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# Essential Role of Superoxide Dismutase on the Pathogenicity of *Erwinia chrysanthemi* Strain 3937

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**The *sodA* gene from *Erwinia chrysanthemi* strain 3937 was cloned by functional complementation of an *Escherichia coli sodA sodB* mutant and sequenced. We identified a 639-bp open reading frame, which encodes a protein that is 85% identical to the *E. coli* manganese-containing superoxide dismutase MnSOD. Promoter elements of this gene were identified by transcriptional mapping experiments. We constructed an *E. chrysanthemi ΔsodA* mutant by reverse genetics. The *ΔsodA* mutation resulted in the absence of a cytoplasmic SOD, which displays the same characteristics as those of MnSOD. The *ΔsodA* mutant was more sensitive to paraquat than the wild-type strain. This mutant could macerate potato tubers, similar to the wild-type strain. In contrast, when inoculated on African violets, the mutant produced, at most, only small necrotic lesions. If the inoculum was supplemented with the superoxide anion-scavenging metalloporphyrin MnTMPyP or purified SOD and catalase, the *ΔsodA* mutant was able to macerate the inoculated zone. Generation of superoxide anion by African violet leaves inoculated with *E. chrysanthemi* was demonstrated with nitroblue tetrazolium as an indicator. Therefore, at the onset of infection, *E. chrysanthemi* cells encounter an oxidative environment and require active protective systems against oxidative damages such as MnSOD to overcome these types of conditions.**

In response to an attack by microbial pathogens, plant cells produce active oxygen species (AOS), which include superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical ($OH^\bullet$). This production of AOS is termed the "oxidative burst." During incompatible interactions, i.e., those involving bacteria that cause a hypersensitive response, there are two phases of AOS production. Phase I is rapid, transient, and nonspecific, whereas the second phase occurs later and yields a much higher concentration of AOS. This specific, biphasic response is believed to be an important component of the plant host defense system (Lamb and Dixon 1997; Van Camp et al. 1998). Furthermore, these reactive oxygen species are involved in the induction of plant immunity (Alvarez et al. 1998; Levine et al. 1994). In contrast, in disease situations (compatible interactions), only phase I occurs. Little is known about the role of AOS produced during pathogenesis (compatible interactions). Is the level of this AOS production too low to be bacteriostatic or bactericidal? Or are the virulent pathogens able to resist this phase of AOS production? A partial answer to these questions now comes from the study of the pathogenicity of *Erwinia chrysanthemi*. This enterobacterium causes soft-rot diseases on a wide range of plants. *E. chrysanthemi* strain 3937 produces and secretes an array of pectinolytic enzymes that degrade plant cell walls and allow bacterial cells to disseminate throughout the plant (Barras et al. 1994; Hugouvieux-Cotte-Pattat et al. 1996). A few years ago, Favey et al. (1995) demonstrated that *E. chrysanthemi* 3937 requires a functional flavohaemoglobin for the successful infection of African violets. This finding remained obscure until it was shown that the flavohaemoglobin of *Escherichia coli* is able to oxidize NO to the less toxic nitrate molecule (Gardner et al. 1998, Hausladen et al 1998). Recently, El Hassouni and collaborators (1999) reported that the peptide methionine sulfoxide reductase, which repairs oxidized proteins, is required for full virulence of *E. chrysanthemi* strain 3937. This result suggests that during pathogenesis, the bacterium has to cope with damages caused by oxidative stress. Thus, the ability to survive in an oxidative environment may be important for pathogenicity of this bacterium. The first line of defense against oxidative stress is mediated by protective enzymes such as superoxide dismutases (SODs), which are metalloproteins that dismutate $O_2^-$ to $H_2O_2$ (Fridovich 1995). There are three general classes of SODs in bacteria, which differ in their metal cofactors (Touati 2000). The manganese- (MnSOD) and iron-containing (FeSOD) enzymes are cytoplasmic, whereas the copper–zinc (Cu,ZnSOD) enzyme is periplasmic. The contribution of SODs to pathogenicity has been well documented for animal pathogens. Secreted FeSODs are virulence factors for the bacterial species *Nocardia asteroides* and *Mycobacterium tuberculosis* (Andersen et al. 1991; Beaman and Beaman 1990). Similarly, the cytoplasmic FeSOD mutants of *Shigella flexneri* and *Bordetella per-*

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*tussis* were shown to be less pathogenic than the wild-type strains (Franzon et al. 1990; Khelef et al. 1996). Deficiency of MnSOD (*sodA* mutant) resulted in severe attenuation of *Yersinia enterocolitica* virulence in the mouse-infection model (Roggenkamp et al. 1997). The periplasmic Cu,ZnSOD also contributes to virulence of *Salmonella typhimurium* and *Haemophilus ducreyi* (Farrant et al. 1997; San Mateo et al. 1998). For phytopathogenic bacteria, the role of SOD in virulence remains unclear. Kim and collaborators (1999) showed that mutants of *Pseudomonas syringae* pv. *syringae* B728a altered in the MnSOD and FeSOD enzymes caused bacterial brown spot disease on bean pods or leaves to the same extent as the wild-type strain, although the mutants were more sensitive to the $O_2^-$-generating compound paraquat than was the wild-type strain. Smith et al. (1996) cloned a *sod* gene encoding the major SOD activity of *Xanthomonas campestris* pv. *campestris*, the metal cofactor of which is Mn. Attempts to construct a SOD-deficient mutant, however, remained unsuccessful. Interestingly, these authors used transcriptional fusions and observed that the *sod* gene was induced in planta within 3 h postinoculation, with similar kinetics during compatible and incompatible interactions (Hugouvieux et al. 1998; Smith et al. 1996). These data suggest that SOD activity may be involved in bacterial protection against oxidative stress occurring at the onset of infection. We investigated the role of SOD in the pathogenicity of *E. chrysanthemi* strain 3937 on its host plant *Saintpaulia ionantha* (African violet).

## RESULTS

### Cloning of the *E. chrysanthemi sodA* homolog.

The *E. chrysanthemi sodA*-like gene was isolated by complementation of the *sodA sodB* mutant strain QC1726 of *E. coli*. A genomic library from strain 3937 of *E. chrysanthemi* constructed in the mobilizable cosmid pAL2917 was introduced by conjugation into strain QC1726. Transconjugants were plated on selective minimal glucose medium containing streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc), and $10^{-9}$ M $O_2^-$-generating compound paraquat. Because the *E. coli* mutant lacks cytoplasmic SOD activity, it cannot grow on this medium (Carlioz and Touati 1986). After 4 days, large colonies were apparent, and 30 of them were purified twice on the same selective medium. The cosmids of these transconjugants were purified and further analyzed. Five cosmids, differing by their restriction map, complement the *E. coli* strain QC1726. A 4.8-kb *Pst*I fragment was present in all of the selected cosmids. In a Southern blot experiment, the 4.8-kb *Pst*I fragment from each cosmid hybridized with a radiolabeled DNA probe containing a part of the *E. chrysanthemi sodA* gene obtained by polymerase chain reaction (PCR; see Materials and Methods). Thus, this fragment was cloned into the pBluescript vector, and the recombinant plasmid pRS101 was introduced by transformation into the *E. coli* strain QC1726. The transformants were able to grow on minimal medium agar plates containing paraquat. A 3.5-kb *Eco*RV fragment from pRS101 was then subcloned into the pBluescript vector, giving rise to pRS101.2. Plasmid pRS101.2 was still able to complement the *sodA sodB* mutant.

### Sequence analysis of the *E. chrysanthemi sodA* locus.

The nucleotide composition of a stretch of 1,860 bp of pRS101.2 was determined by sequencing (Fig. 1). Computer analysis revealed an open reading frame (ORF) of 639 nucleotides, extending from position 729 to 1,367 and showing 70% identity with the *E. coli* gene *sodA* encoding the MnSOD enzyme. This ORF encodes a 213 amino acid protein displaying 85% identity (88.5% similarity) with the *E. coli* MnSOD enzyme. A putative promoter was identified 66 nucleotides upstream from the beginning of this ORF. For confirmation, we performed primer extension on RNA isolated from a cell culture grown in Luria-Bertani (LB) medium reaching an optical density (OD) at 600 nm of 1.5 (see below). The extension reaction product showed that transcription of the *sodA* gene started at nt 670 (A residue), confirming that the anticipated promoter was functional (Fig. 2). The 40-nt sequence encompassing this promoter region is 95% identical to the sequence of the *E. coli sodA* promoter, where we found one putative binding site for the transcription repressor of iron-regulated genes, Fur. This sequence matches the 5′-GATAATGATAATCATTATC-3′ consensus sequence at 13 out of 19 nucleotide positions and overlaps the −35 element (Figs. 1 and 2). There are three potential ATG start codons for the *E. chrysanthemi sodA* ORF. The fact that only the third ATG of the *E. chrysanthemi sodA* gene is preceded by a putative Shine-Dalgarno sequence, GGAGA, suggests that the translation of this gene starts from this position and gives rise to a polypeptide with a theoretical pI and molecular mass of 6.06 and 23,214 Daltons, respectively. At the 3′ untranslated region of the *sodA* gene, an inverted repeat (position 1,536 to 1,572) may form a 15-bp hairpin that could act as a transcription terminator that is consistent with the size of approximately 900 nucleotides from the *sodA* RNA identified by Northern experiments (data not shown). No ORF was detected downstream of the *sodA* gene.

### Construction of an *E. chrysanthemi ΔsodA* gene and *ΔsodA* mutant.

We constructed a *ΔsodA* gene lacking 345 nucleotides from position 801 to 1,145. The 5′ end of the *sodA* gene was amplified by PCR with primers S10 and S11, complementary to position 49 to 68 and 781 to 799, respectively (Fig. 3). Primer S12 (position 1,146 to 1,165) and the M13 reverse primer from the polylinker of pRS101.2 were used to amplify the 3′ end of the *sod*A gene (Fig. 3). Primers S11 and S12 carried a *Bam*HI site at their 5′ extremity. Both PCR products were cloned into vector pCR2.1-TOPO (Invitrogen, Groningen, The Netherlands), giving rise to plasmids pCRS10-11 and pCRS12-13, respectively. These plasmids were sequenced, and no mutation was found. Plasmids pCRS10-11 and pCRS12-13 were hydrolyzed with *Eco*RI–*Bam*HI and *Bam*HI–*Pst*I, respectively. The corresponding restriction fragments were purified and ligated together into the pUC18 vector hydrolyzed with *Eco*RI and *Pst*I. The resulting plasmid, pUCΔsod, thus contains a unique *Bam*HI site at the junction of the two PCR fragments. This site was used to insert the kanamycin (Km) resistance cassette from plasmid pUC4K. The recombinant plasmid was named "pKΔsod." We then attempted to introduce the *ΔsodA* gene disrupted by the cassette coding for Km resistance into the genomic *sodA* locus by reverse genetics. Three independent recombinant clones were isolated (see below). The presence of a disrupted and deleted *sodA* gene in these clones was confirmed by Southern blot hybridization experiments (data not shown). SOD activities of cell extracts from stationary

phase culture of the wild-type strain and three *ΔsodA* mutants were analyzed by native polyacrylamide gel electrophoresis (Fig. 4). *E. chrysanthemi* wild-type extracts showed two bands with SOD activity (Fig. 4). These activities were not detected in the cell extracts from the three mutants, suggesting that they are different oligomers from the same enzyme. The *E. chrysanthemi* SOD activities were not inhibited by $H_2O_2$, suggesting that they are the result of a

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   1  TTTTTCCGGGTTGAAAGCGCGCCGGCGAGCGATGGACGGACGCACCGGCTGCGGGGTCTG   60
  61  CGGGGTGGAACAATTGGAGGAAATCGGCAAACCGGTGTCGCCGCTGCCGTTCACCCAGCA  120
 121  CTTCTCGCTGTCCCGTCTGGAACAGGCGTTGCAGGCGATGAAAAGCGTACAGCAGGCAGG  180
 181  CGCGCTGACCGGTTCAACCCACGCTGCGGCCTGGCTGTCGCCAGAAGGGGAACTGTTGGG  240
 241  CGGCTGCGAAGATGTGGGCCGCCATGTGGCGCTGGACAAACTGCTGGGAACGCGGGCGCG  300
 301  ACAGCCCTGGCAGCAGGGGGCGGCGCTGGTATCCAGCCGCGCCAGCTATGAAATGGTGCA  360
 361  GAAATCCGCCATGTGCGGGGTAGAAATCCTGTTTGCGGTTTCAGCCGCCACCTCGCTGGC  420
 421  GGTAGAAGTGGCCGCGCGCTGCAATCTGACGCTGGTCGGTTTCTGCCGTCCCGGTCGCGC  480
 481  CACCGTTTATACCCACCCGCAGCGGTTGCGGGCATGATGGTCAGGCCAGGCATAGCTTAG  540
 541  ACCGATTTGTTGTTAGCTGGGGCGATTACGGCATAAACGCTTGTAATCTTGGAATAAAGC  600

 601  ACGCCGACAAACCGCCCCGTGCCACGAATAATCATTTTCAAGATCATTTAATTAACTATA  660

 661  ATGAACCTAATGCTTACGCGGCGCTAACACGCAACGGTGCCGCTCAATACTAATTGCGCA  720
                 ↳
 721  CGACAACAATGGAGATGCTAAACATGAGTTATTCACTGCCATCCCTGCCTTATGCTTATG  780
              M  E  M  L  N  M  S  Y  S  L  P  S  L  P  Y  A  Y  D

 781  ACGCACTGGAGCCGCACTTCGACAAGCAAACGATGGAAATCCATCACTCCAAACACCATC  840
       A  L  E  P  H  F  D  K  Q  T  M  E  I  H  H  S  K  H  H  Q
                      *
 841  AGGCTTACGTCAATAACGCTAATGCAGCGCTGGAGTCCCTGCCTGAGTTTGCCGGCCTGT  900
       A  Y  V  N  N  A  N  A  A  L  E  S  L  P  E  F  A  G  L  S

 901  CCGCCGAAGAGCTGATCACCAAACTGGATCAACTGCCGGCCGACAAAAAAGGCCCGCTGC  960
       A  E  E  L  I  T  K  L  D  Q  L  P  A  D  K  K  G  P  L  R

 961  GTAACAACGCCGGCGGCCACGCCAACCACAGCCTGTTCTGGAAAGGCCTGAAACTGGGTA 1020
       N  N  A  G  G  H  A  N  H  S  L  F  W  K  G  L  K  L  G  T

1021  CCACGCTGACAGGCGAACTGAAAGCCGCCATTGAGCGCGATTTCGGCAGCGTTGACGCAT 1080
       T  L  T  G  E  L  K  A  A  I  E  R  D  F  G  S  V  D  A  F

1081  TCAAGGAAAAATTCGAGCAGGCAGCAGCAACCCGTTTCGGCTCCGGCTGGGCATGGCTGG 1140
       K  E  K  F  E  Q  A  A  A  T  R  F  G  S  G  W  A  W  L  V

1141  TACTGAAAGATGACGGCAAACTGGCTGTCGTTTCCACCCCCAACCAGGACAGCCCGCTGA 1200
       L  K  D  D  G  K  L  A  V  V  S  T  P  N  Q  D  S  P  L  M
       *
1201  TGGGCGAAGCGATTTCCGGCGCGTCCGGCTACCCGATCGTCGCACTGGACGTATGGGAAC 1260
       G  E  A  I  S  G  A  S  G  Y  P  I  V  A  L  D  V  W  E  H

1261  ACGCTTACTACCTGCAATACCAGAACCGTCGCCCGGACTACATTAAAGCGTTCTGGTTCG 1320
       A  Y  Y  L  Q  Y  Q  N  R  R  P  D  Y  I  K  A  F  W  F  V

1321  TCGTAAACTGGGACGAAGCGGCTAAACGTTTTGCTGAAGCCAAAAAATAAGTAAAAGGTA 1380
       V  N  W  D  E  A  A  K  R  F  A  E  A  K  K  *

1381  AAAATTAATTTCTATACCCTAAATAATTCGAGTTGCAGGAAGGCGGCGACACAGCGAATC 1440
1441  CCCAGGAGCTTACTCAGGTAAGTAACTGGGGTGAGCGAGGAAAGCCAACGCACCTGCAAC 1500
1501  TTGAAGTATGACGGGTATAAATCAGAAATTAACGCGACAAACCCGGAATAACATTTGTTA 1560
1561  TACCGGGTTTTTTTTATTTTATTTTCCGGATTATCACTACGAAAAGCAGTATAAAGTTTT 1620
1621  ACTATGGTGCGCCGATAGATTCTGCATGTTCTTGTCATAGCCTGCACACAGCCGCCAGTA 1680
1681  TGGCAATTTCACACTCACCGTAAACCAGTTATTAATCACCTTACTTCATCACTCAGCCTA 1740
1741  ATTAATGATGGAAGAAAAAAATGGCTATAAAACTCGAAAATATCAAAGTTGGCAGGAAGT 1800
1801  TAGGATTAGGGTTTTCCTTAATCCTACTGCTGACCGTCATTATTGCTGCAGGAATTCGAT 1860
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**Fig. 1.** Nucleotide sequence of the *sodA* gene. Promoter elements −35 and −10 are in bold. A putative Fur binding site overlapping the −35 promoter element is denoted with a line above. The transcription initiation site identified by the primer extension experiment is indicated by an arrow. The potential traduction start is indicated by a bold methionine residue with its putative Shine-Dalgarno sequence underlined. The boundaries of the polymerase chain reaction-deleted region of the *sodA* gene are indicated by asterisks. A putative transcription terminator is indicated by bold and italic nucleotides. The nucleotide sequence of the *sodA* region has been deposited to the EMBL database as accession no. AJ278262.

MnSOD enzyme (Fig. 4), which is consistent with the sequence relatedness of the cloned gene.

**Growth characteristics of the *E. chrysanthemi* Δ*sodA* mutant.**

Because $O_2^-$ inactivates the enzyme dihydroxyl-acid dehydratase, which contains a 4Fe–4S cluster and catalyzes the penultimate step in the biosynthesis of branched chain amino-acids (Flint et al. 1993), we checked the growth of the Δ*sodA* mutant in minimal glucose medium. This mutant was unable to grow in this type of medium unless the branched amino acids were added to it. In liquid LB medium, the growth of the Δ*sodA* mutant during the exponential phase was slower than that of the wild-type strain. In the same medium containing paraquat at a final concentration of 5 μM, the growth of the wild-type strain was not altered, whereas the growth of the mutant was first reduced and finally stopped (Fig. 5). The addition of Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) at a concentration of 25 μM, however, partially restored the growth of the Δ*sodA* mutant in the presence of 5 μM paraquat (Fig. 5). This metalloporphyrin can be internalized and scavenge intracellular $O_2^-$ (Faulkner et al. 1994). No difference was observed between the strain 3937 and its •*sod*A derivative with a lower concentration of paraquat. We also analyzed the growth of this mutant in intercellular fluids isolated from African violet leaves. In this medium, which mimics the environmental conditions encountered during pathogenesis (Neema et al. 1993), no significant difference between the growth of the wild-type strain and its Δ*sod*A derivative was observed (Fig. 5).

**Pathogenicity of the Δ*sodA* mutant.**

We first examined the behavior of the Δ*sodA* mutant after inoculation on potato tubers. No difference was observed in the symptoms and kinetics of maceration between the wild-type strain and its Δ*sodA* derivative. When pathogenicity was assayed on potted *S. ionantha*, the wild-type strain fully macerated the inoculated leaves and, after 1 week, symptoms spread over the other aerial parts of the plant (Fig. 6). In contrast, the mutant gave 50% negative responses (i.e., no visible symptom at the inoculation site). For the other 50% of the inoculated plants, a small, brown necrotic lesion appeared after 24 h at the inoculation site (Fig. 6). This symptom remained confined at the inoculation site for the next few days. Similar data were obtained when the inoculum of the mutant was amended with the branched chain amino acids, indicating that the lack of pathogenicity was not the result of auxotrophy. Indeed, a twofold increase in viable counts (colony-forming units) relative to the initial inoculum was detected after 2 days in the leaves inoculated with the Δ*sodA* mutant. Within the same time period, the population of the wild-type strain increased tenfold. The addition of the metalloporphyrin MnTMPyP to the inoculum at a concentration of 25 μM, however, partially restored the ability of the Δ*sodA* mutant to macerate *Saintpaulia* spp. leaves. With this SOD mimic, the mu-

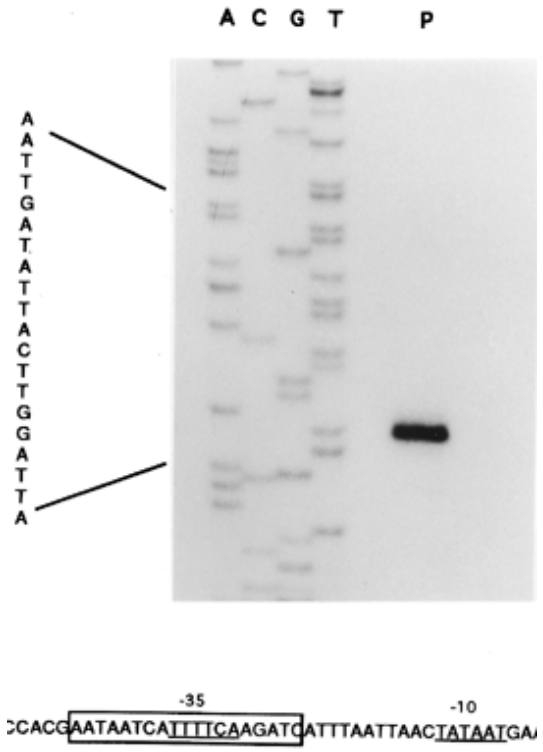


**Fig. 2.** Identification of the transcriptional start site of the *sodA* gene. Lanes A, C, G, and T are sequencing ladders. Lane P is the primer extension reaction (see text). Promoter elements −35 and −10 of the *sodA* gene are underlined, whereas the putative Fur binding site is boxed. The transcriptional start point is indicated by an arrow.

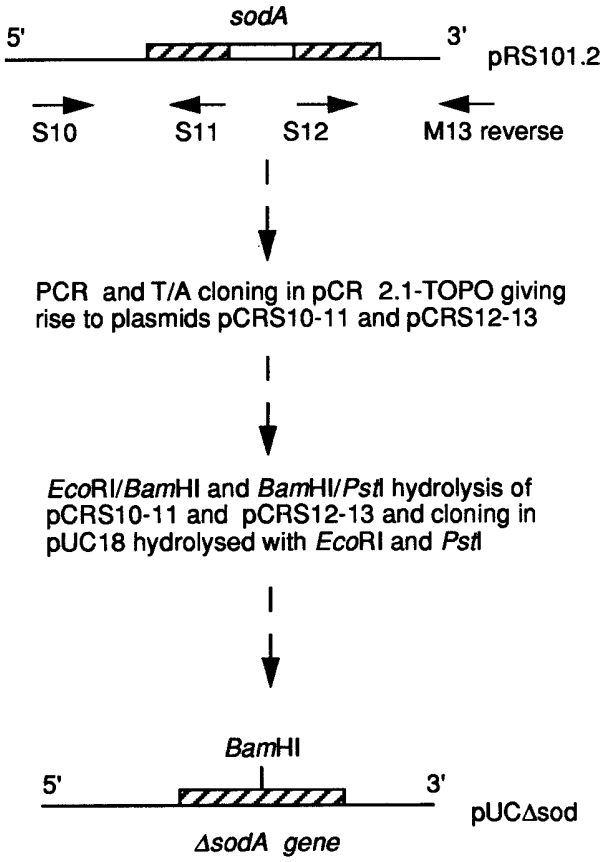


**Fig. 3.** Strategy used for constructing a Δ*sodA* gene by polymerase chain reaction. Primers S11 and S12 contain a *Bam*HI site at their 5′ end. Additional details appear in text.

tant was able to macerate the inoculated area. Within a few days, the macerated tissue dried out with the peripheral tissues, becoming chlorotic (Fig. 6). Because this compound can be internalized by the bacteria, we investigated whether a mixture of purified SOD and catalase added to the inoculum could rescue the mutant. Similar data, i.e., maceration of the inoculated zone, were obtained with these enzymes, which are known to detoxify the $O_2^-$ and $H_2O_2$ (Fig. 6). These results indicate that the *ΔsodA* mutant is subject to oxidative damages during infection and the lack of pathogenicity results from the absence of a functional superoxide dismutase.

**Superoxide production during the *E. chrysanthemi–S. ionantha* interaction.**

Because the presence of $O_2^-$ scavengers restore the capacity of the *ΔsodA* mutant to macerate African violet leaves, a superoxide burst is likely to occur during the *E. chrysanthemi–S. ionantha* interaction. African violet leaves were thus inoculated with the wild-type strain and checked every 30 min for the production of $O_2^-$ with nitroblue tetrazolium as an indicator. A blue formazan precipitate, surrounding the inoculated area, was detected 3 h postinoculation. No coloration could be visualized for shorter times or in leaves infiltrated only with the inoculation buffer (Fig. 7). Thus, when infecting their host, *E. chrysanthemi* cells encounter oxidative conditions to which they have to adapt.

## DISCUSSION

This work aimed to elucidate whether SOD activity was involved in the pathogenicity of *E. chrysanthemi* strain 3937 on African violets. We isolated and cloned an *E. chrysanthemi sodA*-like gene by complementing a *sodA sodB* mutant of *E. coli*. A *ΔsodA* mutant of *E. chrysanthemi* was constructed by reverse genetics. The SOD activity detected in wild-type cell extracts was lacking in those of the *ΔsodA* mutant. *E. chrysanthemi* 3937 thus seems to possess only one cytoplasmic SOD enzyme, which is encoded by the *sodA* gene. This mutant is auxotrophic for the branched chain amino acids and more sensitive to paraquat than the wild-type strain. Paraquat sensitivity could be reduced when the *ΔsodA* mutant was supple-

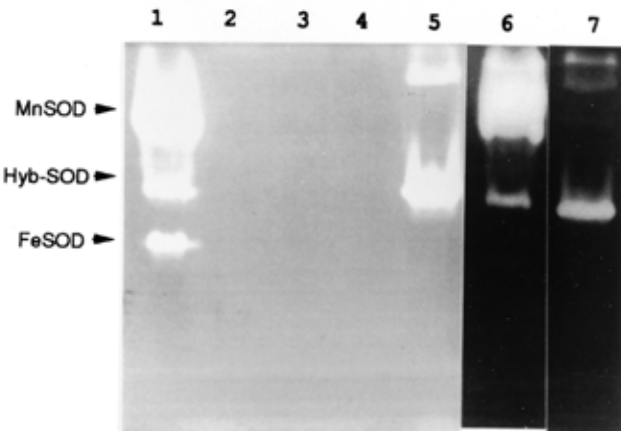


**Fig. 4.** Superoxide dismutase (SOD) activity in crude extracts of *Erwinia chrysanthemi* 3937 and its *ΔsodA* derivatives. Detection was performed on nondenaturating polyacrylamide gels stained for SOD activity (see text). Lane 1: *Escherichia coli* DH5α; lanes 2–4: *ΔsodA* derivatives of *E. chrysanthemi* 3937; lane 5: *E. chrysanthemi* 3937; lane 6: *E. coli* DH5α with 8 mM $H_2O_2$ treatment; lane 7: *E. chrysanthemi* 3937 with 8 mM $H_2O_2$ treatment. The *E. coli* manganese-containing SOD, hybrid SOD, and iron-containing SOD are indicated by an arrow.

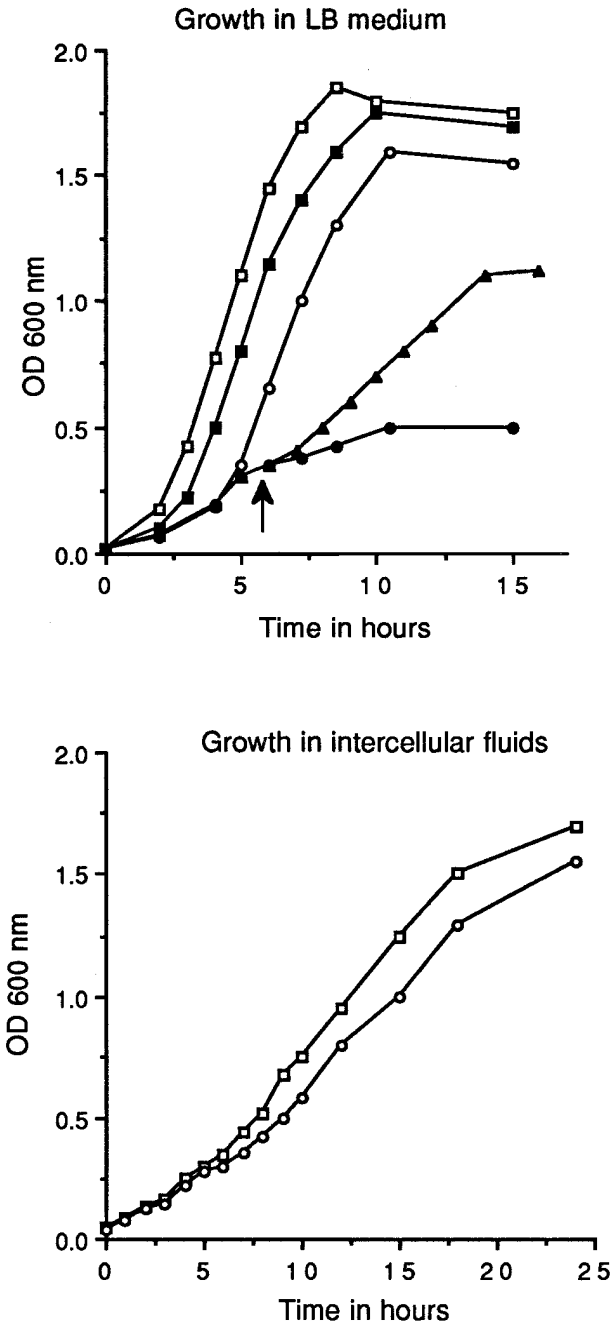


**Fig. 5.** Growth characteristics of the *Erwinia chrysanthemi ΔsodA* mutant. Strains were grown in Luria-Bertani (LB) medium or in intercellular fluids. Bacterial growth of the wild-type strain (squares) and its *ΔsodA* derivative (circles and triangles) was monitored by measuring optical density (OD) at 600 nm. Paraquat was added to the LB medium when $OD_{600}$ was at 0.2. The $O_2^-$-scavenging metalloporphyrin MnTMPyP was added at the time indicated. Open symbols: no addition; solid symbols: paraquat at 5 μM; solid triangles: paraquat at 5 μM plus subsequent addition of 25 μM MnTMPyP. Fivefold-concentrated intercellular fluids from African violet leaves were supplemented with 0.2% glucose to assess bacterial growth. Experiments were performed in duplicate. Results from one representative experiment are shown.

mented with the intracellular $O_2^-$-scavenging metalloporphyrin MnTMPyP. This mutant was able to macerate potato tubers as well as the wild-type strain, indicating that it was not impaired in pectinolytic functions. When tested on potted African violets, however, the *ΔsodA* mutant caused, at most, only small necrotic lesions at the inoculation site, whereas the parental strain gave systemic maceration symptoms. The addition of the SOD mimic MnTMPyP or the mixture of

**Fig. 6.** Symptoms caused by *Erwinia chrysanthemi* strain 3937 and its *ΔsodA* derivative on *Saintpaulia ionantha* leaves 6 days postinoculation. Top left: wild-type strain. Bottom left: *ΔsodA* mutant. Top right: *ΔsodA* mutant amended with 30 units of purified superoxide dismutase (SOD) and catalase per ml. Bottom right: *ΔsodA* mutant supplemented with 25 μM SOD mimic compound MnTMPyP.

purified SOD and catalase to the inoculum restored the capacity of the mutant to macerate the inoculated area, indicating that a superoxide burst occurs at the onset of infection. Indeed, superoxide production could be detected in African violets leaves at 3 h postinoculation around the area infiltrated with *E. chrysanthemi* strain 3937. This plant-generated superoxide is unable to cross the bacterial membrane and probably is converted into $H_2O_2$. It is thus possible that in the presence of $H_2O_2$ produced by the plant, the higher intracellular concentration of $O_2^-$ in the *ΔsodA* mutant leads to the production of the highly toxic free radical $OH^\bullet$ during the Haber-Weiss reaction catalyzed by iron (Halliwell and Gutteridge 1985). Indeed, when this mutant was inoculated only with purified catalase, it was able to macerate the inoculated zone (data not shown). Together these data indicate that bacteria encounter an oxidative environment at the onset of African violet infection. These oxidative conditions could exacerbate the deleterious effects of the high concentration of endogenous $O_2^-$ present in the *ΔsodA* mutant. The production of a maceration symptom by the *ΔsodA* mutant in the presence of different $O_2^-$ scavengers used in this work relies on the ability to partially block oxidative damages caused by the release of plant AOS. Therefore, in *E. chrysanthemi*, the *sodA*-encoded function seems to be of prime importance during the first steps of pathogenesis on African violets. The *E. chrysanthemi* HmpX function, which can metabolize NO, also is critical for pathogenicity on African violet (Favey et al. 1995). The molecule NO, which is an important redox signal involved in plant defense (Delledone et al. 1998; Durner and Klessig 1999; Durner et al. 1998), can interact with $O_2^-$ to form peroxynitrite, a highly toxic and antimicrobial compound. In the presence of NO produced by the plant, the *ΔsodA* mutant could accumulate peroxynitrite. Furthermore, an *E. chrysanthemi fur* null mutant, which accumulates iron because of the lack of the transcriptional repressor Fur, also displayed a reduced pathogenicity on African violets (Expert 1999; Franza et al. 1999). This could be the result of the accumulation of iron into the cell, which is toxic from catalyzing the production of $OH^\bullet$ and feryl radicals during oxidative stress (Nunoshiba et al. 1999; Touati 2000; Touati et al. 1995). In conclusion, during pathogenesis on its host, *E. chrysanthemi* strain 3937 encounters an oxidative environment that is strong enough to exert antibacterial activity. Under these circumstances, this pathogen may require functions involved in detoxification of active oxygen species and iron homeostasis in order to survive and cause disease.

## MATERIALS AND METHODS

### Strains and media.

The bacterial strains, cosmids, and plasmids used in this work are described in Tables 1 and 2, respectively. Nutrient-rich medium was LB (Miller 1972). M9 (Miller 1972) containing 2 g of glucose per liter as a carbon source was used as minimal medium. Amino acids were added at a final concentration of 40 μg per ml. When necessary, the following antibiotics (Sigma, St Louis, MO, U.S.A.) were used per ml: 50 μg of ampicillin (Ap), 30 μg of Cm, 30 μg of Km, 100 μg of Sm, or 25 μg of Tc. For recombinant plasmid selection, 250 of isopropyl-β-D-thiogalactopyranoside and 40 μg of X-gal (Roche Diagnostics, Mannheim, Germany) per ml (each) were added. Methyl viologen (paraquat; Sigma) was added at a final concentration of $10^{-9}$ M or 5 μM in minimal and rich medium, respectively. Amino acid utilization was monitored on M9 agar plates after 4 days of aerobic growth at 30ºC. For genetic marker exchange by homologous recombination, minimal–low phosphate medium was used (Franza et al. 1999). Intercellular fluids from *S. ionantha* were concentrated fivefold and supplemented with 0.2% glucose for growth assay experiments (Neema et al. 1993).

### Mating and transformation methods.

The *E. chrysanthemi* genomic library constructed in cosmid pLA2917 was propagated in the ED8767 strain of *E. coli* (Franza et al. 1999). Triparental matings with helper plasmid pRK2013 were performed as described previously (Franza et al. 1991). Transconjugants were selected on M9 agar plates containing Sm, Cm, Tc, and $10^{-9}$ M $O_2^-$-generating compound paraquat. *E. coli* cells were transformed by the $CaCl_2$ procedure, and *E. chrysanthemi* bacteria were electroporated as described by Sambrook et al. (1989).

### DNA methods.

Cosmid and plasmid DNAs were isolated by the alkaline–lysis method. Additional DNA manipulations (cloning and electrophoresis) were carried out as described by Sambrook et al. (1989). DNA–DNA hybridization analysis was performed by the Denhardt method described by Sambrook et al. (1989). All cloning experiments were performed in the DH5α strain of *E. coli*. Restriction fragments were isolated from Tris-borate-EDTA agarose gels with the QIAquick extraction kit (Qiagen, Hilden, Germany). DNA sequencing was performed on a double-strand plasmid by the dideoxynucleotide chain termination method with Sequenase version 2.0 and [α-$^{35}$S]

**Table 1.** Bacterial strains used in this study

| Strain | Characteristics | Source or reference |
|---|---|---|
| *Erwinia chrysanthemi* | | |
| 3937 | Wild-type strain. | Our collection |
| 3937 *ΔsodA* | *ΔsodA* with the kanamycin resistance gene from pUC4K inserted at the polymerase chain reaction-constructed *Bam*HI site ($Km^r$). | This study |
| *Escherichia coli* | | |
| DH5α | *supE*44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi relA*1 | Sambrook et al. 1989 |
| ED8767 | *supE*44 *supF*58 *hsdS*3 ($r_B^-$ $m_B^-$) *recA*56 *galK*2 *galT*22 *metB*1 | Sambrook et al. 1989 |
| QC1726 | $F^-$ Δ(*argF–lac*) U169 *rpsL ΔsodA* *sodB*::MudPR3 ($Sm^r$ and $Cm^r$) | Touati et al. 1995 |

dATP (Nycomed Amersham, Little Chalfont, Buckinghamshire, U.K.). When necessary, dimethyl sulfoxide (Sigma) was added in the annealing mixture (5% final concentration). Data were analyzed with the UW GCG software package, provided by Bisance (Dessen et al. 1990). The *E. chrysanthemi sodA* gene sequence has been submitted to the EMBL database as accession no. AJ278262.

**PCR amplification of an *E. chrysanthemi sodA* fragment.**

Degenerate primers KHH3 (5′-CCAYCAYGACAAGCAY-C-3′) and DVWEH (5′-TARTANGCRTGYTCCCANACRTC-3′) (Santos et al. 1999), designed according to the conserved amino acid regions of SOD proteins, were used to amplify a fragment of approximately 400 bp from *E. chrysanthemi* 3937 genomic DNA. The sequence of the amplified 444-bp fragment was very similar to the *E. coli sodA* sequence. PCR with degenerate primers KHH3 and DVWEH was performed as described by Santos et al. (1999). Additional PCR was performed in a DNA thermocycler with denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and an extension at 72°C for 1 min, which was followed by an extension reaction at 72°C for 10 min. When necessary, PCR products were cloned into pCR2.1-TOPO according to the manufacturer's instructions.

**RNA isolation and primer extension.**

RNAs were isolated from late exponential cultures of *E. chrysanthemi* cells grown in LB medium according to Babst et al. (1996). Primer extension was performed as described by Uzan et al. (1988) with primer 5′-GCAGGGATGGCAGTGA-ATAA-3′ complementary to positions 749 to 768. The same primer was used to sequence plasmid pRS101. Sequence and primer extension reactions were loaded and run onto a 6% sequencing gel. Extension from this primer yielded one cDNA that comigrated with a T residue (Fig. 2).

**Construction of a *ΔsodA* mutant.**

The cassette from transposon Tn903, which confers resistance to Km, was inserted in the *Bam*HI site of the *E. chrysanthemi ΔsodA* gene cloned in pUC18, generating plasmid pKΔsod (Fig. 3). This plasmid was introduced into *E. chrysanthemi* strain 3937 by electroporation. Transformants were purified once on LB agar plates containing Ap and Km. Overnight cultures in LB with Ap and Km were 20-fold diluted in low phosphate medium supplemented with glucose and all of the amino acids in order to promote plasmid destabilization with exchange recombination of the disrupted DNA insert into the *E. chrysanthemi* chromosome. After 24 h of growth without shaking, the culture was further diluted 20-fold in the same minimal medium. After three passages, the cells were plated on LB agar plates containing Km. Individual colonies were then streaked on plates containing Km plus Ap and Km only. Three $Km^RAp^S$ clones were isolated. One of these *ΔsodA* $Km^R$ mutants was used in all of the experiments described in this work.

**Preparation of cell lysates and visualization of SOD activity.**

Stationary phase cultures of cells from *E. chrysanthemi* or its *ΔsodA*-derivative mutants were harvested by centrifugation and washed in 0.5 ml of 50 mM potassium phosphate and 10 mM $MgCl_2$. Cells were disrupted by sonication, and 20 to 40 μg of proteins were loaded and run onto nondenaturing 10% polyacrylamide gels. SOD activity was visualized by nitroblue tetrazolium negative staining (Beauchamp and

**Table 2.** Cosmids and plasmids used in this study

| Plasmids or cosmids | Characteristics[a] | Source or reference |
|---|---|---|
| Cosmids | | |
| pLA2917 | 21-kb mobilizable cosmid $Tc^r$ and $Km^r$. | Allen and Hanson 1985 |
| pCS3 | 30-kb Sau3A fragment of genomic DNA from *Erwinia chrysanthemi* cloned in the *Bgl*II site of the $Km^r$ gene from pLA2917, $SOD^+$, and $Tc^r$. | This study |
| Plasmids | | |
| pRK2013 | Mobilization helper plasmid; $Km^r$. | Figurski and Helinski 1979 |
| pUC18 | 2.7-kb vector; $Ap^r$. | Sambrook et al. 1989 |
| $pSK^+$ | 2.96-kb pBluescript vector derived from pUC19; $Ap^r$. | Stratagene (La Jolla, CA, U.S.A.) |
| pUC4K | pUC-derived plasmid containing the kanamycin resistance gene from the transposon Tn903; $Ap^r$ and $Km^r$. | Nycomed Amersham (Little Chalfont, Buckinghamshire, U.K.) |
| pCR2.1-TOPO | 3.9-kb ColE1 vector for direct T/A cloning of PCR products; $Ap^r$ and $Km^r$. | Invitrogen (Groningen, The Netherlands) |
| pRS101 | 4.8 kb *Pst*I fragment from pCS3 cloned in $pSK^+$; $Ap^r$ and $SOD^+$. | This study |
| pRS101.2 | 3.5-kb *Eco*RV fragment from pRS101 cloned in $pSK^+$; $Ap^r$ and $SOD^+$. | This study |
| pCRS10-11 | 750-pb fragment from the 5′ part of the *E. chrysanthemi sodA* gene amplified with primers S10 and S11 and cloned in pCR2.1-TOPO; $Ap^r$ and $Km^r$. | This study |
| pCRS12-13 | 700-pb fragment from the 3′ part of the *E. chrysanthemi sodA* gene amplified with primers S12 and M13 reverse and cloned in pCR2.1 TOPO; $Ap^r$ and $Km^r$. | This study |
| pUCΔsod | *Eco*RI–*Bam*HI fragment from pCRS10-11 and *Bam*HI–*Pst*I fragment from pCRS12-13 cloned in pUC18 hydrolyzed with *Eco*RI and *Pst*I; $Ap^r$. | This study |
| pKΔsod | pUCΔsod with the Km resistance gene from pUC4K inserted at the *Bam*HI site of the *ΔsodA* gene; $Ap^r$ and $Km^r$. | This study |

[a] SOD, superoxide dismutase; Tc, tetracycline; Km, kanamycin; Ap, ampicillin; PCR, polymerase chain reaction.

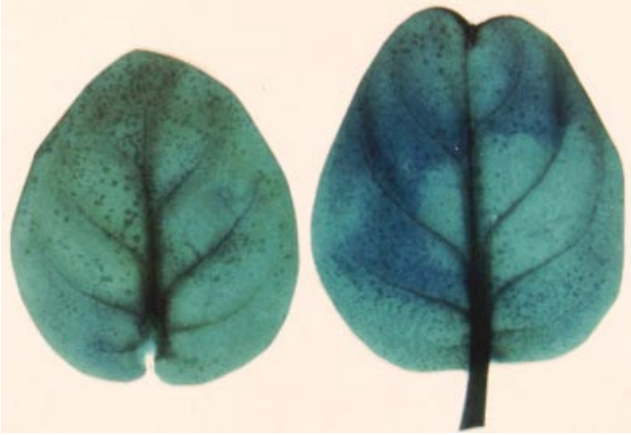

**Fig. 7.** Generation of superoxide anion during the *Erwinia chrysanthemi–Saintpaulia ionantha* interaction. Left: leaf infiltrated with suspension buffer. Right: leaf inoculated with 100 µl of *E. chrysanthemi* 3937 cell suspension. A blue formazan precipitate, which surrounds the inoculated zone, was visualized after 3 h.

Fridovich 1971). The gel was stained in presence of 8 mM $H_2O_2$ for SOD activity inhibition.

**Pathogenicity assays.**

Pathogenicity was tested on potato tubers and potted *S. ionantha* cv. Blue Rhapsody, as reported by Expert and Toussaint (1985). Twenty plants were tested for each condition. Bacterial cells were plated on LB agar medium and incubated for 24 h at 30ºC. Cells were suspended in 9 g of NaCl solution per liter to give an $OD_{600}$ of 0.4. The resulting suspension (3 µl) was used to inoculate potato tubers, whereas 100 µl of inoculum was used for one leaf per African violet plant. When necessary, 40 µg of amino acids per ml, the $O_2^-$-scavenging compound (Faulkner et al. 1994), 25 µM Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP), or 30 units of purified bovine SOD and catalase per ml (Roche Diagnostics) were added to the bacterial suspension just before inoculation. Plant-growth conditions are the same as those described by Sauvage and Expert (1994). Progression of the symptoms was scored daily for 10 days.

**Detection of superoxide production in African violet leaves.**

African violet leaves were inoculated with 100 µl of bacterial suspension or 9 g of NaCl solution per liter. Leaves were cut off every 30 min for 4 h and vacuum infiltrated with 20 mM potassium phosphate buffer containing 0.1% of nitroblue tetrazolium. After 1 h of coloration, leaves were washed overnight in 96% ethanol.

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