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Evidence Against a Direct Antimicrobial Role of H_2O_2 in the Infection of Plants by *Erwinia chrysanthemi*

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We have investigated the role of bacterial resistance to oxidative stress in pathogenesis. The *oxyR* gene from the pathogenic bacterium *Erwinia chrysanthemi* has been characterized. It is closely related to that found in *Escherichia coli* (88% overall amino acid identity). An *E. chrysanthemi oxyR* mutant strain was constructed by marker exchange. After induction with a sublethal dose of H_2O_2, this mutant was more sensitive to H_2O_2 and showed reduced levels of catalase and glutathione reductase activities, compared with the wild type. The *oxyR* mutant was unable to form individual colonies on agar plates unless catalase was added exogenously. However, it retained full virulence in potato tubers and tobacco leaves. These results suggest that the host-produced H_2O_2 has no direct antimicrobial effect on the interaction of *E. chrysanthemi* with the two plant species.

Additional keywords: active oxygen species, phytopathogenic bacteria.

During their interaction with phytopathogenic bacteria, plant cells undergo an oxidative burst that consists of the production of active oxygen species (AOS), including superoxide anion, hydroxyl radical, and hydrogen peroxide (Doke 1983; Adam et al. 1989; Keppler et al. 1989). This burst reflects activation of a membrane-bound NADPH oxidase and resembles that induced in neutrophils activated by animal pathogens (Morel et al. 1991). It has been suggested that the oxidative burst is the first line of defense against pathogens, based on the cytotoxicity of AOS and their rapid induction upon pathogen challenge or elicitor treatment (Keppler and Baker 1989). Thus, it seems that AOS might play a dual role in defense: a direct antimicrobial activity (Baker and Orlandi 1995; Lamb and Dixon 1997) and an indirect effect as mediators of the activation of other defense components, namely, oxidative cross-linking of the cell wall (Hammerschmidt and Kuc 1982; Bradley et al. 1992), hypersensitive cell death (Levine et al. 1994), and elicitation of phytoalexins (Chai and Doke 1987). Although the antimicrobial effect in vitro of O_2^- and H_2O_2 against phytopathogenic bacteria and fungi has been reported (Doke 1987; Ouf et al. 1993), it is unclear whether they have antimicrobial activity in vivo. The hypothesis of an in vivo, direct, antimicrobial effect of AOS has as a corollary that phytopathogenic bacteria must be able to withstand or detoxify AOS to be able to multiply in plant tissues. Detoxification of H_2O_2 is probably an important factor, since this molecule is able to diffuse across membranes and cause multiple biological effects. Indeed, phytopathogenic bacteria possess enzymes that prevent oxidative damage, such as catalases and superoxide dismutases (Katsuwon and Anderson 1989; Klotz and Hutcheson 1992).

The bacterial response to oxidative stress has been extensively studied in *Escherichia coli* and *Salmonella typhimurium*, and it has been shown that several enzymes involved in AOS detoxification, including catalase and glutathione reductase, are under the control of the *oxyR* gene product, a member of the LysR family of transcriptional regulators (Morgan et al. 1986; Christman et al. 1989; González-Flecha et al. 1993). Transient accumulation of *oxyR*-induced proteins upon pretreatment with a nonlethal dose of H_2O_2 results in an adaptation of bacteria to this reagent (Demple and Halbrook 1983). Moreover, strains with inactivating mutations in *oxyR* are unable to induce this regulon and are more sensitive to hydrogen peroxide (Christman et al. 1989).

We have investigated the role of the *oxyR* gene in the pathogenicity of *Erwinia chrysanthemi*, an economically important phytopathogenic bacterium that causes soft-rot diseases in a wide range of crops (Perombelon and Kelman 1980). Active virulence mechanisms are known to contribute to the pathogenesis of this bacterium, especially, the secretion of hydrolytic enzymes that attack the pectic fraction of the plant cell wall (Collmer and Keen 1986) and the induction of plant necrosis elicited by *hrp* (hypersensitive response and pathogenicity) gene products (Bauer et al. 1994). We have recently reported that a different type of mechanism, which enables *E. chrysanthemi* to resist the action of antimicrobial agents from the plant host, makes an important contribution to pathogenicity (López-Solanilla et al. 1998).

We describe here the construction and characterization of an *oxyR* mutant strain of *E. chrysanthemi*. This mutant shows the following features: (i) it is more sensitive to H_2O_2 and produces lower levels of catalase than the wild type, after pre-

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Nucleotide and/or amino acid sequence data can be found at the EMBL data base as accession no. AJ005255.

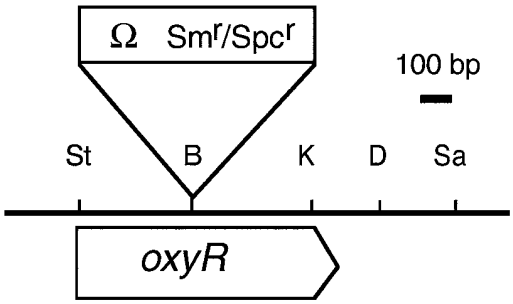
treatment with a sublethal dose of H_2O_2; (ii) it is unable to form individual colonies on solid medium unless catalase is added exogenously; and (iii) it retains full virulence in potato tubers and tobacco leaves. Moreover, the wild-type strain and the *oxyR* mutant are protected from exogenously added H_2O_2 when inoculated into the plant. These results suggest that the oxidative burst does not play a direct, antimicrobial role in these particular plant-bacterium interactions.

RESULTS

Cloning and analysis of the *oxyR* gene of *E. chrysanthemi.*

To investigate the presence of a homolog of the *oxyR* gene, genomic DNA from *E. chrysanthemi* was analyzed by gel blot hybridization with an *oxyR* probe from *E. coli*. To obtain this probe, genomic DNA from *E. coli* DH5α was subjected to PCR (polymerase chain reaction) amplification with two primers (5′CGGCGTGCGGCAGATTCCTG3′ and 5′CAGA TCGGCCATCGGTACGC3′) based on the *E. coli oxyR* sequence (Christman et al. 1989), and the 2.0-kb amplified fragment was used to probe *Eco*RI-digested DNA from the *E. chrysanthemi* strain AC4150. A unique 2.2-kb hybridizing fragment was identified and the corresponding clone was isolated from a genomic library of *E. chrysanthemi* (Fig. 1A). The nucleotide sequence of the pB106 insert was determined (EMBL accession number AJ005255) and found to contain one open reading frame that was homologous to the *oxyR* gene sequence described for *E. coli* by Christman et al. (1989). The percent identity of the two deduced amino acid sequences was 88% (Fig. 1B). This level of similarity, and the fact that the mutant had an altered response to H_2O_2 (see below), justified the designation of the *E. chrysanthemi* gene as *oxyR.*

A



B

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                        10        20        30        40        50        60
                        |         |         |         |         |         |
E.chrysanthemi      MNIRDLEYLVALAEHRHFRRAADSCHVSQPTLSGQIRKLEDELGVMLLERTSRKVLFTQA
E.coli              MNIRDLEYLVALAEHRHFRRAADSCHVSQPTLSGQIRKLEDELGVMLLERTSRKVLFTQA
                    ************************************************************

E.chrysanthemi      GLLLVEQARTVLREVKVLKEMASQQGEAMSGPLHIGLIPTVGPYLLPQIIPMLHRAFPKL
E.coli              GMLLVDQARTVLREVKVLKEMASQQGETMSGPLHIGLIPTVGPYLLPHIIPMLHQTFPKL
                    * *** ******************** ****************** ******  ****

E.chrysanthemi      EMYLHEAQTHQLLAQLDSGKLDCAILAMVKESEAFIEVPLFDEPMKLAIYQDHPWANRER
E.coli              EMYLHEAQTHQLLAQLDSGKLDCVILALVKESERFIEVPLFDEPMLLAIYEDHPWANREC
                    ********************** *** ***** *********** **** ********

E.chrysanthemi      VAMSDLSGEKLLMLEDGHCLRDQAMGFCFQAGADEDTHFRATSLETLRNMVAAGSGITLL
E.coli              VPMADLAGEKLLMLEDGHCLRDQAMGFCFEAGADEDTHFRATSLETLRNMVAAGSGITLL
                    * * ** ********************** ******************************

E.chrysanthemi      PSLAVPQERIRDGVCYLPCYKPEPKRTIALVYRPGSPLRGRYEQLADSVREHMQLHMEKL
E.coli              PALAVPPERKRDGVVYLPCIKPEPRRTIGLVYRPGSPLRSRYEQLAEAIRARMDGHFDKV
                    * **** ** **** **** **** *** ********** ******   *  *  *  *

E.chrysanthemi      SAQSA
E.coli              LKQAV
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Fig. 1. *oxyR* region of *Erwinia chrysanthemi*. **A,** Genetic and physical map of the *oxyR* gene from *E. chrysanthemi*. Insertion of the Ω interposon used for construction of mutant BT109 is represented by a bar. B, *Bal*I; D, *Dra*I; K, *Kpn*I; Sa, *Sac*I; St, *Sty*I. Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance. **B,** Alignment of deduced sequences of OxyR proteins from *E. chrysanthemi* and *Escherichia coli*. Identical amino acids are marked with asterisks. EMBL data bank accession numbers of these sequences: AJ005255 for the *E. chrysanthemi* sequence; J04553 for the *E. coli* sequence.

Insertional inactivation of the *oxyR* gene in *E. chrysanthemi.*

To obtain insertional mutants of the *oxyR* gene, pB106 DNA was digested with *Bal*I endonuclease and the linearized plasmid was ligated to DNA from the Ω interposon (Prentki and Krisch 1984), as represented in Figure 1A. This plasmid, named pB107, was marker exchanged into the *E. chrysanthemi* AC4150 chromosome. Out of several Amp^s Sm^r Spc^r recombinants (data not shown), one mutant strain, named BT109, was selected for further analysis. Marker exchange was verified by DNA gel blot hybridization (data not shown). The *oxyR* mutant was complemented with plasmid pB106, and this complemented strain was named BT110.

The mutant BT109 and the wild type showed essentially the same growth rate in liquid media (Fig. 2A). However, the mutant strain was unable to form individual colonies on agar plates, and this ability was restored to wild-type level in the complemented strain (Fig. 2B). A similar effect has been reported for the *oxyR* mutant of *Haemophilus influenzae* (Maciver and Hansen 1996). These authors proposed that this result was a consequence of the accumulation of hydrogen peroxide produced by the aerobic metabolism of the bacteria. To test this hypothesis, an excess of catalase was added to the agar surface immediately before bacteria were plated. It was observed that catalase restored the ability of mutant BT109 to grow on agar nutrient broth medium to wild-type level (Fig. 2B).

Differential sensitivity to H_2O_2.

Sensitivity of the BT109 mutant strain to hydrogen peroxide was compared with that of the wild type. As shown in Figure 3, the wild type and the BT110 strains showed enhanced tolerance to H_2O_2 upon pretreatment, whereas the BT109 mutant did not respond to it. Without pretreatment, the three strains showed similar sensitivity to H_2O_2 (Fig. 3). A similar effect has been previously described for *E. coli* (Demple and Halbrook 1983) and *S. typhimurium* (Christman et al. 1985).

Since catalase and glutathione reductase activities are known to be controlled by the OxyR regulator in *E. coli*, these enzymatic activities were measured in extracts from log phase cells of the *E. chrysanthemi* strains AC4150, BT109, and BT110, pretreated or not pretreated. As shown in Figure 4, the pretreatment increased five- to sixfold the catalase activity of the wild-type strain and about threefold that of the complemented strain, whereas the activity in the *oxyR* mutant did not significantly increase. These results are in agreement with previous reports of induction of catalase activity in *S. typhimurium* (Christman et al. 1985). In contrast, although the H_2O_2 pretreatment resulted in a 40% induction of gluthathione reductase activity in the wild type, and no induction in the BT109 strain, the complemented BT110 strain constitutively showed a high level of gluthathione reductase activity that was similar to that of the wild type after induction (data not shown).

H_2O_2 induction after infection with *E. chrysanthemi.*

The occurrence of an oxidative burst upon bacterial infection has been well documented in several plant-bacterium interactions (Doke 1983; Adam et al. 1989; Keppler et al. 1989). However, direct evidence for such event had not been reported for *E. chrysanthemi*. We have used a semiquantitative method

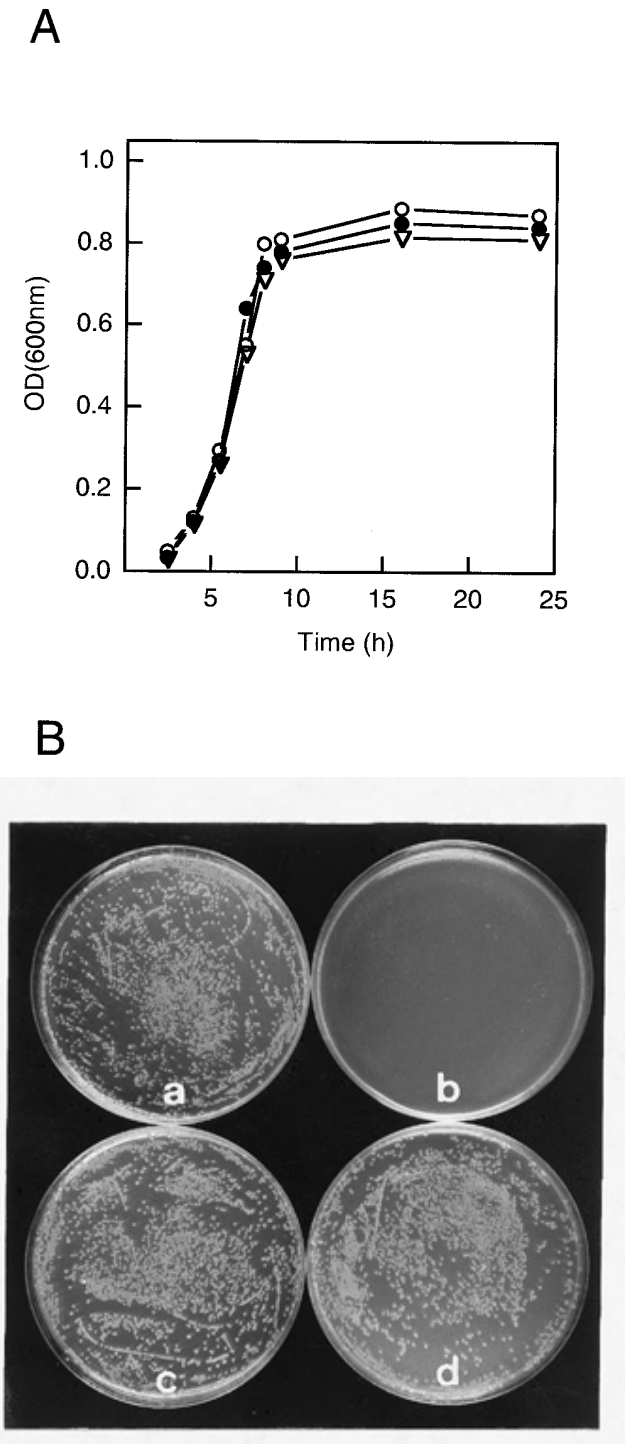

Fig. 2. Effect of *oxyR* mutation on bacterial growth. **A,** Growth in liquid medium. Bacteria cells were inoculated on nutrient broth medium, and growth estimated by monitoring absorbance at 600 nm. Open circles, *Erwinia chrysanthemi* AC4150 (wild type); closed circles, BT109 (*oxyR* mutant); open triangles, BT110 (complemented *oxyR* mutant). **B,** Growth on solid medium. Nutrient agar plates were plated with a bacterial suspension containing around 5×10^2 CFU of the following *E. chrysanthemi* strains: **a,** AC4150; **b,** BT109; **c,** BT110; **d,** BT109 plus an excess of catalase exogenously added.

based on the oxidation of diaminobenzidine (Frederick 1987; Thordal-Christensen et al. 1997; Schraudner et al. 1998), to detect H_2O_2 in tobacco leaves infiltrated with bacterial suspensions of *E. chrysanthemi* AC4150 or mutant BT109, as well as with the *Pseudomonas syringae* strain BT111, used as a control. Figure 5 shows that a similar amount of H_2O_2 is induced upon infection with any of the three strains.

Virulence of the *oxyR* mutant.

To investigate the possible effect on virulence of the observed alteration of in vitro resistance to hydrogen peroxide, potato tubers were inoculated with *E. chrysanthemi* AC4150 or the *oxyR* mutant. Necrotic areas of the developed lesions were measured in all of the tubers after 48 h and no statistically significant differences were found among the lesions produced by the two strains (Table 1; Fig. 6).

The virulence of *E. chrysanthemi* AC4150 and mutant BT109 was also assayed by infiltration in tobacco leaves. Necrotic areas of the developed lesions were measured 48 h after infiltration and no statistically significant differences were found between the mutant and wild-type strains (Table 2).

In planta protection of *E. chrysanthemi* from H_2O_2 stress.

The ability of the wild type and the *oxyR* mutant of *E. chrysanthemi* to resist the effects of exogenously added H_2O_2 in both in vitro and in planta conditions was studied. H_2O_2 concentration of pretreated bacterial suspensions of the wild type or the *oxyR* mutant was adjusted to 10, 20, and 50 mM and immediately inoculated in tobacco leaves. After 5 h, the inoculated areas were excised and ground in 600 µl of 10 mM $MgCl_2$ and the bacterial populations were estimated by colony plating. A control experiment was carried out under exactly the same conditions, except that bacteria were incubated with H_2O_2 in a test tube instead of in the plant tissue. Figure 7 shows that the wild-type strain did not loose viability in planta at 50 mM H_2O_2, whereas, in vitro, all bacteria were essentially killed at 5 mM H_2O_2. The mutant strain was indistinguishable from the wild type in planta, although it was significantly more sensitive in vitro (Fig. 7). These results indicate that the plant tissue protected *E. chrysanthemi* cells from exogenously added H_2O_2. However, it cannot be excluded that oxidative bursts induced by other phytopathogenic bacteria have stronger antimicrobial effects than the exogenously added H_2O_2. To test this possibility, we investigated the effect of coinoculation with *P. syringae* pv. *syringae* BT111 on in planta growth of *E. chrysanthemi* wild type and mutant BT109. This bacterium was chosen because it is a well-known inducer of oxidative burst in plants (Baker and Orlandi 1995). There was no difference in the growth of the mutant, compared with the wild type, in the presence of the second bacterium (data not shown).

Catalase induction in plants after bacterial infection.

The observed in planta protection of bacterial cells from oxidative stress is probably a consequence of the plant H_2O_2 detoxification system. Alternatively, these results could be explained by the possible existence of plant-induced, *oxyR*-independent bacterial catalases. To discriminate between these possibilities, we have determined the total catalase activity in the following situations: (i) activity produced during the infection of tobacco leaves with the wild-type strain (plant plus wild-type bacteria); (ii) activity during the infection with the *oxyR* mutant (plant plus *oxyR*-independent bacterial catalases); (iii) plant catalase activity after treatment with a bacterial filtrate containing the extracellular fraction of either the wild type or the *oxyR* mutant, which represents the eliciting activity

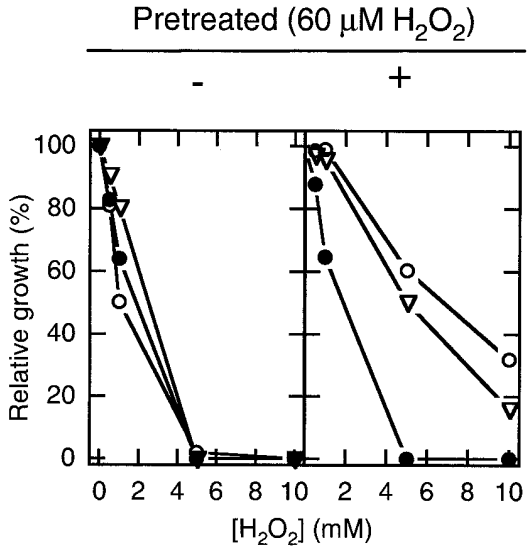

Fig. 3. Susceptibility to H_2O_2 of *Erwinia chrysanthemi* AC4150, mutant BT109, and complemented mutant BT110, before and after pretreatment with 60 µM H_2O_2 for 1 h. Nonpretreated (–) and pretreated (+) bacterial cells were incubated at 28°C in the presence of indicated H_2O_2 concentrations and growth recorded by measuring absorbance at 492 nm in an enzyme-linked immunosorbent assay plate reader. Results correspond to a typical experiment from three independent trials. Magnitude of standard errors was smaller than the symbols in all cases. 100% = bacterial growth in the absence of H_2O_2. Open circles, *E. chrysanthemi* AC4150; closed circles, mutant BT109; open triangles, complemented mutant BT110.

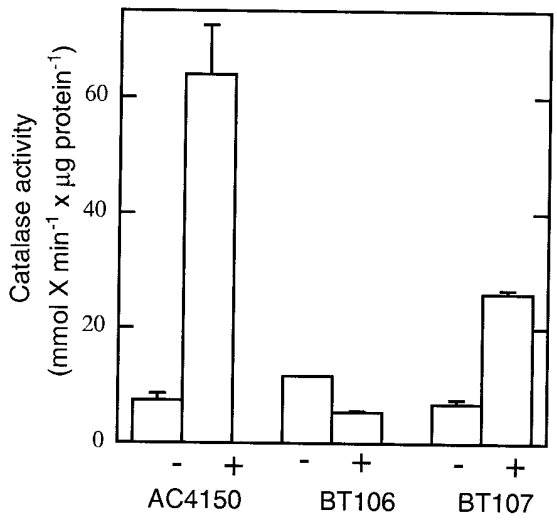

Fig. 4. Catalase activity in bacterial cell extracts from the *Erwinia chrysanthemi* strains AC4150, BT109, and BT110. Bacterial cells pretreated with 60 µM H_2O_2 for 1 h (+) or not pretreated (–), were lysated and centrifuged, and catalase activity was measured in the supernatant. Values are the mean of three independent measurements. Bars represent standard errors.

of the living pathogen (Vidal et al. 1997); and (iv) the catalase activity in the mock-inoculated leaves (constitutive plant enzymes). Figure 8 shows that the activity of the *oxyR*-independent catalases is not detectable in this assay, since the total activity after infiltration with the *oxyR* mutant is not different from the activity after the treatments with bacterial filtrates.

To study catalase induction after infection in other plant tissue, potato tubers were inoculated with wild-type bacteria or mock inoculated, and incubated for 4 h. Then, catalase activity was assayed in the tissue adjacent to the inoculation point. A twofold induction of enzyme activity was found in this tissue of the infected tuber, compared with that of the mock inoculated (6.49 ± 1.09 versus 3.09 ± 0.31 nmol × min^{-1} × µg $protein^{-1}$).

DISCUSSION

It has been suggested that the pathogen-induced oxidative burst might play a direct antimicrobial role and thus represent the first line of defense against pathogen invasion (Keppler and Baker 1989; Peng and Kuc 1992; Legendre et al. 1993; Baker and Orlandi 1995; Bestwick et al. 1997; Lamb and Dixon 1997). However, there is no direct evidence for such a role in vivo. We have used an *oxyR* mutant of *E. chrysanthemi* to investigate the direct antimicrobial role of H_2O_2. Our data suggest not only that H_2O_2 does not play such a role in the interactions investigated but also that both the wild-type strain and the *oxyR* mutant are protected from exogenous H_2O_2 in the apoplastic space of the plant.

Several lines of evidence indicate that infection by *E. chrysanthemi* induces an oxidative burst in the plant: (i) this bacterium secretes several pectic enzymes that degrade the plant cell wall polygalacturonate to oligogalacturonides (Collmer

Table 1. Effects of *oxyR*::Ω mutation on virulence of *Erwinia chrysanthemi* on potato tubers

	Size of lesion (cm^2, mean ± SE)[b]	
Experiment no.[a]	AC4150	BT109 (OxyR⁻)
1	1.783 ± 0.359	1.822 ± 0.424[c]
2	1.809 ± 0.388	1.765 ± 0.478[c]

[a] In each experiment, 20 potato tubers were pair inoculated at two locations with 5×10^6 cells of wild-type and mutant strains; lesions were indicated by browning and maceration around site of inoculation as shown in Figure 4.
[b] Values are product of length and width of the necrotic area.
[c] Differences between parental and mutant strains are not significant according to the Student's *t* test.

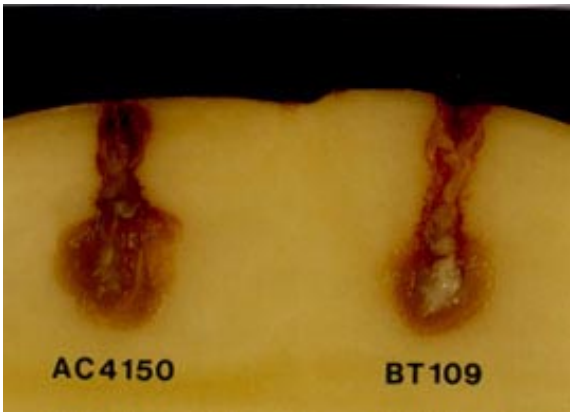

Fig. 6. Soft-rot symptoms produced by *Erwinia chrysanthemi* AC4150 and mutant BT109 on potato tubers. Each tuber was inoculated two times (AC4150 on the left, BT109 on the right) with 50 µl of a suspension containing 5×10^6 cells. Potato tubers were incubated in a moist chamber at 28°C for 48 h.

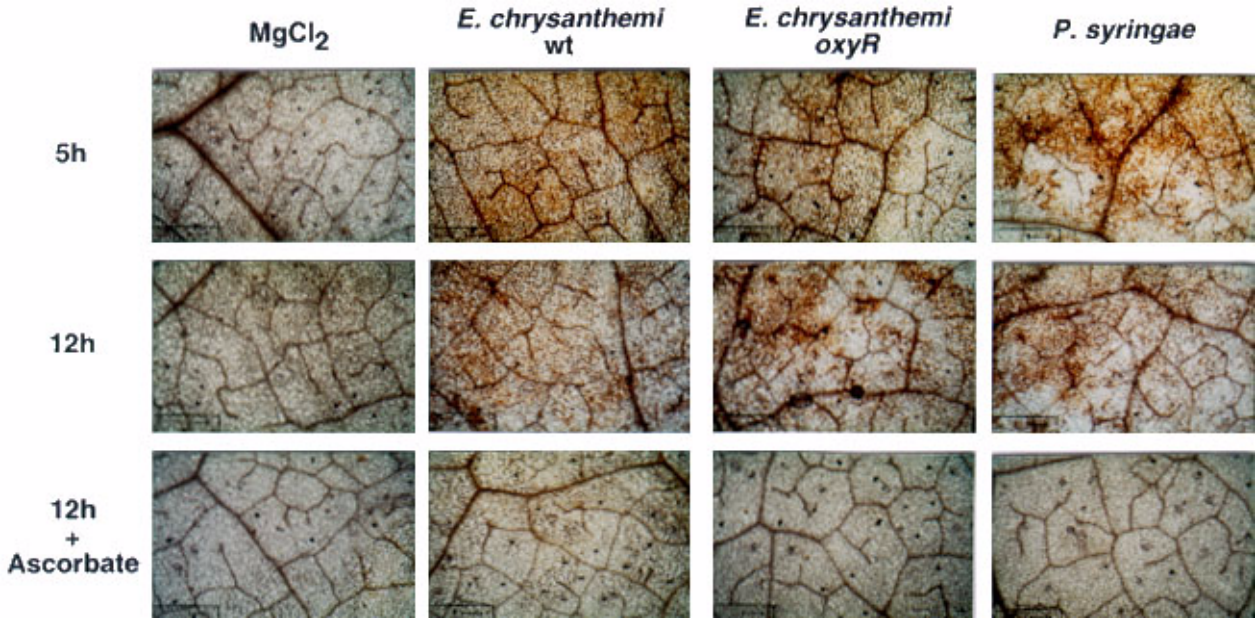

Fig. 5. Histochemical localization of H_2O_2 in tobacco leaves by the "DAB (diaminobenzidine)-uptake" method. A reddish brown pigmentation due to the presence of H_2O_2 was detected in tobacco leaves infiltrated with *Erwinia chrysanthemi* wild type, *E. chrysanthemi oxyR* mutant, and *Pseudomonas syringae* pv. *syringae*, 5 and 12 h after bacteria infiltration. Leaves infiltrated with 10 mM $MgCl_2$ were used as negative controls. In the third row, DAB (1 mg ml^{-1}) was supplemented with sodium ascorbate to 10 mM. Twelve hours after bacteria infiltration, H_2O_2 staining was abolished due to antioxidant effect of the ascorbate. Magnification: bar = 200 µm.

and Keen 1986) and several of these molecules are known to elicit oxidative burst in many plant tissues (Legendre et al. 1993); (ii) *E. chrysanthemi* possesses *hrp* genes that are homologs of those found in *P. syringae* and *Erwinia amylovora* (Bauer et al. 1994) and are able to induce a hypersensitive reaction in tobacco leaves that is apparently similar to the hypersensitive reaction induced by *P. syringae*, although this reaction is normally obscured by the maceration process due to pectolytic activity (Bauer et al. 1994); (iii) we report in this paper the elevation of catalase activity in potato tuber and tobacco leaves extracts infected with *E. chrysanthemi*, compared with the control, and infection with *Erwinia carotovora*, a phytopathogenic bacterium closely related to *E. chrysanthemi*, has been reported to produce elevated levels of a catalase-coding mRNA in potato stems and tubers (Niebel et al. 1995); and (iv) last but not least, the induction of H_2O_2 after infection in tobacco leaves is of a similar magnitude for *E. chrysanthemi* and *P. syringae* (Fig. 5).

Table 2. Effects of *oxyR*::Ω mutation on virulence of *Erwinia chrysanthemi* on tobacco leaves

	Size of lesion(cm^2, mean ± SE)[b]	
Experiment no.[a]	**AC4150**	**BT109 (OxyR⁻)**
1	1.247 ± 0.195	1.169 ± 0.138[c]
2	0.497 ± 0.076	0.436 ± 0.059[c]

[a] In each experiment, 10 leaves were pair inoculated at two locations with 5×10^6 cells of wild-type and mutant strains; lesions were indicated by browning and maceration around site of inoculation.
[b] Values are product of length and width of the necrotic area.
[c] Differences between parental and mutant strains are not significant according to the Student's *t* test.

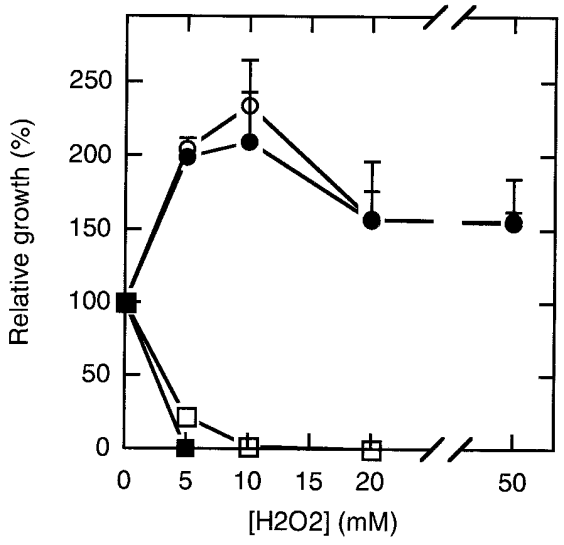

Fig. 7. In planta and in vitro susceptibility of *Erwinia chrysanthemi* AC4150 and Mutant BT109 to H_2O_2. Pretreated (60 µM H_2O_2 for 1 h) bacterial cells were treated with the indicated H_2O_2 concentrations and inoculated into tobacco leaves (in planta) or kept in a test tube (in vitro). After 5 h, plant tissue was excised and bacterial cell viability was monitored by colony plating, both in planta and in vitro. Values are mean of three independent experiments. Bars represent standard errors. Open circles: *E. chrysanthemi* AC4150 in planta. Closed circles: mutant BT109 in planta. Open squares: *E. chrysanthemi* AC4150 in vitro. Closed squares: mutant BT109 in vitro.

Although the production of H_2O_2 has long been known to occur during plant defense responses, an accurate estimate of the concentration in planta has been difficult to achieve. Baker et al. (1995) used a luminol-based technique to detect the hydrogen peroxide produced during the hypersensitive response induced in tobacco upon infection with *P. syringae* pv. *syringae* and Bestwick et al. (1997) attempted a similar measurement with a method based on the oxidation of $CeCl_3$. These authors indicated that the production of H_2O_2 is highly localized and that the plant scavenging activity is considerable. These circumstances make difficult an accurate quantification of this molecule. A semiquantitative technique based on the oxidation of diaminobenzidine (Schraudner et al. 1998) enabled us to estimate that a similar quantity of H_2O_2 is produced in the plant upon infection with *E. chrysanthemi* or *P. syringae* pv. *syringae*.

The occurrence of an H_2O_2 burst upon pathogen infection does not necessarily mean that apoplast-invading bacteria will become exposed to oxidative stress. Plants possess a very effective antioxidant system composed of enzymes, such as catalases and peroxidases, and low molecular weight compounds, such as glutathione and ascorbate, that may decompose H_2O_2 in the tissue as it evolves (Noctor and Foyer 1998). Our finding that H_2O_2 exogenously added in planta did not affect the viability of *E. chrysanthemi* wild type or that of the *oxyR* mutant, even at a concentration as high as 50 mM, suggests that H_2O_2 degradation is rapid enough in this particular environment that bacterial damage is avoided. These concentrations are well above those reported for the oxidative burst in soybean cell suspensions in an incompatible interaction, which were about 1.5 mM (Lamb and Dixon 1997). However, these results could also be explained by the possible existence of plant-induced, *oxyR*-independent bacterial catalases. To contrast this hypothesis we have performed the measurement

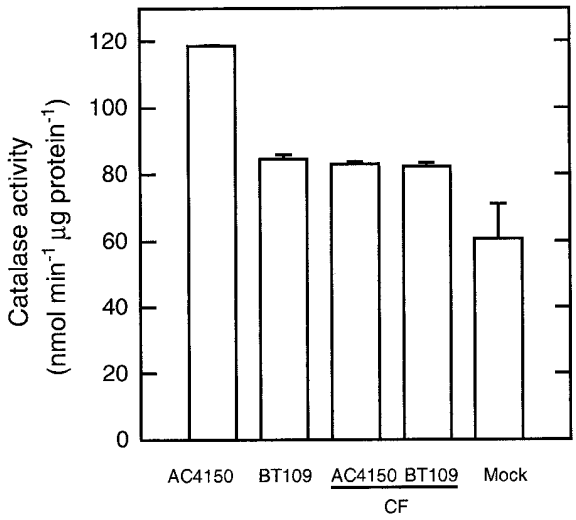

Fig. 8. Total catalase activity in extracts from infiltrated tobacco leaves. Tobacco leaves were infiltrated with 5×10^6 pretreated (60 µM H_2O_2 for 1 h) bacterial cells (*Erwinia chrysanthemi* strains AC4150 and BT109), bacterial extracellular filtrates (CF) of the wild-type and BT109 mutant strains, and mock. After 6 h, infected tissue was excised, ground, extracted, and subjected to French press treatment to break bacterial cells; then, catalase activity was assayed. Values are mean of three independent experiments. Bars represent standard errors.

of the catalase activity upon infection (Fig. 8). These results indicate that most of the catalase produced during the interaction is of plant origin, and that all the bacterial catalase is dependent on *oxyR*. These results are congruent with the hypothesis of a predominant role of the plant in the detoxification of H_2O_2. Plant-induced, *oxyR*-independent catalases, if they exist, could only account for a negligible fraction of the total catalase activity.

The *E. coli oxyR* mutant has been used to investigate the mechanisms by which plants respond to H_2O_2 stress. Gidrol et al. (1996) have reported that an Arabidopsis cDNA coding for an annexin-like protein is able to rescue the *oxyR* mutant of *E. coli* by functional complementation. These authors showed that the complemented *oxyR* mutant had a fivefold increase of catalase activity, essentially similar to the wild-type level. However, the authors did not explain whether annexin had catalase activity by itself or whether it induced this activity in the bacteria, mimicking the effect of the OxyR protein. Annexins constitute a ubiquitous family of membrane and Ca^{2+}-binding proteins whose physiological function is not clear at present, although Gidrol et al. (1996) have proposed that they are potentially involved in protecting the plant cell from oxidative stress. These data raise the possibility, which will merit future investigations, that other annexin-like proteins present in potato tubers and tobacco leaves could actually contribute to the observed rescue of the *E. chrysanthemi oxyR* mutant in planta.

We have observed that *E. chrysanthemi* is capable of adapting to H_2O_2 in a manner similar to that previously described for *E. coli* and *S. typhimurium* (Demple and Halbrook 1983). Also, the phenotype of the *E. chrysanthemi oxyR* mutant with respect to increased sensitivity to H_2O_2 and depletion of catalase and gluthathione reductase activities has been previously reported in *oxyR* mutants of *E. coli* and *S. typhimurium* (Christman et al. 1985). Our observation that the *E. chrysanthemi oxyR* mutant is unable to form individual colonies in LB (Luria-Bertani) medium looked rather puzzling at first. However, a strikingly similar observation has been made for the *Haemophilus influenzae*, *S. typhimurium*, and *E. coli oxyR* mutants (Maciver and Hansen 1996). It must be pointed out that the *E. chrysanthemi oxyR* mutant was able to grow in LB agar plates forming a bacterial grass if plated at high densities (data not shown). Also, it has been reported that an *oxyR* mutant of *S. typhimurium* is not altered in its survival rate following in vitro exposure to human neutrophils (Papp-Szabò et al. 1994).

In conclusion, our data point toward a lack of direct antimicrobial effect of H_2O_2 in the plant defense against *Erwinia* spp. invasion, possibly because the combined effects of antioxidant enzymes and reductant molecules from the plant prevent H_2O_2 from reaching concentrations that are lethal to the bacteria. The isolation of H_2O_2-sensitive mutants from other phytopathogenic bacteria, particularly *P. syringae*, and the characterization of those mutants, await future investigations.

MATERIALS AND METHODS

Microbiological methods, DNA manipulation and sequencing.

Bacterial strains and plasmids used in this work are described in Table 3. Strains of *E. coli* were cultivated at 37°C in LB medium. Strains of *E. chrysanthemi* were cultivated at 28°C in nutrient broth (Difco, Detroit, MI). Antibiotics were added to the media at the following concentrations: ampicillin, 100 µg/ml; spectinomycin, 25 µg/ml; streptomycin, 125 µg/ml (for multicopy plasmid resistance) or 10 µg/ml (for chromosomal resistance). Pretreatment of cells with nonlethal doses of H_2O_2 was performed by diluting an overnight culture into fresh medium until 0.1 OD_{600} and adding hydrogen peroxide to a final concentration of 60 µM for 1 h. Marker exchange in *E. chrysanthemi* was performed as described by Roeder and Collmer (1985). A genomic library of *E. chrysanthemi* was constructed in the λ FIX II (Stratagene, La Jolla, CA). Phagemid pBluescript SK^- (Stratagene) was used for subcloning. Standard molecular cloning techniques employed in this study (small- and large-scale plasmid and genomic DNA purification, restriction enzyme digestion, agarose gel electrophoresis, DNA subcloning, gel blot and hybridization, and colony screening by hybridization) were performed as described by Sambrook et al. (1989). DNA sequencing of both strands was done by the chain termination method on double-stranded DNA templates with an Abiprism Dye Terminator cycle sequencing kit (Perkin-Elmer, Norwalk, CT) in a 377 DNA Sequencer (Perkin Elmer, Norwalk, CT). Sequence alignments were performed at the National Center for Biotechnology Information (on-line) with the BLAST network service (Altschul at al. 1990).

Table 3. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics	Source or reference
Escherichia coli		
DH5α	*supE44 Δlac U169* (ø80 *lacZM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan 1983
XL1-Blue MRA	*Δ (mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene, La Jolla, CA
XL1-Blue MRA (P2)	XL1-Blue MRA (P2 lysogen)	Strategene
Erwinia chrysanthemi		
AC4150	Wild-type strain	Chatterjee et al. 1983
BT109	*oxyR*:: $\Omega Sp^r/Sm^r$ derivative of AC4150	This work
BT110	BT109 complemented with pB106	This work
Pseudomonas syringae pv. *syringae*		
BT111	Wild-type strain	Laboratory collection
Plasmids and phages		
pBluescript SK^-	Amp^r	Stratagene
pB106	pBluescript II carrying AC4150 *oxyR* gene	This work
pB107	pB106::$\Omega Sp^r/Sm^r$	This work
λ FIX II	Phage vector	Stratagene

Enzymatic assays.

Cell lysates were prepared by French press treatment (Dobrogosz 1991) in 50 mM potassium phosphate buffer (pH 7.0). Cell debris was removed by centrifugation at 6,000 × *g* for 2 min, and the clear lysates were filtered through a Macrosep 10K membrane (Filtron, Northborough, MA). The protein content of the lysates was measured by the Bradford procedure (Bradford 1976). Catalase activity in solution was measured as described previously (Beers and Sizer 1952). Glutathione reductase activity was obtained by monitoring glutathione-dependent oxidation of NADPH at 340 nm as described by Carlberg and Mannervik (1985).

Potato tuber extracts (cv. Jaerla) were obtained as follows: the potato tissue was ground in liquid nitrogen and extracted at 4°C for 50 min with a buffer containing 100 mM Tris-HCl, pH 7.5, 10 mM EDTA. The extract was centrifuged at 24,000 × *g* for 10 min and the supernatant was used to assay catalase activity.

To obtain tobacco leaves extracts (cv. Xanthi), the infected areas were excised and ground in 500 µl of a buffer of 100 mM Tris-HCl containing 30 mM $MgCl_2$. The suspensions were filtrated, and cell lysates were prepared by French press treatment as previously described.

Bacterial cell filtrates were obtained as follows: an overnight culture of AC4150 and BT109 mutant strains was diluted into fresh medium until 0.2 OD_{600} was reached. The suspensions were centrifuged at 4,000 × *g* for 10 min and the supernatants were filtered through an acetate filter with a 0.22 µm pore size, to exclude the bacterial cells.

H_2O_2 in situ detection by the "DAB-uptake" method.

Leaf disks of tobacco plants infiltrated with bacterial suspensions containing 5 × 10^6 CFU in 10 mM $MgCl_2$ were removed with a cork borer at 5 and 12 h after infiltration. H_2O_2 detection was performed following the "DAB-uptake" method described by Thordal-Christensen et al. (1997). Leaf disks were placed in 1 mg × ml^{-1} 3,3' diaminobenzidine (DAB-HCl) (Sigma, St. Louis. MO) and incubated at room temperature overnight. For control experiments, the DAB solution was supplemented with ascorbic acid to 10 mM. DAB reactions were examined in leaves cleared in boiling ethanol (96%) for 10 min. The samples were then stored in ethanol (96%) at room temperature or mounted in PBS (phosphate-buffered saline)/glycerol (50%) and kept at 4°C for further examination. H_2O_2 was visualized as a reddish brown coloration.

Susceptibility and virulence assays.

Susceptibility to hydrogen peroxide was measured as follows: appropriate dilutions of H_2O_2 were added to 10^6 pretreated bacterial cells in a microtiter plate and final volume was adjusted to 110 µl with nutrient broth medium, cells were incubated at 28°C, and growth was recorded by measuring absorbance at 492 nm in an enzyme-linked immunosorbent assay plate reader.

Virulence of *E. chrysanthemi* AC4150 wild type and strains BT109 and BT110 was assayed by inoculating 50 µl of a suspension containing 5 × 10^6 pretreated bacteria in a potato tuber, cv. Jaerla, purchased in a local supermarket. The bacteria were contained in a plastic tip that was inserted at a constant depth of 1.5 cm. Three inoculations (wild type, *oxyR* mutant, and complemented strain) were made in each tuber. Potatoes were left at 28°C, 100% relative humidity, for 48 h. After this time, tubers were sliced at the inoculation point and the damage was estimated by measuring the affected area. Differences between wild type and mutant were statistically assessed with a paired Student's *t* test.

Virulence was also assayed in tobacco plants cv. Xanthi. Leaves were infiltrated with 50 µl of a bacterial suspension containing 10^8 CFU per ml in 10 mM $MgCl_2$, as described by Bauer and Beer (1991). Lesions development was scored 48 h post infiltration and the damage was estimated by measuring the macerated area. Differences between wild-type and mutant strains were statistically assessed with a paired Student's *t* test.

Coinoculation experiments of *P. syringae* pv. *syringae* BT111 with *E. chrysanthemi* wild type and mutant BT109 were conducted as follows: bacterial suspensions containing 5 × 10^6 CFU of a *P. syringae* pv. *syringae* BT111-*E. chrysanthemi* AC4150 1:1 mixture, or a *P. syringae* pv. *syringae* BT111-BT109 mutant 1:1 mixture, were inoculated in tobacco leaves. The inoculated areas were excised and ground in 600 µl of 10 mM $MgCl_2$, and the sizes of the bacterial populations were estimated by colony plating. Differences between wild-type and mutant strains were statistically assessed with a paired Student's *t* test.

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