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Physiological and Expression Analyses of *Agrobacterium tumefaciens trxA*, Encoding Thioredoxin▽

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Exposure of *Agrobacterium tumefaciens* to menadione, cumene hydroperoxide, and diamide strongly induced *trxA* expression. The *trxA* mutant showed a reduction in the aerobic growth rate and plating efficiency and was cytochrome *c* oxidase negative. Atypically, the mutant has decreased resistance to menadione but an increased H_2O_2 resistance phenotype.

Agrobacterium tumefaciens is a soilborne gram-negative pathogenic bacterium causing crown gall tumors in many dicotyledonous plants (29). As a plant pathogen and soil bacterium, *A. tumefaciens* is exposed to oxidative stress generated from aerobic metabolism, chemicals in the environment, and plant-generated oxidative bursts during plant-microbe interactions (16). Oxidative stress is highly toxic to bacterial cells, and they have evolved both enzymatic and nonenzymatic pathways to directly detoxify reactive oxygen species and repair oxidative stress-induced damage. This response involves the well-orchestrated coordination of gene expression governed by multiple transcription regulators.

Thioredoxins are small, ubiquitous, and evolutionarily conserved proteins involved in numerous biochemical processes (11). Thioredoxin has the ability to reduce disulfide bonds in target proteins. The two redox-active cysteine residues can be oxidized to form a disulfide and rereduced by the flavoenzyme thioredoxin reductase in an NADPH-dependent reaction. In its function as a thiol:disulfide reductant, thioredoxin, together with glutathione, contributes to the defense against oxidative stress by reducing disulfide bonds in oxidized proteins (4, 25). Thioredoxin is also an electron donor for several enzymes, such as ribonucleotide reductase, methionine sulfoxide reductase, and thiol peroxidases (3, 24). Moreover, the thioredoxin system plays important regulatory roles in cells (17, 33).

Several stresses have been shown to induce thioredoxin expression in prokaryotes. In *Escherichia coli* and *Rhodobacter capsulatus*, *trxC*, encoding thioredoxin 2, is a member of the peroxide-inducible OxyR regulon (26, 34). Moreover, induction of thioredoxin synthesis by oxidative stress has been observed in other bacteria (14, 18, 27, 30).

In this communication, we report the expression analysis and physiological characterization of *trxA* in *A. tumefaciens*. *trxA* has important roles in oxidative stress protection.

***A. tumefaciens trxA*.** An annotated open reading frame, Atu0022, on the *A. tumefaciens* C58 circular chromosome encodes a 106-amino-acid protein product of 11.1 kDa identified as TrxAC1 (32). Its deduced primary structure has 49 and 50% identity to *Bacillus subtilis* (5) and *E. coli* (12) TrxA, respectively (data not shown). The highly conserved thioredoxin active site, Trp-Cys-Gly-Pro-Cys-Lys, is conserved in Atu0022. Atu0022 likely encodes a TrxA protein and is henceforth designated *trxA*. *A. tumefaciens trxA* is located next to *folC* (Atu0021), a convergently transcribed gene encoding a homolog of dihydrofolate synthase. The gene upstream of *trxA* was annotated as *uvrD* (Atu0023), encoding DNA helicase, an important enzyme in DNA repair and replication. *uvrD* and *trxA* are transcribed in the same orientation and are separated by a 77-bp region containing the promoter of *trxA*. The *folC-trxA-uvrD* gene organization is conserved in several *Alphaproteobacteria* (data not shown). Many bacteria have two *trx* genes; thus, an *E. coli* thioredoxin 2 amino acid sequence encoded by *trxC* (20) was used to search with BlastP (2) the *A. tumefaciens* C58 genome (10, 32). No close homologs of the protein could be identified.

Oxidative stress-induced *trxA* expression. Bacterial *trxA* has complex expression patterns. Heat, osmotic, and oxidative stresses have been shown to induce *trxA* expression (14, 18, 27, 30). Hence, *trxA* expression in response to oxidants and a thiol-depleting agent was determined by Northern analysis. RNA samples extracted from *A. tumefaciens* NTL4 (19) exponential-phase cultures uninduced and induced with 250 μM H_2O_2, 200 μM diamide (DA) (a thiol-oxidizing agent), 200 μM cumene hydroperoxide (CHP), and 200 μM menadione (MD) were used. The Northern analysis results reveal a positive hybridization band of 360 bases with the *trxA* probe. This corresponds to monocistronic *trxA* transcripts (Fig. 1A). The levels of *trxA* mRNA show large increases in response to MD treatment (12-fold, as judged from densitometric analysis) and to a lesser extent in response to treatment with DA (7-fold) and CHP (5-fold). By contrast, treatment with 250 μM H_2O_2 did not induce *trxA* expression. The results indicate that *trxA* expression is under oxidative stress regulation and reflect the need of *A. tumefaciens* for thioredoxin molecules in the cells during oxidative stress. The inability of H_2O_2 to induce *trxA* expression suggests that, unlike in other bacteria, *A. tumefaciens* TrxA might not be required as a cofactor for H_2O_2 metabolism and/or protection. The stress-inducible regulation of *trxA* expression is an important strategy enabling *A. tume-*

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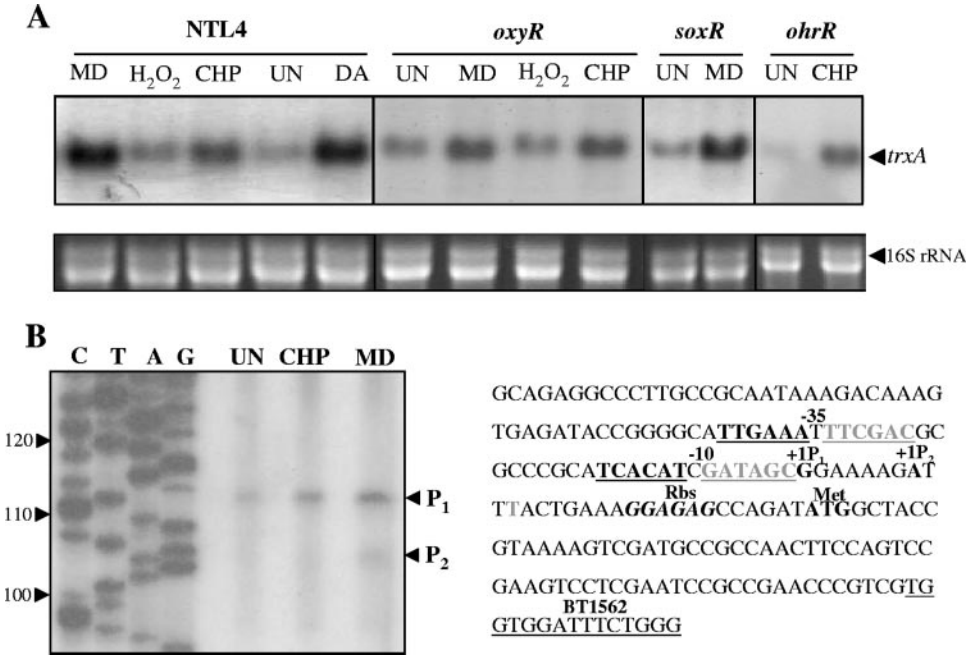

FIG. 1. Expression analysis and promoter localization of *A. tumefaciens trxA*. (A) Northern blot of total RNA (10 μg) samples prepared as previously described from *A. tumefaciens* exponential-phase cultures (7): uninduced (UN) and induced with 250 μM H_2O_2, 200 μM CHP, 200 μM MD, and 200 μM DA for 15 min. The RNA samples were separated, blotted, and hybridized with a ^{32}P-labeled *trxA*-specific probe. 16S rRNA levels were used as loading controls and are shown below the autoradiograph of the Northern blot. The smaller RNA bands running below the 16S rRNA were the result of processing of the 23S rRNA. (B) Primer extension of RNA extracted from uninduced (UN) and CHP- and MD-induced cultures. Primer extension experiments were performed as previously described (7) with the ^{32}P-labeled oligonucleotide primer BT1562 (5′ CCCAGAAATCCACCACGAC 3′). The DNA sequence ladders G, A, T, and C were generated by a PCR sequencing kit with labeled pUC/M13 forward primer and pGEM-3Zf (+) as the template (Applied Biosystems). Numbers next to the ladder represent the length of the fragment from the primer. The arrowheads labeled P1 and P2 indicate *trxA* transcription start sites. The −10 and −35 promoter regions of P1 (black) and P2 (gray) are shown in bold and underlined. A putative ribosome binding site (Rbs) is shown in italics.

faciens to adjust the intracellular level of thioredoxin so that proper cellular functions under stressful conditions can be maintained.

In various bacteria, diverse transcriptional regulators are involved in regulation of *trx* expression. The observed patterns of oxidants inducing *A. tumefaciens trxA* expression have similarity to patterns of *A. tumefaciens* OxyR (a H_2O_2 sensor/transcription regulator)-regulated genes (21). In addition, SoxR (7) and OhrR (6) are members of oxidative stress-responsive transcription regulators that sense increased levels of superoxide anions and organic hydroperoxides, respectively. Northern analyses of MD and CHP induction of *trxA* expression in *A. tumefaciens* NTL4 *oxyR*, *soxR*, and *ohrR* mutants were performed. The results in Fig. 1A illustrate that induction of *trxA* expression by MD and CHP is independent of the known oxidative sensor transcription regulators OxyR, SoxR, and OhrR. Oxidant-induced expression of *trxA* is likely to be regulated by a novel unknown oxidant sensor/transcription regulator. This possibility is being investigated.

Characterization of the *trxA* promoter. Primer extension analysis was performed to localize the 5′ end of *trxA* mRNA and the transcription start site of *trxA* with total RNA from bacteria grown under uninduced and CHP- and MD-induced conditions. Two primer extension products were identified (Fig. 1B). The majority of *trxA* transcripts initiated at a G residue situated 29 nucleotides upstream of the assumed *trxA* ATG codon. *E. coli* RNA polymerase δ^{70} consensus −10- and −35-like motifs were identified as TCACAT and TTGAAA, respectively, and were separated by 16 bp (Fig. 1B). The *trxA* promoter architecture resembles typical strong promoters in *A. tumefaciens* (6, 7). An additional minor 5′ end of the *trxA* mRNA was mapped to a T residue located 10 nucleotides downstream of the major *trxA* transcription start site. The putative −35 (TTCGAC) and −10 (GATAGC) regions were atypical of *A. tumefaciens* promoters, a finding which is reflected in the low levels of transcription initiation for this minor promoter. Alternatively, the minor 5′ end of *trxA* mRNA could arise from premature termination of reverse transcriptase. The primer extension results indicated that both the *trxA* major promoter and the putative *trxA* minor promoter were induced by CHP and MD pretreatments. The putative binding sites for the *A. tumefaciens* oxidative stress sensor transcription regulators OxyR, OhrR, and SoxR have been reported (6, 7, 21). The binding sites for these regulators could not be found in the vicinity of either the major or minor *trxA* promoter; this result further supports the Northern blot analysis of *trxA* expression, which shows the gene to be regulated by a novel unknown regulator.

Physiological analysis of the *trxA* mutant. In many bacteria, inactivation of *trxA* has resulted in pleiotropic changes in bacterial phenotypes, including hypersensitivity to stresses, growth defects, and cell death (4, 14, 25, 27). In order to evaluate the function of *trxA* in *A. tumefaciens*, a *trxA* mutant was constructed by targeted insertion inactivation of the gene by

pKnocktrxA, performed as previously described (1, 6), and the mutant was confirmed by Southern analysis. First, we examined the growth rate of the *trxA* mutant and the isogenic wild-type strain NTL4 grown in a rich medium (Luria-Bertani [LB]). The *trxA* mutant displayed a growth defect in LB medium by a prolonged exponential phase, with the mutant having a doubling time of 99 min, compared with 83 min for the NTL4 parental strain (data not shown). Moreover, the *trxA* mutant showed a defect in the aerobic plating efficiency. The mutant had an aerobic plating efficiency 2 orders of magnitude lower than that of NTL4 (Fig. 2A). However, when the plating efficiency was assessed under a microaerobic atmosphere (6% oxygen and 12% carbon dioxide) generated from the CampyGen system (Oxoid, United Kingdom), the mutant plating efficiency was less than 1 order of magnitude lower than that of the wild type (data not shown). This suggests that oxygen under aerobic conditions caused the reduced plating efficiency of the *trxA* mutant. Both the retarded growth rate and reduced plating efficiency phenotypes of the *trxA* mutant could be completely restored by *trans* expression of *trxA* from pTrxA (*trxA* in the broad-host-range plasmid vector pBBR1MSC-2 [15]), as illustrated in Fig. 2A (*trxA*/pTrxA), indicating that these phenotypes resulted from lack of thioredoxin. Thus, we further tested the ability of dithiothreitol (DTT), a disulfide reductant capable of crossing membranes, to complement the growth defect phenotypes of the *trxA* mutant. The results show that addition of 100 μM to 5 mM DTT to the culture medium failed to alleviate growth defects in the *trxA* mutant (data not shown), suggesting that the inability to reduce disulfide bonds of cytoplasmic proteins and small molecules such as oxidized coenzyme A and oxidized glutathione were not key factors in causing the growth defect in the *trxA* mutant.

Thioredoxin is involved in many enzymatic systems and has diverse functions, both in cellular processes and antioxidant defenses (4). The reduced aerobic plating efficiency phenotype has been shown previously to be associated with defects in antioxidant defense systems that cause accumulation of oxidants and subsequent cellular damage. This led us to test whether high-level expression of genes encoding antioxidant enzymes from an expression vector could rescue the aerobic growth defects of the *trxA* mutant. High levels of the *Agrobacterium* catalase-peroxidase KatA (*trxA*/pKatA) (22), organic hydroperoxide resistance peroxidase (*trxA*/pOhr) (6), and a cytoplasmic iron superoxide dismutase (SOD) (*trxA*/pSod1) (S. Mongkolsuk et al., unpublished data) partially rescued the reduced plating efficiency phenotype of the *trxA* mutant (Fig. 2A). It appears likely that the reduced aerobic plating efficiency phenotype was at least in part due to intracellular accumulation of different types of reactive oxygen species, including H_2O_2, organic hydroperoxides, and superoxide anions. In addition, decreased levels of some essential reductase enzymes that require thioredoxin for their activities, such as ribonucleotide reductase, could have adverse effects on bacterial growth (25). Methionine sulfoxide reductase, an enzyme that repairs oxidized proteins, also requires thioredoxin as a reducing equivalent. Thus, reduction in these enzyme activities could be partially responsible for the *trxA* phenotype.

TrxA not only is involved in the reduction of protein disulfides in the cytoplasm but also is a component of the electron transfer pathway from the cytoplasm to the periplasmic thiol-

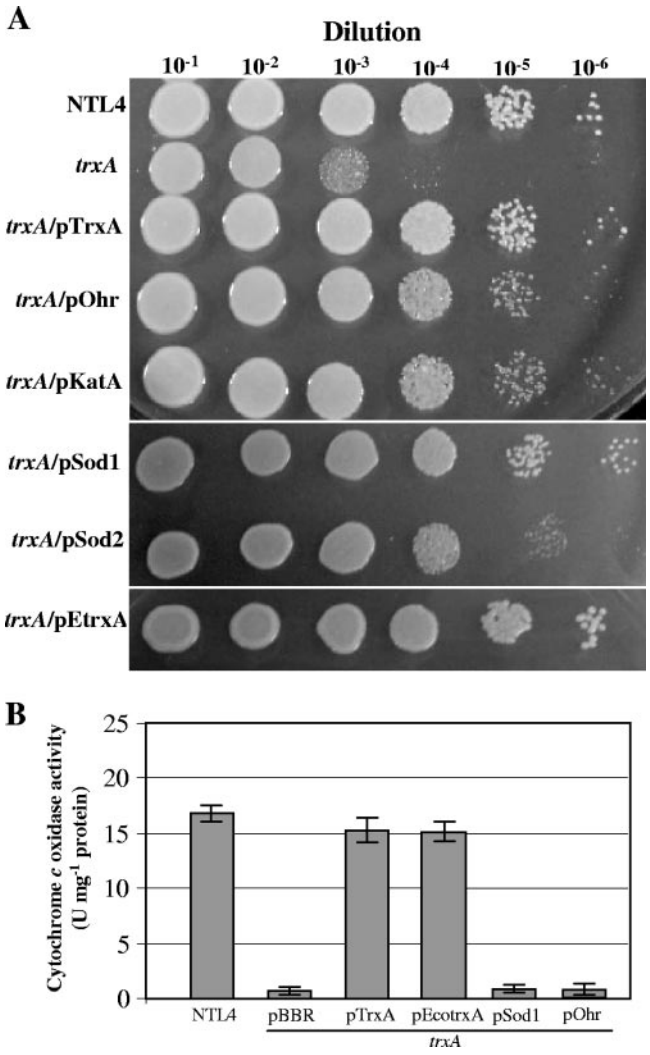

FIG. 2. Plating efficiency and oxidase tests of *A. tumefaciens* strains. (A) Exponential-phase *A. tumefaciens* cultures in LB medium were adjusted to an optical density at 600 nm of 0.1 before 10-fold serial dilutions were made. Then, 10 μl of each dilution was spotted onto an LB agar plate and incubated at 30°C for 24 h. (B) Quantitative analysis of cytochrome *c* oxidase activity was measured as previously described (28). Essentially, 10 μl of clear lysate was added to 940 μl of assay buffer (10 mM Tris-HCl [pH 7.0], 120 mM KCl). The reaction was initiated by the addition of 50 μl of reduced cytochrome *c* solution (218 μM in 0.5 mM DTT), and the reduction in the A_{550} was measured kinetically for 1 min. A unit of cytochrome oxidase activity is expressed as the amount required to oxidize 1 μmol of ferrocytochrome *c* per min at 25°C and pH 7.0. The calculation is based on the fact that the difference in the extinction coefficient ($\Delta\varepsilon^{mM}$) between ferrocytochrome *c* and ferricytochrome *c* at 550 nm is 21.84 (28).

disulfide oxidoreductases, a cascade of enzymes responsible for disulfide bonding of periplasmic proteins and maturation of cytochrome *c* (8). Inactivation of *trxA* renders *E. coli* unable to assemble *c*-type cytochrome (23). Hence, we determined whether TrxA was required for cytochrome *c* assembly by measuring the cytochrome *c* oxidase activity (28) in the *A. tumefaciens* strains. As shown in Fig. 2B, the *trxA* mutant produced drastically reduced cytochrome *c* oxidase activity (0.3 ± 0.2 U mg of protein^{-1} [mean ± standard deviation]) compared

with the isogenic wild-type strain NTL4 (16.8 ± 0.9 U mg of protein^{-1}) and the mutant complemented with pTrxA (15.0 ± 2.4 U mg of protein^{-1}). Although pKat, pSod1, and pOhr partially suppressed the growth defects of the mutant, they failed to complement the cytochrome *c* oxidase deficiency of the mutant (Fig. 2B), indicating that the reduced aerobic plating efficiency and oxidase deficiency could arise from different pathways.

Because we identified *A. tumefaciens trxA* by its homology to known thioredoxin genes, an argument could be raised concerning whether the observed phenotypes in the *A. tumefaciens trxA* mutants are actually due to the mutants' inability to produce thioredoxin. We use the rationale that if various phenotypes of the *A. tumefaciens trxA* mutant arose from lack of thioredoxin, they should be complemented by a well-characterized thioredoxin gene from other bacteria. Thus, an expression plasmid, pEcotrxA, which contains the well-characterized *E. coli trxA* gene (12) in the broad-host-range vector pBBR1MSC-2 (15), was transformed into the *trxA* mutant, and the ability to complement various phenotypes of the mutant was tested. The results in Fig. 2A and B show that expression of *E. coli trxA* restored the altered phenotypes of the *trxA* mutant. This confirms that the observed *trxA* mutant phenotypes result from inactivation of *trxA*.

Alteration of oxidative stress resistance in the *trxA* mutant. Thioredoxin is important to many antioxidant enzymes. Thus, the resistance levels against various oxidants in the *A. tumefaciens trxA* mutant and its parental strain, NTL4, were determined by the inhibition zone method. As the mutant showed growth defects under an aerobic atmosphere, the oxidant resistance tests were performed under microaerobic conditions (6% O_2 generated from CampyGen [Oxoid, United Kingdom]) to minimize growth rate differences between the strains. We found that under this condition, the defect in bacterial growth of the *trxA* mutant has no significant effects on the tests, as shown by all strains having similar zones of inhibition against imipenem antibiotic disks (30 μg) (Oxoid, United Kingdom). For the parental strain, the zones of growth inhibition for H_2O_2 (1.0 M), MD (1.0 M), CHP (1.0 M), and DA (1.0 M) were 13.2 ± 0.6, 18.2 ± 0.5, 22.0 ± 0.4, and 28.2 ± 0.8 mm, respectively, compared to zones of 10.0 ± 0.4, 21.3 ± 0.7, 22.5 ± 0.6, and 28.0 ± 0.7 mm, respectively, for the *trxA* mutant (Fig. 3A). The phenotypes of increased resistance to H_2O_2 and decreased resistance to MD in the *trxA* mutant could be complemented by expression of *trxA* from pTrxA (Fig. 3A).

Menadione is thought to exert its toxicity by generating intracellular superoxide anions via a redox cycling reaction (9). Hence, the levels of total SOD activity in the *trxA* mutant were determined (21). The total SOD level in the *trxA* mutant was 0.28 ± 0.05 U mg of protein^{-1}, similar to that found in the isogenic wild-type strain NTL4 (0.27 ± 0.06 U mg of protein^{-1}). Thus, the decreased MD resistance phenotype of the *trxA* mutant is independent of SOD activity. In addition, inactivation of *trxA* resulted in defects in cytochrome *c* maturation (Fig. 2B). The disruption of electron transfer could be responsible for the leakage of electrons from the respiratory chain, leading to increased production of superoxide anions (13). Consequently, the *trxA* mutant generates and accumulates more intracellular superoxide anions, which renders it hypersensitive to a superoxide generator, MD. We attempted to verify this assumption by introducing *sod1*, a major cytoplasmic iron SOD (FeSOD) (pSod1), and *sod2*, a periplasmic FeSOD (pSod2), on the expression vector pBBR1MSC-2 into the *trxA* mutant, and we determined the MD resistance levels in the transformants. The results show that neither pSod1 nor pSod2 was able to complement the decreased MD resistance phenotype of the mutant (Fig. 3B). Thus, accumulation of intracellular superoxide anions is not involved in the phenotype of increased sensitivity to superoxide, even though it is partially responsible for aerobic growth defects.

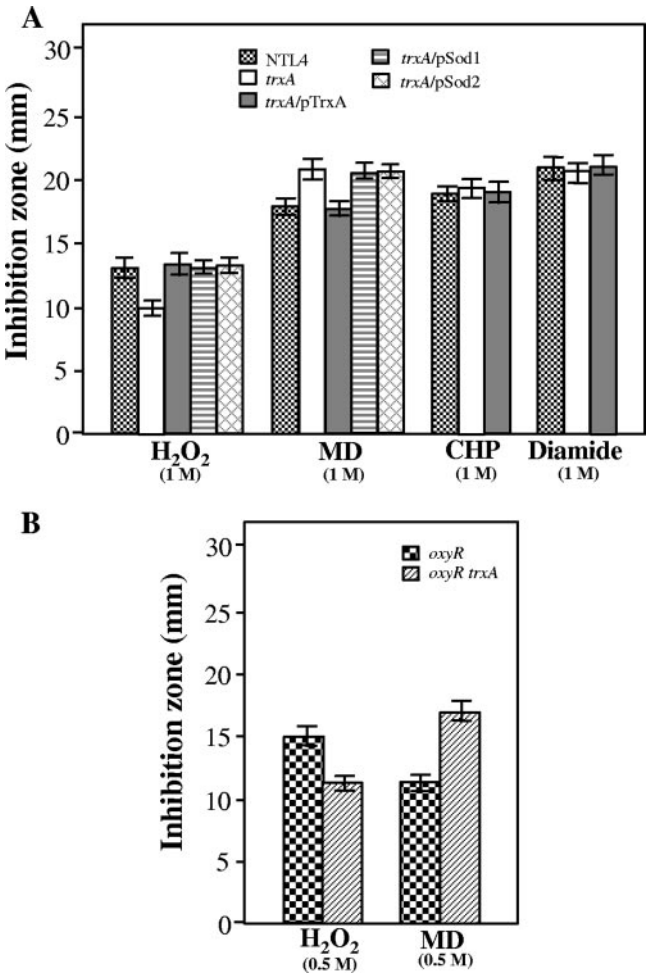

FIG. 3. Determinations of oxidant resistance in *A. tumefaciens* strains. (A) The resistance levels to oxidants of *A. tumefaciens* NTL4, the *trxA* mutant, and the mutant complemented with *trxA* (*trxA*/pTrxA), *sod1* (*trxA*/pSod1), and *sod2* (*trxA*/pSod2) on the expression vectors were measured by zones of growth inhibition around 6-mm-diameter paper disks (prepared from Whatman filter paper no. 3) soaked with 5 μl of 1 M H_2O_2, 1 M CHP, 1 M MD, and 1 M DA, as described previously (21). Inhibition zones were measured after 24 h of incubation at 30°C under a microaerobic atmosphere generated from CampyGen (Oxoid, United Kingdom). Values are means and standard deviations of four replicates. (B) Resistance levels to oxidants of the *A. tumefaciens oxyR* mutant and the *oxyR trxA* double mutant against 0.5 M H_2O_2 and 0.5 M MD.

Increased resistance to H_2O_2 in the *trxA* mutant was unexpected. Generally, the level of H_2O_2 resistance is directly correlated with total catalase activity, as is also the case in *A. tume-*

faciens (31). The *trxA* mutant had total catalase activity of 7.7 ± 0.6 U mg of protein^{-1}, which was comparable to the enzyme level in the parental strain NTL4 (7.5 ± 0.7 U mg of protein^{-1}). OxyR is directly involved in determining the H_2O_2 resistance in *A. tumefaciens* (21); hence, we tested whether it had a role in the increased H_2O_2 resistance in the *trxA* mutant. A *trxA-oxyR* double mutant was constructed by introducing pKNOCKoxyR (21) into the *trxA* mutant. The double mutant was more resistant to H_2O_2 than was the *oxyR* single mutant (Fig. 3B). Thus, the increased H_2O_2 resistance phenotype of *trxA* is independent of OxyR-regulated genes and the catalase enzyme. However, the finding that overexpression of catalase partially compensated for the reduced plating efficiency of the *trxA* mutant suggested that the mutant does accumulate intracellular H_2O_2 (Fig. 2A). Inactivation of *trxA* could lead to increased expression of OxyR-independent genes, which are involved in repaired or limited H_2O_2-induced damages to macromolecules. It is likely that the high resistance to external H_2O_2 of the *trxA* mutant is not due to enhanced H_2O_2 degradation.

Conclusions. *A. tumefaciens trxA*, encoding thioredoxin, has important roles in a variety of cellular functions. The expression of *trxA* is oxidative stress inducible and regulated by unknown regulators of the oxidative stress response. Inactivation of *trxA* severely affected bacterial growth under the conditions of an aerobic atmosphere and cytochrome *c* maturation. The mutant had unusual oxidative stress sensitivity phenotypes, being more sensitive to MD and less sensitive to H_2O_2 than was the wild type.

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