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A Gene-Tagging System for Monitoring of *Xanthomonas* Species

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A novel chromosomal gene tagging technique using a specific fragment of the fatty acid desaturase-like open reading frame (*des*-like ORF) from the *tox-argK* gene cluster of *Pseudomonas syringae* pv. *phaseolicola* was developed to identify *Xanthomonas* spp. released into the environment as biocontrol agents. *X. campestris* pv. *convolvuli* FB-635, a pathogen of *Convolvulus arvensis* L., (bindweed), was chosen as the organism in which to develop and test the system. A 0.52 kb DES fragment amplified from *P. syringae* pv. *phaseolicola* C-199 was inserted into pGX15, a cosmid clone containing a 10.3 kb *Eco*RI-*Hin*dIII fragment derived from the xanthomonadin biosynthetic gene cluster contained in plasmid pIG102, to create a *pig*G::DES insertion. The 10.8 kb *Eco*RI-*Bam*HI fragment carrying the *pig*G:: DES insertion was cloned into pLAFR3 to generate pLXP22. pLXP22 was then conjugated into *X. campestris* pv. *convolvuli* FB-635 and the *pig*G::DES insertion integrated into the bacterial chromosome by marker exchange. Rifampicin resistant, tetracycline sensitive, starch hydrolyzing, white colonies were used to differentiate the marked strain from yellow pigmented wild-type ones. PCR primers specific for the unique DES fragment were used for direct detection of the marked strain. Results showed the marked strain could be detected at very low levels even in the presence of high levels of other closely related or competitive bacteria. This PCR-based DES-tagging system provides a rapid and specific tool for directly monitoring the dispersal and persistence of *Xanthomonas* spp. released into the environment.

Keywords : *des*-like ORF, gene tagging, monitoring, PCR, *Xanthomonas campestris* pv. *convolvuli*.

The adverse environmental effects of continued reliance on the use of chemical herbicides are well known and show the necessity of developing effective alternative weed control strategies. One alternative is the use of host-specific newly introduced foreign plant pathogens or genetically modified microorganisms (GEM). The release of foreign and/or GEMs into the environment is being pursued with some caution, in part because very little is known about their fate. A prerequisite to the release of any pathogen, regardless of its origin or determined host specificity, should be the availability of a simple, reliable method to identify the pathogen without any ambiguity. This is critical to assessing accurately the risk of its release. Accurate identification and monitoring of a released organism into the ecosystem is more reliably performed using genetically marked organisms. These genetic markers should be highly specific and detectable under conditions in which the released organism may be present at very low levels or in samples containing a large excess of other organisms. A marker system often used for tagging involves the generation of mutants resistant to certain antibiotics. Unfortunately, such mutations can cause deleterious effects on other characteristics of the organism, including environmental fitness of the GEMs. In addition, an indigenous soil organism resistant to antibiotics can be present in small numbers. A relatively sensitive and selectable system using *lacZY* genes from *Escherichia coli* has been developed for monitoring fluorescent pseudomonads (Drahos et al., 1986). However, the *lacZY* system loses selectivity when indigenous lactose-catabolizing microorganisms exist in the ecosystem.

Other tagging methods using gene probes (Bej et al., 1991; Bej et al., 1990), PCR-based *moc*-tagging system for *Pseudomonas fluorescens* (Hwang et al., 1994), bioluminescence (*lux*) (Shaw et al., 1992) and green fluorescent protein (GFP) (Egener et al., 1998) have been developed to detect GEMs released into the environment. While very useful, these markers do not allow for direct selection and enumeration of bacteria. Insertion of a *lux* cassette into *Xanthomonas campestris* pv. *campestris* (Shaw et al., 1992) and GFP-tagging of *Azoarcus* (Egener et al., 1998) have been used for this purpose, also. Although the *lux* system is highly specific and apparently unique among rhizosphere and phyllosphere bacteria, bioluminescence is an energy demanding process, and successful detection of *lux*-tagged bacteria requires that they be metabolically active. Another disadvantage of both of these systems is that the genes for *lux* and *gfp* are carried on a transposable element. Such

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genes would therefore have a much higher probability of horizontal transfer to another organism with an eventual concomitant loss of specificity.

In this study, we describe the development of a novel PCR based gene tagging system that utilizes the unique DES fragment of the phaseolotoxin gene of *P. syringae* pv. *phaseolicola* (Hatziloukas et al., 1995) as a PCR tag. We tested the strategy on a strain of *X. campestris* pv. *convolvuli* (FB-635) that is being considered for the biological control of bindweed, *Convolvulus arvensis* L.

Materials and Methods

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani (LB) broth at 37°C. *X. campestris* pv. *convolvuli* strains were cultured in nutrient broth-yeast extract (NBY) broth medium and nutrient starch agar (NSA) or yeast-extract dextrose calcium carbonate (YDC) agar plates at 28°C (Schaad, 1989). When necessary, the following antibiotics were used: rifampicin (100 μg/ml), tetracycline hydrochloride (50 μg/ml), kanamycin (25 μg/ml), and ampicillin (100 μg/ml).

DES fragment. A 523 bp fragment containing the DES sequences (Hatziloukas et al., 1995), was amplified from *P. syringae* pv. *phaseolicola* C-199 with the tailed primers 5605fx (5'-GCGC**CCCGGG**TGTTCGCCAGAGGCA-3') and 6105rx (5'-CGCG**CCCGGG**AGCTTCTCCTCAAAACAC-3'), (the *Xma*I restriction site-incorporated into primers are underlined). Five μl of whole cell suspensions, denatured at 95°C for 10 min, were added to a PCR reaction mixture containing each primer at 4 pmole, 1x Thermo buffer, each dNTP at a concentration of 500 nM, and 0.5 U of Vent DNA polymerase (NEB, UK). PCR amplification was performed with a Perkin Elmer PE9600 by using "Touch-down" PCR. After 10 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and elongation at 75°C for 1 min, the annealing temperature was decreased 1°C each cycle until it reached annealing temperature of 56°C. The PCR was completed with an additional 25 cycles of denaturation at 94°C for 30 s, annealing 53°C for 30 s and elongation at 75°C for 1 min. Ends were filled in by a final 7 min incubation at 72°C.

Bacterial mating and marker exchange. For bacterial conjugations, the donor *E. coli* strains and helper *E. coli* strain HB101 containing pRK2013 (Ditta et al., 1980) were grown overnight on LB medium with appropriate antibiotics at 37°C. *X. campestris* pv. *convolvuli* was grown in LB broth medium for 24 hr at 30°C. Cells were washed twice in RGMC broth (10.0 g tryptone, 1.0 g yeast extract, 8.0 g NaCl, 1.0 g glucose, 5 mM $MgCl_2$, 2 mM $CaCl_2$), combined, and resuspended together in RGMC broth. The suspension was spoted onto RGMC plates and incubated at 30°C for 12 to 16 hr. Transconjugants were selected on NSA and LB agar plates containing rifampicin (100 μg/ml) and tetracycline (50 μg/ml) at 30°C.

Marker exchange of the insertional mutants into the chromosome of strain FB-635 was done in successive cultures of transconjugants in LB broth containing 100 μg/ml rifampicin only and plating onto NSA and YDC agar plates. Marker-exchanged cells were selected for Rif^r, starch hydrolysis and typical mucoid, white colonies (wild-type colonies are yellow).

Recombinant DNA techniques. For restriction analysis, plasmid DNA of each clone was prepared by using Quantum Prep Plasmid miniprep kit (Bio-Rad Lab, Richmond, CA, USA).

Table 1. Bacterial strains and plasmids used in this study

Strain and Plasmid	Genotype, Phenotype, or Description	Reference or Source
E. coli		
DH5α	F^- 80d*lacZ*Δ M15Δ (*lacZYA-argF*)*U169 deoR recA1 endA1 phoA hsdR17*(r_K^-, m_K^+) *supE44*λ^- *thi-1 gyrA96 relA1*	GIBCO BRL
HB101	F^- *mcrB mrr hsdS20*(r_B^-, m_B^-) *recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20*(Sm^r) *xyl5*λ^-*leu mtl1*	GIBCO BRL
Xanthomoas campestris pv. *convolvuli*		
635R12, 21 & 22	Rif^r 635	This study
635pt1 - 7	635R12::0.5 kb DES	This study
635pt8 - 14	635R21::0.5 kb DES	This study
635pt15 - 22	635R22::0.5 kb DES	This study
Plasmids		
pLAFR3	Derivative of pLAFR1, Tet^r	Staskawicz et al., 1987
pRK2013	IncP Km^r traRK2 repRK2 repE1	Figurski and Helsinki, 1979
pGEM-7Zf(+)	Amp^R	Promega
pGX15	10.3 kb *Eco*RI-*Hind*III fragment from pIG102 in pGEM-7Zf(+)	This study
pGXP6	pGX22 containing a PCR-amplified 0.5 kb DES fragment of *P. syringae* pv. *phaseolicola*	This study
pLXP22	10.8 kb *Eco*RI-*Bam*HI fragment from pGXP6 in pLAFR3	This study

Restriction digests and ligations were performed using standard techniques (Sambrook et al., 1989). When indicated, DNA fragments were purified from agarose gels by using QIAquick gel extraction kit (QIAGEN, Valencia, CA, USA). For confirmation of the presence of the DES fragment insert on the plasmids and marker-exchanged weed bacteria, PCR was performed with primers 5605fx and 6105rx, as above.

Stability of tagged strains. A culture of each FB-635 strain tagged with the DES insert was transferred for 10 generations on YDC agar. After incubating for 3 days the culture was streaked to a new plate. After the 10th transfer, samples of whole cells were tested by PCR with the DES primers, p 3.1 and p. 5.1 (Prosen et al., 1993).

Growth characteristics and pathogenicity tests. Marker-exchanged strains were tested and compared to the parent strain for growth *in vitro* and pathogenicity to bindweed plants. Cultures were grown overnight in NBY, adjusted to 0.1 OD at 600 nm and diluted 100 fold in 0.85% NaCl. Aliquots of 100 µl were added to each of three 250 ml flasks containing 50 ml NBY and incubated on a shaker at 28°C. After 2, 6, 15, and 24 hrs, samples were diluted and 100 µl assayed onto each of four plates of YDC agar to determine colony morphology and growth rate (generation times). Colony morphology was determined by streaking cultures onto NSA, also. Seedlings of bindweed at the 4-6 leaf stage were atomized with a 100 fold dilution of the adjusted suspension and placed in a lighted dew chamber with a 12 hr day (30°C) and a 12 hr night (20°C) for 10 days. Wild-type strain FB-635 was used as a control. All 22 tagged strains (Table 1) were tested for colony morphology and starch hydrolysis. Strain, FB-635 pt 22 and wild-type strain FB-635 were tested for growth rate.

Detection of target strains by PCR and BIO-PCR. For classical PCR, samples of 10 µl of cells were tested by direct PCR without extraction of DNA (Prosen et al., 1993). For BIO-PCR (Schaad et al., 1995) 100 µl samples were plated onto each of three plates of NSA and YDC agars. After 48 hr at 30°C, cells from the resulting pin point size colonies were collected by washing each plate three times with 1.0 ml saline. Thirty µl of the pooled plate washings from each medium were then used for direct PCR as described above.

Results

Construction of a vector. A 0.52-kb DES fragment was amplified from the *tox-argK* gene cluster of *P. syringae* pv. *phaseolicola* C-199 using the tailed primers, 5605fx and 6105rx, (Fig. 1. DES/PCR-amplification). A 10.3 kb *Eco*RI and *Hin*dIII fragment containing *pig*G was excised from pIG102 (Poplawsky and Chun, 1997) and ligated into pGEM-7Zf (+) to generate pGX15 (Fig. 1. pGX).

The DES fragment was then inserted into pGX15 as an *Xma*I fragment. The resulting plasmid, pGXP6, carries the DES fragment inserted into the unique *Xma*I site within *pig*G (Fig. 1. pGXP). The *pig*G::DES construct was isolated from pGXP6 as a 10.8 kb *Eco*RI and *Bam*HI fragment and ligated into similarly digested pLAFR3 to create pLXP22 (Fig. 1. pLXP). The *pig*G::DES insertion carried by pLXP22 was verified by restriction mapping and classical PCR using DES primers P3.1 and P5.1 as above.

Construction of DES-containing Xanthomonas strains by marker exchange. pLXP22 was mobilized by triparential mating from *E. coli* DH5α into *X. campestris* pv. *convolvuli* FB-635. Three independent FB-635(pLXP22) transconjugants were selected and used for marker exchange mutagenesis. Tetracycline resistant and starch hydrolyzing transconjugants were purified and then grown in LB for several generations in the absence of antibiotic selection for the plasmid. To identify *pig*G::DES mutants, diluted bacterial suspensions were spread onto YDC and NSA plates containing rifampicin. After 5 days, starchhydrolysing white colonies on the plates were picked and screened for tetracycline resistance. Rifampin resistant, tetracycline sensitive, starch-hydrolyzing, white colonies were considered marker-exchanged, DES-insertion clones.

From these experiments, 22 apparent marker-exchanged mutants were selected and subsequently verified by using PCR-amplification with DES-specific P3.1 and 5.1 primers. The strains containing this mutation are designated FB-635 pt 1 to 22.

Stability of tagged strains. Each of the 22 tagged strains resulted in a 0.52 kb band when tested by PCR after the 10th generation.

Characteristics of *pig*G::DES *X. campestris* pv. *convolvuli* strains. All 635 pt mutant strains grew normally on NSA and YDC agar media and showed starch hydrolysis activity on NSA medium similar to that of the wild-type strain. Insertion of the DES fragment into the genomic DNA had no detectable effect on the growth rates of the tagged strain. The generation times (minutes) were 130 and 133 after 15 hr for wild-type strain FB-635 and tagged strain FB-635 pt 22, respectively. After 24 hr the generation times for FB-635 and FB-635 pt were 122 and 120, respectively.

All of these strains caused typical water-soaked lesions and subsequent yellowing and leaf drop of bindweed leaves (data not shown) which were indistinguishable, with respect to timing and severity, from those caused by the parent strains, as described previously (Schaad et al., 1997).

Detection of the target fragment by PCR amplification. The 0.52 kb region that was inserted into *X. campestris* pv. *convolvuli* DNA was chosen as the target for PCR-based detection methods because it represents a DNA sequence unique to this bacterium. Two primers, P3.1 and P5.1, specific to the inserted DES fragment, amplified a 0.52 kb DNA fragment when whole cells of strain 635pt20 was used as a template (Fig. 2). No amplification products of any size were detected when these two primers were used with wild-type *X. campestris* pv. *convolvuli* strain. The

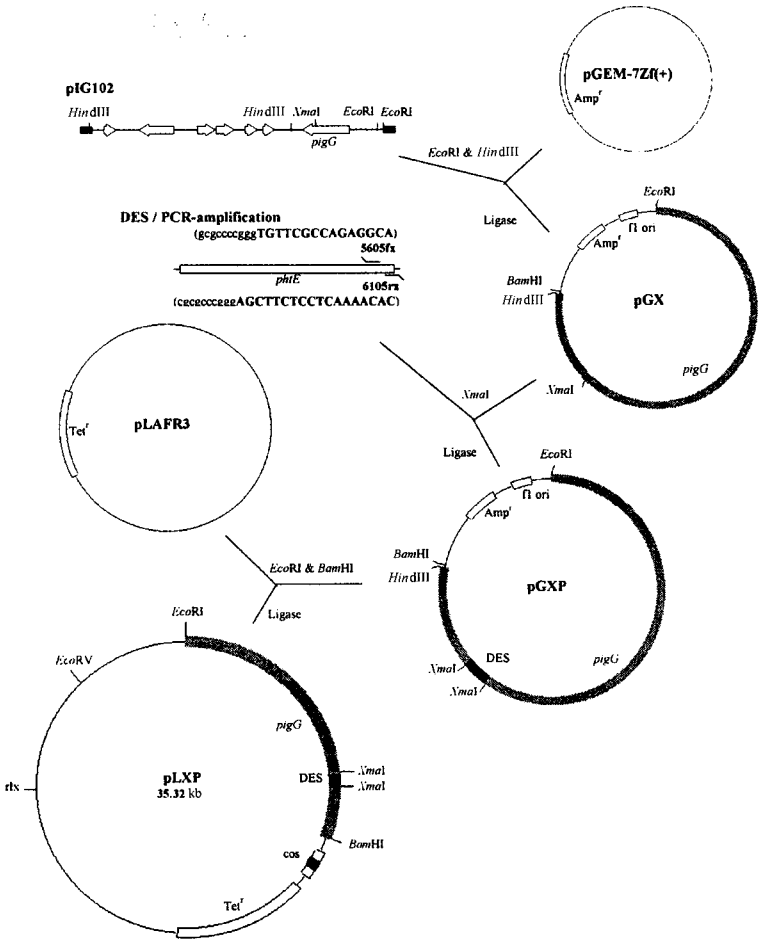

Fig. 1. Construction of pLXP. Mutted arrows and text indicate the construction manipulation. The 0.52 kb *Xma*I fragment amplified from *P. syringae* pv. *phaseolicola* C-199 that contains specific priming site, DES, for P3.1 & 5.1 primers was transferred into the unique *Xma*I site within *pig*G region of 10.3 kb *Eco*RI-*Hin*dIII xanthomonadin gene cluster that resides on pGX. 10.82 kb *Eco*RI-*Bam*HI fragment from pGXP was inserted into *Eco*RI-*Bam*HI-digested pLAFR3 to make resulting pLXP. Circular plasmids are not to scale.

0.52 kb DNA fragment was detectable with BIO-PCR when pure culture samples containing as few as 10 to 30 cells per ml were used as template (Fig. 2).

To determine whether an excess amount of heterologous cells from the parental strain, *X. campestris* pv. *convolvuli* FB-635 would affect specificity and sensitivity for amplification of the target, 10-20 CFU of 635pt20 was mixed with numbers of wild-type *X. campestris* pv. *convolvuli* cells ranging from 1-3×10^5 CFU of wild-type *X campestrix* pv. *convolvuli* cells. The mixture was then subjected to PCR-amplification of DES region using primers 3.1 and 5.1. The 0.52 kb amplification product was detected from all reaction mixtures. There were no apparent differences in the intensity of the bands in the gel and no other amplified products were detected (Fig. 3).

Discussion

To monitor released organisms one must be able to identify the organism from other strains of the same organism and/or from closely related organisms without any ambiguity

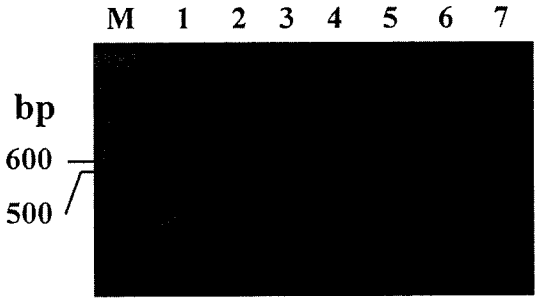

Fig. 2. Specific detection of the DES fragment amplified by PCR using whole cells of *X. campestris* pv. *convolvuli* FB635pt20. Lanes contain amplified product from 800-1,200 CFU (lane 1), 400-800 CFU (lane 2), 200-400 CFU (lane 3), 100-200 CFU (lane 4), 10-20 CFU (lane 5), and 1-3 CFU (lane 6) and no addition of the cells (lane 7). Lane M contains a 100-bp ladder marker (GIBCO BRL).

(Steffan and Atlas, 1988). The ability to reliably and reproducibly monitor an organism in the environment is critical in assessing the risks from its release (Edwards, 1993). Strategies for detecting released organisms to determine risk assessment must include specific markers that will allow for the direct selection of the tagged organism from closely related microorganisms. Although several pathogenic bacteria have been described for biological control of weeds (Elliot and Kennedy, 1991; Kremer et al., 1990; Johnson, 1994), no risk assessment studies were included. Our detection scheme will allow for much simplified, accurate, and sensitive risk assessment studies.

We have chosen a xanthomonad pathogenic to a weed as our model organism for testing the DES-tagging system because most pathovars of *X. campestris* are highly host specific and therefore good choices for weed biocontrol. Very few xanthononads will infect plants of another family. For example, the type species, *X. campestris*, infects only plants in the family Crucifereae. Another advantage of choosing a xanthomonad is that they generally survive in soil only for as long as the host tissue is intact. Our preliminary host range tests with *X. campestris* pv. *convolvuli* showed that the organism failed to infect tomato, potato, cotton, wheat, corn, beans (Schaad et al., 1997). A fragment of the phaseolotoxin gene cluster was chosen to tag our target bacterium because the gene has not been found in any other organism (Peet et al., 1986; Prosen et al., 1993), except for the related pathovar *P. syringae* pv. *actinidiae* (Sawada et al., 1997). Because the phaseolotoxin gene cluster is unique to *P. syringae* pv. *phaseolicola*, and pv. *actinidiae* only, we should expect absolute identity of any released biocontrol agent to which we have inserted our DES fragment. The only organism which could result in a PCR false positive would be *P. syringae* pv. *phaseolicola* and pv. *actinidiae*. Since the host range of these organisms is relatively well established, and includes beans, kudzu, pigeon pea (*Cajanus cajan*), and mung bean (*Vigna radiata*) for pv. *phaseolicola* (Mitchell, R. E., 1984) and actinidia for pv. *actinidae*, the chance of finding it on other plants would be remote. Phenotypically our tagged strain is easily differentiated from *Pseudomonas*.

BIO-PCR (Schaad et al., 1995) is well suited for use in detecting the tagged organism because only viable cells are detected; and false positives from free DNA or dead cells and false negatives from possible presence of PCR inhibition will not occur. As few as 2-3 cells per ml of sample are detectable by BIO-PCR (Schaad et al., 1995) even in the presence of numerous other bacteria. Our results confirm these earlier results that detection is highly sensitive. Using BIO-PCR we were able to detect the marked organism at very low numbers even when in the presence of large numbers of competing bacteria (Fig. 3). Bindweed can be a serious problem in tomato production fields. Since beans are not normally grown in areas where tomatoes are grown, release of a marked strain of *Xanthomonas* for biological control should not be a problem. Even if *P. syringae* pv. *phaseolicola*, or pv. *actinidiae* could somehow be present, they can easily be discriminated from *X. campestris* pv. *convolvuli* strains carrying the DES fragment by isolation on semiselective agar media. *P. syringae* pv. *phaseolicola* grows on MSP medium (Mohan and Schaad, 1987), whereas *X. campestris* pv. *convolvuli* does not. Furthermore, *X. campestris* pv. *convolvuli* grows and hydrolyses starch on NSCAA medium (Schaad, 1989) whereas these two pseudomonads do not. Our tagged strain is white whereas most wild-type xanthomonads are yellow (a known exception is *X. campestris* pv. *manihotis* which infects *cassava*). In addition to this, our marker strategy lends itself to use in other detection methods. The fusion junction between the *X. campestris* pv. *convolvuli* host DNA and the DES marker DNA should constitute a unique sequence, probably not present anywhere in nature. Specific primers covering one of the junctions could be readily designed, to address this point.

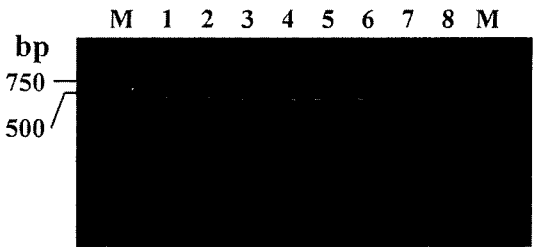

Fig. 3. Detection of the 0.52 kb DES fragment by PCR-amplification in the presence of heterologous strain. Lane 1 to 6 contain 10 CFU of *X. campestris* pv. *convolvuli* FB635pt20 in addition to various CFU number of wild-type *X. campestris* pv. *convolvuli* FB635 as follows: 1-3 CFU (lane 1); 10-20 CFU (lane 2); 100-200 CFU (lane 3); 1,000-2,000 CFU (lane 4), 10,000-20,000 CFU (lane 5), no addition of FB635 (lane 6) and no addition of FB635pt20 but 1,000 CFU of FB635 (lane 7) and no additions of cells (lane 8). Lanes M contain a PCR marker (Promega, USA).

Several systems using carrier transposons have been described to construct such insertions (Shaw et al., 1992; Egener et al., 1998). Marker systems designed for organisms to be released into the environment must be stable within the bacterium. In addition, marker genes should not be transmissible to other indigenous microbes via horizontal transfer; that is, they should be genetically stable and contained. These two requirements are best met by inserting the genes into the genomic DNA of the target organism rather then into extrachromosomal DNA such as a plasmid. In this work, we designed an insertion system based on homologous recombination using 10.3 kb *Eco*RI-*Hind*III

fragment that carries genes involved in the biosynthesis of xanthomonadin, a pigment unique to xanthomonads (Poplawsky et al., 1993). We believe this to be a better system then a transposon-based marker system. In previous reports, seven transcriptional units involved in xanthomonadin production were identified (Poplawsky et al., 1993; Poplawsky and Chun, 1997). Insertional inactivation of *pig*G caused a complete loss of visible pigmentation. In a comparison of pathogenicity between *pig*G insertion mutation strains and parent strains, all mutant strains caused typical symptoms which were indistinguishable from those caused by the parent strain (Poplawsky and Chun, 1997). Our results are the same.

To identify genetic problems associated with introduction of foreign DNA it is normally necessary to conduct extensive phenotypic tests to determine if the insertion event has altered the strain in any phenotypic character (Vahjen et al., 1997). With our novel *pig*G gene recombination system the modified strains should not need to be tested to determine whether the insertion event disrupted any normal trait of the tagged organism. The pigment gene has been shown to be neutral with respect to other functions (Poplawsky and Chun, 1997). This allows us to insert virtually any determinant into the *Pig* gene without concern over possible effects on the disruption of relevant characteristics of the bacterium. Our marking system should prove useful for determining the survival of economically important xantho-monads on plant surfaces or in soil. Presence of the recombinant DES fragment is a perfect target for specific detection of the marked xanthomonad against a high background of many other microorganisms. Detection can be performed using specific PCR primers to the DES fragment using whole cells directly extracted from environmental samples, without extracting DNA or from semiselective agar media (Schaad, 1989) when combined with BIO-PCR.

As a tagging system for *Xanthomonas* spp., the DES system has several advantages over other systems including the following aspects: 1) its presence is restricted to only two other organisms, 2), it has no effect on other important functions of the target bacterium other than colony color, 3) it can easily be adopted to sensitive detection methodologies including direct PCR-amplification using whole cells without any further treatment, and 4) it can be used for the tagging of any *Xanthomonas* species or pathovar.

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