CONSTRUCTION OF A *PSEUDOMONAS SYRINGAE PV. SYRINGAE* REPORTER VECTOR FOR THE STUDY OF THE EXPRESSION OF THE *recA* GENE IN RESPONSE TO UV LIGHT

A Senior Honors Thesis

By

JULIE ANN COTTON

Submitted to the Office of Honors Programs & Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the UNIVERSITY UNDERGRADUATE RESEARCH FELLOWS

April 2000

Group: Molecular Genetics
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RESEARCH FELLOW

Approved as to style and content by:

George Sundin
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April 2000

Group: Molecular Genetics
ABSTRACT


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Responsible for such diseases as brown spot of bean, *Pseudomonas syringae* pv. *syringae* is an inhabitant on the leaf surface (phyllosphere) of plants and must maintain large population numbers in order to infect host plants. The ability to repair genetic damage caused by UV-B light, that part of the solar spectrum from 290-320 nm, is essential to the survival and success of the bacterium. The *recA* gene is the initiating gene of the regulatory network, known as the SOS repair mechanism, which is responsible for repairing genetic damage blocking DNA replication in many species of bacteria.

We have cloned and characterized a putative promoter region of the *P.s. syringae recA* gene by DNA subcloning and sequencing techniques. We constructed a *recA::gfp* reporter vector using an improved green fluorescent protein cloning cassette shown to be effective in *P.s. syringae* (Miller and Lindow, 1997). We hope this vector will allow us to quantify the expression of the *recA* gene in response to UV-B light in two *gfp* fusion strains of *P.s. syringae*. The strains containing this plasmid may be used in several *in vitro* and environmental studies in order to understand the response of the *recA* gene and its role within the SOS repair mechanism, and to investigate the habitat of the bacteria within the phyllosphere.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td></td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td></td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td></td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td></td>
<td>v</td>
</tr>
<tr>
<td>SECTION</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>MATERIALS AND METHODS</td>
<td>3</td>
</tr>
<tr>
<td>2.1</td>
<td>Bacterial strains and plasmids</td>
<td>3</td>
</tr>
<tr>
<td>2.2</td>
<td>DNA subcloning and sequencing</td>
<td>3</td>
</tr>
<tr>
<td>2.3</td>
<td>Vector formation</td>
<td>7</td>
</tr>
<tr>
<td>3.</td>
<td>RESULTS</td>
<td>10</td>
</tr>
<tr>
<td>3.1</td>
<td>Sequencing results</td>
<td>10</td>
</tr>
<tr>
<td>3.2</td>
<td>Confirming the recA-gfp vector</td>
<td>12</td>
</tr>
<tr>
<td>4.</td>
<td>CONCLUSIONS</td>
<td>13</td>
</tr>
<tr>
<td>4.1</td>
<td>Use of the green fluorescent protein as a prokaryotic reporter gene</td>
<td>13</td>
</tr>
<tr>
<td>4.2</td>
<td>Future research possibilities</td>
<td>14</td>
</tr>
<tr>
<td>REFERENCES</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>VITA</td>
<td></td>
<td>20</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Diagram of sequenced subclones</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Map of the recA-gfp reporter vector pJAC3</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>Results of the KpnI end sequence of pGWS171</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>Sequence of pJAC1 (5'-3')</td>
<td>11</td>
</tr>
</tbody>
</table>

LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacterial strains and plasmids</td>
<td>4</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

*Pseudomonas syringae* pv. *syringae* is the causal agent of bacterial brown spot of snap beans, an economically significant disease for many producers (Hirano et al., 1995). A frequent problem for growers, *P. s. syringae* causes necrotic lesions on bean pods that reduce the quality of the product and can render entire fields unmarketable (Hirano et al., 1995). The bacteria can inhabit the leaves of the plant without causing disease until environmental factors, such as rain, favor bacterial population growth (Hirano et al., 1995). A threshold population size of $10^9$ calls per gram of leaf tissue has been determined to be predictive of disease on snap bean (Hirano et al., 1995). The ability to tolerate high doses of UV radiation is critical for the maintenance of *P. s. syringae* populations in the phyllosphere; tolerance of UV solar radiation may favor pathogen population expansion and lead to the occurrence of brown spot disease.

Solar radiation is the primary source of energy for life on earth. However, in addition to visible light, deleterious UV radiation, which induces DNA damage, is present in solar radiation. UV-B radiation, that part of the natural light spectrum from 290-320 nm, induces DNA damage in the form of pyrimidine dimers that can be deadly to microorganisms through the blockage of DNA replication (Friedberg et al., 1995). A widely researched bacterial survival mechanism following irradiation with UV wavelengths is the SOS inducible response. The *recA* gene has been characterized and determined to play an important role in cellular UV protection (Miller and Kokjohn, 1990). This is effected through the induction and the expression of the SOS regulatory network, a system comprised of at least 20 unlinked genes, many of which are involved in DNA repair (Miller and Kokjohn, 1990).
The SOS regulatory network, first suggested by Miroslav Radman in the early 1970's, allows DNA replication to continue, even following severe damage from UV radiation or other chemical mutagens (Friedberg et al., 1995). The LexA protein normally represses the expression of genes in the SOS regulatory network. The SOS inducing signal is generated by single-stranded regions produced when the cell attempts to replicate damaged DNA (Friedberg et al., 1995). The RecA protein recognizes this signal, and cleaves the repressor, LexA, causing SOS genes including recA to be expressed at increased levels (Friedberg et al., 1995). The expression of the DNA systems that are part of the SOS network is responsible for cellular survival and recovery following DNA damage. After the damage has been repaired, LexA again accumulates and represses the genes in the system (Friedberg et al., 1995).

In this report, we describe the subcloning process and sequencing results used to isolate a putative recA promoter region in P.s. syringae. A recA-gfp reporter vector was constructed using this region to facilitate the study the expression of the recA gene in P.s. syringae after exposure to UV light. The significant role that the recA gene may play in the survival of such pathogens is important to the understanding of phyllosphere habitat and may contribute to future beneficial biocontrol methods.
2. MATERIALS AND METHODS

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* and *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Difco) at 28°C and 37°C, respectively. *Pseudomonas*-selective minimal media was obtained from Gibco-BRL. Ampicillin (Ap) was added to media at the concentration of 25 μg ml⁻¹ for *P. syringae* and 75 μg ml⁻¹ for *E. coli*. Kanamycin (Km) was added to media at 25 μg ml⁻¹, and tetracycline (Tc) was added to media at 25 μg ml⁻¹, according to resistance requirements. Competent *E. coli* cells were prepared from strain DH10B cultured in 2 x YT by treatment with Tris-HCl, calcium chloride and magnesium chloride for 30 minutes in ice, then transferred to a Tris-HCl, CaCl₂ and MgCl₂ with 20% glycerol solution for storage at -70°C. Sterile saline solution was made at the concentration of 8.5g L⁻¹ NaCl in water.

2.2. DNA subcloning and sequencing

The *recA* gene and flanking sequences of *P. syringae* was obtained from Dr. Kyle Willis (University of Wisconsin), on the plasmid pKW11. The *recA* gene had been marked with the Tn5 insert (5.7-kb), a commonly used DNA transposon encoding for kanamycin resistance. The *recA* gene, which the insert interrupts, is known to be about 1,040-bp (1.04 kb) according to the conservation of the gene sequence between species (Friedberg et al., 1995).
The promoter region was tentatively identified by subcloning and sequencing (see Table 1 for a list of bacterial strains and plasmids employed). Although the recA gene was known to be interrupted by Tn5 marker, the direction of transcription of the gene was unknown. The pKW11

Table 1. Bacterial strains and plasmids used in this study and their relevant characteristics.

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Relevant genotype or characteristic</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>$\Delta(lacZYA-argF)_{X74}$ Competent cells, contain no plasmids</td>
<td>Ausubel et al. (1987) Grant et al. (1990)</td>
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<td>DH10B</td>
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<td><strong>Pseudomonas syringae</strong></td>
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<tr>
<td>pv. syringae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B86-17</td>
<td>one plasmid 61-70 kb</td>
<td>DE Legard</td>
</tr>
<tr>
<td>FF5</td>
<td>UV sensitive, contains no plasmids</td>
<td>Sundin and Bender (1993)</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCR2.1</td>
<td>PCR cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km resistant, Mob$, Tra$</td>
<td>WG Miller</td>
</tr>
<tr>
<td>pProbe KT</td>
<td>Km resistant. 11 kb gfp cloning cassette for prokaryotic transcriptional transfusions</td>
<td>Stratagene</td>
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<tr>
<td>pBluescript SK+</td>
<td>Ap resistant, lacZ’. Cloning and transcription vector</td>
<td>Promega</td>
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<tr>
<td>pGem 3zf</td>
<td>Ap resistant, lacZ’. Cloning and transcription vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pGem 7zf</td>
<td>Ap resistant, lacZ’. Cloning and transcription vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pKW11</td>
<td>Tc, Km, Ap resistant. <em>P. syringae</em> (recA4::Tn5 insertion)</td>
<td>DK Willis</td>
</tr>
<tr>
<td>pGWS166</td>
<td>8.8-kb SphI from pKW11 in pBluescript (recA4::Tn5 insertion)</td>
<td>This study</td>
</tr>
<tr>
<td>pGWS168</td>
<td>Ap, Km resistant. 1.0-kb IS50-T7 PCR product from pGWS166 in pCR2.1</td>
<td>This study</td>
</tr>
<tr>
<td>pGWS169</td>
<td>Ap, Km resistant. 2.2-kb IS50-T7 PCR product from pGWS166 in pCR2.1</td>
<td>This study</td>
</tr>
<tr>
<td>pGWS171</td>
<td>1.2-kb SphI-KpmI from pGWS169 in pGem 3zf</td>
<td>This study</td>
</tr>
<tr>
<td>pJAC1</td>
<td>0.84-kb SphI-EcoRV (Blunt cut and ligated to SmaI pGem 7zf) from pGWS171 in pGem 3zf</td>
<td>This study</td>
</tr>
<tr>
<td>pJAC2</td>
<td>~0.5-kb SphI to HinII (HinII-EcoRII removed) from pJAC1</td>
<td>This study</td>
</tr>
<tr>
<td>pJAC3</td>
<td>0.84-kb EcoRI (added restriction site)-HindIII segment from pJAC1 in pProbe-KT</td>
<td>This study</td>
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</tbody>
</table>
plasmid DNA was isolated using the Qiagen plasmid purification kit according to manufacture's instructions. Restriction endonuclease digestion using the SsrI restriction enzyme was carried out to cut the DNA encompassing the Tn5 insert, excising an 8.8-kb fragment of DNA. After running the reaction on a 1-% agarose gel (Gibco-BRL), the fragment was extracted from the gel using the Qiagen gel extraction kit, according to manufacturer's protocol. The 8.8-kb fragment of DNA was ligated into the pBluescript SK vector, forming pGWS166, was transformed into E. coli DH10B competent cells using the protocol in the Promega Protocols and Applications Guide (Doyle 1996).

The transformed E. coli was plated to LB media amended with Ampicillin with X-gal, which selects for lacZ expression. Colonies that did not express the lacZ gene were selected from the media. The colonies were grown and cultured with Ap. pGWS166 DNA was isolated using the methods above. Two polymerase chain reactions (PCR) were carried out in order to identify the beginning of the recA gene. Known primer sites within the Tn5 insert and on either side of the inserted DNA, in the pBluescript SK vector plasmid transcription initiation site, were utilized by PCR methods. This isolated 1-kb and 2.2-kb fragments, each including a section of the recA gene and possible flanking regions of DNA on either side of the insert. The synthetic oligonucleotide primers used for PCR runs and the Taq polymerase were supplied by New England Biolabs.

The PCR products were run on a 1-% agarose gel, and the 1-kb and 2.2-kb fragments were identified and cut from the gel. The two DNA fragments were extracted from the gel using the Qiagen gel extraction kit, according to manufacture's protocol. The two fragments were individually ligated into the pCR2.1 plasmid vector (Invitrogen), forming pGWS168 and pGWS169, respectively. PCR sequencing reactions were carried out using the ABI Sequencing
Big Dye kit, according to manufacturer's specifications. DNA sequences were run at the Texas A&M University Gene Technologies Laboratory. Homology analysis, using the raw DNA sequence were done using the National Center for Biotechnological Information Gene Bank Blast search online system.

The 2.2-kb subclone was found to include the beginning sequence of the *recA* gene, indicated with the ATG start codon and highly conserved *recA* DNA comparative sequences, as well as the end sequence of a preceding gene. Therefore, the promoter region was predicted to lie between the end of the preceding gene and the beginning of the *recA* gene. This region was further localized for sequencing using the following methods.

A 1.2-kb region was cut from pGWS169 using *KpnI* and *SphI* was ligated into the pGEM 3Zf vector forming pGWS171, and transformed into the *E. coli* strain DH10B as above. The plasmid was sequenced by methods similar to those listed above. The results from the *KpnI* direction of the plasmid were readable, but the sequence from the *SphI* side was unreliable. The results of the sequencing from the *KpnI* side was adequate, as it included the *recA* start sequence and upstream sequence (see the solid arrow in Figure 1).

Subsequent plasmids (see diamond-ended lines on Figure 1) were constructed and sequenced using the same methods as described for pGWS171. pJAC1 was constructed by subcloning the 0.84-kb *SphI-EcoRV* fragment from pGWS169 to reduce excess *recA* gene sequence and to place the DNA in the appropriate 5' to 3' direction. The pJAC2 plasmid was formed by extracting the *HincII - EcoICRI* region of pJAC1 in order to confirm the sequencing results of pJAC1. A novel oligonucleotide Mdl (5' GGGGCAAGGATGATGCGC 3') was created (Gene Technologies Laboratory, Texas A&M University) to recognize a region before the
HincII site of the plasmid pJAC1, and compared with results from the 3' (EcoRV) end of the pJAC1 plasmid to confirm the remaining sequence of the pJAC1 fragment.

![Diagram of sequenced subclones](image)

**Figure 1.** Diagram of sequenced subclones. Diamond-end lines represent the subclones pGWS171, pJAC1, and pJAC2. The circle of the solid arrow denotes the recA gene start and the arrow points in the direction of transcription. The binding site of the Md1 oligonucleotide is denoted with a diamond and the dashed arrow points in the direction of the sequence. Major restriction enzyme sites are shown above pGWS171.

2.3 Construction of a recA::gfp transcriptional fusion

The pProbe-KT vector was supplied by W.G. Miller in *E. coli* strain DH5α. The strain was cultured in a LB amended with Km. A large scale plasmid preparatio of pProbe-KT cut with HindIII and EcoRI. The vector pJAC1 did not have matching cloning sites to allow for the direct cloning of the 0.84-kb fragment of DNA. Therefore, the fragment of DNA was replicated by PCR using the SP6 primer and a novel oligonucleotide T7-EcoRI (5' GCGAATTCTAATACGA-CTCACTATAGGCG 3') consisting of the T7 primer with an EcoRI restriction site added to the
end. Originally obtained from the Texas A&M University Gene Technologies Laboratory, the oligonucleotide concentration could not be determined and several PCR reactions using different primer concentration were ineffective. After reordering the oligonucleotide from New England Biolabs, with concentration specified, the reaction worked properly and the fragment was replicated. This allowed the PCR-generated DNA to be cut with EcoRI and HindIII and ligated by the method above into the pProbe-KT vector, forming the pJAC3 plasmid (see Figure 2). pJAC3 was transformed into DH10B E. coli as described in section 2.2, and Km resistant colonies were selected.

Tri-parental matings of the E. coli host strain containing pJAC3 and the recipient P. s. syringae strains B86-17 or FF5 were performed. This method includes a conjugation proficient donor E. coli strain containing pRK2013 that assists in transferring the plasmid from the host strain to the recipient bacteria of interest (Figurski and Helinski, 1979). Each strain of bacteria was cultured in LB with selective antibiotics when required. The cultures were spun in a centrifuge to collect the bacteria, and resuspended in sterile saline solution. The host, donor, and recipient, strains were mixed in microfuge tubes in different proportions. The mixture was spot plated on plain LB media, dried briefly in a laminar flow hood, and incubated overnight at 28°C. Transconjugants were selected by resuspending the mating mix in sterile saline solution and plating to minimal medium amended with Ap (25 μg ml⁻¹), which selects for P. syringae, and Km which selects for the Km marker of pProbe-KT. These plates were incubated for several days. The mating was most successful when the recipient strain of P. syringae was three times or more that of the donor E. coli strain. The resulting larger colonies were streaked to LB medium amended with Km. The isolates were then cultured and the plasmid DNA isolated as specified above. Gel electrophoresis of the plasmids cut with EcoRI suggested that the pJAC3 vector had
been transferred to the *P. syringae* strains. Similar procedures were carried out to generate *P. syringae* strains with only the pProbe-KT vector for use as controls.

**Figure 2.** Map of the *recA-gfp* reporter vector pJAC3. T1 represents transcription-blocking regions. Note that the vector has multiple regions before the *recA* promoter insert site and reporter gene to reduce the production of the green fluorescent protein when the promoter sequence has not been induced.
3. RESULTS

3.1 Sequencing results

The sequencing of the KpnI end of the fragment yielded a 557-bp sequence was retrieved and entered into the National Center for Biotechnological Information Blast search system for comparison with other identified gene sequences. Regions of recA promoter and recA gene similarity were ranked. The last 408 bases in the sequence encode for the first 136 amino acids in the RecA protein. The Blast system found this gene sequence to be 100% identical to the recA gene of P. flourescens, a closely related Pseudomonad.

A putative promoter region beginning 51-bp downstream of the search-identified recA start codon was determined by visual comparison of this DNA sequence to the consensus sequence LexA protein binding sites (Walker 1984). When recA is in the uninduced state, the LexA protein binds to the promoter region characterized by the CTG and CAG sequences. The putative LexA binding region is located at the base pair 70-85 (see Figure 3). The recA start codon, ATG, are the 136-138th nucleotides in the sequence.
Figure 3. Results of the KpnI end sequence of pGWS171.

The pJAC1 sequencing results were combined with those obtained from the sequencing of pJAC2 and Mdl sequencing reaction (see Figure 4). This allowed for a review and confirmation of the 0.84-kb sequence of pJAC1 that was ligated into the pProbe-KT vector to form the pJAC3 reporter vector construct.

Figure 4. Sequence of pJAC1 (5'-3'). The putative LexA binding is highlighted. The ATG start codon appears in bold letter. Other sites of importance are underlined and identified by number. The beginning amino acid sequence is shown above the rec4 gene region.

1 = Mdl primer site
2 = HindII restriction site
3.2 Concreting the recA-gfp vector

The *P. s. syringae* bacterial strains B86-17/p JAC3, FF5/pJAC3, B86-17/pProbe KT and FF5/pProbe KT were grown overnight in LB broth amended with Ap. The culture was diluted to $10^6$ and 50μl aliquots were spot plated to Ap amended LB media. The plates were incubated at 28°C for 24 hours. The plates were then opened and exposed to 500 UV-B light provided by an XX-15L lamp (UVP Products). Duplicate plates were not exposed. The plates were incubated for one hour, and then exposed to 490 nm light to excite GFP.

Unfortunately, the equipment available did not allow for definite confirmation of GFP production. I will soon utilize equipment better suited to evaluate GFP fluorescence emissions.
4. CONCLUSIONS

4.1 Use of the green fluorescent protein as a prokaryotic reporter gene

The green fluorescent protein (gfp) isolated from the jellyfish Aequorea victoria is able to produce fluorescence without additional cofactors or damage to the organism in which it is used (Plautz 1996). For these reasons, it is being used increasingly as a reporter gene to indicate gene expression and regulation.

The cloning cassette designed for prokaryotic transcriptional fusions provided by W. G. Miller (1997) was used in the formation of the vector. In our plasmid construct, the GFP will be produced when the recA promoter sequence is induced after DNA damage has occurred. The GFP cloning vector contains a 'red shift' mutation that allows the excitation maximum to shift to 490 nm, a wavelength near common laser excitation sources, which allows the reporter vector to be viewed with less specialized equipment (Miller and Lindow, 1997). Another mutation causes increased protein solubility, a characteristic that reduces erroneous long-term expression results (Miller and Lindow, 1997). gfp is expressed at increased levels comparative to the wild-type gfp, possibly allowing weakly transcribed genes to be measured (Miller and Lindow, 1997), which may be of benefit if low UV doses (such as solar radiation) are used to test expression levels.

I hope to be able to utilize the above features in future testing such as the possible studies listed below.
4.2 Future research possibilities

(1) Further subcloning of the unidentified region of the pJAC1 sequence may be done to
determine the minimum sequence needed for recA promoter function.

(2) In vitro UV-B exposure studies using the P. s. syringae strains containing pJAC3 are planned
to quantify the time frame of expression of the recA gene, as well as the level of expression of
recA in response to varying exposure to UV light.

(3) Plant studies may be carried out to determine habitat of the P. s. syringae bacteria on leaves.
Research suggests that solar radiation has great significance to the microbial ecology of the
phyllosphere (Sundin and Jacobs, 1999). It has been suggested that the ability to access
protected sites of leaves contributes to the survival of P. s. syringae in the phyllosphere and
may increase the opportunity for pathogenicity (Wilson et al., 1999). Plants can be set under
emitting lamps and GFP may be viewed without destroying the physical habitat of the
bacteria. This experiment may reveal the specific leaf structures P. s. syringae prefer.

(4) Long-term research plans involve an examination of the regulation of the recA gene of P.
syringae and of the rulAB locus (Sundin and Murillo, 1999), which encodes tolerance to UV
radiation. rulAB is part of the cellular SOS system in P. syringae (Sundin et al., 1996),
activated after exposure to UV radiation. To our knowledge, the role of solar radiation in the
regulation of recA and downstream components of a SOS system has not been examined
previously.

(4) Increased expression of the recA and rulAB genes has been confirmed in vitro by exposure to
a UV radiation (Sundin and Murillo 1999), but the rate of expression when exposed to
incident solar radiation outdoors has not yet been determined. Future plans also include
outdoor solar studies using specially designed enclosed boxes which will allow us to see the response of the bacteria to actual solar radiation. A special Mylar film (Kodak) which blocks UV-B light can be added to provide an UV-B control. The use of the film would provide useful insight in comparison to the full spectrum exposure. The boxes must be kept on ice to prevent heat effects and constantly agitated on a platform in order to expose all of the bacteria in suspension within the box.

The investigation of rulAB and recA expression is significant to others working with bacterial repair mechanisms, as many of the genetic traits of these repair mechanisms are conserved throughout many bacterial strains. A broad-host-range plasmid investigation using the E. coli recA\(^{+}\) gene showed that at least 20 different genera of gram-negative bacteria were able to repress the expression of the E. coli recA\(^{+}\) gene to some extent in the absence of DNA damage and to induce it after DNA damage, suggesting that the bacteria possess an SOS regulatory system and that the LexA binding site is significantly conserved in these various systems (Friedburg and others 1995). Homologous rulAB genes have also been found, such as umuDC in E. coli, mucAB in naturally occurring plasmids, and samAB and in Salmonella typhimurium (Miller and Kokjohn 1990).

Such observations as habitat on the leaf phyllosphere and response to solar radiation are critical to an understanding of the ecology this plant pathogen. Microorganisms that inhabit UV-stressed environments such as the leaf phyllosphere but do not cause disease may be a biocontrol possibility in the future. Understanding the survival process such as the SOS repair mechanism of these competing bacteria may contribute to the development of future biocontrol strategies.
REFERENCES


VITA

After graduating from John Marshall High School in San Antonio, Texas, in 1996, I began my higher education at Texas A&M University, College Station. I look forward to receiving a bachelor of science in Bioenvironmental Science and a minor in English upon graduation in December of 2000. I plan to graduate with University and Foundation Honors.

In addition to the Undergraduate Research Fellows Program, I served in the Agricultural and Natural Resource Policy Congressional Internship Program in the office of Congressman Ruben Hinojosa, and became a member of the Golden Key National Honors Society. I have earned several University scholarships, including the Academic Excellence Award and the Aggie Spirit Collegiate Award, and am grateful to the Texas Seed Trade Association for their support through the Ed Hefley Memorial Scholarship Fund.

I gained much experience as an assistant in the laboratory of Dr. George Sundin, where I have been employed for almost two years. I look forward to graduate school in the fall of 2001.

Please feel free to send correspondence to Julie Ann Cotton, 11235 Javalin Trail, Helotes, Texas 78023.