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A Gene for Superoxide Dismutase from *Xanthomonas campestris* pv. *campestris* and Its Expression during Bacterial-Plant Interactions

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A recombinant plasmid selected from a library of *Xanthomonas campestris* pv. *campestris* genomic DNA by functional complementation of a superoxide dismutase (SOD)–deficient strain of *Escherichia coli* contained a gene encoding the major SOD activity of *X. campestris* pv. *campestris*. Inhibition and renaturation studies suggested that manganese was the metal cofactor for this SOD. Examination of the nucleotide sequence of an active subclone revealed a 612-bp open reading frame that encodes a protein with high amino acid sequence homology to a range of SOD enzymes. The *sod* gene was mutagenized with Tn*5-lacZ*. None of the insertions that abolished SOD-conferring activity were in the correct orientation for *lacZ* expression. Repeated attempts to introduce these insertions into the chromosome of *X. campestris* pv. *campestris* were unsuccessful and it was concluded that the *sod* gene may be essential for viability. In order to monitor the expression of the *sod* gene, a *sod-gus* transcriptional fusion was constructed. Expression of the *sod* gene varied according to the growth stage of the organism in culture. In planta, the *sod* gene was induced within 3 to 4 h of inoculation, with similar kinetics during compatible and incompatible interactions with turnip and pepper, respectively.

Additional keyword: active oxygen species.

Active oxygen species (AOS), which include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^\cdot$), can be formed by a variety of mechanisms in biological systems (Sutherland 1991). AOS are endogenously generated in all aerobic organisms as a result of respiration. Many aerobes also come into contact with exogenous sources of AOS as a result of specific environmental conditions. For example, a flux of AOS is generated by both plant and animal cells in response to attack by microbial pathogens. This specific production of AOS, termed the oxidative burst in plant cells and the respiratory burst in animal cells, is believed to be an important component of host defense.

The oxidative burst consists of two phases of AOS production. Phase I AOS is rapid, transient, and nonspecific, occurring almost immediately after challenge with most saprophytic and pathogenic bacteria. Phase II AOS, in contrast, occurs much later, is larger and longer-lived, and is specifically stimulated only by bacteria that cause a hypersensitive response (Baker et al. 1991). Accumulating evidence suggests that stimulation of the oxidative burst may mediate a whole range of plant responses that, directly or indirectly, contribute to various defense mechanisms. These responses include changes in membrane permeability (Adam et al. 1989; Keppler and Baker 1989), cross-linking of cell wall proteins (Bradley et al. 1992; Brisson et al. 1994), lignification (Gross 1980; Peng and Kuc 1992), phytoalexin accumulation (Montillet and Degousee 1991; Sharma and Mehdy 1992), direct killing of plant cells (Levine et al. 1994), and direct killing of pathogens (Keppler and Baker 1989; Peng and Kuc 1992). Recent evidence also suggests that AOS may act as second messengers involved in the induction of systemic acquired resistance (Chen et al. 1993; Conrath et al. 1995) and cellular protectant genes (Levine et al. 1994).

In order to protect themselves from the potentially deleterious effects of AOS, aerobic bacteria have evolved a whole battery of defensive antioxidant enzymes. The first line of defense is mediated by protective enzymes such as superoxide dismutase (SOD) and catalase, which scavenge O_2^- and H_2O_2, respectively, and in combination catalyze their reduction to nontoxic compounds.

SODs are metalloproteins that catalyze the dismutation of O_2^-, the first reactive intermediate generated during the reduction of molecular oxygen, to H_2O_2 and O_2. Structural genes for SOD have been cloned from a wide range of organisms and have been found to encode three types of enzyme, differentiated by their metal cofactors, which can be either iron, manganese, or copper plus zinc. CuZnSOD, encoded by *sodC*, occurs primarily in the cytosol of eukaryotic cells and in the chloroplasts of higher plants (Bannister and Rotilio 1987). A few bacteria also contain CuZnSODs that show homology to the cytosolic CuZnSODs found in eukaryotes but contain an additional leader peptide indicative of a periplasmic location (Beyer et al. 1991). FeSOD, encoded by *sodB*, is primarily found among prokaryotes but has also been found within the chloroplasts of some plant species (Bannister and Rotilio 1987). MnSOD, encoded by *sodA*, is commonly found both among prokaryotes and in the mitochondria of eukaryotic cells. *Escherichia coli* contains all three types of SOD: FeSOD and MnSOD, which are located in the cytoplasm, and

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CuZnSOD, which is located in the periplasm (Benov and Fridovich 1994).

SODs have been established as virulence factors for several animal pathogens. For example, virulent strains of *Nocardia asteroides* possess an extracellular SOD activity that has been shown to protect cells against oxidative killing in vivo (Beaman et al. 1983; Beaman and Beaman 1990). *Mycobacterium tuberculosis* also possesses an extracellular SOD activity that is thought to be an important virulence factor (Kusunose et al. 1976; Anderson et al. 1991). Similarly, FeSOD mutants of *Shigella flexneri* and CuZnSOD mutants of *Brucella abortus* were shown to be significantly less virulent than the corresponding wild-type originals (Franzon et al. 1990; Tatum et al. 1992). Since the production of AOS is a common feature of both plant and animal defense responses, it is clearly possible that SODs may also act as virulence factors in plant pathogens, although there is no evidence of this to date. As an approach to answering this question, we have cloned the gene for the major SOD activity of *X. campestris* pv. *campestris*. This was achieved by functional complementation of an *E. coli* strain deficient in SOD activity (QC1891; Carlioz and Touati 1986), an approach that has been successfully used to clone *sod* genes from a variety of organisms (Hassett et al. 1993; Haas and Goebel 1992; Natvig et al. 1987; Van Camp et al. 1990). Furthermore, we describe the construction of a *sod-gus* transcriptional fusion and its use to study SOD expression during growth in culture and in plant tissues.

RESULTS

Cloning of a *sod* gene from *X. campestris* pv. *campestris*.

E. coli QC1891 transconjugants resulting from the transfer of a library of *X. campestris* pv. *campestris* genomic DNA were selected for functional complementation of the *sod* phenotype on rich media containing high levels of paraquat (PQ, 10^{-4} to 10^{-6} M). No colonies grew on plates containing 10^{-4} M PQ. Twenty-seven large colonies, clearly visible on a background of very poorly growing bacteria, were picked from plates containing both 10^{-5} M and 10^{-6} M PQ onto each of a second set of minimal media selection plates containing lower levels of PQ (10^{-8} to 10^{-11} M PQ). Sixteen of the 27 colonies grew on one or more of the lower concentrations of PQ.

Extracts from each of these 16 transconjugants were analyzed for specific SOD activity following native PAGE. All exhibited a single, identical, SOD activity that ran slightly faster than that of the *E. coli* FeSOD (Fig. 1). Corresponding analysis of bacterial extracts from wild-type *X. campestris* pv. *campestris* revealed three SOD activities, two discrete activities running slightly faster than the *E. coli* FeSOD and MnSOD activity bands and a third SOD band running approximately equidistant between the two in the same relative position as the hybrid SOD in *E. coli* (Fig. 1). Restriction enzyme analysis with *Eco*RI and *Hin*dIII of plasmid DNA extracted from transconjugants revealed that all of the clones conferring resistance to PQ were identical. One plasmid, subsequently referred to as pIJ3093, was chosen as a representative for further work. Extraction and re-introduction of pIJ3093 into QC1891 and "curing" of the plasmid from QC1891 following introduction of the incompatible plasmid pPH1JI clearly demonstrated that the presence of pIJ3093 was associated with the ability to direct SOD synthesis in *E. coli* QC1891.

To characterize the metal cofactor requirement of the enzyme encoded by pIJ3093, inhibitor studies were performed. One of a duplicate set of native gels containing extracts of *X. campestris* pv. *campestris* or QC1891/pIJ3093 was soaked in H_2O_2 prior to staining for SOD activity. In addition, extracts were treated with 2 mM KCN for 30 min before electrophoresis. The SOD activity encoded by the cloned *X. campestris* pv. *campestris* SOD gene was not inactivated by H_2O_2 nor by KCN, indicating that the activity was not due to FeSOD or CuZnSOD. The apoenzyme of the SOD produced by QC1891/pIJ3093 was generated by treatment with guanidinium hydrochloride at low pH and was renatured in the presence of 2 mM $FeSO_4$ or $MnCl_2$, exactly as described by Yamakura et al.

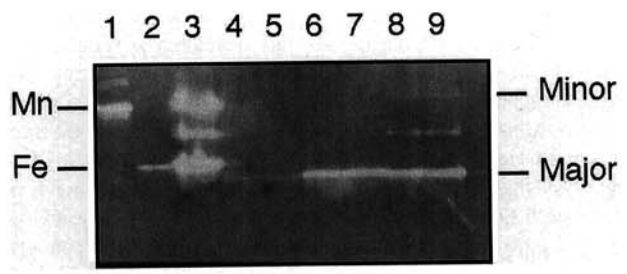

Fig. 1. Superoxide dismutase (SOD) activity in extracts of *Escherichia coli* and *Xanthomonas campestris* pv. *campestris*. Analysis of SOD activity was by specific enzymatic visualization following separation by native polyacrylamide gel electrophoresis. Lane 1: *E. coli* MnSOD standard (Sigma); lane 2: *E. coli* FeSOD standard (Sigma); lane 3: *E. coli* TG1 (wild type); lanes 4 and 5: *E. coli* QC1891 (*sodA sodB*); lanes 6 and 7: *E. coli* QC1891/pIJ3093 (pLAFR3 containing 22.5 kb of *X. campestris* pv. *campestris* DNA); and lanes 8 and 9: *X. campestris* pv. *campestris* 8004 (wild type). Bands running between the MnSOD and FeSOD in lane 3 and the major and minor bands in lanes 8 and 9 are believed to be hybrid SOD activities.

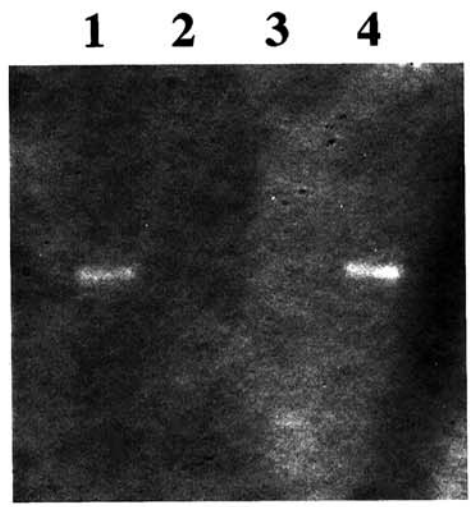

Fig. 2. Metal ion requirement of *Xanthomonas campestris* pv. *campestris* superoxide dismutase (SOD) expressed by *Escherichia coli* QC1891/pIJ3093. The SOD apoenzyme was generated by treatment with guanidinium hydrochloride at low pH and reconstituted with $FeSO_4$ or $MnSO_4$. SOD activities were assessed by specific visualization following native polyacrylamide gel electrophoresis. Lane 1: Native SOD; lane 2: apoenzyme; lane 3: Fe-reconstituted enzyme; and lane 4: Mn-reconstituted enzyme.

(1995). Only Mn^{++} restored SOD activity to the inactive apoenzyme (Fig. 2). These results indicate that the gene in pIJ3093 encodes the MnSOD of *X. campestris* pv. *campestris*. This is consistent with sequence relatedness (see below).

Localization of the *X. campestris* pv. *campestris sod* gene within the cosmid pIJ3093.

pIJ3093 contained 22.5 kb of *Xanthomonas* DNA. Subclones were generated by restriction enzyme digestion and were cloned into pIJ3200, a broad host range vector that can be transferred to *E. coli* by conjugation. Each of the subclones generated was transferred to the SOD-deficient *E. coli* strain QC1891 to test for functional complementation of the *sodA sodB* phenotype. SOD activity of transconjugants was analyzed by specific enzyme visualization following native PAGE. SOD-conferring activity was sequentially localized to a 4.0-kb *Hin*dIII/*Bam*HI fragment (pIJ3095; Fig. 3) and a 2.5-kb *Hin*dIII/*Kpn*I fragment (pIJ3096; Fig. 3).

Sequence analysis of the *sod* gene.

Examination of the nucleotide sequence of the insert DNA from pIJ3096A revealed a 612-bp open reading frame that would encode a protein with high amino acid sequence homology to a range of SOD enzymes. The nucleotide sequence, approximately 200 bp of sequence preceding the start of the coding region, and the predicted 204 residue amino acid sequence are shown in Figure 3. The predicted amino acid sequence of the *X. campestris* pv. *campestris* SOD was submitted to a FASTA homology search of the Swissprot database. The deduced protein product was most similar to the Fe/MnSOD from *Methylomonas J* (81% identity), and showed extensive homology to MnSOD sequences from a range of bacteria (57 to 66% identity) and sequentially lower levels of homology to bacterial FeSODs (46 to 47% identity), eukaryotic MnSODs (45 to 48% identity) and FeSODs isolated from plants (32 to 44% identity).

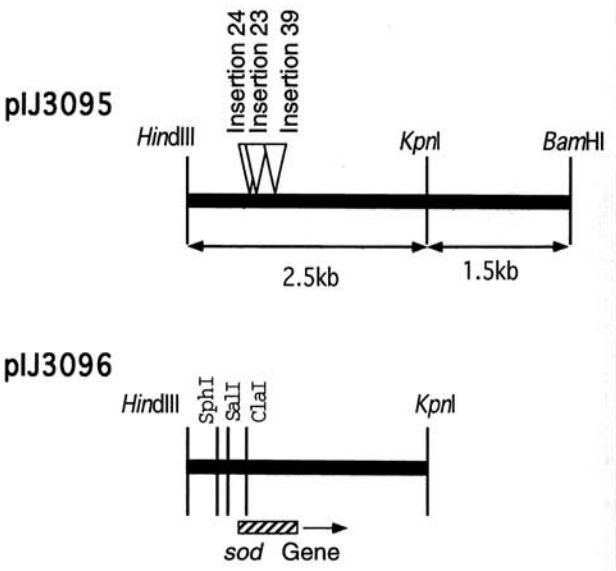

Fig. 3. Subclones of pIJ3093 generated during localization of *Xanthomonas campestris* pv. *campestris sod* gene. Relevant restriction sites and the position of Tn*5-lacZ* insertions that abolished superoxide dismutase–conferring ability are indicated.

Primer extension analysis of RNA revealed a single primer extension product, which mapped the transcriptional start site of the *X. campestris* pv. *campestris sod* gene to 41 bp upstream of the ATG start codon (see Figures 4 and 5). The ribosome-binding or Shine-Dalgarno sequence and putative promoter bases in the –10 and –35 regions are indicated in Figure 4.

Tn*5-lacZ* mutagenesis of pIJ3095.

Plasmid DNA was extracted from 10 single colonies chosen at random from each of 10 pools of independently mutagenized cells and digested with *Hin*dIII and *Sac*I. Clones in which the 4.0-kb fragment was replaced with a fragment of 12.3 kb contained a Tn*5-lacZ* insertion within the target region. Fifteen clones with Tn*5-lacZ* insertions within the target DNA were identified and transferred into *E. coli* QC1891 by conjugation. Bacterial extracts from each of the transconjugants were analyzed for SOD activity following native PAGE. Three independent insertions, 23, 24, and 39, eliminated the ability of pIJ3095 to confer SOD activity to *E. coli* QC1891. These insertions were respectively mapped to 1.9 kb, 2.0 kb, and 1.7 kb from the internal *Kpn*I site (see Figure 3). None of the insertions were in the correct orientation for *lacZ* expression.

Transfer of Tn*5-lacZ* insertions into the *X. campestris* pv. *campestris* genome.

Attempts made to marker exchange Tn*5-lacZ* insertions 23, 24, and 39 into the chromosome of wild-type *X. campestris* pv. *campestris* 8004 by the standard method used in our laboratory (Turner et al. 1985) were unsuccessful. It was hypothesized that *X. campestris* pv. *campestris* 8004 may be severely compromised or unable to survive without an intact copy of the *sod* gene. Consequently, further attempts to marker exchange Tn*5-lacZ* insertion 39 were made with a range of modified procedures designed to reduce the damaging effects of endogenously produced oxygen radicals. Selection at lower temperatures, on minimal media (MMXC) or in the presence of 0.1 or 0.5 mM ascorbate, an antioxidant, did not allow recovery of any marker exchange mutants.

Expression of the *X. campestris* pv. *campestris sod* gene in bacterial cultures.

As none of the Tn*5-lacZ* insertions were in the correct orientation for *lacZ* expression, it was necessary to construct a *sod-gus* transcriptional fusion to follow SOD expression. *sod* expression, measured as GUS activity per cell of *X. campestris* pv. *campestris* 8004/pIJ3099-3, was followed in cultures grown in rich and minimal media. Cultures grown in both types of media grew to similar densities although bacteria grew slightly better in rich media than in minimal media (Fig. 6). *sod* expression appeared to be growth phase-dependent, peaking about 20 h after inoculation, at an OD_{600} of 0.9 to 1.0, and was significantly greater (on a per cell basis) in cultures grown in rich media (Fig. 6).

Expression of the *X. campestris* pv. *campestris sod* gene in bacteria in planta.

Leaves of turnip and pepper plants were infiltrated with a suspension of *X. campestris* pv. *campestris* 8004/pIJ3099-3 in water at a concentration of 5×10^7 CFU/ml. At intervals after inoculation, disks were excised from the leaves and homoge-

nized in sterile water. The bacterial numbers and the level of GUS in these homogenates were measured as described in Materials and Methods. The *sod* gene expression was calculated as GUS activity per 10^8 CFU of recoverable bacteria. In both plants, *sod* expression increased after a lag of approximately 2 h (Fig. 7). Little difference was seen between expression in turnip (compatible interaction) and pepper (incompatible interaction) in terms of the kinetics or magnitude of the response. This experiment was repeated several times with longer time courses but with essentially the same result.

DISCUSSION

The cloned *sod* gene from *X. campestris* pv. *campestris* encodes the major activity in this organism, which is probably a MnSOD. Similarly, Chamnongpol et al. (1995) reported that the major SOD enzyme of *X. oryzae* pv. *oryzae* is a MnSOD based upon inhibitor studies. Although the amino acid sequence of the *X. campestris* pv. *campestris sod* gene is more similar to bacterial MnSOD sequences than to FeSOD sequences, it shows the highest level of similarity to the cambi-

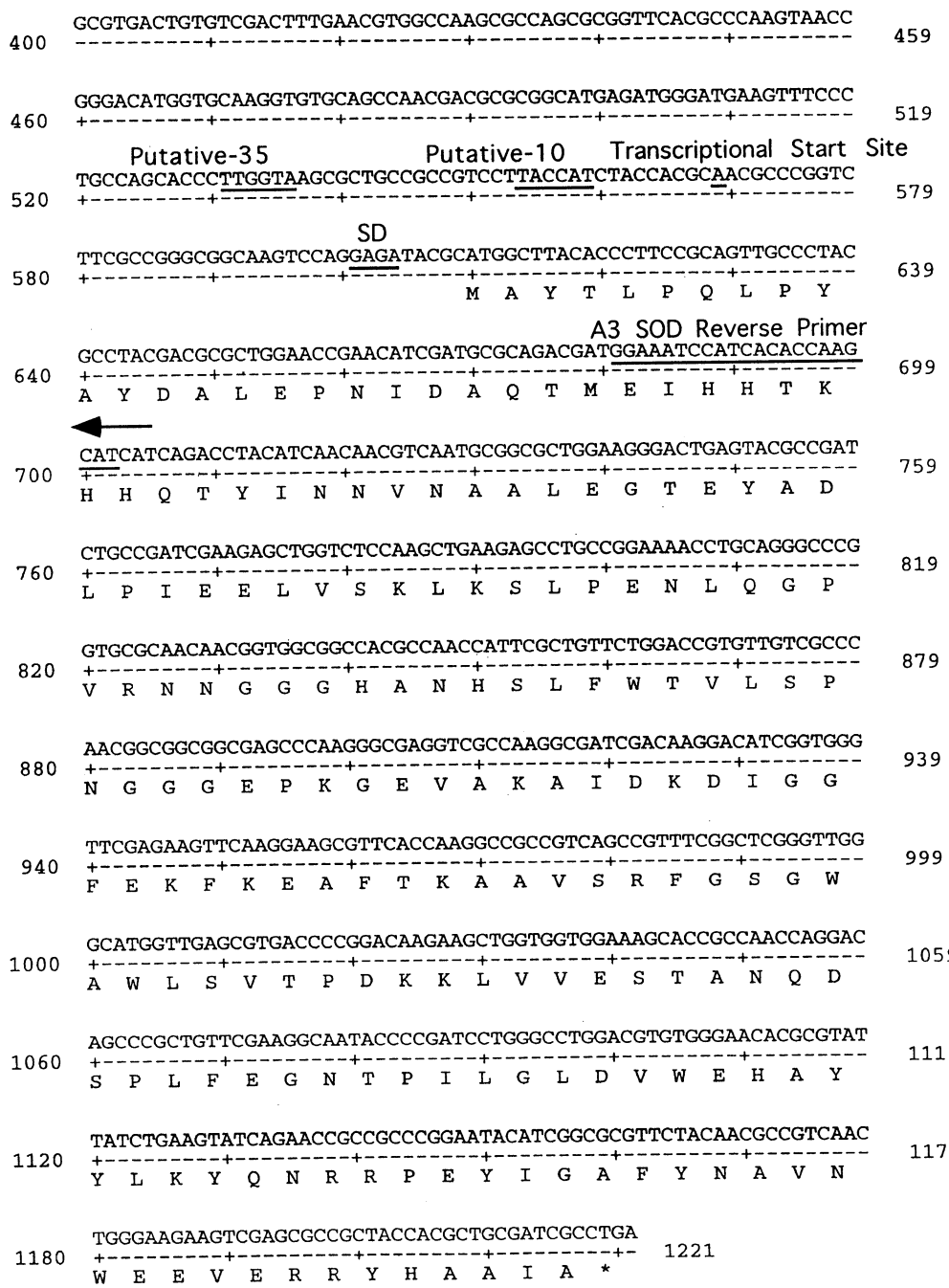

Fig. 4. Part of the nucleotide sequence of insert DNA from pIJ3096A including the complete *sod* gene from *Xanthomonas campestris* pv. *campestris*. The transcriptional start site, putative –10 and –35 sequences, Shine-Dalgarno sequence, and the predicted amino acid sequence are indicated.

alistic Fe/MnSOD from *Methylomonas J*. Despite considerable sequence homology, most FeSODs and MnSODs are only active when the catalytic site is occupied by the metal found in the native enzyme. However, a few species of bacteria, including *Bacteroides fragilis* (Gregory and Dapper 1983; Gregory 1985), *Streptococcus mutans* (Martin et al. 1986), and *Methylomonas J* (Matsumoto et al. 1991) produce a single SOD protein that can contain and be active with either iron or manganese at the active site. These enzymes have been designated "cambialistic" SODs (Martin et al. 1986). The high degree of relatedness between the *X. campestris* pv. *campestris* SOD and the Fe/MnSOD from *Methylomonas J* may imply that the *X. campestris* pv. *campestris* SOD is also cambialistic. The specific activity of cambialistic SOD enzymes is much lower with Fe than with Mn as the cofactor (Matsumoto et al. 1991), which could explain the apparent absence of activity seen in the *X. campestris* pv. *campestris* SOD apoenzyme reconstituted with Fe. However, if the *X. campestris* pv. *campestris* SOD is cambialistic, the reconstitution and inhibition experiments suggest that Mn is the cofactor in the native enzyme under the growth conditions used.

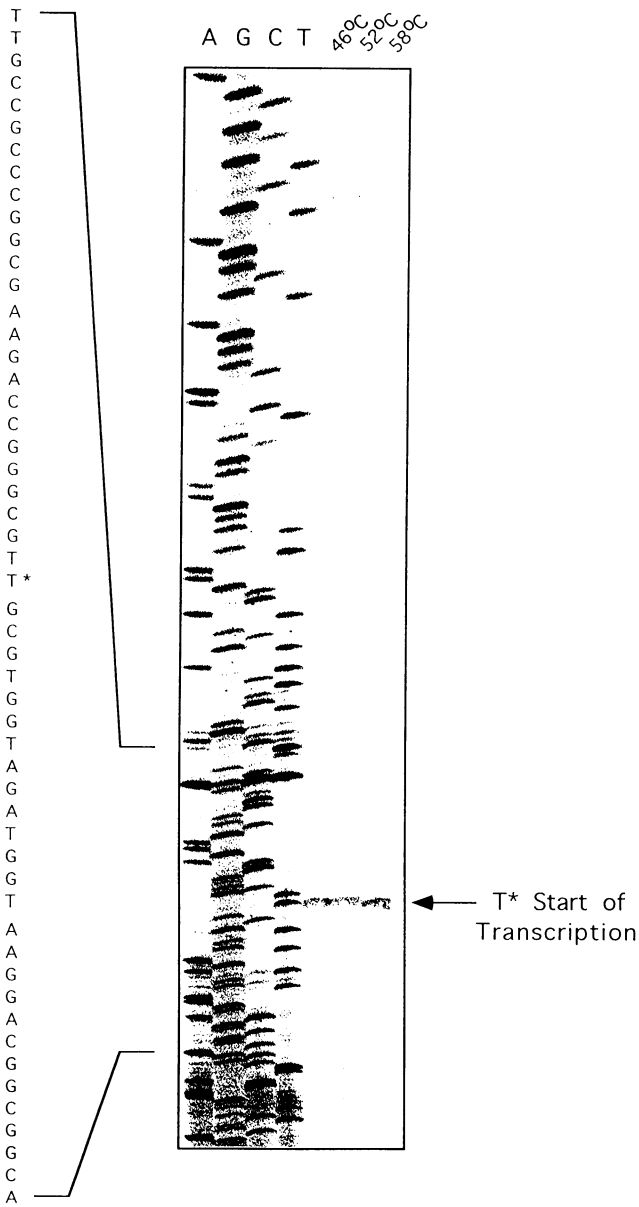

Fig. 5. Primer extension analysis of the *sod* gene from *Xanthomonas campestris* pv. *campestris*. The first four lanes represent the sequencing ladder based on reactions using the A3SOD primer. The annealing temperatures for primer extension are given above each lane.

Repeated attempts to introduce a mutated copy of the *sod* gene into the chromosome of wild-type *X. campestris* pv. *campestris* were unsuccessful. This may suggest that the *X. campestris* pv. *campestris* SOD is essential for viability and that the minor SOD activity seen on native gels cannot compensate for the loss of the major activity. Similar work carried out by Sadosky et al. (1994) on *Legionella pneumophila* strongly suggests that the gene product encoded by *sodB*, FeSOD, is required for viability of *L. pneumophila*.

The level of transcription of the *sod* gene was significantly lower in cells grown in minimal media (MMXC) than in cells grown in rich media (NYGB; Fig. 6). This difference is probably due, at least in part, to the different metabolic rates of *X. campestris* pv. *campestris* 8004/pIJ3099-3 during growth in NYGB and MMXC. The doubling time of bacteria grown in NYGB, 2.37 h, was significantly lower than that of bacteria grown in MMXC (3.13 h), indicating that the metabolic rate and thus the requirement for SOD activity were also probably greater in cells grown in rich media. This lower level of *X. campestris* pv. *campestris sod* gene expression observed in minimal media compared with that in rich media contrasts with work on other microorganisms. In *Pseudomonas putida*, a root-colonizing saprophyte, SOD activity in extracts from cells grown in rich or sugar-deficient nitrogen media are similar, whereas SOD activity of both *E. coli* and *S. typhimurium* is repressed during growth in media containing free sugar such as sucrose (Katsuwon and Anderson 1989).

Chamnongpol et al. (1995) have reported that the specific activity of SOD increases in the earliest phase of growth after dilution of late log phase cultures of *X. oryzae* pv. *oryzae* and then declines. This is an unusual pattern of expression; the levels of SOD in many other bacteria are highest during the early stationary phase with the lowest activities in the early log phase. This more commonly observed pattern for the levels of SOD is consistent with our measurements of the level of transcript for the *sod* gene in *X. campestris* pv. *campestris* using the *sod-gus* transcriptional fusion. We do not understand the apparent contradiction between our results and those of Chamnongpol et al. (1995). Detailed differences in experimental protocol, particularly in the dilution factor of the cells used to establish the culture, may be critical, however.

The *sod* gene from *X. campestris* pv. *campestris* 8004 was up-regulated following introduction of the bacteria into plants. However, no differences in kinetics or magnitude of the response were seen between compatible and incompatible interactions with turnip and pepper, respectively. We do not know what factors are responsible for this increased *sod* expression. One possibility is that it is a response to the generation of AOS by the plants. Since *X. campestris* pv. *campestris* 8004 is compatible with turnip and incompatible with pepper, bacterial infiltration presumably elicits phase I AOS production in turnip and both phase I and II in pepper. The absence of differential induction of the *sod* gene in the two plants could be explained if bacteria respond only to the nonspecific phase I

AOS generation but with no further response to phase II in pepper. However, exposure of *X. campestris* pv. *campestris* to exogenous AOS generated by a xanthine-xanthine oxidase system had no effect on the induction of the *sod* gene (J. M. Dow, unpublished results), suggesting that induction in planta is not a direct response to extracellular O_2^- or H_2O_2 alone. In culture, the major SOD enzyme of *X. campestris* pv. *campestris* can be induced by addition of PQ (S. G. Smith, unpublished results), which generates O_2^- intracellularly by redox cycling. This raises a second possibility: that the *sod* gene induction observed in planta is a response to endogenous plant compounds (such as plumbagin from *Plumbago*) that are active as redox cycling agents. It is not known how widespread such agents are within the plant kingdom. A third possibility is that induction is simply a response to the new nutritional or environmental conditions within the plant intercellular spaces. This response to a changed environment may mirror in some way the transition from mid-log to late log/early stationary phase in liquid cultures.

The expression of the *X. campestris* pv. *campestris sod* gene in planta could be studied more rigorously by monitoring GUS activity of *X. campestris* pv. *campestris* 8004/pIJ3099-3 in near isogenic lines of a suitable host plant during both compatible and incompatible interactions. Complementary studies of the production of AOS by the host plant in analogous compatible and incompatible interactions with *X. campestris* pv. *campestris* 8004 would test whether any correlation exists between the amount and timing of AOS production by the plant and the level and duration of *sod* gene induction in the inoculated bacteria. However, adequately characterized plant material is not available for such experiments.

We were unable to create a *sod* mutant of *X. campestris* pv. *campestris* to test definitively the role of the enzyme in plant pathogenesis. Nevertheless, the results presented do support the notion that the SOD activity encoded by the cloned *sod* gene is important in bacterial metabolism and protection/adaptation during both compatible and incompatible interactions. Further investigation of the role of antioxidant enzymes in protection against oxidative stress in *X. campestris* pv. *campestris* should prove to be both exciting and informative.

MATERIALS AND METHODS

Growth of bacteria and plants.

Bacterial strains and plasmids used in this work are listed in Table 1. Growth media, cultural conditions, and procedures for plasmid transfer by conjugation were as described by Turner et al. (1984, 1985). Turnip (Just Right) and pepper (Early Calwonder ECW 10R) were grown in a greenhouse with 16 h light at a temperature of 15 to 20°C or 20°C, respectively.

DNA manipulation.

Plasmid purification, restriction endonuclease mapping, gel electrophoresis, and DNA ligation were performed by standard procedures (Sambrook et al. 1989). DNA restriction fragments were isolated from agarose gels following the procedure of Heery et al. (1990).

Complementation of *E. coli sodA sodB* mutant QC1891 and visualization of SOD activity.

A useful characteristic of *E. coli sodA sodB* mutants, which forms the basis of the selection employed in complementation

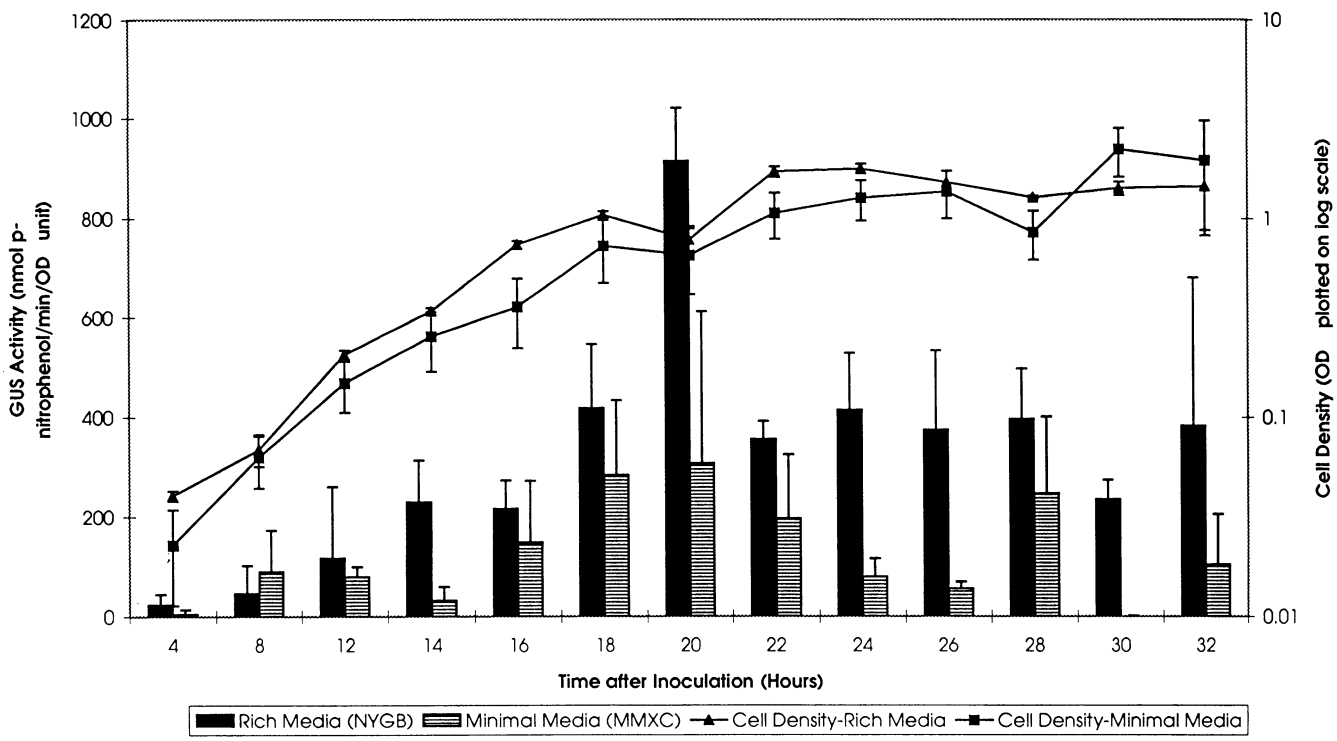

Fig. 6. Expression of the *Xanthomonas campestris* pv. *campestris sod* gene during growth in vitro. Cultures of *X. campestris* pv. *campestris* 8004/pIJ3099-3 (pLAFR3 containing *sod-gus* transcriptional fusion) grown in rich and minimal media were assayed for GUS activity with *p*-nitrophenyl-β-D-glucuronide by the procedure of Wilson et al. (1992).

experiments, is their inability to grow on aerobic minimal medium (M63; Miller 1972) unless certain amino acids are supplied (Carlioz and Touati 1986). Although SOD$^+$ clones cannot be screened on minimal media alone (since "pseudo revertants," which can grow at modest rates under these conditions, appear at quite a high frequency as a result of spontaneous mutation), they can be successfully screened on media containing PQ, a generator of superoxide, since SOD-deficient pseudo revertants remain sensitive to PQ.

A library of wild-type *X. campestris* pv. *campestris* genomic DNA, cloned into the broad host-range cosmid vector pLAFR1 (Daniels et al. 1984), was transferred to a *sodAsodB* mutant of *E. coli*, QC1891 (Carlioz and Touati 1986). SOD$^+$ transconjugants were selected on rich media containing appropriate antibiotics and high levels of PQ (10^{-4} M to 10^{-6} M). Cell extracts were prepared for native polyacrylamide gel electrophoresis (PAGE) as described by Hassett et al. (1993). Samples were applied to 10% nondenaturing gels and stained for SOD activity according to the procedure of Salin and McCord (1974). To characterize the SOD enzyme encoded by the cloned *X. campestris* pv. *campestris sod* gene, one of a duplicate set of native gels containing extracts of *X. campestris* pv. *campestris* (separated by native PAGE) was soaked in 8 mM H_2O_2 for 15 min prior to staining for SOD activity. This technique is widely used to characterize different types of SOD since FeSOD and CuZnSOD are inactivated by hydrogen peroxide but MnSOD is not (Matsumoto et al. 1991).

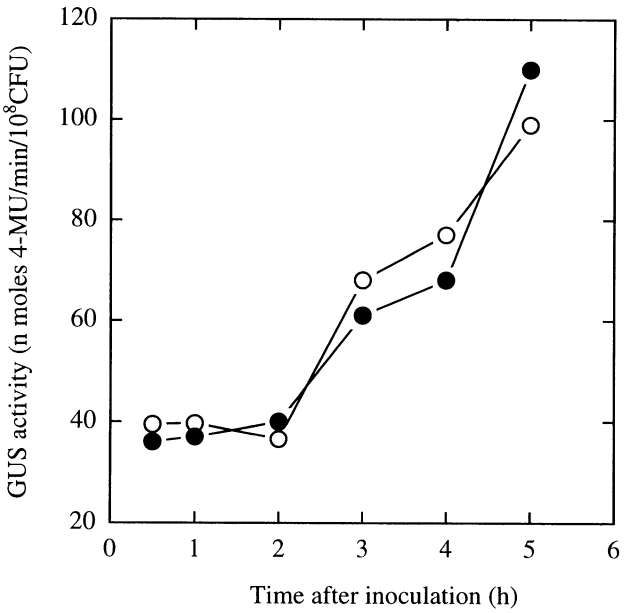

Fig. 7. Expression of the *Xanthomonas campestris* pv. *campestris sod* gene during the compatible interaction with turnip (open circles) and incompatible interaction with pepper (closed circles). Leaf tissue was inoculated with *X. campestris* pv. *campestris* 8004/pIJ3099-3 (pLAFR3 containing *sod-gus* transcriptional fusion) at 5×10^7 CFU/ml. GUS activity of homogenates of leaf disks was assayed fluorimetrically by release of 4-methylumbelliferone (4-MU). Bacterial numbers were determined by plating serial dilutions on nutrient agar. Data are expressed as GUS activity per 10^8 CFU and are the means of replicate measurements.

DNA sequencing and primer extension.

The 2.5-kb *Hin*dIII/*Kpn*I fragment in pIJ3096 was cloned into pUC18 to produce pIJ3096A. Both ends of the insert contained in pIJ3096A were sequenced, using forward and reverse universal primers. The DNA sequences generated from these reactions were translated in all six reading frames and compared with the protein sequences held on the Swissprot database in a FASTA homology search. One of the deduced amino acid sequences was found to be highly homologous to sequences of a range of structural genes for SOD. Thus, the *X. campestris* pv. *campestris sod* gene was predicted to lie toward the *Hin*dIII site at the end of the 2.5-kb insert in pIJ3096A. Additional clones for sequencing were generated by cutting pIJ3096A with *Sph*I or *Sal*I, followed by religation to create two deletion derivatives, pIJ3096AΔSph and pIJ3096 AΔSal. DNA was sequenced with forward and reverse universal primers and a range of reverse primers that were synthesized on the basis of the already determined sense strand sequence. Automated sequencing was carried out with an Applied Biosystems (Foster City, CA) 373A DNA Sequencer. Double-stranded DNA was prepared by means of a Wizard miniprep system (Promega, Madison, WI) from pUC18 plasmids maintained in *E. coli* XL1Blue, and sequenced as described in the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit Protocol (Applied Biosystems). Sequence data were analyzed by computer with Applied Bio-

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Reference
Escherichia coli DH5α	Wild type	Hanahan 1983
E. coli XL1Blue	*recA1* Tc^r	Bullock et al. 1987
E. coli BW313	F′ *dut1 ung1*	Tye et al. 1978
E. coli TG1	Wild-type F′	Gibson 1984
E. coli QC1891	TG1 *sodA sodB* Km^r Cm^r	Carlioz and Touati 1986
Xanthomonas campestris pv. *campestris* 8004	Wild-type Rif^r	Turner et al. 1984
pBluescriptKS+	Amp^r	Short et al. 1988
pUC18	Amp^r	Yanisch-Perron et al. 1985
pLAFR1	Tra$^-$ Mob$^+$ IncP replicon Tc^r	Friedman et al. 1982
pIJ3200	As pLAFR1 with M13 polylinker	Liu et al. 1990
pLAFR3	As pLAFR1 with pUC8 polylinker	Staskawicz et al. 1987
pPH1JI	Tra$^+$ Mob$^+$ IncP replicon Gm^r Sp^r	Beringer et al. 1978
pIJ3093	22.5-kb *Eco*RI fragment of *Xanthomonas* DNA cloned in pLAFR1, confers SOD activity	This work
pIJ3095	4.0-kb *Hin*dIII/*Bam*HI fragment cloned in pIJ3200, confers SOD activity	This work
pIJ3096	2.5-kb *Hin*dIII/*Kpn*I fragment cloned in pIJ3200, confers SOD activity	This work
SLJ4D4	35S-*gusA* fusion in pUC118	Jones et al. 1992

systems SeqEd software version 1.03 and compiled by means of the GCG fragment assembly program (NEWGELSTART).

Total RNA was extracted from *X. campestris* pv. *campestris* 8004/pIJ3095 following the procedure described by Gilman (1987). The location of the 5′ end of *X. campestris* pv. *campestris* sod RNA was determined using an AMV Reverse Transcriptase Primer Extension System (Promega) and a reverse primer, A3SOD: TGC TTG GTG TGA TGG ATT TCC, which specifically binds 69 nucleotides downstream of the start codon of the *sod* coding sequence. To determine the exact position of the 5′ end, the same end-labeled primer was used concurrently in a manual DNA sequencing reaction. The products of this reaction, which was carried out with a *fmol* DNA sequencing kit (Promega), were run adjacent to the primer extension product on a 6% sequencing gel.

Tn*5-lacZ* mutagenesis and marker exchange.

Tn*5-lacZ* mutagenesis of pIJ3095 was carried out as described by Arlat et al. (1991), using the transposon Tn*5*-B20 (Simon et al. 1989). Single colonies were selected from each of 10 independent mutagenesis experiments. Initial attempts were made to introduce Tn*5*-B20 insertions into the genome of *X. campestris* pv. *campestris* 8004 by the marker exchange procedure of Turner et al. (1985). Marker exchange was also attempted with a range of modified procedures designed to alleviate problems that may be caused by mutation of the chromosomal copy of the *sod* gene (see Results). The position and orientation of insertions were mapped following digestion with *Bam*HI or *Kpn*I and verified by sequencing with a primer, Tn5lac: TGG AAA ACG GGA AAG GTT CCG TT, that binds 51 bp from the end of Tn*5*-B20.

Oligonucleotide-mediated site-directed mutagenesis.

A *Kpn*I/*Cla*I fragment of DNA containing the target sequence of the *X. campestris* pv. *campestris sod* gene was cloned from pIJ3096A into pBluescriptKS$^+$ by means of the *Kpn*I site of the vector. The resulting recombinant plasmid, pIJ3097, contained 668 bp of *X. campestris* pv. *campestris* DNA encompassing the entire upstream sequence and 56 bp of the coding sequence of the *sod* gene (bases 1 to 668; Fig. 3). pIJ3097 was transformed into *E. coli* BW313 (*dut ung*) and in vitro mutagenesis was carried out according to a procedure based on the Kunkel method (Sambrook et al. 1989), using the mutagenic primer: GTA AGC CAT GGG TAT CTC C. Since the mutation creates a new and unique *Nco*I site, putative mutated plasmids were screened by restriction enzyme analysis. The presence of the new *Nco*I site was verified by DNA sequencing. The plasmid containing the mutagenized DNA sequence was called pIJ3098.

Construction of the *sod-gus* transcriptional fusion.

The subclones generated during construction of the *sod-gus* fusion are listed in Table 2. The *gus* expression plasmid SLJ4D4 (Jones et al. 1992) was digested with *Nco*I and *Hin*dIII to release a 2.6-kb fragment containing the coding sequence of the *gusA* gene from *E. coli* and the octopine synthase 3′ polyadenylation sequences. This 2.6-kb *Nco*I/*Hin*dIII fragment was gel purified, ligated into *Hin*dIII/*Nco*I cut plasmid DNA from pIJ3098 (containing pBluescript vector plus *X. campestris* pv. *campestris* DNA up to the introduced *Nco*I site), and subsequently transformed into wild-type *E. coli* DH5α. Colonies were screened on plates containing Amp and X-Gluc (40 μg/ml) to detect β-glucuronidase activity. 1% (wt/vol) glucose was also added to the plates to suppress the intrinsic GUS activity present in *E. coli*. Plasmid DNA extracted from five blue colonies (putative GUS$^+$) was digested with *Kpn*I and *Hin*dIII to verify the presence of the 2.9-kb vector (pBluescript) and the 3.2-kb insert containing 2.6-kb *gusA* DNA plus 0.6 kb *X. campestris* pv. *campestris* DNA. The plasmid that contained the *sod-gus* fusion was called pIJ3099-1. To allow ligation of the *sod-gus* fusion into pLAFR3, pIJ3099-1 was digested with *Kpn*I and *Hin*dIII and the fusion construct was gel purified and ligated into pUC18. Plasmid DNA from putative pUC18/*sod-gus* transformants, selected as before, was verified by restriction enzyme analysis. The plasmid containing the correct vector and insert fragments was called pIJ3099-2. The *sod-gus* fusion was subsequently cloned into pLAFR3 after digestion with *Eco*RI and *Hin*dIII and gel purification. The pLAFR3 plasmid containing the *sod-gus* fusion, called pIJ3099-3, was transferred to *X. campestris* pv. *campestris* strain 8004 (wild type) by conjugation. Since pLAFR3 lacks promoter sequences adjacent to the cloned DNA and *X. campestris* pv. *campestris* does not contain an indigenous *gusA* gene, any GUS activity present in the resultant transconjugants should reflect the activity of the *X. campestris* pv. *campestris sod* promoter alone.

Assay of *sod* gene expression.

Five-hundred-milliliter flasks containing 100 ml of rich media (NYGB) or minimal media (MMXC) and appropriate antibiotics were inoculated with 1.0 ml of an overnight culture of *X. campestris* pv. *campestris* 8004/pIJ3099-3 diluted to an OD_{600} of 0.5. The activity of GUS per bacterial cell was measured as described by Wilson et al. (1992). The average and standard deviation of three replicate measurements were subsequently calculated. For assay of the expression of the *sod* gene in bacteria in planta, leaves of 4- to 5-week-old turnip and pepper plants were inoculated with a suspension in water of *X. campestris* pv. *campestris* 8004/pIJ3099-3 at 5×10^7 CFU/ml as described by Newman et al. (1994). Leaf disks (0.9 cm in diameter) were taken from the inoculated area at different time points after inoculation. These were homogenized in sterile, distilled water and the homogenates assayed for GUS activity with the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide after permeabilization of the bacterial cells by addition of sodium dodecyl sulfate (SDS)

Table 2. Summary of subclones generated during construction of *sod-gus* fusion

Plasmid	Relevant characteristics
pIJ3096A	2.5-kb *Kpn*I fragment in pUC18 (contains *Xanthomonas campestris* pv. *campestris sod* gene)
pIJ3097	*Kpn*I/*Cla*I subclone of pIJ3096A containing 668 bp of *X. campestris* pv. *campestris* DNA cloned in pBluescriptKS$^+$
pIJ3098	As pIJ3097 with introduced *Nco*I site at ATG start of *sod* gene
pIJ3099-1	*Kpn*I/*Hin*dIII fragment carrying *sod-gus* fusion cloned in pBluescriptKS$^+$
pIJ3099-2	*Kpn*I/*Hin*dIII fragment carrying *sod-gus* fusion cloned in pUC18
pIJ3099-3	*Hin*dIII/*Eco*RI fragment carrying *sod-gus* fusion cloned in pLAFR3

and chloroform (Wilson et al. 1992). Fluorescence was measured on a Titertek Fluoroskan II microtiter plate reader with reference to a standard curve of 4-methylumbelliferone in 0.4 M sodium carbonate. Bacterial numbers were measured after plating serial 10-fold dilution of 10-μl aliquots of the homogenate (before addition of SDS and chloroform) on NYG agar plates with rifampicin (50 μg per ml) and tetracycline (5 μg per ml). The growth of *X. campestris* pv. *campestris* 8004/pIJ3099-3 after inoculation into turnip and pepper showed similar kinetics to those described previously by Newman (1995) for the wild-type strain 8004.

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