after the treatment divided by the number of CFUs obtained prior to treatment multiplied by 100. In some experiments, cells were treated with arsenite in the presence of 200 µM $FeCl_3$ or in the presence of an iron chelator, 200 µM 2,2′-dipyridyl. All assays were performed at least three times and representative data are shown.

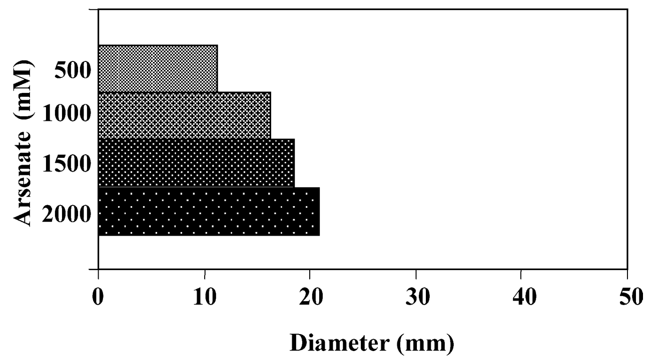
3. Results and discussion

3.1. The dose-response of wild-type Xp to arsenical compounds

To determine the basal level of resistance of wild-type *Xp* to arsenic, inhibition zone assays of cells exposed to filter disks soaked in varying concentrations of sodium arsenite (25, 50, 100, 200, 400, and 800 mM $NaAsO_2$) and sodium arsenate (250, 500, 1000, 1500, and 2000 mM Na_2AsO_4) were performed. No clear inhibition zone was observed at 25 mM arsenite and 250 mM arsenate. Clear inhibition zones were observed at concentrations above 25 mM arsenite and 250 mM arsenate, the diameters of which increased with increasing concentrations of arsenical compounds (Fig. 1). *Xp* showed a higher tolerance to arsenate than to arsenite. An



(A)



(B)

Fig. 1. Determination of the basal levels of resistance to arsenic in wild-type *Xanthomonas*. Sensitivity to arsenic was determined by inhibition zone assay as described in Section 2. Exponential phase cells were spread on SB agar plates containing filter disks impregnated with 5 µl of the indicated concentrations of trivalent sodium arsenite (A) or pentavalent sodium arsenate (B). The inhibition zones were measured after incubation at 28 °C for 24 h. The diameters of the inhibition zones are indicated in mm. Results represent the means of triplicate experiments.

inhibition zone could be detected at 500 mM of sodium arsenate (Fig. 1B) whereas a clear inhibition zone could be observed at a much lower concentration of sodium arsenite (50 mM) (Fig. 1A). These results are consistent with the well-documented fact that arsenite is more toxic than arsenate [15]. Sodium arsenite and sodium arsenate at concentrations of 100 mM and 1 M, respectively, which yielded manageable inhibition zone sizes, were chosen for use in subsequent experiments.

3.2. *Role of oxidative-inducible genes and regulatory genes in response to killing concentration of arsenic*

Arsenic has been shown to induce ROS, which mediate its toxicity [5,13]. In order to investigate the effects of peroxide-scavenging enzymes (KatA, AhpC, and Ohr) and peroxide response regulatory proteins (OxyR and OhrR) on arsenic resistance, the resistance levels to 100 mM sodium arsenite and 1 M sodium arsenate were determined in wild-type *Xp* and various mutant strains (Table 2). Inactivation of

Table 2
Inhibition zone assays[a] of mutant strains compared to those of wild type at lethal concentrations of arsenic

Strain	100 mM As(III)	1 M As(V)
Xp	21.0 ± 0.8	16.1 ± 0.8
Xp oxyR	26.4 ± 1.0	19.5 ± 1.5
Xp katA	21.1 ± 0.9	15.4 ± 0.7
Xp ahpC	21.1 ± 0.8	16.5 ± 1.2
Xp ohrR	22.3 ± 1.2	16.3 ± 0.5
Xp ohr	21.3 ± 1.0	17.0 ± 0.6
Xp HR	21.8 ± 0.8	ND
Xp/pKatA	22.2 ± 0.7	15.9 ± 1.4
Xp/pAhpCF	21.2 ± 0.9	16.6 ± 0.7
Xp/pOhr	20.8 ± 0.9	16.1 ± 1.0
Xp oxyR/pOxyR	22.6 ± 0.06	ND
Xp oxyR/pOxyRC199S	28.3 ± 0.15	ND
Xp oxyR/pOxyR5	22.8 ± 0.07	ND
Xp oxyR/pOhrR	26.0 ± 0.14	ND
Xp oxyR/pKatA	26.9 ± 0.9	ND
Xp oxyR/pAhpCF	28.3 ± 1.2	ND
Xp oxyR/pOhr	25.5 ± 1.6	ND

[a] Inhibition zone assays were performed as described in Section 2. Exponential phase cells were spread on SB agar plates containing filter disks impregnated with 5 µl of 100 mM of sodium arsenite As(III) or 1 M sodium arsenate As(V). The inhibition zones were measured after incubation at 28 °C for 24 h. The diameters of the inhibition zones are indicated in mm. Results represent the means and standard errors of triplicate experiments. ND: not determined.

katA, *ahpC*, *ohr*, and *ohrR* (*Xp katA*, *Xp ahpC*, *Xp ohr*, and *Xp ohrR* strains, respectively) resulted in no significant alterations in arsenic resistance levels compared to wild-type *Xp* as judged by the diameter of the inhibition zones against either arsenite or arsenate. By contrast, an *oxyR* mutant (*Xp oxyR*) showed increased sensitivity to both arsenite and arsenate relative to wild-type *Xp*. In addition, the arsenite-sensitive phenotype of *Xp oxyR* could be complemented by the plasmid-borne expression of functional OxyR, as observed in *Xp oxyR*/pOxyR. These data indicated that OxyR plays a crucial role in protection of *Xanthomonas* from arsenic toxicity.

3.3. *Overexpression of* katA, ahpCF, ohr *did not confer increased protection against arsenic toxicity*

Overproduction of the peroxide-scavenging enzymes, catalase, alkyl hydroperoxide reductase, and organic hydroperoxide resistance protein in strains *Xp*/pKatA, *Xp*/pAhpCF, and *Xp*/pOhr, respectively, did not enhance arsenic tolerance compared to the parental strain *Xp* (Table 2). Additionally, inhibition zones obtained from the multiple peroxide-resistant mutant *Xp* HR and wild-type *Xp* were similar (Table 2), despite the fact that *Xp* HR has 100-fold-increased levels of catalase [3]. These results suggested that wild-type levels of these protective enzymes are sufficient to counter the ROS that are accumulated as a consequence of arsenic exposure.

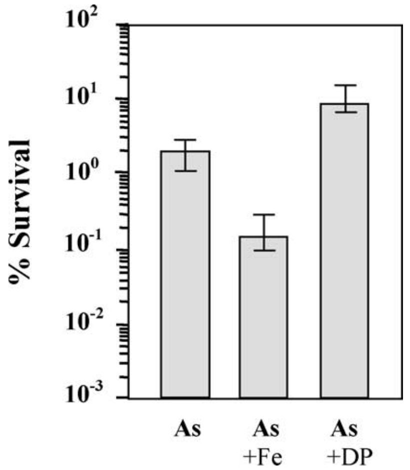


Fig. 2. Effect of iron level on arsenite toxicity in the *Xp oxyR* mutant. Survival assays were performed as described in Section 2. Exponential phase cells were treated with either 150 mM sodium arsenite (As), 150 mM sodium arsenite in the presence of 200 μM $FeCl_3$ (As + Fe) or 200 μM 2,2′-dipyridyl (As + DP). Values presented are means and standard deviations of three replicates.

3.4. *Arsenite resistance requires the active oxidized form of OxyR*

It has been reported that the redox status of OxyR determines its function such that expression of *oxyR*-regulated genes is repressed by reduced OxyR while the oxidized form acts as a transcriptional activator [11]. It is also known that a conserved cysteine residue at position 199 of OxyR is involved in the redox sensing [11]. We tested the ability of two mutant OxyRs, OxyRC199S, containing a cysteine 199 to serine substitution, and OxyR5, that is permanently locked in the oxidized form, to complement the arsenite sensitive phenotype of *Xp oxyR*. As shown in Table 2, *Xp oxyR* expression of OxyRC199S from pOxyRC199S had no effect on the arsenite-sensitive phenotype. However, the arsenite-sensitive phenotype of *Xp oxyR* was complemented when OxyR5 was expressed from pOxyR5 [11]. These data confirm that the oxidized form of OxyR is required for the maintenance of wild-type levels of arsenite resistance in *Xp*. Furthermore, the arsenite-sensitive phenotype in *Xp oxyR* could not be rescued by the introduction of plasmids encoding *katA*, *ahpCF*, or *ohr*. This suggests the existence of unidentified OxyR-regulated genes that are involved in conferring arsenic resistance to *Xp*.

3.5. *Role of iron in arsenite toxicity in* Xanthomonas

Studies have provided experimental evidence that superoxide anions and H_2O_2 are generated in various cellular systems in the presence of arsenite [13]. Iron is known to be involved in the generation of highly toxic hydroxyl radicals from superoxide anions and H_2O_2 via the Fenton reaction [4]. In addition, arsenic-induced release of iron from ferritin has been reported and likely contributes to arsenic toxicity [1]. We determined the effect of iron on arsenite toxicity using percent survival assays in a *Xp oxyR* mutant. Survival of arsenite-treated cells was negatively affected by iron (Fig. 2). The *Xp oxyR* mutant, treated with 150 mM arsenite in the presence of 200 μM $FeCl_3$ showed a 10-fold lower rate of survival than cells treated with arsenite alone. By contrast, co-treatment of cells with arsenite and an iron chelator (200 μM 2,2′-dipyridyl) had a protective effect against arsenite toxicity (Fig. 2). Similar findings were observed in the wild-type *Xp* (data not shown). These data provided evidence that iron plays an important role in arsenite toxicity in *Xanthomonas*.

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NOTES

Important Role for Methionine Sulfoxide Reductase in the Oxidative Stress Response of *Xanthomonas campestris* pv. phaseoli

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A methionine sulfoxide reductase gene (*msrA*) from *Xanthomonas campestris* pv. phaseoli has unique expression patterns and physiological function. *msrA* expression is growth dependent and is highly induced by exposure to oxidants and *N*-ethylmaleimide in an OxyR- and OhrR-independent manner. An *msrA* mutant showed increased sensitivity to oxidants but only during stationary phase.

Xanthomonas spp. are soil bacteria that are the causative agents of bacterial blight diseases in many economically important crops. Bacteria are constantly exposed to harmful reactive oxygen species (ROS) that originate from many sources, such as aerobic respiration, chemical pollutants in the environment, and the initial defense responses of plants to microbial invasion. ROS are highly reactive and can damage biological macromolecules, including proteins, nucleic acids, and lipids. Methionine residues in proteins are particularly susceptible to oxidation by ROS resulting in formation of racemic mixtures of methionine-*S*-sulfoxide and methionine-*R*-sulfoxide. Most eukaryotic and prokaryotic cells possess repair enzymes, such as peptide methionine sulfoxide reductases (Msr proteins), which catalyze the thioredoxin-dependent reduction of either free methionine sulfoxide [Met(O)] or protein-bound Met(O) to methionine. *Escherichia coli* and several other bacteria have two methionine sulfoxide reductases, namely, MsrA and MsrB, encoded by two structurally unrelated genes (20). MsrA and MsrB have distinct substrate specificities. MsrA uses only the S epimer, while MsrB uses the R epimer of Met(O) as a substrate (6, 17).

In bacteria, the physiological function of the Msr proteins has not been fully elucidated. In general, *msrA* is recognized as a gene required for bacterial virulence and survival under some stressful conditions (4, 18, 20). Examination of *msrA* expression patterns could give important clues as to its physiological function(s). While different bacteria appear to display different *msrA* expression patterns in response to various conditions, in no case has a regulator of *msrA* expression been identified. Moreover, there is little correlation between the gene expression pattern and any possible physiological role for the gene. For example, MsrA has been shown to play a significant role in the protection of several microorganisms from oxidative stress, and yet in none of these bacteria has the gene been shown to be oxidative stress inducible (5, 18–20, 22, 26). In many microorganisms, the mechanism of regulation of *msrA* expression and the physiological function(s) of the gene product remain to be elucidated.

In this paper, the expression patterns of *msrA* in *Xanthomonas campestris* pv. phaseoli were examined. The gene has novel patterns of growth-phase-dependent and oxidative-stress-inducible expression. The oxidative-stress-inducible expression of *msrA* is not regulated by known stress sensors and transcriptional regulators. Physiological analysis of an *msrA* mutant indicated that the gene plays an important role in the protection against oxidative stress.

Nucleotide sequence accession number. The nucleotide sequence determined in this study was assigned GenBank accession number AF404824.

Cloning, genome organization, and transcription of the *msrA* locus from *X. campestris* pv. phaseoli. The isolation of a genomic clone (pA301) containing *talA*, encoding a transaldolase, from *X. campestris* pv. phaseoli was reported previously (24). Analysis of the nucleotide sequence downstream of *talA* revealed the presence of an unidentified open reading frame (ORF) and a truncated ORF with high homology to the C-terminal region of MsrA. A fragment containing this truncated gene (0.45-kb SphI fragment from pA301) was used as a probe to isolate a DNA fragment containing full-length *msrA* from an existing genomic library constructed in λZip-lox (11). A positively hybridizing plaque was purified and excised into plasmid pA8. Analysis of the nucleotide sequence revealed that the fragment contained the putative *msrA* that was predicted to encode a 216-amino-acid polypeptide with a molecular mass of 23.5 kDa and a pI of 5.39. The deduced amino acid sequence of *Xanthomonas* MsrA showed a high degree of identity to both eukaryotic and prokaryotic peptide methionine sulfoxide reductases (MsrA). Analysis of the *Xanthomonas* MsrA amino acid sequence showed the presence of a conserved consensus sequence, GCFWG, that is thought to comprise the active site

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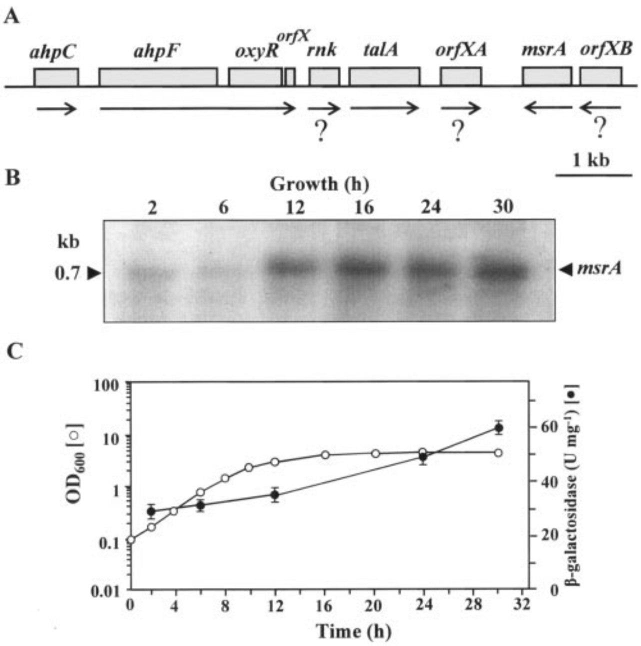

FIG. 1. Gene organization and growth-phase-dependent expression of *msrA*. (A) Physical and transcription maps of *msrA* in *X. campestris* pv. phaseoli. The arrows indicate the orientations and lengths of the transcripts. Question marks indicate uncharacterized genes. (B) Results of a Northern blot hybridization showing *msrA* expression at different growth phases. At the indicated times, RNA samples were prepared from cultures of *X. campestris* pv. phaseoli. RNA isolation, gel electrophoresis, and Northern blotting were done as previously described (15). Ten micrograms of total RNA was loaded into each lane. The blot was probed with a radioactively labeled *msrA* fragment (15). (C) Growth curve and expression analysis of *X. campestris* pv. phaseoli strain Xp08 containing an *msrA* promoter-*lacZ* fusion. The growth curve (○) of Xp08 (*msrA*::*lacZ*) in SB medium was determined at 28°C with continuous shaking at 150 rpm. At the indicated times, samples were removed and crude lysates were prepared and assayed for β-galactosidase activity (14). OD_{600}, optical density at 600 nm.

of the enzyme (17), and two cysteine residues at the C terminus which correspond to Cys-198 and Cys-206 of *E. coli* MsrA that have been shown to be involved in catalysis (17, 23).

msrA was located between two ORFs of unknown function (*orfXA* and *orfXB*) on the *X. campestris* pv. phaseoli genome (Fig. 1A). Nonetheless, the genes in this region showed an interesting organization. Comparison of the sequence of the *msrA* region of *X. campestris* pv. phaseoli with those of *X. campestris* pv. campestris and *Xanthomonas axonopodis* pv. citri showed that the gene organization *hemK-ahpC-ahpF-oxyR-orfX1-rnk-talA-orfXA-msrA-orfXB* (Fig. 1A) was conserved among the three bacteria (2). *msrA* is located in a region rich in genes involved in the oxidative stress response. We have shown that, in addition to *msrA*, *ahpC* and *ahpF*, encoding the catalytic and the reductase subunits of alkyl hydroperoxide reductase, respectively, and the peroxide sensor and transcription regulator OxyR are essential for the peroxide stress protection response (10, 13). Moreover, *talA* also plays an important role in protecting the bacteria from a superoxide generator, menadione (MD) (24).

A partial transcription map of the region is also shown (Fig. 1A). The transcripts encoding *ahpC*, *ahpF-oxyR-orfX1*, and *talA* have been previously determined (13, 24). Northern analysis, using a 340-bp SmaI-SphI *msrA*-specific fragment as a probe, indicated that *msrA* was transcribed on a 0.7-kb monocistronic mRNA (Fig. 1B).

Growth-phase-dependent expression of *msrA*. The expression of *msrA* during the different stages of bacterial growth has not been well studied. In some bacteria, *msrA* appears to play important roles in oxidative stress protection (4, 18, 20, 26). Hence, the timing of its expression is likely to be important, since the levels of resistance to oxidative stress vary significantly at different stages of growth (11). The growth-phase-dependent expression patterns of *msrA* were investigated by use of both Northern blot analysis and *msrA* promoter-*lacZ* fusion analysis. The results of Northern hybridizations showed that *msrA* was expressed at low levels during exponential-phase growth (Fig. 1B). The expression increased eightfold (as judged by densitometer analysis of Northern blots) as the culture entered the stationary phase and during the stationary phase. These results were independently confirmed by use of an *msrA* promoter-*lacZ* fusion construct. A promoterless *lacZ* was transcriptionally fused to *msrA* on the *X. campestris* pv. phaseoli chromosome (*msrA*::*lacZ*) to yield strain Xp08 by using the R6K-derived suicide plasmid pVIK112 (7) inserted with the 273-bp DNA fragment of the *msrA* coding region (corresponding to nucleotides 121 to 393) at EcoRI and SmaI sites. The plasmid was introduced into *X. campestris* pv. phaseoli by electroporation. Xp08 was selected by its kanamycin resistance and was confirmed by Southern blot analysis (data not shown). *msrA* promoter activity (β-galactosidase activity) was monitored in Xp08 throughout the different growth phases. As shown in Fig. 1C, the β-galactosidase activity increased twofold (from 30 to 60 U mg^{-1} protein) as growth proceeded from exponential to stationary phase, with peak β-galactosidase levels being attained as cells entered the stationary phase and during the stationary phase of growth. These results are consistent with those of the Northern hybridization analysis and indicated that the expression of *msrA* is stationary phase dependent. A similar pattern of *msrA* expression in *E. coli* has been observed (18). Generally, soil bacteria spend long periods in a nutrient-limited state and have evolved mechanisms to survive under starvation conditions that involve increasing the expression of genes that protect them from the various starvation-associated stresses (21). The growth-phase-dependent expression pattern of *msrA* suggests that it belongs to the starvation stress response genes. *msrA* is likely to play an important physiological role(s) during stationary phase. The mechanism(s) controlling stationary-phase-dependent gene expression in *Xanthomonas* is not known; analysis of the bacterial genome did not show any ORFs with high homology to RpoS, suggesting that other sigma factors or additional regulatory mechanisms may control stationary-phase-dependent *msrA* expression. Interestingly, the regulator of stationary-phase expression of *E. coli msrA* is also not known, but it has been shown that the regulator is not σ^S (18).

Oxidative stress induction of *msrA* expression. In several bacteria, *msrA* has been shown to be important in protecting bacteria from oxidative stress, probably by repairing oxidized Met residues (27). However, in the bacteria thus far investigated, *msrA* expression has not been shown to be induced by

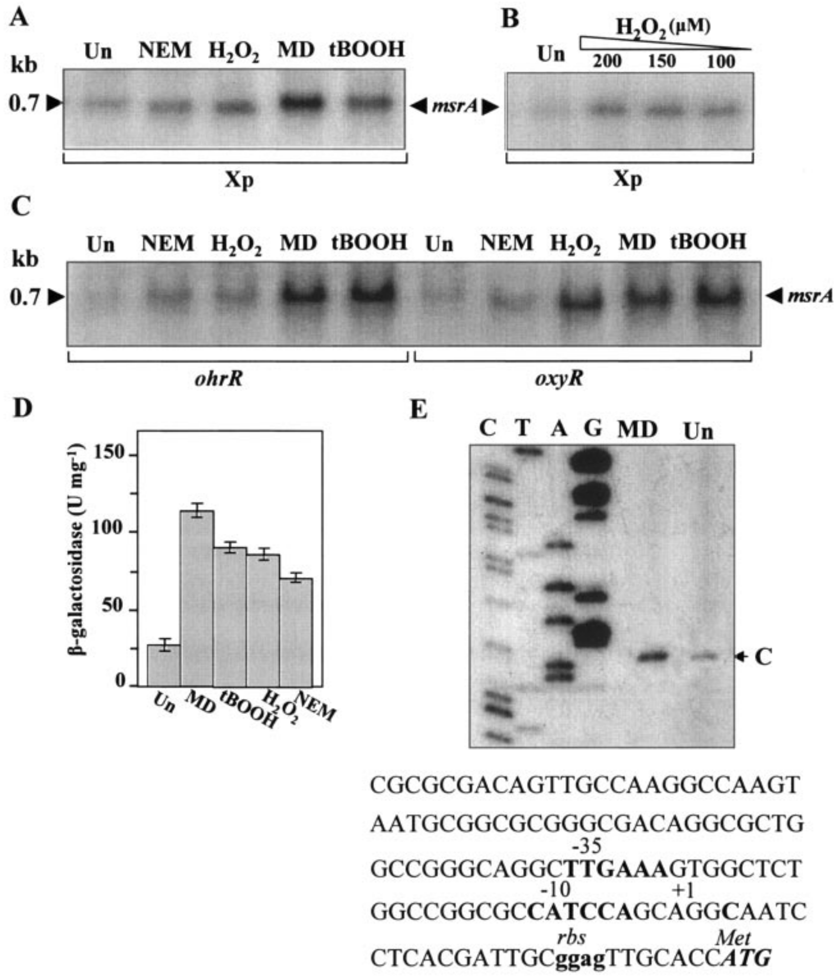

FIG. 2. Oxidant-inducible *msrA* expression and localization of the *msrA* promoter. *X. campestris* pv. phaseoli exponential-phase cultures were treated with 100 μM NEM, 200 μM H_2O_2, 200 μM MD, or 200 μM tBOOH for 10 min before total RNA was isolated, and Northern blots were prepared and probed with a radioactively labeled *msrA*-specific probe. Ten micrograms of total RNA was loaded into each lane in all Northern blot hybridization experiments. (A) Northern blot showing *msrA* expression in response to oxidant treatments in *X. campestris* pv. phaseoli (Xp). The arrow indicates the size of the *msrA* mRNA. Un, uninduced. (B) Northern blots of *msrA* expression in response to various concentrations of H_2O_2. (C) Northern blot showing *msrA* expression in *X. campestris* pv. phaseoli *oxyR* and *ohrR* mutants treated with various oxidants. The *ohrR* and *oxyR* mutants were grown and treated with oxidants as described for panel A with the exception that the *oxyR* mutant was treated with 100 μM H_2O_2, 100 μM MD, or 100 μM tBOOH. (D) Xp08 (*msrA-lacZ*) was grown and treated with various oxidants. Crude lysate preparation and β-galactosidase levels were determined as previously described (14). (E) Primer extension of RNA extracted from uninduced (Un) and MD-induced cultures. The experiment was performed using ^{32}P-labeled oligonucleotide primer BT110 (5′CTAACGTTGTTTGAAGGCG3′) as previously described. C, T, A, and G are sequence ladders generated by using the same primer. The arrowhead indicates the *msrA* transcription start site. Putative −35 and −10 regions are shown in bold, capital letters. A putative ribosome binding site (*rbs*) is marked in bold, lowercase letters, and the translation initiation codon ATG is in bold italics.

oxidative stress (5). This suggests that the constitutive basal expression of *msrA* is sufficient to confer protection against oxidative stress generated from internal and external sources. In *X. campestris* pv. phaseoli, as in other bacteria, exposure to sublethal levels of oxidants leads to a severalfold increase in the expression of oxidative-stress-protective enzymes, such as catalase (KatA), alkyl hydroperoxide reductase (AhpC), and organic hydroperoxide resistance thiol peroxidase (Ohr) (1, 13, 15). This inducible response plays an important role in protecting the bacterium against stresses. Thus, *msrA* expression in response to exposure to various oxidants was investigated by Northern blot analysis. Exponential-phase cultures of *X. campestris* pv. phaseoli grown in SB medium (15) were treated with *N*-ethylmaleimide (NEM), H_2O_2, MD, or *tert*-butyl hydroperoxide (tBOOH) for 10 min. RNA samples were extracted (15) and analyzed by Northern blot hybridization using a radioactively labeled *msrA* probe. The results in Fig. 2A show that pretreatment of the cultures with MD induced *msrA* expression 10-fold, while tBOOH, H_2O_2, and NEM pretreatments produced intermediate levels of induction of sixfold, threefold, and twofold, respectively. The oxidant induction of *msrA* promoter was done using an *msrA* promoter-*lacZ* fusion. A similar pattern of oxidant induction of *msrA* promoter was obtained in Xp08 (*msrA*::*lacZ*), with menadione being the most potent inducer, followed by H_2O_2, tBOOH, and NEM (Fig. 2D). In *Xanthomonas*, MD, H_2O_2, and tBOOH have all been shown to be potent inducers of genes in the OxyR regulon, while tBOOH also induces genes in the OhrR regulon (13, 15). NEM induction of *msrA* probably results from the depletion of thiol antioxidant molecules and the inactivation of oxidant scavenging enzymes that lead to oxidative stress. The observed pattern of oxidant-inducible *msrA* expression in *Xanthomonas* differs from previous reports of other bacteria, in which induction of *msrA* expression has been observed in response to a shift in pH (26), exposure to phenolic compounds (22), and treatment with cell wall-active antibiotics but not to oxidative stress (19).

Since *X. campestris* pv. phaseoli *msrA* displayed a unique oxidant-inducible expression pattern, we attempted to identify the regulator involved in controlling the expression of the gene. The oxidant-inducible expression pattern of *msrA* was similar to the patterns observed for many OxyR-regulated genes (10, 12). In *Xanthomonas*, OxyR is a peroxide sensor and a global transcriptional regulator of peroxide stress and OxyR-regulated genes are involved in the detoxification of H_2O_2 (*katA*) and organic hydroperoxides (*ahpC*) (1, 10, 13). Thus, analyses of the effects of oxidants on the expression of *msrA* in the wild type and an *oxyR* mutant were performed. The results shown in Fig. 2C clearly showed that the pattern of oxidant-induced *msrA* expression was not affected by inactivation of *oxyR*, since *msrA* transcription was highly induced by tBOOH. It should be noted that the inducing concentrations of oxidants were lowered to 100 μM for H_2O_2, MD, and tBOOH and 50 μM for NEM due to the *oxyR* mutant's inherent hypersensitivity to oxidants relative to the wild type (16). The effect of inactivation of the organic-hydroperoxide-sensing transcription repressor, *ohrR*, on *msrA* expression was also tested (14). The results of Northern blot analyses using the parental strain and an *ohrR* mutant showed that the profiles of oxidant induction of *msrA* in the two strains were similar (Fig. 2A and C). From these results, we concluded that both *oxyR* and *ohrR* are not responsible for the regulation of *msrA* expression. The evidence suggests the existence of an unidentified regulator(s) that could sense and respond to oxidative stress by increasing transcription of *msrA*.

The relative levels of peroxide induction in the wild type and *oxyR* and *ohrR* mutants reveal interesting patterns. The magnitude of H_2O_2 and tBOOH induction of *msrA* was lower in the parental strain than in either the *oxyR* or *ohr* mutant strain (Fig. 2B and C). This is due to the inability of the *oxyR* mutant to induce expression of the catalase and alkyl hydroperoxide reductase genes, which are responsible for H_2O_2 and organic hydroperoxide detoxification, respectively. Similarly, the *ohrR* mutant that has decreased *ohr* expression due to a polar effect of the mutation in *ohrR* (14) thus has a reduced capacity to metabolize organic hydroperoxide. Thus, in the regulatory mutants, intracellular peroxide levels were higher due to lower levels of peroxide detoxification enzymes. This would result in increased protein oxidation in the mutants relative to the parental strain, which in turn may stimulate higher levels of *msrA* induction. At present, the regulator(s) of oxidant-induced *msrA* expression has not been identified, and it remains to be seen whether such a regulator directly or indirectly senses oxidants and/or oxidized proteins.

In order to localize the *msrA* promoter region, the transcription initiation sites of *msrA* mRNA, isolated from uninduced and MD-induced cultures, were mapped by primer extension. The results shown in Fig. 2E showed a single predominant primer extension product corresponding to a transcription initiation site located 27 nucleotides upstream of the *msrA* translation start. Analysis of the sequence upstream of the transcription start site revealed the presence of a −10 promoter sequence, TTGAAA, separated by 17 nucleotides from a −35 promoter sequence, CATCCA. The *msrA* promoter −35 and −10 regions matched the consensus sequences for *X. campestris* promoters at 6/6 and 4/6 nucleotides, respectively (8). No sequences similar to the consensus binding sites for either OxyR or OhrR were found in the vicinity of the *msrA* transcription start. This was consistent with the results of the Northern blot analyses that indicated that OxyR and OhrR are not involved in the regulation of *msrA*. In addition, the primer extension results clearly showed that MD pretreatment increased *msrA* transcription initiation (Fig. 2E). Thus, the increase in the steady-state level of *msrA* mRNA after MD treatment is at least in part due to increased transcription of *msrA*.

Analysis of the physiological role of *msrA*. As mentioned previously, the physiological roles of *msrA* appear to differ in different bacteria. In order to determine the physiological role of *msrA* in *X. campestris* pv. phaseoli, an *msrA*-disrupted mutant was constructed by insertional inactivation using the nonreplicative plasmid pKStet (a tetracycline resistance derivative of pBluescript KSII [Stratagene]) containing a 220-bp internal fragment of *msrA*. The *msrA* mutant strain Xp07 was isolated, and the insertional inactivation of the gene was confirmed by both PCR and Southern blot analysis (data not shown). First, the aerobic growth rates of the mutant and the parental wild-type strain were determined in complex medium (Silva Buddenhagen [SB]) and minimal medium (M9). No significant difference between the growth rates of the strains was observed (data not shown). Thus, the loss of *msrA* function caused no adverse effects on bacterial growth. Recent reports suggested that in some bacteria, inactivation of *msrA* led to increased sensitivity to oxidative stress, indicating the importance of the gene in protecting bacteria from the stress. The level of resistance of the *msrA* mutant against various oxidants was determined as previously described (3) and compared with that of the parental strain. Exponential- and stationary-phase cultures were serially diluted and overlaid on SB agar plates containing the appropriate concentrations of oxidants, including H_2O_2, tBOOH, MD, and NEM. The surviving colonies were counted after 48 h of incubation at 28°C. During exponential-phase growth, Xp07 and the parental strain had similar levels of resistance to all oxidants tested (Fig. 3A). However, high-level expression of *msrA* from the plasmid pMsrA (broad-host-range plasmid pBBR1MCS-2 [9] containing the *msrA* gene) in Xp07 resulted in a small (7- to 10-fold) increase in the levels of resistance to H_2O_2, tBOOH, and NEM (Fig. 3A) relative to those of the parental strain. This observation suggested that the enzyme does not play a major role in protecting *X. campestris* pv. phaseoli from oxidant killing during the exponential phase of growth. Nonetheless, high-level expression of *msrA* does provide additional protection against oxidant killing.

Analysis of growth-phase-dependent oxidant resistance levels indicated that *msrA* played an important protective role against oxidant killing during the stationary phase of growth.

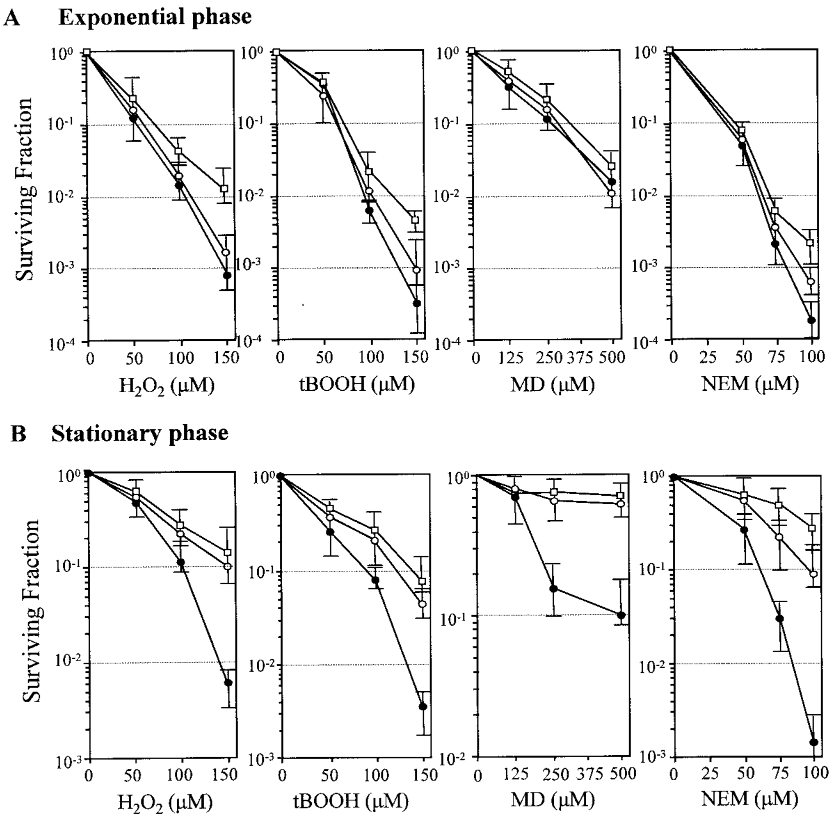

FIG. 3. Determination of oxidant resistance levels in *X. campestris* pv. phaseoli strains. *Xanthomonas* strains were grown in SB medium for 4 h for exponential-phase experiments and 30 h for stationary-phase experiments. Determination of oxidant resistance levels was done using a plate sensitivity assay as previously described (3) with some modifications. Essentially, cell aliquots were serially diluted in 50 mM sodium phosphate buffer, pH 7.0, prior to being plated on SB agar alone and SB agar containing the indicated concentration of oxidants. The colonies were scored after incubation at 28°C for 48 h. The surviving fraction was defined as the number of CFU from the oxidant-containing plate divided by the number of CFU from the SB agar plate. Experiments were repeated three times, and means and standard deviations are shown. The survival curves of the *msrA* mutant (Xp07 [●]), Xp07 harboring pMsrA (□), and the parental wild type (○) are shown.

The stationary-phase cells of *msrA* mutant strain Xp07 were 10- to 100-fold more sensitive to H_2O_2, tBOOH, MD, and NEM treatments than the parental strain (Fig. 3B), and the oxidant sensitivity phenotype of Xp07 could be complemented by pMsrA. Analysis of related *X. campestris* pv. campestris genome has shown the existence of *msrB*. The phenotypes of *msrA* mutant could not be complemented by expression of *msrB* in an expression vector (data not shown).

In *Xanthomonas*, as well as in other bacteria, stationary-phase cells are highly resistant to oxidant killing (11). The mechanisms responsible for stationary-phase resistance to oxidants are not fully understood but are thought to be independent of the levels of scavenging enzymes. It has been previously shown that the activities of oxidant-scavenging enzymes, such as catalase and superoxide dismutase, decreased as *Xanthomonas* cultures entered into the stationary phase and during the stationary phase of growth (11, 25). This is likely to lead to intracellular accumulation of oxidants and a subsequent increase in the oxidation of macromolecules. Thus, during stationary phase, enzymes that are involved in the various repair processes, such as MsrA, become important in protecting cells from intracellular oxidants. *Xanthomonas msrA* is the only protein oxidation repair system thus far studied that shows a good correlation between the gene expression pattern and its physiological role. During normal growth, exponential-phase cells are less likely to be damaged by oxidants, due to the presence of high levels of oxidant-scavenging enzymes. However, during exponential phase, the bacteria are still highly susceptible to extracellular oxidants. The oxidant-inducible expression of *msrA* during exponential phase provides the cells with additional MsrA to repair damage caused by exposure to extracellular oxidants. This is reflected in the low level of *msrA* expression during exponential phase. As growth continues into stationary phase, a decline in scavenging enzyme activities (11, 25) leads to an increase in the intracellular accumulation of oxidants and hence the need to increase *msrA* expression to repair oxidized proteins.

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NOTES

Multinucleated Giant Cell Formation and Apoptosis in Infected Host Cells Is Mediated by *Burkholderia pseudomallei* Type III Secretion Protein BipB

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Here we have assessed the role of a type III translocator protein, BipB, in the cell biology and virulence of *Burkholderia pseudomallei*. Genetic inactivation of *bipB* reduced multinucleated giant cell formation, cell-to-cell spreading of bacteria, and induction of apoptosis of J774A.1 macrophages. The *bipB* mutant was also significantly attenuated following intranasal challenge of BALB/c mice, whereas virulence was fully restored by complementation with a functional *bipB* gene.

Burkholderia pseudomallei, the etiological agent of melioidosis in humans and animals, is a gram-negative bacterium. Melioidosis is endemic in southeast Asia and tropical Australia and has been reported sporadically elsewhere (6). Currently, there is no vaccine against melioidosis. Uniquely among intracellular bacterial pathogens, *B. pseudomallei* induces host cell fusion leading to multinucleated giant cell (MNGC) formation in tissue culture models of infection (14). This novel phenotype may be relevant to pathogenesis, since granuloma formation and generation of MNGC are also found in tissues of humans with melioidosis (23). In addition to inducing MNGC formation, *B. pseudomallei* is able to spread from cell to cell and induce apoptotic death in infected host cells (14). The molecular mechanisms of these pathogenic characteristics have not been elucidated.

Analysis of the *B. pseudomallei* genome and several other studies have demonstrated the presence of a type III secretion system (TTSS) (for reviews, see references 3, 12, 17, 20, and 22). A knockout mutant of *B. pseudomallei* lacking a functional *bipD* gene, a homologue of *Salmonella enterica* serovar Typhimurium *sipD*, on the TTSS3/*bsa* cluster of TTSS exhibited reduced replication in murine macrophage-like cells (20), was significantly attenuated in BALB/c mice and gave partial protection against subsequent challenge with wild-type *B. pseudomallei* (19). These data correlated with the recent report that the TTSS3/*bsa* cluster is required for the pathogenicity of *B. pseudomallei* (21). In addition to BipD, *B. pseudomallei* BipB and BipC (46 and 30% amino acid identity to *Salmonella* SipB and SipC, respectively) have been identified in the TTSS3/*bsa* cluster (3). Here, we report on the role of BipB in the pathogenesis of infection with *B. pseudomallei*. With *Salmonella* organisms, purified SipB integrates into artificial membranes and induces liposome fusion (10), and it is required for inducing apoptosis in murine macrophages (11). By analogy with SipB, therefore, we investigated the role of BipB for MNGC formation, cell-to-cell spreading, and induction of apoptosis in infected host cells. We also examined the virulence of a *B. pseudomallei bipB* mutant in a murine model of melioidosis.

Construction of a *B. pseudomallei bipB* mutant. Analysis of the *B. pseudomallei* genome (http://www.sanger.ac.uk/Projects/B_pseudomallei), by use of the *sipB* sequence from *S. enterica* serovar Typhimurium as the query in a TBLASTX search, identified a coding sequence of 1,860 bp encoding the predicted BipB protein of 620 amino acids. In order to determine the function of BipB in *B. pseudomallei*, a chromosomal *bipB* mutant of *B. pseudomallei* was constructed. In brief, a 250-bp internal fragment of the *bipB* gene was amplified from *B. pseudomallei* K96243 genomic DNA by use of primers BipB-45 (5′-AACCAGGCCACGCAGCAG-3′) and BipB-46 (5′-CGTCTTCTGCATCTCCTC-3′). The amplified fragment was cloned into a suicide vector, pKNOCK-Tc (1), kindly provided by M. F. Alexeyev. This constructed plasmid was introduced from *Escherichia coli* S17-1λpir (7) into *B. pseudomallei* K96243 by conjugation. Transconjugants were selected by plating on pseudomonas agar supplemented with SR103 (Oxoid, United Kingdom) containing tetracycline. The isolated mutant, designated *B. pseudomallei* BS46 (*bipB*::pSSB-1), was verified by PCR and Southern blot hybridization to ensure insertion of the *bipB* suicide plasmid at the correct location (data not shown). For complementation analysis, the amplified *bipB*

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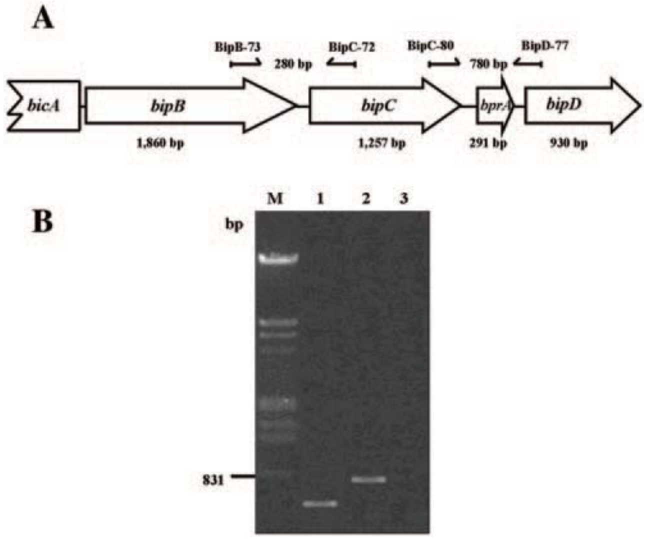

FIG. 1. The *B. pseudomallei bipB* operon. (A) Physical map of *bipB-bipC-bprA-bipD* gene organization together with locations of primer pairs BipB-73–BipC-72 and BipC-80–BipD-77 for RT-PCR analysis of *B. pseudomallei bipB* operon. (B) Ethidium bromide-stained gel showing the amplified DNA of RT-PCR products from primer pairs BipB-73–BipC-72 (lane 1) and BipC-80–BipD-77 (lane 2). Lane 3 is an RNA sample subjected to PCR to ensure no DNA contamination in the RNA preparation. Lane M shows lambda DNA markers.

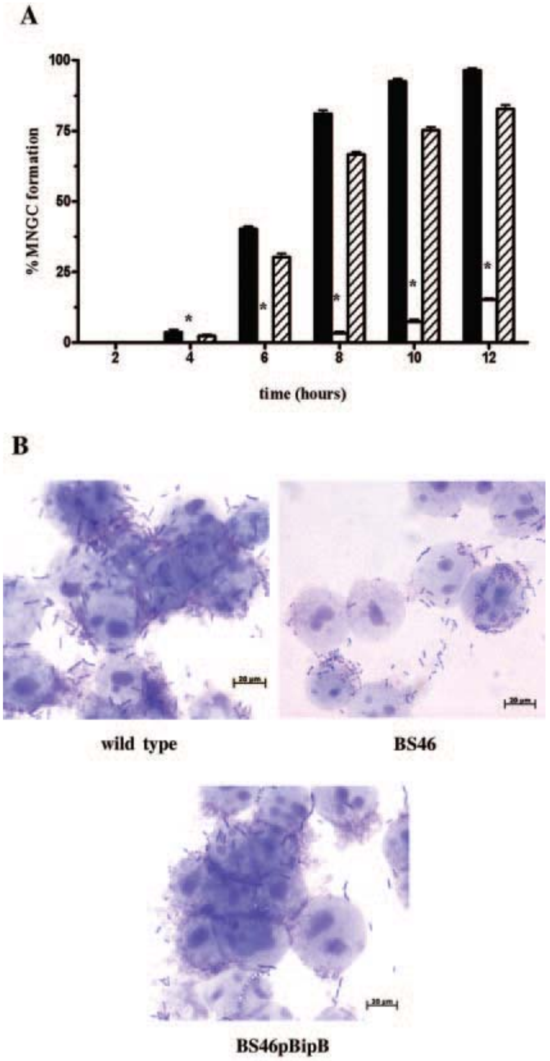

FIG. 2. MNGC formation of *B. pseudomallei*. (A) The percentages of MNGC formation of J774A.1 cells infected with *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), and BS46pBipB (BS46 harboring pBipB; striped bars) were determined every 2 h. Asterisks indicate significant differences ($P < 0.05$, t test) between the wild type and BS46 at 4 h ($P = 0.0142$) and 6 to 12 h ($P < 0.0001$) and between BS46 and BS46pBipB at 4 h ($P = 0.0155$) and 6 to 12 h ($P < 0.0001$). Percentage of MNGC formation was determined by the following equation: MNGC formation = (number of nuclei within multinucleated giant cells/total number of nuclei counted) $\times$ 100. Error bars represent standard errors of the means for experiments performed in triplicate. (B) Giemsa staining of MNGC formation of J774A.1 cells infected with wild type, BS46, or BS46pBipB. Bars, 20 μm.

gene was cloned into pBBR1MCS (15) and introduced into *B. pseudomallei* BS46. To confirm that *B. pseudomallei* BS46pBipB contained the *bipB* gene, the DNA plasmid was extracted and sequenced (data not shown).

To determine whether *bipB* was cotranscribed with the downstream genes *bipC-bprA-bipD*, reverse transcription-PCR (RT-PCR) was undertaken. Extraction of total RNA, by use of the modified hot acid phenol method, was carried out as described previously (2). In brief, mid-exponential-phase cultures were harvested and extracted with hot acid phenol. Total RNA was precipitated and resuspended with RNase-free distilled water. For RT-PCR analysis, *bipB-bipC-bprA-bipD* was reversed transcribed into cDNA (Invitrogen) and then amplified with different primers, namely, BipB-73 (5′-CTGCTCGGCGATCTGCTCAA-3′), BipC-72 (5′-ACCGCCTTGTCGCCCTG-3′), BipC-80 (5′-GAGCAGAAAGAGGACGAGA-3′), and BipD-77 (5′-CGCAGATCGTCGTCGGTCA-3′) (Fig. 1A).

As depicted in Fig. 1B, *B. pseudomallei bipB-bipC-bprA-bipD* was transcribed in a single transcriptional unit. It is likely that *B. pseudomallei* BS46 is a polar *bipB* mutant. To investigate whether this mutation does not have effect on expression of other secreted proteins, Western blot analysis using anti-BopE (kindly provided by M. P. Stevens, United Kingdom) to detect BopE in whole-cell and secreted protein fractions of *B. pseudomallei* BS46 and wild-type strains was undertaken. BopE, homologous to the *Salmonella* SopE, was an effector protein secreted by the *B. pseudomallei* TTSS (18). BopE was detected in both whole-cell and secreted protein fractions of *B. pseudomallei* BS46 (data not shown). This suggests that the TTSS of *B. pseudomallei* BS46 is still functional to express and secrete other proteins such as BopE.

The polar *bipB* mutant is defective in MNGC formation. To investigate the potential role of BipB in MNGC formation, *B. pseudomallei* K96243 (wild type), BS46 (*bipB*::pSSB-1), and BS46pBipB (BS46 harboring pBipB) were used to infect J774A.1 murine macrophage-like cells as described previously (14). At different times after initiation of the challenge, the infected cells were fixed, Giemsa stained, and evaluated for MNGC formation. Figure 2 shows that BipB protein plays a role in *B. pseudomallei*-induced MNGC formation. At 12 h postinfection (Fig. 2A), wild-type bacteria induced extensive MNGC formation (96.46%), while BS46 did not (15.12%). The formation of MNGC was restored in a complementation assay using strain BS46pBipB (82.7%). Figure 2B shows that MNGC loaded with numerous bacilli could be readily observed at 6 h after infection with wild-type bacteria but that this was abolished in the *bipB* mutant BS46. However, this defective phenotype was transcomplemented by reintroduction of the plasmid-born *bipB* gene. However, when the observation period was extended to 24 h, formation of MNGC in BS46-infected macrophage did occur but was still significantly less than the wild-type strain. Thus, BipB is necessary for optimal MNGC induction, but BipB-independent fusion can also occur, albeit at a reduced efficiency.

The mechanism for the MNGC formation is still unknown, and to our knowledge, this altered phenotype has not been observed in other intracellular bacteria that possess the TTSS. Based on the *Salmonella* SipB-induced fusion events in vitro (10) and those that would be transient in vivo (9), we hypothesize that BipB may have membrane fusion activity as well. It may act in concert with other proteins to induce fusion of host cell membranes. A combination of biochemistry, cell biology, and proteomics will be required to unveil the detailed pathways of MNGC formation.

The polar *bipB* mutant is defective in cell-to-cell spread and invasion into epithelial cells. The observation of MNGC led us to look closely at cell-to-cell spread of infected host cells by using a plaque assay previously described (14). HeLa cells were infected with *B. pseudomallei* and overlaid with an agarose medium containing kanamycin (250 μg/ml). To enhance visualization, plaques were overlaid with agarose containing an additional 0.01% neutral red and observed 4 h later. Figure 3A demonstrates that plaque-forming efficiencies for *B. pseudomallei* wild type (2.66) and BS46pBipB (0.68) were significantly higher than that for BS46 (0.2). It is possible that only partial complementation in BS46pBipB could have resulted from a polar effect that disrupted downstream *bipC* and *bipD* genes also participating in cell-to-cell spreading. This hypothesis is supported by a previous report, from Stevens et al. (20), that a *bipD* mutant exhibited an inability to escape from endocytic vacuoles, a requirement for cell-to-cell spread. If so, it would indicate that BipB works cooperatively with BipC and BipD in a manner similar to that of SipABCD in *Salmonella* (4).

The strategies that intracellular bacteria, i.e., *Listeria* sp. and *Shigella* sp., use to spread from cell to cell via interepithelial protrusion are quite similar (8). The process depends on the efficiency of bacterial invasion into the epithelial cytosol, protrusion formation, and the lysis of the double-membrane-bound protrusion vacuole to release bacteria into the adjacent cell. To investigate whether defective cell-to-cell spread (as detected by plaque assay) was due to an invasion defect, invasion efficiency was determined by using human respiratory epithelial cell line A549 challenged with *B. pseudomallei* as described earlier. This cell line was chosen because it is more susceptible to invasion than HeLa cells. Intracellular bacteria were counted after lysing of infected cells. Invasion efficiency of BS46 was severely restricted (0.09%) when compared to that of the wild type (0.39%), but invasion efficiency was restored to nearly normal levels in BS46pBipB (0.28%) (Fig. 3B). These data correlated with those for the *bipD* mutant that exhibited impaired entry into nonphagocytic host cells (18). In this scenario, we believe that several effector proteins, such as BopE, that contribute to invasion (18) would not be delivered into the host cell cytoplasm, even though it was expressed. This proposed mechanism is based on the study of *Salmonella* in which inactivation of *sip* genes resulted in impaired invasion effi-

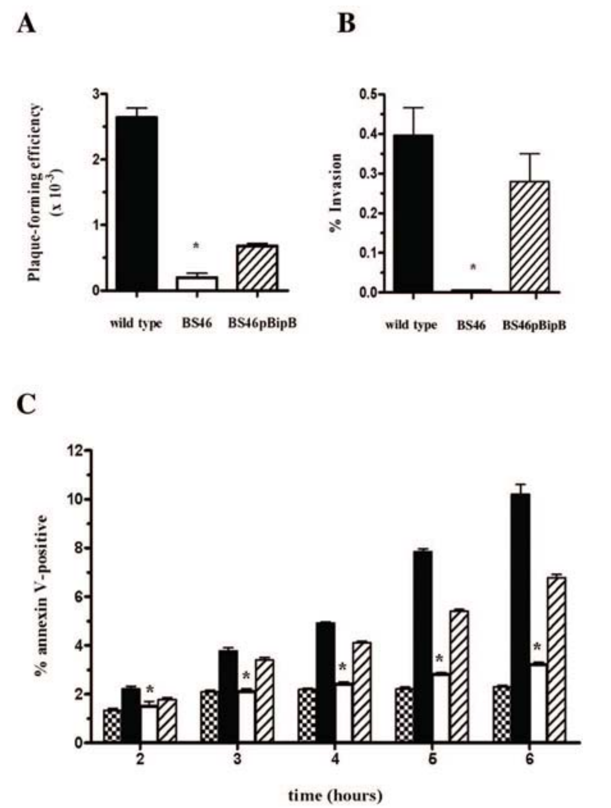

FIG. 3. Plaque formations, invasion, and apoptosis induction. (A) Plaque formations of HeLa cells by *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), and BS46pBipB (BS46 harboring pBipB; striped bars). Asterisks indicate significant differences ($P < 0.05$, *t* test) between wild type and BS46 ($P = 0.0001$) and between BS46 and BS46pBipB ($P = 0.0031$). Plaque-forming efficiency was determined by the following equation: plaque-forming efficiency = number of plaques/bacterial CFU added per well. Error bars represent standard errors of the means for experiments performed in triplicate. (B) Invasion of A549 cells by *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), and BS46pBipB (BS46 harboring pBipB; striped bars) strains. Asterisks indicate significant differences ($P < 0.05$, *t* test) between wild type and BS46 ($P = 0.0050$) and between BS46 and BS46pBipB ($P = 0.0173$). Percent invasion was determined by the following equation: invasion = (number of intracellular bacteria postinfection/number of CFU added) × 100. Error bars represent standard errors of the means for experiments performed in triplicate. (C) Effect of *bipB* mutation on induction of apoptosis. J774A.1 cells were infected with *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), BS46pBipB (BS46 harboring pBipB; striped bars), and uninfected cells (checkered bars). The percentages of J774A.1 cells stained fluorescein isothiocyanate positive and propidium iodide negative by flow cytometry were analyzed. Asterisks indicate significant differences ($P < 0.05$, *t* test) between wild type and BS46 at 2 h ($P = 0.0123$), 3 h ($P = 0.0004$), and 4 to 6 h ($P < 0.0001$) and between BS46 and BS46pBipB at 2 h ($P = 0.1064$), 3 h ($P = 0.0006$), and 4 to 6 h ($P < 0.0001$). Error bars represent standard errors of the means for experiments performed in triplicate.

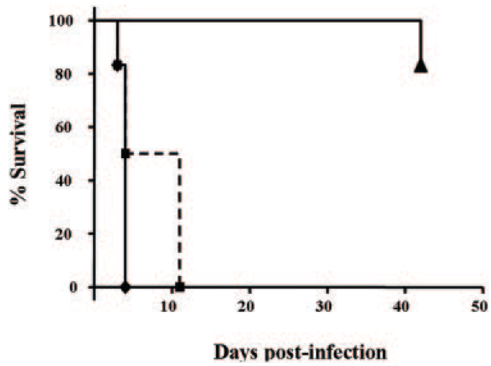

FIG. 4. Survival of BALB/c mice (six mice per group) inoculated intranasally with 10^3 CFU of *B. pseudomallei* K96243 (■) or BS46 (▲) or BS46pBipB (◆). Mice were observed daily, and percent survival was plotted against time.

ciency due to the lack of translocation of effector proteins, such as SopE, into host cells (4, 13, 24). In addition to invasion, BipB may play a role in other steps involved in cell-to-cell spreading. Further experiments are required to investigate this possibility.

The polar *bipB* mutant is defective in induction of apoptosis. *B. pseudomallei* can induce apoptotic death in infected macrophages (14). To determine the role of BipB in this process, J774A.1 cells were infected with *B. pseudomallei* strains. At different time intervals, the supernatant and cells were collected to quantify the apoptosis level by using an annexin V-fluorescein isothiocyanate detection kit (BD Biosciences, CA). At 6 h postinfection (Fig. 3C), cells infected with wild-type *B. pseudomallei* yielded significantly higher numbers of positive cells (10.20%) than those infected with BS46 (3.21%). Infection with BS46pBipB restored cytotoxicity (6.77%). These data indicated that BipB was required for efficient induction of apoptosis in host cells, although a low level of apoptosis may occur via a BipB-independent mechanism, since the level of apoptosis in uninfected cells is 2.3%. This is the first report identifying a *B. pseudomallei* virulence factor that mediates apoptosis. Interestingly, this finding joins a growing list of bacteria, including *Pseudomonas aeruginosa*, *Yersinia* sp., *Salmonella* sp., and *Shigella flexneri*, that kill host cells via apoptotic death through a type III secretion-mediated mechanism. In *Salmonella* and *Shigella*, SipB and IpaB have been shown to induce macrophage apoptotic death by activating caspase-1 (11, 25). Here, we also expect that apoptosis induced by *B. pseudomallei* will involve BipB interaction with the caspase pathway (14).

Effect of *bipB* mutation on virulence of *B. pseudomallei* in vivo. The finding that BipB is important in induction of MNGC, plaque formation, bacterial invasion, and killing of phagocytic cells in vitro led to the hypothesis that a mutant unable to produce this protein could be less virulent than the wild-type strain in vivo. We therefore assayed the virulence of the *bipB* mutant in a pulmonary model of melioidosis in BALB/c mice as previously described (19). *B. pseudomallei* strains were administered via the intranasal route. Viable counts were performed to confirm the inoculation dose, and the mice were monitored twice daily for signs of infection. There was a significant difference in percentage survival (the P value was <0.05, as determined by a log rank test) for mice infected with wild-type *B. pseudomallei* versus mice infected with BS46 (Fig. 4). All mice given the wild-type strain died within 5 to 11 days, whereas five of six mice infected with the *bipB* mutant survived until day 42 (termination of experiment). To confirm that attenuation resulted from the inactivation of *bipB*, we also challenged mice with strain BS46pBipB, and all died by day 4 postchallenge (Fig. 4), which was not significantly different from the wild-type strain. These observations indicated that a functional *bipB* gene was required for full virulence of *B. pseudomallei* in mice. This result is supported by previous reports (19, 21) that TTSS3/Bsa plays an important role for maximal virulence in all of its animal hosts.

Delivery of virulence-associated effector proteins into eukaryotic cells requires a set of translocator proteins. The translocons are components of oligomeric protein channels that insert themselves into the eukaryotic cell membrane to form a pore which effector proteins can pass through to gain access to the cytosolic host targets (5, 16). We have shown here that BipB translocator plays a critical role in the intracellular lifestyle of *B. pseudomallei* (i.e., MNGC formation, invasion of nonphagocytic cells, and induction of apoptotic death). We hypothesize that the *bipB* mutant is unable to deliver the effector proteins into the host cell cytoplasm and was thus impaired in invasion efficiency and ability to induce apoptosis. However, it is also possible that BipB acts as an effector protein to induce apoptotic death. Deletion of BipB clearly also reduces the efficiency of MNGC formation; however, the relationship between BipB protein and the fusion process is still under investigation. In vivo, BipB was required for full virulence of *B. pseudomallei* in mice, thus further confirming the importance of BipB for virulence in murine models of melioidosis.

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