RESEARCH LETTER

Copper ions potentiate organic hydroperoxide and hydrogen peroxide toxicity through different mechanisms in *Xanthomonas campestris* pv. *campestris*

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Abstract

Copper (Cu)-based biocides are important chemical controls for both fungal and bacterial diseases in crop fields. Here, we showed that Cu ions at a concentration of 100 μM enhanced *t*-butyl hydroperoxide (*t*BOOH) and hydrogen peroxide (H_2O_2) killing of *Xanthomonas campestris* pv. *campestris* through different mechanisms. The addition of an antilipid peroxidation agent (α-tocopherol) and hydroxyl radical scavengers (glycerol and dimethyl sulphoxide) partially protected the bacteria from the Cu-enhanced *t*BOOH and H_2O_2 killing, respectively. Inactivation of the alkyl hydroperoxide reductase gene rendered the mutant vulnerable to lethal doses of copper sulphate, which could be alleviated by the addition of an H_2O_2 scavenger (pyruvate) and α-tocopherol. Taken together, the data suggest that Cu ions influence the killing effect of *t*BOOH through the stimulation of lipid peroxidation, while hydroxyl radical production is the underlying mechanism responsible for the Cu-ion-enhanced H_2O_2 killing effects.

Introduction

Xanthomonas campestris is an important phytopathogen that causes damaging diseases in economically important crops worldwide. During plant–microorganism interactions, the rapid production and accumulation of reactive oxygen species (ROS) is an initial defence response against the infecting microorganisms (Levine *et al.*, 1994). Plant lipoxygenases that catalyse the formation of fatty acid hydroperoxide have been shown to be induced by microbial invasion and are involved in plant–microbial defence responses (Croft *et al.*, 1993; Kolomiets *et al.*, 2000; Jalloul *et al.*, 2002). These ROS are highly toxic and exert detrimental effects on the invading microorganisms through their ability to stimulate lipid peroxidation and protein and DNA damage that eventually lead to cell death (Farr & Kogoma, 1991).

Copper (Cu) is required as a cofactor for a variety of enzymes, such as terminal oxidases, monooxygenases, and dioxygenases. An excess of Cu in aerobic cells generates ROS through a Fenton-like reaction, in which Cu (I) ions react with hydrogen peroxide (H_2O_2) to form hydroxyl radicals (Gunther *et al.*, 1995). Nonetheless, the precise mechanisms by which Cu ions exert lethal effects on bacterial cells remain ambiguous. A study in *Escherichia coli* revealed that membrane injury caused by lipid peroxidation is one of the factors responsible for Cu-induced cell death (Lebedev *et al.*, 2002). Macomber *et al.* (2007) demonstrated that intracellular Cu failed to catalyse the formation of oxidative DNA damage. Indeed, excessive intracellular Cu suppressed iron-mediated oxidative killing (Macomber *et al.*, 2007). More recently, experimental data suggest that the iron–sulphur clusters of dehydratase enzymes are the primary intracellular targets of Cu toxicity in *E. coli* (Macomber & Imlay, 2009). The mechanisms for Cu toxicity in *Xanthomonas* spp. are not yet fully elucidated, even though Cu-based biocides containing copper hydroxide ($Cu(OH)_2$), copper sulphate ($CuSO_4$), and copper oxychloride are widely used in

agricultural settings to limit the spread of phytopathogenic fungi and bacteria (Hopkins, 2004). Cu-based bactericides are effective control measures for plant diseases caused by *X. campestris* (McGuire, 1988). Here, we show experimentally that the presence of Cu synergistically increased the killing effects of H_2O_2 and organic hydroperoxide.

Materials and methods

Bacterial strains and growth conditions

Xanthomonas campestris pv. *campestris* (Xcc) was grown aerobically in Silva-Buddenhagen (SB) medium (0.5% sucrose, 0.5% yeast extract, 0.5% peptone, and 0.1% glutamic acid, pH 7.0) (Chauvatcharin *et al.*, 2005) at 28 °C. Overnight cultures were inoculated into a fresh SB medium to yield an $OD_{600\,nm}$ of 0.1. Exponential-phase cells ($OD_{600\,nm}$ of 0.5 after 4 h) were used in all the experiments.

Molecular genetics techniques

General molecular techniques for bacterial genomic and plasmid DNA preparations, PCR, restriction endonuclease digestion, DNA ligation, transformation of *E. coli*, gel electrophoresis, and Southern blotting analysis were performed using standard protocols (Sambrook & Russell, 2001). The transformation of Xcc was performed using electroporation. Competent cells were prepared from an exponential-phase culture in SB medium. Cells were harvested, washed once with 10% (v/v) glycerol, and resuspended in this same solution. Electroporation was conducted in a 0.2-cm electrode gap cuvette with a Gene Pulser electroporator (Bio-Rad) using the following settings: 2.5 kV, 200 Ω, and 25 μF. DNA sequencing was performed using an automated sequencer (ABI 310, Applied Biosystems).

Oxidant killing experiments

Oxidant killing experiments were performed as described previously (Banjerdkij *et al.*, 2005). Bacterial cultures were grown to the exponential phase before aliquots of cells were removed and treated for 30 min with lethal concentrations of H_2O_2 (50 mM) or *t*-butyl hydroperoxide (*t*BOOH) (50 mM) that would reduce bacterial survival by 10- to 100-fold. Treatments with oxidant plus Cu included the addition of $CuSO_4$, at a final concentration of 100 μM, to the killing mixture. In antioxidant protection tests, ROS scavengers, i.e., 0.4 M dimethyl sulphoxide (DMSO), 1.0 M glycerol, or 1 mM α-tocopherol, were added to bacterial cultures 10 min before the addition of oxidants (Mongkolsuk *et al.*, 1998; Vattanaviboon & Mongkolsuk, 1998). After the treatment, cells were removed and washed once with a fresh SB medium before the determination of cell survival by plating appropriate dilutions on SB agar. Colonies were counted after 48 h of incubation at 28 °C. The survival percentage was defined as the number of CFU recovered after the treatment divided by the number of CFU before treatment multiplied by 100.

Determination of Cu resistance levels

Cu resistance was determined as described previously, with some modifications (Sukchawalit *et al.*, 2005). Briefly, $CuSO_4$ at a final concentration of 1 mM was added to an exponential-phase culture of Xcc. The culture was further incubated for 1 h with continuous shaking. In antioxidant protection experiments, 1 mM α-tocopherol, 10 mM pyruvate, and 1.0 M glycerol were added to bacterial cultures 10 min before the addition of $CuSO_4$. The number of surviving cells was determined using viable plate counts and expressed as per cent survival.

Construction of the *ahpC* mutant and pAhpC

The insertional inactivation of *ahpC* (*xcc0834*, da Silva *et al.*, 2002) was achieved using the pKNOCK suicide vector system (Alexeyev, 1999). An *ahpC* gene fragment was PCR amplified using BT2684 (5′-CGCAGCGTCTCGGTGACG-3′) and BT2685 (5′-AGTGGAAGACGCCGCTGA-3′) oligonucleotide primers and Xcc genomic DNA as a template. The 300-bp PCR product was cloned into pGem-T-easy (Promega) and then an EcoRI fragment was subcloned into pKNOCK-Km cut with the same enzyme to generate pKNOCKahpC. The recombinant plasmid was electroporated subsequently into wild-type Xcc. The mutant, which was selected for its kanamycin resistance phenotype, was confirmed by Southern blot analysis using an *ahpC*-specific probe (data not shown).

The pAhpC plasmid used for the plasmid-borne expression of *ahpC* was constructed by PCR amplification of full-length *ahpC* using BT3026 (5′-CAGGGATGCGAGGCGGCT-3′) and BT3027 (5′-AGGAAACTCAATGTCTCT-3′) primers. PCR was performed using *Pfu* DNA polymerase with proofreading activity (Promega), and the product was directly cloned into the broad-host-range plasmid vector, pBBR1MCS-4 (Kovach *et al.*, 1995), at the EcoRV site, to form pAhpC. The *ahpC* gene was expressed in *Xanthomonas* under the control of the *lacUV5* promoter of the vector.

Results and discussion

Cu ions enhance organic hydroperoxide killing

Exposure of an exponential-phase culture of Xcc to 50 mM *t*BOOH for 30 min resulted in roughly 10% survival compared with the untreated culture (Fig. 1). The effect of Cu ions in *t*BOOH killing was investigated. $CuSO_4$ at concentrations below 0.5 mM exerted no adverse effects on Xcc

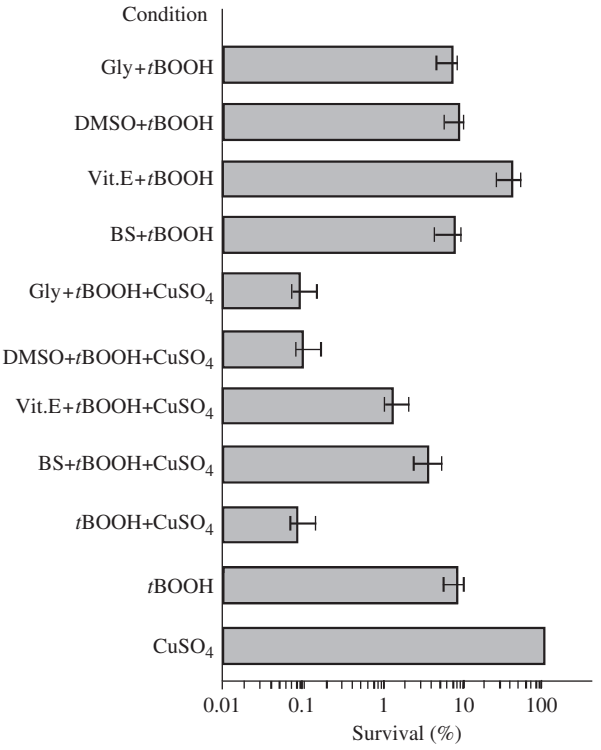

Fig. 1. The effect of $CuSO_4$ on *t*BOOH toxicity. The culture of *Xanthomonas campestris* pv. *campestris*, *t*BOOH killing, and a combination of $CuSO_4$ treatments were performed as described in Materials and methods. The concentrations of the substances used in the experiments were 50 mM *t*BOOH, 100 μM $CuSO_4$, 200 μM bathocuproine sulphonate (BS), 1 mM α-tocopherol (Vit. E), 0.4 M DMSO, and 1.0 M glycerol (Gly). The per cent survival was defined as the number of CFU recovered after the treatment divided by the number of CFUs before treatment multiplied by 100. Error bars indicate the mean ± SD of four independently performed experiments.

growth in rich medium (SB). The addition of 100 μM $CuSO_4$ to the *t*BOOH killing treatment resulted in a 100-fold decrease in the per cent survival compared with only *t*BOOH treatment (Fig. 1). The enhanced killing effect of *t*BOOH by $CuSO_4$ was abolished by the addition of the Cu chelator, bathocuproine sulphonate, at a final concentration of 200 μM (Fig. 1). Generally, organic hydroperoxide toxicity is a result of lipid peroxidation reactions (Farr & Kogoma, 1991). In the presence of redox metals, organic hydroperoxides can be reduced to form alkoxyl radicals that can initiate lipid peroxidation and generate lipid alkyl radicals. The alkyl radical then reacts with oxygen to produce lipid peroxyl radicals. The reaction is then perpetuated as lipid peroxyl radicals further react with another unsaturated fatty acid to form fatty acid hydroperoxide, which contributes to the chain reaction of lipid peroxidation (Farr & Kogoma, 1991). Among membrane fatty acids, polyunsaturated fatty acids are highly susceptible to peroxidation. The majority of the cellular fatty acids of *X. campestris* (Wells *et al.*, 1992) cultivated under physiological conditions are saturated fatty acids, while around 15% are monounsaturated fatty acids, such as palmitoleic acids ($C_{16:1}$), which can undergo lipid peroxidation (Rael *et al.*, 2004). However, it remains unknown whether Xcc grown under the test conditions produce polyunsaturated fatty acids.

Because exposure to Cu ions has been shown to increase membrane lipid peroxidation that leads to cell death (Lebedev *et al.*, 2002), we speculated that Cu ions might initiate lipid peroxidation by reacting with *t*BOOH. The resulting alkoxyl radicals could then participate in the chain reaction of lipid peroxidation. The hypothesis that Cu potentiates *t*BOOH toxicity via lipid peroxidation was tested by the addition of 1 mM α-tocopherol (vitamin E), which possesses antilipid peroxidation activity, to the bacterial suspension before treatment with *t*BOOH plus $CuSO_4$. As shown in Fig. 1, α-tocopherol alleviated the Cu-enhanced *t*BOOH killing effect by 20-fold, indicating that, at least in part, Cu was capable of triggering *t*BOOH-mediated lipid peroxidation. In addition, α-tocopherol also substantially increased the survival percentage of treatment with *t*BOOH alone by fourfold (Fig. 1). We also examined the ability of the hydroxyl radical scavengers DMSO and glycerol to protect cells from the $CuSO_4$-enhanced *t*BOOH killing effect. The addition of either DMSO or glycerol at concentrations of 0.4 and 1.0 M (Vattanaviboon & Mongkolsuk, 1998), respectively, before the treatment with *t*BOOH and $CuSO_4$, had no protective effect (Fig. 1). It is likely that hydroxyl radicals are not involved in *t*BOOH plus $CuSO_4$ toxicity.

We have reported previously a synergistic killing effect of superoxide anions and organic hydroperoxide. The combined treatment of a superoxide generator and *t*BOOH drastically increased the ability to kill cells compared with the single-substance treatments (Sriprang *et al.*, 2000). Recently, it has been shown that iron–sulphur cluster-containing dehydratases are intracellular targets of Cu toxicity, probably due to increased production of superoxide anions (Macomber & Imlay, 2009). Thus, the possibility that Cu-mediated *t*BOOH toxicity involves superoxide anion generation activated by Cu ions cannot be ruled out.

Cu ions enhance H_2O_2 killing

Although a previous *in vitro* study has shown that Cu ions are able to react with H_2O_2 in a Fenton-like reaction to generate hydroxyl radicals (Gunther *et al.*, 1995), it is still controversial whether this reaction occurs *in vivo*. Experiments were performed to test the effect of Cu ions on H_2O_2-mediated killing of Xcc. The treatment of Xcc cultures with 50 mM H_2O_2 for 30 min resulted in approximately 10% survival (Fig. 2). The addition of $CuSO_4$ (100 μM) to the H_2O_2 killing mixture was highly lethal to cells and reduced

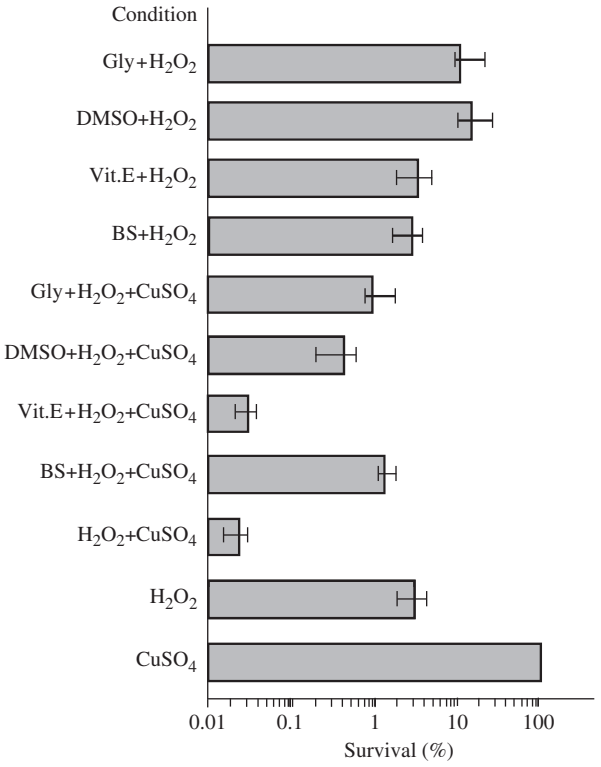

Fig. 2. The effect of $CuSO_4$ on H_2O_2 toxicity. The culture of *Xanthomonas campestris* pv. *campestris*, H_2O_2 killing, and a combination treatments were performed as described in Materials and methods. The concentrations of the substances used in the experiments were 50 mM H_2O_2, 100 μM $CuSO_4$, 200 μM bathocuproine sulphonate (BS), 1 mM α-tocopherol (Vit. E), 0.4 M DMSO, and 1.0 M glycerol (Gly). The survival percentage was defined as described in Fig. 1. Error bars indicate the mean ± SD of four independently performed experiments.

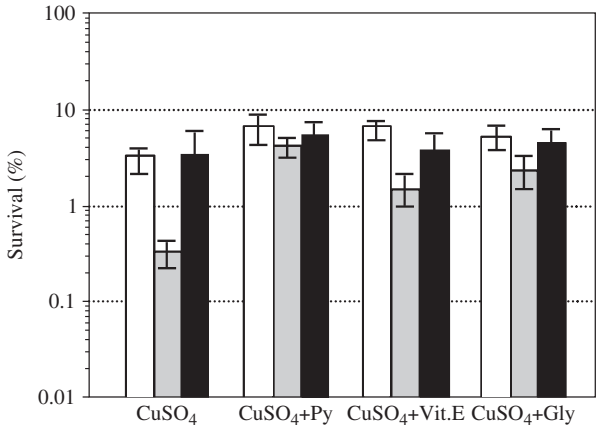

Fig. 3. Cu resistance levels in the *Xanthomonas campestris* pv. *campestris ahpC* mutant. Exponential cultures of wild-type *X. campestris* pv. *campestris* (open bar), the *ahpC* mutant (shade bar), and the complemented strain (closed bar) were treated with 1 mM $CuSO_4$ for 1 h in the presence or absence of either 10 mM pyruvate (Py), 1 mM α-tocopherol (Vit. E), or 1.0 M glycerol (Gly). The survival percentage was calculated from the CFUs on SB plates containing $CuSO_4$ divided by the CFUs on SB plates without $CuSO_4$ multiplied by 100. Error bars indicate the mean ± SD of three independently performed experiments.

the per cent survival to 0.05% (Fig. 2). The synergistic effect of $CuSO_4$ and H_2O_2 was abolished when a Cu chelator (200 μM bathocuproine sulphonate) was added to the cell suspension before the combined treatment of $CuSO_4$ and H_2O_2 (Fig. 2). This observation suggests the possibility that an elevated level of Cu ions could react with H_2O_2 to produce hydroxyl radicals, which lead to increased cell death. This speculation was supported by experiments in which the addition of hydroxyl scavengers DMSO (0.4 M) and glycerol (1.0 M) to bacterial cultures, before treatment with $CuSO_4$ and H_2O_2, significantly protected bacterial cells from the killing effects (Fig. 2). We then determined whether lipid peroxidation contributes to $CuSO_4$ and H_2O_2 toxicity. The ability of α-tocopherol (1 mM) to reduce the lethal effects of $CuSO_4$ and H_2O_2 treatment was tested. As illustrated in Fig. 2, α-tocopherol was unable to alleviate $CuSO_4$ and H_2O_2 killing. The evidence indicates that Cu ions potentiate H_2O_2 toxicity in a manner different from *t*BOOH. While lipid peroxidation is a major factor responsible for the Cu ion-mediated enhancement of *t*BOOH toxicity, hydroxyl radicals likely account for Cu ion-dependent H_2O_2 toxicity.

An *ahpC* mutant is vulnerable to Cu

Alkyl hydroperoxide reductase, encoded by *ahpC*, is a member of the peroxiredoxin enzyme family. AhpC not only plays a role in the detoxification of organic hydroperoxides by converting them to their corresponding alcohols, but the enzyme is also necessary for the degradation of endogenously generated H_2O_2 due to its much lower k_{cat}/K_m compared with catalase (Seaver & Imlay, 2001). Thus, the *ahpC* mutant accumulates intracellular H_2O_2 and organic hydroperoxides produced as byproducts of normal aerobic metabolism (Seaver & Imlay, 2001; Charoenlap *et al*., 2005; Wang *et al*., 2006). If Cu toxicity is partly due to the stimulation of oxidative stress production, we would expect that the Cu resistance level in the *ahpC* mutant might be altered. An Xcc *ahpC* mutant was constructed using the pKNOCK system (Alexeyev, 1999). The *ahpC* mutant was more sensitive to *t*BOOH killing treatment than the wild-type Xcc (data not shown). The Cu resistance of the *ahpC* mutant was measured using a killing assay (Sukchawalit *et al*., 2005), and the results showed that the mutant was more than 10-fold more sensitive to $CuSO_4$ (1 mM) than the wild-type Xcc (Fig. 3). The ectopic expression of *ahpC* from the expression plasmid, pAhpC, complemented the $CuSO_4$-sensitive phenotype of the *ahpC* mutant (Fig. 3, *ahpC*/pAhpC). The lack of a functional *ahpC* rendered Xcc

vulnerable to elevated levels of $CuSO_4$. This phenotype is presumably due to the loss of *ahpC* function, which causes the accumulation of organic hydroperoxide and H_2O_2, which could react with Cu ions to generate more harmful oxygen radicals.

We then tested whether the addition of the H_2O_2 scavenger, 10 mM pyruvate (Mongkolsuk *et al.*, 1998), the lipid peroxide inhibitor, 1 mM α-tocopherol (Aoshima *et al.*, 1999), or the hydroxyl radical scavenger, 1 M glycerol (Vattanaviboon & Mongkolsuk, 1998), could diminish the Cu killing effect in the *ahpC* mutant. Pyruvate, α-tocopherol, or glycerol was supplemented in the cultures before treatment with 1 mM $CuSO_4$. Supplementation with pyruvate, α-tocopherol, or glycerol rescued the *ahpC* mutant from death by Cu treatments (Fig. 3). The presence of pyruvate increased the survival percentage of the *ahpC* mutant by more than 10-fold compared with Cu killing without pyruvate. Likewise, the prior addition of α-tocopherol and glycerol led to a five and sevenfold increase in the survival of the *ahpC* mutant, respectively, after the Cu treatment relative to the control experiments. The protective effect of the scavengers in the *ahpC* mutant was consistent with the idea that the mutant accumulates ROS. Additionally, the data indicate that a principal Cu toxicity mechanism towards Xcc involves oxidative stress. In addition, investigations in Cu efflux machinery mutants, in which the intracellular Cu level is elevated, also showed enhancement of bacterial sensitivity to ROS (Sitthisak *et al.*, 2007; Nawapan *et al.*, 2009). This evidence supports the link between Cu exposure and oxidative stress.

In conclusion, the *in vivo* data presented here suggest that the toxic effect of Cu ions in the presence of organic hydroperoxides, either endogenously generated or from an exogenous source, which could arise from lipid peroxidation, while increased the production of hydroxyl radicals, is associated with Cu ion-enhanced H_2O_2 toxicity.

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