Sensitivity of *Xanthomonas campestris* pv. *citri* and Some Other Phytopathogenic Bacteria to Superoxide, Ultraviolet Light and Methylviologen

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Abstract

Incubation of *Xanthomonas campestris* pv. *citri*, pv. *campestris*, pv. *pruni*, and pv. *vesicatoria* with an extracellular O_2^- generating system, xanthine-xanthine oxidase (XOD) reaction mixture, resulted in extensive death of these bacteria. However, other tested bacteria, *Agrobacterium tumefaciens*, *Erwinia herbicola* and *Corynebacterium michiganense* were more tolerant to the O_2^- generating system. The toxicity of the O_2^- generating system to each bacterium was positively correlated with sensitivity to ultraviolet light irradiation, and negatively correlated with the activity of the bacterial superoxide dismutase (SOD). Killing of *X. campestris* pv. *citri* by the O_2^- generating system occurred with a linear dependency on log concentration of XOD. Exogenously added SOD partially negated the effect with a linear dependency on the log concentration. *X. campestris* pv. *citri* was also highly sensitive to methylviologen, which is known to produce superoxide in living cells. Co-injection into citrus leaves of a virulent isolate of *X. campestris* pv. *citri* together with methylviologen, at a concentration causing no host tissue damage, resulted in marked inhibition of the bacterial growth in the host leaf tissue. Co-injection with SOD and catalase restored the growth of the bacteria. A spray application of methylviologen onto citrus leaves, which was sprayed at 1, 7, and 24 hr after the bacteria were inoculated by spray, protected the leaves from occurrence of citrus canker lesions.

(Received February 10, 1987)

Key words: superoxide, *Xanthomonas campestris* pv. *citri*, ultraviolet light, methylviologen.

Introduction

Recent observations concerning O_2^- generation by some plant tissues reacting hypersensitively to infection with phytopathogenic fungi^{6,7)} have suggested that O_2^- , and its derivatives may function as direct or indirect components of resistance to the microorganisms. Hypersensitivity has also been implicated as a resistance response to phytopathogenic bacteria^{3-5,16,17)}. Effectiveness of superoxides in killing of fungi or bacteria may be dependent on the potential of the microorganisms to protect themselves from the activated oxygen species.

An *in vitro* system generating O_2^- , the xanthine-xanthine oxidase reaction, was demonstrated to kill some nonphytopathogenic bacteria^{2,11)}. Ultraviolet light irradiation¹⁴⁾ or methylviologen treatment¹²⁾, which are known to generate or propagate O_2^- in living cells, are also shown to be bactericidal. However, aerotolerant organisms may be generally

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protected by superoxide dismutase and associated superoxide-catabolizing enzymes¹⁵⁾.

So far there is little knowledge concerning the sensitivity of phytopathogenic bacteria to superoxides. In the present experiments, sensitivity of *Xanthomonas campestris* pv. *citri* and several other phytopathogenic bacteria to superoxides was determined using a xanthine-xanthine oxidase reaction system as an extracellular O_2^- generating system. Correlation between the sensitivity of these bacteria to $O_2^{12)}$ and internal generators of superoxide, ultraviolet light¹⁴⁾ and methylviologen¹²⁾, was examined. A brief report of this work has been published¹⁸⁾.

Materials and Methods

Bacteria and culture. *Xanthomonas campestris* pv. *citri* (U9-1, U6, U37 and U9-1CM), *X. campestris* pv. *campestris* (1076), *X. campestris* pv. *pruni* (1092), *X. campestris* pv. *vesicatoria* (1256), *Erwinia herbicola* (V2), *Corynebacterium michiganense* (C1-1-1), *Agrobacterium tumefaciens* (A277) and *Escherichia coli* (PL22) were used. U9-1, U6, U37, 1092, and V2 were kindly provided by Dr. M. Goto, C1-1-1 by Dr. A. Shirata, A277 by Dr. C. Matsui, and PL22 by Dr. H. Yamagata. U9-1CM was isolated as a colony mutant of U9-1 producing little extracellular polysaccharides. All bacteria used, except A277, were resistant to streptomycin, and were cultured on a potato-extract-sucrose agar medium (hot water extract from 60 g tuber tissue, 20 g sucrose and 15 g agar per liter) containing streptomycin at 100 μ g/ml at 28 C in the dark. In the case of A277 culture, streptomycin was omitted from the culture medium.

Bacterial cell suspensions were obtained by suspending bacteria, which were grown for 1 to 2 days on the above medium, in sterilized distilled water. The concentration of each bacterium was determined by correlating absorbance at 600 or 540 nm of the suspension with colony forming units (CFU).

Bacterial killing assay. The effects of the xanthine-xanthine oxidase (XOD) system were determined by mixing bacterial suspensions (0.2 ml of 1×10^6 /ml) with 0.02 ml of XOD in 2 M $(NH_4)_2SO_4$ at various concentrations, 0.6 ml of 0.2 M potassium-phosphate buffer (pH 7.8) and 0.38 ml of 3.16 mM xanthine solution. After incubation at 25 C for 2 hr, the reaction mixture was transferred to a ice bath, diluted 200 times with cold sterilized distilled water and colony forming units determined on PSA. The assay of O_2^- generating activity at the various concentrations of XOD involved spectrophotometric measurement of absorbance at 550 nm at 25 C from 30 to 60 sec after initiation of the reaction in reaction mixtures containing 10 μ M cytochrome c without bacteria¹³⁾.

Bacterial killing by methylviologen involved suspension of 1×10^6 cells/ml in a solution of 1 g peptone and 0.5 g NaCl per liter and varying concentrations of methylviologen. The mixture were incubated at 25 C in the light (ca. 3,000 lux) for 3 days. After filtration of each suspension through a filter (pore size: 0.45 μ), the bacteria remaining on the filter were transferred to PSA medium to determine CFU.

Bacterial killing by ultraviolet light was assayed by spreading 0.1 ml of bacterial suspension (5×10^3 cells/ml) onto PSA. Plates were incubated at 28 C for 2 hr, and

then the surface was exposed to ultraviolet light from an ultraviolet lamp (Toshiba GL-15) at distance of 30 cm for 10 sec. After growth for 3 days, bacterial colonies were counted.

Assay of superoxide dismutase activity in bacteria. A bacterial suspension (1×10^8 cells/ml) was sonicated using a cell disrupter (Heat System Ultrasonic Inc. Model W-225 at 30 W for 15 min in an ice bath. The sonicate was filtered through a membrane (pore size: 0.45μ) and the filtrate assayed for SOD activity according to a modified method of McCord and Fridovich¹³. The reaction mixture consisted of 0.2 ml of the test sample, 0.04 ml of XOD in 2 M $(\text{NH}_4)_2\text{SO}_4$, 1.2 ml of 0.1 M potassium-phosphate buffer containing 0.2 mM EDTA (pH 7.8), 0.2 ml of 120 μ M cytochrome c and 0.76 ml of 3.16 mM xanthine solution. Concentration of XOD was adjusted to that which produced a reduction of cytochrome c of O. D. 0.030/min at 550 nm. Protein content of the test sample was determined by the method of Miller¹⁶.

Bacterial colonization in citrus leaves. A bacterial suspension of 1×10^6 cells/ml was injected into the intracellular space of detached leaves by a syringe or sprayed on the lower surface of the leaves. The petioles of the inoculated leaves were placed into water and the leaflets were incubated in light (ca. 3,000 lux) or dark at 25 C.

For determination of bacterial colonization in leaf tissues disks (10 mm in diameter) were prepared from leaves which had been injected with a bacterial suspension. The tissue was homogenized in sterile distilled water and the homogenate was spread onto PSA to determine CFU.

Results

Sensitivity to an exogenous O_2^- generating system

All *Xanthomonas* spp. tested were highly sensitive to the exogenous xanthine-XOD O_2^- generating system. A few percent of each xanthomonad survived in the reaction system generating 0.95 nmoles O_2^- /min. Less than 30% of each xanthomonad survived when 0.47 nmoles O_2^- /min were generated. However, *E. herbicola*, *C. michiganense* and *A. tumefaciens* appeared to be more highly tolerant to both levels of O_2^- production (Fig. 1).

The percentage of survival of isolated U9-1 of *X. campestris* pv. *citri* and of *E. coli* were linearly dependent on log concentration of XOD, but demonstrated different dosage effects (Fig. 2). LD_{50} of *X. campestris* pv. *citri* and *E. coli* was given by concentrations of XOD providing 0.15 and 0.95 nmoles O_2^- /min, respectively (see Fig. 5).

Correlation between sensitivity to O_2^- and ultraviolet light treatment

The bacterial species displayed differential sensitivity to ultraviolet light. The bacteriocidal effect of ultraviolet light was significantly correlated to the survival rate upon treatment with a 0.47 nmole/min O_2^- generating system ($r=0.79$, $p<0.05$) (Fig. 3).

Correlation between sensitivity to O_2^- and endogenous SOD activity

Extracts from each bacterium showed substantial but variable SOD activity. SOD activity of each bacterium except *E. herbicola* was significantly correlated with percentage of survival against exogenous O_2^- generating system ($r=0.98$, $p<0.05$) (Fig. 4). *E.*

Fig. 1. Differential sensitivity of phytopathogenic bacteria to the bactericidal action of xanthine-xanthine oxidase reaction systems. Bacteria were suspended in the reaction mixtures generating O_2^- at 0.47 (open bars) and 0.95 (filled bars) nmoles/min (initial velocity), respectively. Bacterial killing was determined as described in Materials and Methods. An isolate of *Escherichia coli* was used as a reference bacterium. Survival(%) = (colony forming unit in xanthine-xanthine oxidase mixture/that in the assay mixture containing no xanthine oxidase) $\times$ 100. N. T.: not tested. Each value represents the average of three experiments.

Fig. 2. Killing of *Xanthomonas campestris* pv. *citri* (U9-1) (●) and *Escherichia coli* (○) as a function of xanthine oxidase concentration. Experiments were carried out as described in Fig. 1, except that xanthine oxidase was added at different concentrations.

Fig. 3. Correlation between bacteriocidal effects on phytopathogenic bacteria of ultra-violet light (UV) and xanthine-xanthine oxidase reaction system. Survival against UV (%) = $a/b \times 100$, where a and b are populations of viable cells exposed to UV and not exposed, respectively. Survival against O_2^- (%): see Fig. 1. A: *Xanthomonas campestris* pv. *citri* (U9-1), B: *X. campestris* pv. *citri* (U9-1CM), C: *X. campestris* pv. *vesicatoria*, D: *X. campestris* pv. *campestris*, E: *Escherichia coli*, F: *Erwinia herbicola*, G: *Corynebacterium michiganense*, H: *Agrobacterium tumefaciens*.

herbicola displayed very low SOD activity, but was relatively insensitive to the O_2^- generating system.

Protection by SOD and catalase of *X. campestris* pv. *citri* against exogenous- O_2^-

Isolate U9-1 of *X. campestris* pv. *citri* was protected against a xanthine-XOD reaction system, which provide O_2^- at a rate giving LD_{50} , by the presence of SOD, an enzyme scavenging O_2^- to H_2O_2 and O_2 . SOD protected against bacterial killing (Fig. 5) in a linear fashion with log increases in SOD concentration from 1 to 100 $\mu\text{g/ml}$. At a high concentration of SOD (100 $\mu\text{g/ml}$) less than 50% protection was achieved. Catalase (50 $\mu\text{g/ml}$) protected against the bacterial killing by almost 100% and 30% in the case of presense of SOD (100 $\mu\text{g/ml}$) and absense of SOD, respectively (data not shown).

When *E. coli* was incubated with a xanthine-XOD reaction system providing O_2^- at LD_{50} and the same concentrations of SOD as those for *X. campestris* pv. *citri*, no protective effect was found at concentrations of SOD lower than 10 $\mu\text{g/ml}$. Protection for *E. coli* was linearly dependent on log concentration of SOD from 10 to 100 $\mu\text{g/ml}$. At the highest concentration (100 $\mu\text{g/ml}$) of SOD, nearly 100% protection was achieved in the absence of catalase (Fig. 5).

Killing of *X. campestris* pv. *citri* by methylviologen

The isolated U9-1 of *X. campestris* pv. *citri* was sensitive to methylviologen treatment. *X. campestris* pv. *citri* cells were killed at concentrations of methylviologen higher than 1 μM , depending on log concentration of the compound in the range from 0.01 to 1 μM

Fig. 4. Correlation between superoxide dismutase (SOD) activity in the extracts from bacteria and sensitivity to the bactericidal effect of xanthine-xanthine oxidase reaction system. SOD activity: assayed as described in Materials and Methods. Survival against O_2^- : see Fig. 1. A: *Xanthomonas campestris* pv. *citri* (U9-1), B: *X. campestris* pv. *pruni*, C: *Escherichia coli*, D: *Erwinia herbicola*, E: *Agrobacterium tumefaciens*.

Fig. 5. Protection of *Xanthomonas campestris* pv. *citri* (U9-1) (●) and *Escherichia coli* (○) against xanthine oxidase as a function of superoxide dismutase (SOD) concentration. Each assay mixture contained xanthine oxidase generating 0.15 and 0.47 nmoles O_2^- /min for giving LD_{50} to *X. campestris* pv. *citri* and *E. coli*, respectively, and SOD at different concentrations.

(Fig. 6). In comparison, nearly 50% of *E. coli* survived at 1 μ M.

To compare the sensitivity of the *X. campestris* pv. *citri* to methyl viologen with that of its host plant leaves (*Citrus natsudaidai*), 50 μ l of solution of various concentration were injected into leaves in the light. A small necrotic lesion appeared around the needle-wound applied with 10 μ M methylviologen, but no visible changes were observed at concentrations lower than 1 μ M. At 100 μ M, a large necrotic area appeared in the light while no visible changes were observed in the dark (data not shown).

Methylviologen (1 μ M) reduced multiplication of *X. campestris* pv. *citri* (U9-1) in citrus leaves by about 70% of control when inoculated in the light, and by 23% in the dark (Fig. 7). When SOD (100 μ g/ml) or catalase (100 μ g/ml) was coinjected, some inhibition of the methylviologen effect was observed in the light but not in the dark (Fig. 7). Co-injection with a mixture of SOD and catalase stimulated bacterial multiplication (Fig. 7) in both the light and dark to above the control values.

Inoculation of citrus leaves of *X. campestris* pv. *citri* produced citrus canker lesions at 10 ± 2 lesions/leaf ($n=25$) around 10 days after inoculation in the light. However, no canker lesions appeared in the inoculated leaves that were subsequently sprayed with methylviologen solution at 1 μ M 1, 7, and 24 hr after inoculation (data not shown). Spraying with methylviologen did not cause any visible changes in the citrus leaves.

Fig. 6. Killing of *Xanthomonas campestris* pv. *citri* (U9-1) and tissue collapse of citrus leaves (*Citrus natsudaidai*) by methylviologen as a function of its concentrations. Necrotic area (bar): determined 3 days after application of 50 μ l of methylviologen solution (0.01–100 μ M) on the surface of leaf tissues with one needle wound. % inhibition of bacteria (●): $a/b \times 100$, where a and b are the populations of bacteria incubated in 0.1 % peptone containing 0.05 % NaCl for 3 days in the presence and absence of methylviologen, respectively.

Fig. 7. Effect of methylviologen on the multiplication of *Xanthomonas campestris* pv. *citri* (U9-1) in citrus leaves and recovery of its effect by superoxide dismutase (SOD) (S) and catalase (C). Bacterial suspension containing 1 μ M methylviologen (M) was injected together with SOD (100 μ g/ml) (MS), catalase (100 μ g/ml) (MC) or SOD plus catalase (100 μ g/ml, each) (MSC). The inoculated leaf disks were incubated for 3 days in the light or dark at 28 C. Multiplication rate (% control): $a/b \times 100$, where a and b are increased population of viable bacteria in leaf tissues inoculated with or without the enzymes, respectively.

Discussion

The bactericidal effect of O_2^- produced by xanthine-XOD reaction^{9,10)} was first demonstrated by Green and Pauli¹¹⁾. In the present experiments phytopathogenic bacteria *Xanthomonas* spp. were shown to be more sensitive to this O_2^- generating system, than *Erwinia*, *Corynebacterium* and *Agrobacterium* isolates.

The nature of the activated oxygen species active as a bactericide is uncertain. With *E. coli*, the inability of SOD to protect against O_2^- has led to the proposal that H_2O_2 may be the bactericidal agent²⁾. The present experiments also showed that SOD at low concentrations did not protect against the killing of *E. coli*, although complete protection was observed at high concentrations. *X. campestris* pv. *citri*, although it was more sensitive to xanthine-xanthine oxidase reaction system, was partially protected from killing by SOD even at high concentrations. These data suggest that the killing of *X. campestris* pv. *citri* may take place by a different mechanism from that for *E. coli*. The great protection conferred on *X. campestris* pv. *citri* by a mixture of SOD and catalase as well as the partial protection by SOD or catalase alone, indicates that killing may be partly mediated by $\cdot OH$, a products of a reaction between O_2^- and H_2O_2 , in addition to O_2^- or H_2O_2 alone, or both.

A positive correlation between sensitivities of phytopathogenic bacteria to the exogenous xanthine-XOD reaction system and ultraviolet light irradiation, which generate O_2^- within the cell¹⁴⁾, was revealed. These studies also demonstrated differences between the bacterial phytopathogens in sensitivity to superoxide. A negative correlation of SOD activity in extracts from the bacteria with sensitivity to the xanthine-XOD reaction system suggests that the differential sensitivity to superoxides among bacteria may be dependent on their activity to catabolize superoxides. However, one isolate of *Erwinia* showed very low activity of SOD although it was relatively tolerant to the xanthine-XOD reaction system and ultraviolet light. Its tolerance to superoxide presumably depends on mechanisms other than SOD to escape from the toxicity of superoxides.

As expected from the sensitivity of *X. campestris* pv. *citri* to superoxides, growth of this species was inhibited by methylviologen, which is known to propagate O_2^- by coupling with endogenously generated O_2^- ⁸⁾. The bacterium was nearly 100 fold more sensitive to methylviologen than leaves of its susceptible host, citrus, even in light when O_2^- can be extensively propagated by coupling with photosynthetic electron transfer. Indeed, the multiplication of *X. campestris* pv. *citri* in host leaves was greatly inhibited by injection with the methylviologen although there was no visible damage to the host tissues. The reduction of the methylviologen-inhibition of bacterial growth by SOD and catalase suggested that bactericidal levels of superoxides were being generated in host leaf tissues. A greater reduction of it by mixture of them than each suggested that an active superoxide species may be $\cdot OH$ produced by reaction between O_2^- and H_2O_2 *in situ*. The observed protection of citrus leaves from incidence of citrus canker by spraying methylviologen subsequent to *X. campestris* pv. *citri* inoculation, also supports O_2^- production in leaf tissue. The exact mechanism of bactericidal effect in leaf tissue

treated with methylviologen remain to be further investigated. The superoxide-sensitive nature of xanthomonads may possibly be utilized in control measures by applying superoxide-producing agents to plant tissues at concentrations which are bactericidal but not phytotoxic.

The authors wish to thank Drs. M. Goto, C. Matsui, A. Shirata and H. Yamagata for kind supply of the bacteria isolates and Dr. Anne J. Anderson for her critical review of this manuscript.

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和 文 摘 要

道家紀志・柴田俊浩 : *Xanthomonas campestris* pv. *citri* および数種の植物病原細菌の活性酸素、紫外線およびメチルビオロジェンに対する感受性

Xanthomonas campestris pv. *citri*, pv. *campestris*, pv. *pruni* および pv. *vesicatoria* を O_2^- 生成系であるキサントン-キサントン酸化酵素反応系に混合すると、強い殺菌効果がみられた。しかし、他に供試した *Agrobacterium tumefaciens*, *Erwinia herbicola*, *Corynebacterium michiganense* はその O_2^- 生成系に対して比較的耐性であった。各々の細菌の O_2^- 生成系に対する感受性は、紫外線感受性と正の相関を示し、また、細菌のスーパーオキシドジスムターゼ活性と負の相関を示した。*X. campestris* pv. *citri* に対する殺菌作用は、キサントン酸化酵素の対数濃度と直線的相関を示し、その反応系に加えたスーパーオキシドジスムターゼ及びカタラーゼによって部分的に回復した。本菌は生体内で O_2^- を増産するメチルビオロジェンに強い感受性を示し、本菌を宿主植物のカンキツ葉に薬害を与えない濃度のメチルビオロジェンと共に注射接種すると、顕著な増殖抑制効果がみられた。また、本菌をスプレー接種したカンキツ葉に、接種後一日以内にこの薬剤をスプレー処理すると、発病が顕著に抑制された。