1991

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Molecular Genetic Analysis of the Interaction Between the Bacterial Pathogen *Xanthomonas campestris pv. vesicatoria* and the Tomato Plant

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May 3, 1991

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X. campestris pv. vesicatoria (Xcv) is a bacterial pathogen of tomato and pepper. Xcv is able to infect most tomato lines, causing bacterial spot disease, which can lead to death. A strain of Xcv exists, however, which elicits a resistance response in a particular tomato line. This avirulent strain carries a single locus, the avirulence gene avrRxv, which specifies the resistance inducing activity. According to Flor's gene for gene model, a corresponding gene must exist in the resistant plant. Evidence exists for this gene but it has not yet been cloned. In this study, the role of the avrRxv gene product has been examined using various techniques. Western blotting and Northern blotting have been employed to study expression of AvrRxv. Large quantities of a fusion protein were produced using a β-galactosidase expression vector and used in immunizations to create polyclonal antibodies. Isolation of AvrRxv specific antibodies is underway.

This study is concerned with the mechanisms of plant disease resistance, specifically the mechanism by which a certain line of the tomato plant (Lycopersicon esculentum) is resistant to infection by certain strains of the bacterial pathogen Xanthomonas campestris pv vesicatoria (Xcv). Xcv is a gram negative, rod shaped bacterium that secretes a polysaccharide slime. Xcv causes bacterial spot disease in tomato, resulting in widespread crop loss each year (Whalen, et al 1988). Bacterial spot disease is characterized by the appearance of small "wet" spots on the leaves of tomato, called watersoaked lesions. These lesions are regions of infection and bacteria in these lesions will eventually spread to other leaves.

Resistance to infection has been shown in one line of tomato, Hawaii 7998. This line is resistant to some strains of Xcv. Resistance is
characterized by brown spots on the leaves which do not grow or spread. In this case, the spots are regions of plant cell death. This resistance is known as a hypersensitive resistance response. The result is that the tomato plant does not die and Xcv does. It is assumed that this plant line contains the corresponding resistance gene to \textit{avrRxv}. This gene has not yet been cloned, in fact no resistance genes in any system have been cloned to this day.

Genetic studies indicate that resistance results when the protein product from a plant resistance gene interacts either directly or indirectly with the product of a bacterial avirulence gene (Keen and Staskawicz, 1988). Any infection of tomato with Xcv where both genes are not present results in disease, whereas when both genes are present the contact results in a hypersensitive resistance response (Fig. 1). This system is an example of Flor's gene for gene hypothesis (Flor, 1971). Somehow the combination of the avirulence gene product and the resistance gene product causes cell suicide in the plant cells, which is an effective defense mechanism. As the plant cells die, spread of the Xcv is halted and the Xcv eventually dies. This is known as a gene for gene resistance reaction because the interaction between the plant and pathogen, each of which contains a specific gene, determines the outcome of infection. The simplest model we can imagine to explain this reaction on the molecular level is that the avirulence gene product is a secreted protein and the resistance gene product is a receptor on the plant cell surface. Upon contact, the avirulence product from Xcv would bind to the resistance gene receptor of tomato and in some way cause cell death, by either an active or passive killing mechanism.

From an evolutionary standpoint it is important to note that although
the avirulence gene is detrimental to the survival of Xcv in the resistant plant it remains in the gene pool. In prokaryotes, in general, a gene that is detrimental to the survival of an organism should not remain in the gene pool. The fact that this gene does remain in the gene pool may be explained by the fact that the gene may have some other useful function for Xcv as well.

The avirulence gene involved in this interaction has been cloned from Xcv and is named \textit{avrRxv} (Whalen, \textit{et al} 1988). The presence of this gene in Xcv in the interaction of Xcv with the resistant plant produces resistance, with no known exception. The lack of this gene in the interaction produces infection, with no known exception. These observations indicate that this gene is the avirulence gene. This reasoning was supported by an experiment in which \textit{avrRxv} was cloned into a plasmid and transformed into a normally virulent strain of Xcv. The result was that the transformed Xcv became avirulent to Hawaii 7998 (unpublished data). This demonstrates that \textit{avrRxv} is the avirulence gene corresponding to the resistance in Hawaii 7998.

The purpose of this study is to examine the role of the avirulence gene \textit{avrRxv} in the interaction between Xcv and tomato. The molecular function of this gene is completely unknown. The only evidence of its role is the phenotypic evidence in the interaction between Xcv and tomato and the study of the protein sequence. The phenotypic evidence is that the gene is necessary for resistance in the interaction between Xcv and Hawaii. The presence or absence of the gene determines the fate of the infection. Computer studies of the protein sequence that have been performed in this lab indicate that the protein produced by this gene is generally hydrophilic with a few short regions of hydrophobicity and that there is no consensus
sequence for transfer of the protein through the cytoplasmic membrane. This evidence suggests that the AvrRxv protein is not a membrane-bound protein. It is also probably not an secreted protein, although this is not known for sure because secreted proteins do not always contain consensus sequences.

My interest is in the expression of the avrRxv gene in Xcv and in the localization of the AvrRxv protein in the interaction of Xcv and tomato. The study of expression is the study of the production of the AvrRxv protein or avrRxv mRNA with respect to time, conditions, and various effectors. It first must be found whether or not the gene is expressed constitutively. If so the AvrRxv protein would always be present and ready to react. But if not, the induction of expression of the gene is important because it should give evidence as to what causes production of the AvrRxv protein, and therefore what turns on the response. The expression may be induced by the lack or presence of sugar molecules, proteins, or other important factors such as coenzymes, changes in temperature or pH, or even in response to some type of cell stress. Once understood, these steps should be important in understanding the puzzle of the cell-cell interaction.

Next, once the expression of the gene is understood, then the localization of the protein can be studied. This study will be important in determining the function of the protein. If the avirulence protein is soluble and secreted, then it may bind a receptor on the plant cell. If it is soluble but not secreted, it may be responsible in an internal reaction of Xcv or may cause the release of a secondary molecule. If it is hydrophobic and membrane-bound it may be a receptor or recognition molecule. Localization of the avirulence protein may help to answer these and other questions.
I have chosen to write this paper in a review article format as opposed to a scientific article. This has been done because most of the work is still underway and results are not yet available. This format should be more effective for explaining the techniques and the details of the system rather than revealing results.

**Biological Materials**

Hawaii 7998 is the only tomato line known to contain resistance to Xcv. Two susceptible tomato lines which have been used are called Bonny Best and Walter. The strain of Xcv from which the avirulence gene was isolated and cloned is named 75-3. This gene was cloned using the cosmid cloning technique (Whalen, et al. 1988). The clone containing *avrRxv* is called pXV9006 and is approximately 2.1 bp in length. The virulent strain of Xcv that has been used is known as 56. A transconjugant strain of 56 has been developed which contains the pXV9006 fragment. Also, an *E. coli* strain has been developed which contains the pXV9006 fragment in a pUR289 lacZ fusion plasmid, this strain is called JM107 (pUR289 exo 3-3) and is used as an expression vector.

**Northern Blotting**

Northern blotting is the study of the mRNA levels of a specific gene using a DNA single strand as a probe. Since I already had the clone of the gene to use as a probe I began my work with Northern blotting. Northern blotting involves four main steps: RNA purification, running samples on a formaldehyde-agarose gel, transfer of the RNA in the gel to a filter, and then probing with a radioactively labelled DNA clone. The purpose was to study the RNA expression of a particular gene under varying conditions. Northern blots are very specific because of the complementarity of the RNA and the
DNA clone. Conditions that affect expression of mRNA can be varied by simply treating the cells before RNA purification. These variables include use of different strains, time, stimulation with various molecules or contact between plant and pathogen cells. In each case the conditions under which cells are grown can be varied and then the cells are simply lysed and RNA is extracted.

RNA purification can be done in a variety of ways. The general procedure calls for the lysing of the cells, then separation of RNA from the rest of the lysate and collection of the RNA. This all must be accomplished under RNase-free conditions. RNases are ubiquitous proteins responsible for the breakdown of RNA. Therefore RNase inhibitors such as Diethylpyrocarbonate (DEPC) and Guanidine thiocyanate (GTC) must be used. Other conditions must be controlled as well, such as the amount of divalent cations (Ca++, Mg++) and the pH. These are controlled, respectively, by using sodium EDTA and a Tris buffer.

I used an RNA extraction used by Thanh Huynh for work on *Pseudomonas*. (personal correspondence). It involved lysing the cells in an SDS buffer by sonication. This combination of a detergent and sonication is sufficient to lyse the cell wall and membrane. The nucleic acid is then extracted from the proteins and lipids using a series of phenol and chloroform extractions. Then, the RNA is selectively extracted from the DNA using lithium acetate. Finally the RNA is precipitated using sodium acetate and isopropanol. This procedure is relatively standard for prokaryotic systems. Agarose mini gels and OD absorbance readings were used to quantitatively measure the total RNA levels and to test for purity. Equal amounts of total RNA are needed in each sample of a Northern blot.
My goal was to study the RNA expression of the *avrRxv* transcript under various potentially inductive conditions. I first wanted to test whether transcription was constitutive (constantly transcribed) by simply running a Northern blot using the 75-3 (containing *avrRxv*) and the 56 (no *avrRxv*) strains. If the gene is constitutively expressed hybridizing mRNA would be evident in the 75-3 mRNA and not in the 56 mRNA when probed with pXV9006. I then planned to look at the effect of growth on various sugar sources on *avrRxv* expression. Specific sugar sources have been shown to turn on other avirulence genes (Huynh, 1989). I then planned to treat 75-3 with the Hawaii 7998 plant intercellular wash fluid (IWF). This is to examine whether soluble ions or factors in the intercellular space of the tomato leaf are responsible for induction of *avrRxv* expression. Intercellular wash fluid is collected by infiltrating the plant leaves with a solution of magnesium chloride (MgCl₂) under a vacuum, then cutting into the leaf along the midvein and centrifuging the leaves to collect the liquid. Finally a high concentration of *Xcv* was actually injected into tomato, allowed to incubate inside the plant, and then isolated from leaves using the IWF protocol described above. This is to test if actual contact between plant and bacterial cells was necessary for induction of expression.

Each of the previously described variations in growth conditions were attempted before RNA was extracted. Each technique for variation of the conditions affecting expression, but the result of the intercellular wash fluid experiment and the injection of the cells into the plant was that no RNA at all was extracted from the cells. This result was probably due to RNase contamination. These were discouraging results.

The results of the Northern blotting as a whole were relatively poor.
The main problem was in extracting RNA from Xcv. I performed seven RNA preparations, with four to ten samples per preparation, but had only marginal success. Some preparations were complete failures, while some were partially successful (Fig. 2). It was interesting to note, however, when RNA extractions were attempted on the JM107 E. coli strain, they were usually much more successful than when using Xcv. This led me to believe that the problem was not in the procedure itself but was specific to the Xcv uses. Three possible explanations as to why RNA extraction of Xcv is problematic are that Xcv RNA is more fragile than E. coli for some reason, that Xcv has more RNases present, or the copious polysaccharide slime produced by Xcv has an interfering effect. It is known that Xcv produces this slime. Xcv is the commercial source of Xanthan gum, a gummy substance used in candles. I began to consider whether the polysaccharides of the slime may be responsible for the lack of success in RNA extraction. Many polysaccharides would extract along with nucleic acids in a phenol or chloroform extraction. These extractions are generally responsible for separating lipids and proteins from nucleic acids. It is also possible that polysaccharides could precipitate along with nucleic acids in the presence of sodium acetate and ethanol or isopropanol, although they should not precipitate in high quantity in lithium acetate. Some of the RNA mini gels which I ran show evidence of polysaccharide contamination. It is not known exactly how polysaccharide contamination affects RNA preparations, but it does confuse attempts to quantitatively measure the RNA and it hinders the migration of RNA in agarose gels. This caused many problems in trying to do Northern blots.

From all of the RNA samples prepared, only two Northern blots were
performed, containing 8 samples. When the gels were run only four of the eight samples in one Northern seemed adequate. Both gels was transferred anyway and then probed. The probing produced large smears of binding and the blot was a failure. I interpreted these smears as some sort of nonspecific binding. I then decided to move on to Western blotting to study expression.

**Immunization and Antibody Production**

Another goal of this project was to produce monospecific polyclonal antibodies against the AvrRxv protein (antigen). Antibodies are highly specific proteins which are produced in animals as a recognition and defense mechanism. Antibodies recognize a small part of the structure of the antigen in three dimensional space. Monospecific antibodies are antibodies that bind only one protein under standard conditions. Polyclonal antibodies are different antibodies with different recognition sites that all recognize the same antigen. Antibodies against a given antigen can be experimentally produced by repeatedly immunizing an animal with the antigen. The animal’s defense system then produces high levels of the antibodies to aid in the breakdown of these antigens in the blood. These antibodies can then be purified from the serum of the animal’s blood and used experimentally. Two rabbits were obtained for use in antibody production. Rabbits are commonly used for polyclonal antibody production because of their relatively large size (compared to mice) and their large ears which are good for obtaining blood. The antibodies produced were to be used in Western blotting and immunoelectron microscopy as a specific probe for AvrRxv protein.

The first thing that needs to be done for immunizations to occur is to
produce a large amount of pure antigen. This was achieved through use of the pUR expression vector (Sambrook, et al 1989). The pUR plasmid, grown in *E. coli*, is a ligation of a plasmid containing the *lacZ* gene responsible for producing the β-galactosidase protein (which contains an inducible promoter) with the *avrRxv* clone (Fig. 3). This fusion was produced at the University of California at Berkeley (personal communication). The important feature of this plasmid is that the promoter can be induced to express itself strongly by the addition of IPTG, a lactose analog. Addition of this effector causes high level transcription of the *lacZ* gene which results in the production of a large fusion protein (Sambrook, et al 1989). This protein contains the amino acid sequence of β-galactosidase as well as that of *avrRxv* all in one continuous protein. This is called a β-gal fusion.

In studying the production of this fusion protein and searching for a way to collect it, I discovered that it was being produced in inclusion bodies inside the *E. coli* cells (Fig. 4). Inclusion bodies are large masses of usually hydrophobic protein that accumulate during high level production of that protein (cite). I found these inclusion bodies through light microscopy of these cells one hour after IPTG stimulation. This was important because it made collection and purification of the protein more simple.

The procedure for collection of the protein involved lysing the cells using an SDS buffer and sonication and then simply spinning down the precipitate and resuspending in SDS-PAGE buffer (Laemmeli, 1970). A test were performed to make sure the correct construct was in hand. Unstimulated strains and stimulated strains that did not contain the *avrRxv* insert were run on an SDS-polyacrylamide gel. A unique band was found in
the gel in the lane that contained the stimulated JM107 (pUR289 exo 3-3), this band was identified as the fusion protein. This protein is about 155 kD; β-gal is 115 kD and the avrRxv protein is predicted to be about 40 kD. Large quantities of this strain were then grown, stimulated and then the protein was collected. This protein solution was then frozen at -80°C for later use.

For each immunization, 1 ml. of protein solution was used. This 1 ml. was run on a large 1% SDS-polyacrylamide gel, loading 100 μl/ lane. Upon completion of running the gel, the gel was precipitate stained in 0.1 M potassium chloride (KCl). Other staining procedures could not be used because they would destroy the antigenicity of the proteins. This staining procedure was used because it was helpful in showing the protein bands, while not usually harming antigenicity. The protein amount in the band was estimated to be between one and two mg, which is sufficient to immunize two rabbits. The band containing the fusion protein was then removed for use. At this point, the protein should have been sufficiently pure for use in immunization. The cut out band was split in half for immunization of the two rabbits.

Each half of the gel band was resuspended 0.5 ml. of phosphate buffered saline (PBS) and 0.5 ml. of Freund’s adjuvant. Adjuvant is a non-antigenic suspension which acts as support to display the antigen, thereby increasing antigenicity. Adjuvant also causes the antigen to remain embedded in the injected region for a time, which helps the antigen to be released slowly, increasing the immune response in the animal. The resulting mixture was then injected into the rabbits subcutaneously using sterile syringes and 20 gauge needles.
Both rabbits were immunized five times with equivalent amounts of antigen. Each immunization was performed five weeks after the last. Approximately nine days after the fifth immunization, about 30 ml. of blood was collected. Blood is collected from the marginal artery of the ear. This can be done using a small gauge needle.

Serum was harvested from the whole blood by allowing the blood to clot at 65 degrees C, then pouring off the supernatant. This supernatant was then centrifuged to remove undissolved particles and cells. The clot was then placed at 4 degrees C for 1/2 hour and then the supernatant was again poured off. This supernatant was then spun and added to the previous liquid fraction. This "milking" of the clot can be repeated several times. I did it twice. This preparation resulted in 15 ml. of serum from each animal.

**Antibody Purification and Depletion**

Once the blood serum had been processed, antibodies must be extracted. More specifically, the IgG form of antibodies needed to be obtained. IgGs are the primary form of antibody produced in a secondary immune response. These antibodies must be separated from the serum solution and from the rest of the dissolved proteins. There are a few techniques available to accomplish this task, but I chose to use high salt protein A affinity chromatography because it has highly specific IgG binding properties as well as potential for high yield of IgG. The combination of these two factors make protein A chromatography the technique of choice for work with rabbit IgGs.

Protein A is a cell wall component of *Staphylococcus aureus*, which has been shown to bind selectively to the Fc region of antibodies, particularly IgG molecules (Pierce instruction manual, 1988). Protein A columns consist
of agarose covalently bonded to protein A. Serum is simply run through the column at pH 8, in which case the IgG molecules should bind to the protein A through weak-noncovalent interactions and become trapped in the column, while the rest of the serum elutes from the column. A buffer of pH 2.8 is then added, which disrupts the protein A-IgG binding and allows the IgG to elute from the column (Fig. 5). The resulting eluate should be a relatively pure solution of IgGs. This eluate can then be added to a desalting column and eluted with the buffer of choice.

Columns were run using 0.5 ml. of each of the two serum samples. The protein A column eluate containing the IgGs was collected in five 1 ml. fractions. Of these fractions, 2 ml. were desalted in the desalting column and the result was 20 ml. of antibody solution in Tris buffered saline solution for each of the two serum samples. The antibody dilution was estimated at 1:40 relative to the concentration in serum. This concentration is very useful for Western blots provided that the titer (antibody concentration) in the serum was high.

After running columns to get pure solutions of IgG, the next step was to remove non-specific antibodies by running an antibody depletion. This was necessary for two reasons. One reason was that the antigen used was a fusion protein containing AvrRxv and β-galactosidase. Since these are both foreign proteins to the rabbit, the antibodies produced should be specific for both β-gal and AvrRxv. I wished to get rid of the antibodies specific for β-gal. Also, there may be other contaminating antibodies in the solution. These antibodies could be the result of an infection in the rabbit, impure antigen used for immunization, as well as common constitutively produced antibodies. The presence of these contaminating antibodies would cause the
solution to be polyspecific and would make interpretation of the Western blots difficult or impossible.

The primary depletion protocol was also adapted from a procedure used by Thanh Huynh (Huynh, et al. 1989). A second depletion procedure was devised which was a variation of the first and will be discussed later. The main procedure involved the use of the JM107 E. coli strain containing the pUR289 plasmid without the avrRxv fragment. This strain was grown up and stimulated with IPTG just as in the avrRxv expression protocol. In this case, the result was high level expression of β-galactosidase. These cells were then lysed and added to the antibody solution. The result was that antibodies specific for β-gal or any other of the E. coli proteins bound these proteins and precipitated out of solution. This procedure should subtract all antibodies from the solution except those specific for AvrRxv.

This procedure was performed on 10 ml. of each of the two antibody samples while 10 ml. of each was left undepleted. Four different Western blots were run each using one of the four antibody samples as a probe. I decided to use Western blots to test the specificity and titer of the antibodies rather than ELISA or any other technique because of the relative technical ease of the Western blot and because the antibodies are to be used on Western blots eventually.

**Western Blotting**

I then planned to study the protein AvrRxv levels, using Western blotting. Western blotting is very similar to Northern blotting except that it is the study of protein levels as opposed to RNA levels. Western blotting is the study of the levels of a particular protein. Western blotting consists of four basic steps that are similar to Northern blotting: protein extraction,
running samples on a polyacrylamide gel, transfer to filter and then probing with a monospecific antibody (Fig. 6). Both techniques are used to study levels of expressed gene products and both are specific for one particular gene product. I decided to use Western blots for the same reasons as I had decided to use Northern blots. I simply decided that protein extraction would be preferable to RNA extraction because of the problems with Xcv RNA extraction and therefore Western blots would be more successful than Northern blots.

The Western blot procedure I used was adapted from Huynh, et al 1989. This procedure was relatively standard. The first step is to run protein samples on a standard vertical SDS-polyacrylamide gel. Once the gel is completed, the proteins must be transferred to nitrocellulose. This was done using an electrophoretic transfer apparatus which uses an electric current to cause the proteins to migrate out of the gel and to become stuck on the nitrocellulose membrane. The membrane was then blocked for one hour using a blocking solution that was 3% powdered milk, 3% bovine serum albumin (BSA), 0.015% Antifoam A, and 0.001% sodium azide in tris-buffered saline. This solution serves the purpose of binding to all non-proteinaceous regions of the filter to exclude antibody binding. This blocking eliminates non-specific antibody binding. The filters were then washed with Tris-buffered saline (TBS) containing 0.05% Tween 20, a detergent. Next the primary antibody was added in a 1:500 ratio as compared to the concentration in serum, diluted in TBS with Tween 20 and BSA, and incubated for 30 minutes to overnight. The blot was then washed in TBS with Tween 20 twice and TBS once for five minutes each. The secondary antibody was then added and incubated for 2-4 hours. The
antibody used was a goat-anti rabbit IgG with an alkaline phosphatase conjugate. This antibody binds any rabbit antibodies on the filter and can be visualized due to its alkaline phosphatase conjugate. To visualize the antibodies 30 ml of an alkaline phosphatase buffer were used per blot. This buffer contains 100 mM Tris-HCl at pH 9.5, 100 mM NaCl, and 5 mM MgCl₂ in water. To this was added 200 µl of 50 mg./ml. nitro blue tetrazolium (NBT) in dimethyl formamide (DMF) and 100 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 70% DMF. The result is that the BCIP is dephosphorylated and a colored product is formed. This coloration only occurs at sites where secondary antibody is present, so bands of antibody binding are obvious on the filter.

The first series of Western blots was performed to determine the concentration and specificity of the four antibody samples. Four gels were run, each containing a molecular weight standard and four samples. Lane 1 contained the molecular weight standard, while lanes 2 through 4 used the two different JM107 strains: with and without the avrRxv insert and also with and without IPTG stimulation. The stimulated JM107 (pUR289) samples would be expected to produce high levels of β-gal and the JM107 (pUR289 exo 3-3) with avrRxv should produce large amounts of the fusion protein. Four gels, each with five samples, were transferred to nitrocellulose and probed with one of the four antibody samples. The desired result was that the two filters probed with the depleted antibody samples would contain a single band in lane 5. This lane is the only lane that should contain the fusion protein and if it is the only lane that has a band of antibody binding, then the antibodies in the solution should be monospecific for AvrRxv. The two filters probed with the undepleted
antibody solution should be expected to contain the same band or bands as in the undepleted solution-probed filters as well as possibly more bands, signifying contaminating antibodies.

The result of this first series of Western blots were very similar to what was expected (Fig. 7.8). The filters probed with undepleted antibody solution showed many bands of binding while the two filters probed with depleted antibody solution showed a few very light bands, a dark band in lane 3 at about 110 kD and two dark bands in lane 5 at about 110kD and 150 kD. The filters probed with undepleted serum appeared as expected and the filters probed with depleted antibody solution were very close to my expectations. I interpreted the depleted antibody blots in the following way: the light bands that appeared in each lane at various molecular weights were the result of small amounts of contaminating antibodies that were not removed in the depletion procedure; the dark bands in the two stimulated sample lanes were the result of antibodies specific for β-galactosidase that were not removed after the depletion procedure. This band signifies a partial success in antibody production.

The next Western blot was run using 10 lanes, including the five lanes previously described, three Xcv lanes and two more molecular weight standards (Fig. 9). This Western was performed using the same procedure as the first, except that the protein transfer procedure was more successful, as judged by transfer of the colored molecular weight markers. When this blot was developed and visualized, however, there were greater numbers of significantly dark bands than on the previous depleted-antibody blots. This blot looked similar to the undepleted antibody-probe blots in the previous experiment. This was a discouraging result.
To determine if this result was due to the amount of antibody used, I then decided to run a new series of blots containing the same 5 lanes as in the first set, but varying the amount of antibody probe. I used 500:1, 250:1, and 125:1 dilutions of antibodies to probe these filters. The result, again, was that there were many dark bands despite antibody dilution (Fig. 10). I then decided to try a new depletion procedure.

Since the previous depletion seemed to be only partially successful, as demonstrated by the fact that the antibodies in solution were polyspecific, I decided to use a variation of the depletion procedure on the previously depleted serum in hopes of removing non-specific antibodies. In this depletion I essentially ran a Western blot, but all of the lanes in the gel contained either β-galactosidase or a JM107 (pUR289) stimulated sample. I then treated the filter containing this protein with 1 ml. of diluted antibody, incubated it for four hours, and then collected the bathing antibody solution. The purpose of this depletion was the same as that of the last depletion, to remove antibodies specific for anything other than AvrRxv. I tried this new approach to depletion instead of repeating the initial approach because I was unsure how variable protein conformations affect antigenicity. The first depletion procedure was performed with soluble proteins. The potential antigens were in solution when they were placed in contact with the antibodies. The problem that may be responsible for the lack success in the depletion procedure is that when running Western blots, the antigens are not only stuck to a filter but also denatured using SDS. I theorized that the different molecular conditions that exist in the Western blot may account for the polyspecificity of the antibodies in solution. If a depletion could be performed with all of the proteins in their "Western blot conformation".
then that may provide a more specific depletion. I performed this depletion using two western filters each consisting of 8 lanes of the JM107 (pUR289) stimulated sample and 2 lanes of β-galactosidase sample.

I then performed a Western blot to test the success of this second depletion procedure using the same 5 lanes as in the previous depletion check as well as a β-galactosidase lane. This Western blot still showed many bands of antibody binding, indicating polyspecificity.

My latest Western blot is the fourth in the series and the results were somewhat better, but still not a success (Fig. 14). This time I included Xcv samples as well as the JM107 samples. The problem of polyspecificity still exists and I am still working to deplete the antibody solution.

**Conclusion**

My work has been interested in the interaction between Xcv and tomato at the molecular genetic level. This work used relatively standard molecular genetic techniques to study a system whose mechanism is a mystery. The actual gene responsible for avirulence in Xcv has been found and cloned, and is called *avrRxv*. This gene has been shown to be responsible for avirulence in Xcv. My study has been to determine the mechanism by which the product of this gene elicits resistance in resistant plants. This consisted of quantitative and localization studies of this protein and its precursors under varying conditions. One of my projects involved creating antibodies which specifically bind to the avirulence gene product. I then used these antibodies to visually locate and quantify the amount of avirulence gene protein that exists in Xcv. Another project involved using the *avrRxv* clone to study the amounts of avirulence gene RNA that exists in Xcv. These studies are intended to help in understanding the role of the
avirulence gene product. My project as a whole has yielded some success, but have not yet yielded any answers to the mystery of the role of the avirulence gene product. I have created antibodies and studied protein and RNA levels, but have encountered more problems than I anticipated. Therefore, much of my work is still underway.

This work has been very fulfilling and satisfying for me for several reasons. For one thing, I enjoyed the freedom of working independently. Although I had guidance from my tutor, as well as quite a bit of encouragement, I have spent a great deal of time on my own, either performing lab work or doing book or computer work. I have also learned quite a lot about molecular genetics as well as host pathogen interactions. This should be useful for my future, especially if I decide to go into medical research. Finally, I got the opportunity to work on original research in the basic sciences which is both exciting and rewarding. It is a great feeling to study a system and process that has never been studied; to be a pioneer, essentially exploring the unknown. This work could also someday have applications in the agricultural industry. Although my work in this small area has not yet yielded important results, I am confident that what I have done will lead to these results in the future.


Kane, J. F., and D. Hartley. 1988. Formation of Recombinant Protein Inclusion


**BACTERIAL STRAIN**

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Fig. 1. Diagram of potential results of contact between strains of *Xanthomonas campestris* pv. *vesicatoria* and cultivars of tomato. WS denotes leaf tissue water soaking which is a susceptible reaction. HR denotes a hypersensitive resistance response.
Fig 2. Two agarose mini-gels containing RNA samples obtained from *E. coli* (top) and *X. campestris* pv. *vesicatoria* (bottom) showing the difference in purity. Lane 1 in each gel is a molecular weight marker and lanes 2-5 are various samples. The *E. coli* samples are relatively pure as demonstrated by the well defined bands and lack of smearing. The *X. campestris* samples are not as pure due to the streaking the less defined bands and the sample that remains in the wells.
Fig. 3. Diagram depicting the pUR plasmid vectors. These vectors have various cloning sites. Once a gene has been cloned, it can be expressed due to the inducible lacZ promoter. (Sambrook 1989)
Fig. 4. An example of inclusion bodies in *E. coli*. Inclusion bodies may be produced when high levels protein production, as in the case of pUR expression vectors. They consist of large amounts of protein. (Kane 1988)
Collect blood (~30ml) 
Allow to clot at 4°C 
Save serum 

Elute IgG's and collect pH 2.8 

Ab's remain bound to protein A, other serum proteins elute 

Take small amount of serum and pour into protein A column. Protein A binds to Fc region of IgG in pH 8 buffer

**Fig. 5.** IgG purification scheme. This procedure was used to purify IgG antibodies from whole serum solution.
Grow up cell culture

Run SDS-Polyacrylamide gel electrophoresis

Add Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (AP substrates)

Filter Paper GEL Filter Paper NITROCELLULOSE FILTER
Transfer proteins to nitrocellulose

Treat filter with secondary antibody specific for the Ab region of rabbit IgG's (these Ab's are conjugated to alkaline phosphatase)

Fig. 6. Western blotting. Standard procedure used to run Western blots on E. coli and X. campestris samples. (Huynh, 1988)
Fig. 7. JM107 with pUR289-probed with undepleted antibody serum.

Fig. 8. JM107 with pUR289-probed with depleted antibody serum.

Fig. 9. Xcv and E. coli samples probed with undepleted antibody serum.

Fig. 10. JM107 with pUR289-probed with twice depleted antibody serum.