AN ABSTRACT OF THE THESIS OF

Yuqi Zhao for the degree of Doctor of Philosophy in Genetics Presented on May 3rd, 1991.

TITLE: Molecular Genetic Characterization of a Pathogenicity-attenuated Mutant of Pseudomonas syringae pathovar syringae.

Abstract approved: Redacted for Privacy

Dallice Mills

A pathogenicity locus of Pseudomonas syringae pv. syringae identified by Tn5 mutagenesis was investigated. The mutant strain PS9024 is attenuated for disease expression in its host, Phaseolus vulgaris, but produces the hypersensitive reaction (HR) in the nonhost, tobacco (Nicotina tabacum). A cosmid clone carrying 16 kilobases (kb) of contiguous genomic DNA partially complements this mutant. Altered growth of the mutant in planta was also partially restored. Marker exchange mutagenesis with Tn3-HoHo1 at two other sites within this locus results in mutants with attenuated and severely reduced pathogenicity. The locus is complex and contains repetitive DNA sequences. Northern analysis reveals that this locus is expressed in planta, but is not expressed in

a rich growth medium, and the transcript is larger than 10 kb, suggesting that the locus is transcribed as a polycistronic mRNA. Comparison of total cellular protein profiles of R32 and PS9024 using SDS-PAGE analysis further reveals that at least nine protein bands ranging from approximately 100 kD or above in size are present in the wild type strain R32, but absent from the mutant. Additionally, a protein of approximately 45 kD is absent from the mutant. The site of Tn*5* insertion has been partially sequenced. The initial search of the data banks suggested a gene or genes related to the ornithine biosynthetic pathway map to this locus. Further study strongly suggest a gene that encodes a membrance-associated protein and under the control of a promoter identical to *app*A gene promoter maps at this site and it is involved in the process of pathogenesis.

Molecular Genetic Characterization of a Pathogenicity-attenuated Mutant of *Pseudomonas syringae* pathovar *syringae*

by

Yuqi Zhao

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed May 3rd, 1991

Commencement June 1991

APPROVED:

Redacted for Privacy

Professor of Genetics and Botany and Plant Pathology in charge of major

Redacted for Privacy

Director of Genetics Program

Redacted for Privacy

Dean of Graduate School

Date thesis is presented May 3rd, 1991

Typed by Yuqi Zhao for Yuqi Zhao

THIS THESIS IS DEDICATED

To my mother, Guizhen Ma

For her love and faith in me.

To my father, Fenghe Zhao

For his love and teaching of life principles.

Especially to my wife, Wanjia

For her love, comfort and encouragement to make this possible.

To my dearest daughter, Jennie and son, Andrew

Their births are the best things to ever happen in my life.
They brought me laughter, sweetness and richness of life.

ACKNOWLEDGEMENTS

My sincere thanks are expressed to my major professor, Dr. Dallice Mills, for the research support and for his valuable time, suggestions and encouragement during the course of the research.

Thanks go to Drs. George Pearson, Joyce Loper, Carol Rivin and Christine Snow-Harter for serving on my committee.

I am especially indebted to my friends who have provided ideas, hints and suggestions during the frustrated experiments; They made it a little easier. They are: Drs. Pradip Mukhopadhyay, Jim Litts, Mohammad Zuber, Marilyn Ehrenshaft, Greg Bertoni, Martin Romantschuk, and especially Janet Williams Hanus and Chee Harn.

Finally, to all the friends who have kept it all in perspective, I am thankful, including Kevin McCluskey, Brian Russell, Grace Richter, Tina Hoffman and John Sollinger.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

MOLECULAR GENETIC CHARACTERIZATION OF A PATHOGENICITY-ATTENUATED MUTANT OF *PSEUDOMONAS SYRINGAE* PATHOVAR *SYRINGAE*

CHAPTER ONE

GENERAL INTRODUCTION

The *Pseudomonas syringae* group of Gram negative phytopathogenic bacteria is composed of about forty pathovars that are defined by several criteria including host range, the host plant from which they were originally isolated, and biochemical and physiological characteristics (Sands *et al.*, 1980). *Pseudomonas syringae* pv. *syringae* Van Hall (hereafter referred to as Pss) has a broad host range. It attacks more than forty plant genera that include both monocots and dicots (Stapp 1961). Typically Pss causes bacterial leaf spot diseases on the fruit and leaves of host plants, as well as stem cankers. It grows as an epiphyte and invades its host through natural wounds, stomates and hydathodes, and multiplies in the intercellular matrix of plant parenchymatous tissues. Yield losses have been reported for crop plants it infects, which include corn (*Zea mays*), bean (*Phaseolus vulgaris*) and stone fruit trees such as sweet cherry (*Prunus avium* L.), (for further details, see Hoitink *et al.*, 1968; Gross *et al.*,

1984; Lindemann et al., 1984).

The development of molecular genetic techniques, especially recombinant DNA technology, has greatly enhanced studies of genetic mechanisms controlling host-parasite interactions, and characterization of genes that confer pathogenicity. A great body of literature has accumulated in the last ten years through studies of various pathovars of P. syringae, and of other phytopathogenic bacteria. Comprehensive reviews of various aspects of the molecular genetics of plant pathogenic bacteria have recently appeared. Mills (1985) has described the use of transposon tagging as an approach to cloning pathogenicity determinants. Chatterjee and Vidaver (1986) have produced a comprehensive general review of research on a variety of phytopathogenic bacteria. Kotoujansky (1987) has reviewed work with Erwinia spp. Panopoulos and Peet (1985) and Coplin (1989) have highlighted work on indigenous plasmids in pathogenic bacteria. Keen and Staskawicz (1988) have reviewed host range determinants in plant pathogens and symbionts, and Daniels et al., (1988) have reviewed the molecular genetics of pathogenic determinants. The focus of this chapter will be limited primarily to Pss. It is an attempt to provide a summary of research from various laboratories

that have used molecular genetic approaches in studies of pathogenicity determinants of Pss.

DETERMINANTS OF PATHOGENICITY IN PSEUDOMONAS SYRINGAE PV. SYRINGAE

As with any other plant pathogen, several steps have been arbitrarily delineated to describe the infection process in susceptible host plants. First, there is need for attachment of bacteria onto the host, which is followed by penetration and multiplication. Disease symptoms then occur in susceptible hosts. The uncoupling of any one of the steps during the disease cycle will cause failure of the pathogen to incite disease on its host plant. In order to understand the genetic mechanisms underlying this process, researchers have attempted to obtain mutants that block specific steps in the disease cycle and study the genes that are involved.

Role of Bacterial Piliation in Attachment and Pathogenicity

Bacterial pili are nonflagellar, proteinaceous filaments protruding from the surface of cell membranes. They are known to function in adhesion of the bacteria

to other cells and to solid surfaces (Clegg and Gerlach 1987). Romantschuk and Bamford (1986), using scanning electron microscopy, have reported that piliation, as measured by the ability of strains to adsorb phages to their pili, is directly correlated with the adhesive capacity of P. s. pv. phaseolicola. They have observed that a super-piliated strain adhered more efficiently to plant surfaces than a wild-type strain, and that a non-piliated mutant adhered poorly. Furthermore, the piliated strain adhered preferentially around the stomata of the bean leaves, whereas the non-piliated strain was distributed evenly on bean leaves. Interestingly, they found no difference in pathogenicity between piliated and non-piliated strains once inoculum of either strain was injected directly into the plant tissue. However, disease symptoms were not observed when the non-piliated strain was sprayed onto the plant leaves. These results suggest that bacterial pili are required for pathogenicity by ensuring successful attachment of the bacteria to the host plant. A similar experiment was also carried out by Romantschuk (1987) using Pss HS191, a piliated corn pathogen. Comparisons of piliated and non-piliated derivatives revealed no preference for binding to the stomata of corn leaves, although the adhesion to the whole leaf surface in

general was reported elsewhere to be correlated with the presence of pili (Korhonen et al., 1986).

Flagellar Motility Confers Epiphytic Fitness Advantages

Haefele and Lindow (1987) have determined the role of flagellar motility in bacterial fitness of P. syringae on the phylloplane by comparing the wild-type strain with a nonmotile mutant. The nonmotile mutant strain, 3Mot$^-$, was isolated using 5%(v/v) methanesulfonic acid-ethyl ester. Results of this study have indicated that the loss of flagellar motility reduces the epiphytic fitness of a normally motile strain of Pseudomonas as measured by its growth, survival, and competitive ability on bean leaf surfaces. That the parental strain, 31R, was nonpathogenic, precluded any correlation of pathogenicity and motility. Motility has been reported to increase the infectivity of P. s. pv. phaseolicola (Panopoulos and Schroth 1974). Hattermann and Ries (1989), however, have demonstrated that there is no difference in pathogenicity between flagellar and nonflagellar strains of P. s. pv. glycinea. Bacterial adhesion to plant surfaces has been reported to be independent of motility (Leben and Whitmoyer 1979).

Bacterial Outer Membrane (OM) Proteins and their Role as Virulence Determinants

That outer membrane proteins could function as possible virulence factors was first proposed by Garrett et al., (1974). The premise was based upon the observation that selection for resistance to phage A7 resulted in the attenuation of virulence of cherry (Prunus avium) strains of P. s. pv. morsprunorum. Resistance to phage infection often involves a change in one or more of the OM components (Osborn & Wu 1980). Evidence has been presented by Expert and Toussaint (1985) that in Erwinia chrysanthemi, an enterobacterial phytopathogen, the loss of one to three iron-regulated OM proteins was correlated with the loss of pathogenicity. In strain HS191 of a corn pathogen of Pss, Hurlbert and Gross (1983) have also found that the loss of OM protein 5, as measured by SDS-PAGE and isoelectric focusing methods, was associated with acquisition of avirulence (strain AO111) and reduced virulence (strain PSG100).

Outer membrane proteins are also known to be involved in mediating iron uptake via pyoverdin$_{pss}$, the fluorescent siderophore produced by Pss (Cody and Gross 1987). Comparison of the OM proteins that were expressed in bacteria under low and high iron concentrations and detected by two-dimensional electrophoresis revealed nine OM proteins that were

iron-regulated. A mutant strain, B301D, that lacks the major iron-regulated protein, 4a [MW 74,000 kilodaltons (kD)], was incapable of iron uptake (IU^-) through ferric $pyoverdin_{pss}$. In contrast, however, the OM protein profile of a non-florescent mutant (Flu^-) and the wild-type strain were essentially similar. It is suggested that these OM proteins could act as receptors on the cell membrane that are involved in iron acquisition *via* siderophore activity. The expression of these OM proteins could be intricately regulated by the environment.

Production of Siderophores by Pss may not be Associated with Pathogenicity

The common feature for all the fluorescent pseudomonads is the production of extracellular, water-soluble, yellow-green pigments which fluoresce under UV irradiation. These fluorescent pigments are known to function as siderophores because they are synthesized only under iron-limiting conditions (Lenhoff 1963). Siderophore production in *P. aeruginosa*, an animal pathogen, is known to be correlated with virulence (Cox 1982; Crosa 1984). It has been reported that *P. aeruginosa* PAO1 produces two siderophores, $pyoverdin_{pa}$ and $pyochelin_{pa}$, during the infection process (Ankenbauer *et al.*, 1985; Cox and Adams, 1985). Studies on

pyochelin$_{pa}$ (Cox 1982) have indicated its production is directly associated with virulence of P. aeruginosa. It has also been shown that pyoverdin$_{pa}$ stimulates growth of the bacterium in human serum (Ankenbauer et al., 1985). However, the role of pyoverdin$_{pa}$ in the process of bacterial infection is not well characterized. It is possible, therefore, that siderophore production in Pss may play a similar role in plant pathogenesis.

Gross (1985) has suggested that the siderophore from Pss is primarily responsible for iron acquisition from the host by sequestering Fe(III) in a form that can be readily and specifically utilized by the pathogen during growth in planta. However, another study has shown no correlation between the in situ fluorescent siderophore production by Pss and pathogenesis on bean leaf surfaces (Loper and Lindow 1987). This latter study has also shown no significant difference in growth, survival or pathogenicity between wild-type Pss strain B728a and a non-fluorescent (Flu^-) mutant derivative I-1. To date, chrysobactin produced by E. chrysanthemi (Persmark et al., 1989), a pathogen of soft rot disease of African violet (Saintpaulia ionantha), is the only siderophore produced by a plant pathogen that has been identified as a virulence factor in pathogenesis. Whether the fluorescent pigment of Pss plays a role in pathogenesis has yet to be determined.

It appears that it would influence iron-limited growth and confer protection to growing cells exposed to UV irradiation in culture as suggested by Loper and Lindow (1987). However, the role of fluorescent pigment production in pathogenesis on the phylloplane in field-grown plants remains unknown. A comprehensive discussion of siderophores in microbial interaction on plant surfaces was presented in a recent review (Loper and Buyer, 1991).

Plasmalemma K^+/H^+ Exchange Promotes Multiplication of the Pathogen in Plants

Following entry into host tissue, little is known about the molecular mechanisms that enable Pss to multiply within leaf inter-cellular spaces and subsequently cause various types of leaf-spotting symptoms. Also it is unclear what pathogen-induced changes are required of the host in order to allow for a successful infection. Atkinson et al., (1985) have discovered that induction of the hypersensitive reaction (HR) in tobacco by P. s. pv. pisi is associated with the activation of a specific plant plasmalemma K^+ efflux and H^+ influx exchange. The K^+/H^+ exchange is characterized by a rapid, transient polarization of the plant plasmalemma. A similar phenomenon was also observed by Atkinson et al., (1986) when

suspension-cultured tobacco cells were treated with a pectate lyase isozyme from E. chrysanthemi. Furthermore, it has been demonstrated (Baker et al. 1986) that low levels of the pectate lyase from E. chrysanthemi suppress both the multiplication and the HR-inducing activity of P. s pv. pisi in tobacco. It is interesting that many phytopathogenic bacteria, including Pss, that are pathogenic and induce necrosis in one host plant, also induce the HR in non-host plants. In fact, the ability of various strains of bacteria to cause the HR in tobacco has been used as a criterion for establishing pathogenicity of Pseudomonas species (Klement 1963).

Molecular genetic studies have indicated that some genes that control pathogenesis in host plants are also responsible for the induction of the HR in the non-host plants. A mutation that spontaneously affects both the HR and pathogenesis is now considered to have occurred in a hrp gene (to be discussed later). It has been discovered that H^+/K^+ ion exchange can be induced both in host and non-host plants. It is possible, therefore, that the mechanism by which Pss induces leaf-spot disease in its host plant is analogous to the induction of the HR in the non-host plant. Indeed, Atkinson and Baker (1987) have reported that a Tn5-induced mutant of Pss fails to induce the plasmalemma K^+/H^+ exchange in

tobacco, and will neither multiply in bean leaves nor have any effect on sucrose transport. With regard to ion exchange, the primary distinction between non-host plants and host plants is the rapid K^+/H^+ exchange and temporary induction of the HR that occurs in non-host plants. The K^+/H^+ exchange also precedes the onset of growth of the bacterial population. These results indicate that continued exchange of ions elevates host inter-cellular fluid pH from 5.5 to 7.5, with the consequent reversal of the H^+ gradient. The reversed H^+ gradient results in interferences with the ATPase-driven transport system that is believed to lead to a net accumulation of sucrose, amino acids, and inorganic ions within host inter-cellular spaces where bacteria reside. It is surmised that multiplication of bacteria is therefore promoted by the increased inter-cellular pH and nutrient level.

Role of Pss Phytotoxins and Bacteriocins in Pathogenesis

There are two major phytotoxins that are known to be produced by Pss. Syringomycin is produced by many strains of Pss which have a broad host range, whereas syringotoxin is produced only by isolates that incite disease in citrus. Both of these phytotoxins cause specific physiological and biochemical effects in host

plant cells which are manifested as necrotic symptoms (DeVay et al., 1978). Pss is the only pathovar known to produce necrosis-inducing rather than chlorosis-inducing toxins. Both phytotoxins cause alteration of oxidative metabolism in isolated maize mitochondria (Surico and DeVay 1982).

Syringomycin

The synthesis of syringomycin (SR) has been shown to be determined by chromosomal genes (Currier and Morgan 1983; Gonzalez et al., 1984). The chemical structure of SR is not yet fully determined, but it is known to contain a positively charged hexapeptide moiety consisting of arginine, phenylalanine, serine, and diaminobutyric acid in 1:1:2:2 molar ratios (Gross and DeVay 1977a; Zhang and Takemoto 1987). Little is known about its biosynthetic pathway, although it is possible that its synthesis may resemble the peptide antibiotic synthetase system of Bacillus spp. (Durbin 1981). This system involves enzymatic assembly by a peptide synthetase rather than involvement of template mRNA on ribosomes.

Early studies (Gross and Devay 1977b) demonstrated a strong correlation between the systemic necrosis produced in inoculated hypocotyls of cowpea and maize, and the synthesis of SR as measured by the standard fungal (Geotrichum candidum) inhibition bioassay.

Syringomycin concentrations as low as 1 μg/ml when applied to parenchymatous tissues will elicit necroses resembling symptoms caused by the bacterium. Paynter and Alconero (1979) have successfully detected syringomycin production in infected peach leaves and stems using a fluorescent immunological tagging method. The involvement of syringomycin in pathogenesis was further elucidated recently by Xu and Gross (1988). Transposon Tn*5* was delivered into a Tox^+ strain, B301D-R, using the suicide vector, pGS9, and the resultant singular Tn*5* insertion mutants were screened for changes in syringomycin production by testing for antibiosis against *G. candidum*. Four major Tox^- groups (*i.e.*, A-D) were differentiated by pathogenicity and ability to cause a hypersensitive reaction in tobacco. Members from three of the groups (B, C and D) were nonpathogenic ($Path^-$), whereas mutants from group A remained pathogenic in cherry fruit assays, but the disease index was 17 to 66% lower than the parental wild-type strain, B301D-R. These data suggest that syringomycin is not essential for pathogenicity but contributes significantly to virulence. One intriguing observation regarding the Tox^- strains in groups C and D is that Tn*5* insertion resulted in a phenotype that is similar to *hrp* gene mutants, *i.e.*, HR^- and $Path^-$. This indicates the possible association between phytotoxin production and

some *hrp* gene activities. Whether *hrp* genes are structural genes for syringomycin biosynthesis, or genes that regulate their biosynthesis, is not yet known.

Syringotoxin

The structure of syringotoxin (ST) is similar to syringomycin and is composed of a pentapeptide consisting of theronine, serine, glycine, ornithine, and a modified basic amino acid in equal molar ratio (Gross and Devay 1977c; Gonzalez *et al.*, 1981). Syringotoxin is unique to citrus isolates of Pss (Gonzalez *et al.*, 1981). Strains of Pss are known to produce either syringomycin or syringotoxin, but not both. In addition to differences in chemical composition, syringotoxin can also be distinguished from syringomycin by its slower migration in polyacrylamide gels, and by its different spectrum of biological activity against bacteria and fungi (Gonzalez *et al.*, 1981). Tn*5*-induced Tox^- mutants have been produced using pSUP1011, a transposon suicide vector (Morgan and Chatterjee 1985), and they are severely attenuated in virulence. The loss of syringotoxin production was associated with the insertion of Tn*5* into three *Eco*RI chromosomal fragments of 10.5, 17.8 and 19.3 kilobases (kb) in size. Southern hybridization analysis of DNA fragments suggests that the 10.5 and 17.8 kb *Eco*RI fragments may be contiguous.

Recent evidence (Morgan and Chatterjee 1988) indicates that the DNA fragments that are involved in syringotoxin production are unique only to ST-producing Pss strains. It was also suggested that the genes are clustered, and that they encode two large proteins (ST1, ca. 470 kD, and ST2, ca. 435 kD) which may be peptide synthetases similar to those involved in the biosynthesis of peptide antibiotics in Bacillus spp. (Chatterjee et al., 1989).

Syringostatins

The third group of toxins produced by Pss was reported by Isogai et al., (1989) and designated as syringostatins. The toxins were isolated from a Pss strain, SY12, a pathogenic isolate that causes bacterial blight of lilac (Syringa vulgaris L.). The chemical structure of these toxins was obtained using HPLC (High Performance Liquid Chromatography) and MS (Mass Spectrum). They are structurally similar to syringomycin and syringotoxin, but clearly different from them in their amino acid compositions. All of the toxins identified (syringostatins A to H) contain the basic structure of threonine, serine, 2,4,-diaminobutanoic acid and ornithine in the molar ratios of 1:1:2:1. Variation in structure within these toxins were detected. The role of these toxins in pathogenesis is yet to be studied.

Syringacin W-1

Bacteriocins of fluorescent phytopathogenic pseudomonads can be separated into at least sixteen groups based on the spectra of strains that are killed (Vidaver and Buckner 1978). Syringacin W-1, a bacteriocin produced by some Pss strains, is a 20 x 75 nm rod-shaped particle composed of an inner core and an outer sheath (Smidt and Vidaver 1986). It consists principally of protein (67.2%) and carbohydrate (10-35%). Bacteriocins are foreseen to be useful as bio-control agents because they effectively reduce the populations of plant bacterial pathogens on plant tissue, especially when used in combination (Vidaver 1976; Smidt and Vidaver 1982).

USE OF TRANSPOSON TAGGING IN THE STUDY OF PATHOGENICITY-RELATED GENES

Definition of Pathogenicity Genes

Conventionally, mutations that lead to auxotrophy or to poor growth <u>in vitro</u> are generally discarded in pathogenicity studies. It has become a concern, however, that some of these genes may accurately be defined as pathogenicity genes. In the broadest sense,

Daniels et al., (1988) have suggested that any gene that is involved in the adaptation of a pathogen to the physiochemical environment within the host may be considered as an essential component of pathogenicity. Accordingly, the genes that have been identified by their involvement in the synthesis of known determinants of pathogenicity probably represent only a small fraction of the genes required for full pathogenicity. Consequently, new approaches are needed for screening pathogenicity-related genes of a broader spectrum. Transposon mutagenesis is a relatively new development in molecular biology that offers a great opportunity to study pathogenicity-related genes in plant pathogens (reviewed by Mills 1985). The general principle of this method is schematically described in Figure 1-1. The advantage of transposon mutagenesis is that the transposon can randomly transpose into the bacterial genome and inactivate a structural gene where it resides. Transposition often results in an obvious change of phenotype, and location of the transposon within the genome can be mapped to the position of the gene in question. This approach provides the potential to identify and clone all the possible pathogenicity genes.

Disease Specific Genes (dsg) and Genes that Control the Hypersensitive Response and Pathogenicity (hrp)

Transposon mutagenesis has been used in a wide variety of organisms, including Pss, to isolate mutants affected in their virulence or pathogenic behavior in planta. These studies have led to the identification of different types of genes that are involved in plant-pathogen interactions. The acronym dsg (Disease Specific Gene) has been assigned to genes responsible only for disease symptom development on host plants, whereas the acronym hrp, (Hypersensitive Reaction and Pathogenicity) has been assigned to genes that control the elicitation of the HR on non-host plants and the expression of disease symptoms on host plants (Lindgren and Panopoulos 1986).

Recently, a gene designated as lemA1 was identified by Tn5 transposon mutagenesis of Pss strain B728a (Willis, et al., 1990). The mutation affects only pathogenicity. Its lesion-forming ability on both the pods and leaves of bean (Phaseolus vulgaris) was found to be genetically separable from other phenotypic traits in planta. Strain NPS3136, which contains the lemA1 mutation, displays growth dynamics similar to its parental strain on bean, but does not incite lesions. Eight overlapping cosmid clones isolated from the

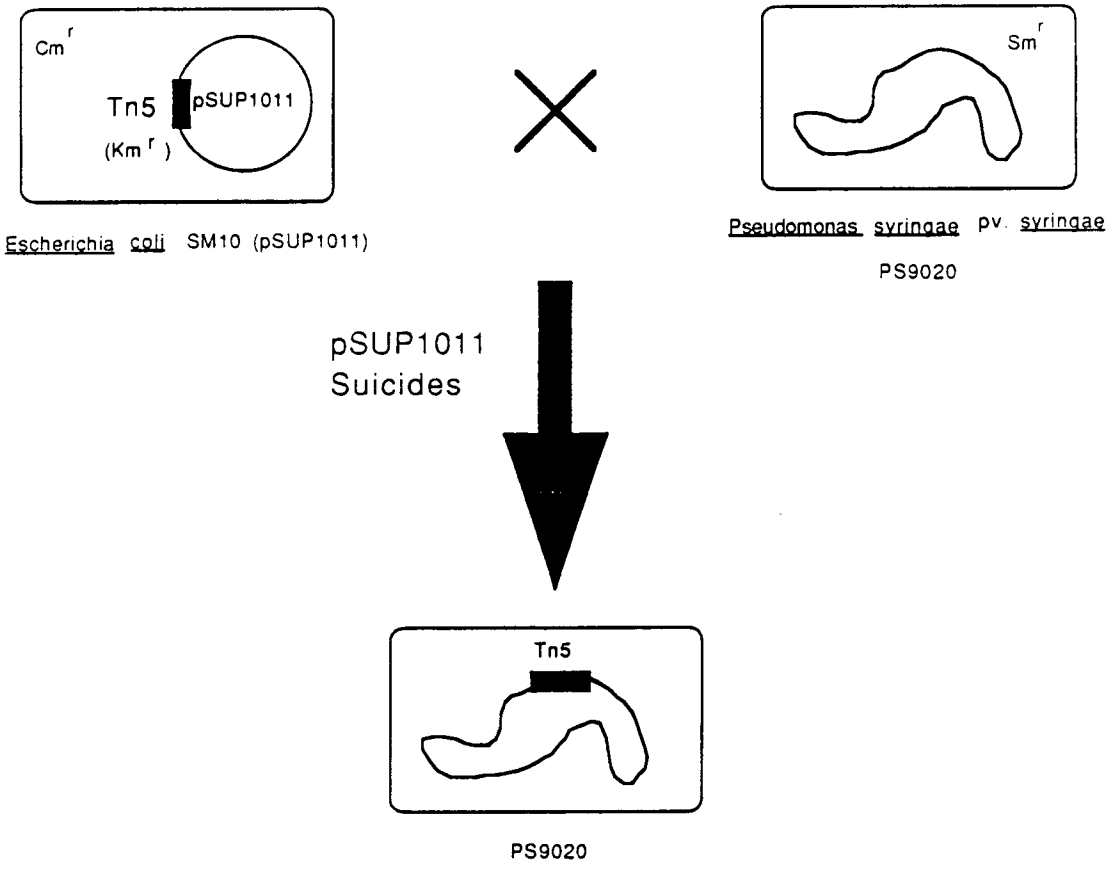


Figure 1-1. Tn5 mutagenesis and screening for non-pathogenic mutants in P. s. pv. syringae PS9020 (adapted from Mills and Niepold, 1987).

parental genomic library restore lesion formation in NPS3136. The lemA locus was localized to a 6.7 kb region.

Four Tn5-induced pathogenicity-deficient mutants of Pss strain PS9020 have been identified by Anderson and Mills (1985). Endonuclease restriction site mapping analyses have indicated that all four mutants resulted from single Tn5 insertion events because each resides within a different EcoRI fragment in the genome (Mills and Niepold, 1987; Zhao and Mills, unpublished data). Three of the mutants appear to have Tn5 inserted in dsg loci, whereas the insertion in the remaining mutant, PS9021, has identified a hrp gene locus. PS9021 differs from the parental wild-type pathogenic strain by its inability to cause symptoms in bean (Phaseolus vulgaris L. cultivar "Eagle") and to induce the HR in tobacco Nicotina tabacum cv. "Samsun NN". It also has a mucoid colony morphology rather than the smooth colony morphology of its parental strain, PS9020. The three dsg$^-$ mutants, PS9022, PS9023 and PS9024 produced reduced symptoms in susceptible beans.

The in planta growth of PS9022 and PS9023 is similar to the parental strain (Zhao and Mills, unpublished data). In contrast, PS9021, the hrp mutant, completely fails to grow in planta even though its growth kinetics in minimal growth medium are similar to

the parental strain (Bertoni and Mills 1987). The Tn*5*-induced *hrp* mutation of PS9021 (designated *hrp*M) has been complemented by a cosmid from the genomic library of wild-type DNA (Niepold *et al.*, 1985; Mills *et al.*, 1987). A 3.9 kb *Hind*III fragment which encompasses the *hrp*M locus has been sequenced (Mills *et al.*, 1988; Mukhopadhyay *et al.*, 1988) and shown to contain two open reading frames (ORFs) on one strand which could encode putative polypeptides of 40 kD and 83 kD. Two ORFs that could encode polypeptides of 81 kD and greater than 39 kD were also present on the opposite strand. Hydropathy and homology analyses indicate that the 40 kD and the 83 kD polypeptides have properties of DNA binding proteins and transmembrane proteins, respectively. A fusion protein consisting of a portion of the 83 kD polypeptide and the β-galactosidase gene from *Escherichia coli* has been constructed and expressed in *E. coli* (Mills *et al.*, 1985). Recent evidence suggests that the *hrp*M gene may be involved in the uptake of nitrogenous compounds, their metabolism or both (Mills *et al.*, 1991).

A regulatory function of another *hrp* locus in Pss has been reported recently (Lu and Hutcheson, 1989). A 4.3 kb regulatory region from a 31 kb *hrp* gene cluster has been detected by marker exchanging a *hrp*-*lux* gene fusion into Pss61. The resultant HR^- mutant, Pss61-143,

exhibited constitutive expression of the hrp-lux fusion. However, another mutant Pss61-145 which contained the identical fusion, but was lacking this 4.3 kb region downstream of this insertion site, exhibited poor lux expression. Attenuation of lux expression was restored by the 4.3 kb fragment on a cosmid provided in trans. These results suggest that this 4.3 kb region encompasses a regulatory element that controls expression in trans of at least some hrp genes within the cluster. Recent work indicates that this regulatory locus may reside within a region of approximately 1 kb of DNA (Y. Lu, personal communication).

A regulatory function for hrpS, from P. s. pv. phaseolicola, has been recently reported (Grimm et al., 1989; Grimm and Panopoulos 1989). Its predicted protein product shares significant homology with a highly conserved domain found in several prokaryotic regulatory proteins, such as NtrC, NifA, TyrR and DctD. Structural and functional homologues of hrp genes are also known to exist in several pathovars of P. syringae. The hrpM locus of Pss is known to share homology with the hrpF locus of P. s pv. phaseolicola (Lindgren and Panopoulos 1986; Lindgren et al., 1988a). DNA probes made of hrpS cross-hybridize with DNA sequences of several other pathovars (Grimm et al., 1989). Furthermore, hrp mutations in this bacterium can be

complemented by the corresponding genes from closely related pathovars, such as P. s. pv. savastanoi and P. s. pv. glycinea (Lindgren et al., 1988b).

Moreover, hrp::Tn5 mutations in P. s. pv. phaseolicola can also be marker exchanged into the genomes of different pathovars, such as P. s. pv. tabaci and P. s. pv. glycinea, and give rise to hrp mutants. One interesting observation by Lindgren et al., (1988b) regarding hrp gene function is that the elicitation of the HR in incompatible race/cultivar specific necrosis incited by P. s. pv. glycinea in soybean cultivars requires a fully functional hrp region in addition to an avr gene function. It was speculated (Grimm et al., 1989) that the expression of the HR requires either individual hrp or avr gene products or the interaction of both gene products as a complex.

Interaction between hrp and avr genes was also suggested for Pss (Huang et al., 1988). A cosmid clone, pHIR11, which contains a 31 kb insert from Pss strain 61, was reported to restore the wild-type phenotypes to all the Tn5-induced HR^- mutants of this strain when tested in tobacco plants. Interestingly, this cosmid clone enables P. fluorescens strain 55, a nonpathogen that normally causes no plant reaction, to elicit the HR in tobacco plants. It also converts pathogenic strains of Pss 226, a tomato (Lycopersicon esculentum Mill)

pathogen that causes the HR in tobacco, and P. s. pv. tabaci, a pathogen of tobacco that causes the HR in tomato, into nonpathogenic strains on their respective host plants. Typical K^+/H^+ ion exchange responses (XR) accompanied these HR elicitations. It is postulated that the 31 kb insert of pHRI11 contains a hrp gene cluster and an avr gene. A single TnphoA-induced mutation in pHIR11, pHIR11-18, no longer conferred the HR phenotype to P. fluorescens 55. Mutation of Pss 61 and 226 derived by gene replacement of pHIR11-18 abolished their ability to incite any plant reactions in all plants tested.

A more detailed review on hrp genes of phytopathogenic bacteria has recently appeared (Wills, et al., 1991).

Host Range and Avirulence (avr) genes

The inducible hypersensitive reaction of plants is a major factor that restricts the host range of pathogens (Keen and Staskawicz 1988). Two kinds of plant resistance to pathogens are defined. A general resistance of the plant confers the HR to entire pathogen species; whereas the HR in a specific resistance of the plant is induced only by certain genotypes of a single pathogen species. In specific resistance, if the pathogen harbors a dominant gene and the plant host contains a complementary disease

resistance gene, the infected plant responds with a hypersensitive reaction. The dominant genes in the pathogen that are genetically complementary to disease resistance genes in the plant host are called avirulence (avr) genes. This very specific interaction that is characteristic of many plant/pathogen interactions follows the gene-for-gene concept (Flor 1942, 1971) and will result in an incompatible interaction (Ellingboe 1979). Avirulence (avr) genes that modulate the HR have been cloned and characterized from several bacterial pathogens, including P. s. pv. glycinea (Staskawicz et al., 1984). The function of avr genes in bacteria and the mechanism by which avr genes lead to plant recognition of the bacteria and further induction of the HR, however, are unclear. No avr gene has yet been cloned from Pss.

SUMMARY

Considerable information has accumulated in recent years through molecular genetic studies of Pss (Table 1-1). These results indicate that uncoupling or mutation in any step of the disease cycle of Pss could result in

an unsuccessful infection of the pathogen on its susceptible host plant.

Transposon tagging has provided a direct method to identify all the possible pathogenicity genes that otherwise are very difficult to identify using conventional means.

This dissertation is concerned with the use of molecular genetic approaches to study a transposon Tn*5*-induced mutant of Pss which shows attenuated-pathogenicity in its susceptible host plant, *Phaseolus vulgaris* L.

Table 1-1. Summary of Studies on Molecular Biology and Pathogenicity of Pseudomonas syringae pv. syringae

Subject	Disease Cycle/Mutants/ /Gene Involved	Reference
Piliation	Attachment /non-piliated mutant	Romantschuk and Bamford (1986)
Flagella	Penetration /Flagella-less mutant	Haefele and Lindow (1987)
OM Proteins	Virulence factors /OM Proteins /Pyoverdinpss	Hurlbert and Gross (1983) Cod and Gross (1987)
Plasmalemma	Multiplication/HR	Baker et al., (1986)
H+/K+ ion exchange	Multiplication/HR	Atkinson and Baker (1987)
	Multiplication/Path	Atkinso et al., (1986)
Syringomycin	Virulence factors	Gross et al., (1977b)
	/Chemical structure	same (1977c)
	/Antibody detection	Paynter and Alconero (1979)
	/Plasma membrane	Zhang and Takemoto (1987)
	/Toxin-less mutants	Xu and Cross (1988)
Syringotoxin	Virulence factors/ST	Morgan and Chatterjee (1985)
	Virulence factors/ST	same (1988)
Bacteriocin	Syringacin/Antibiosis	Smidt et al., (1986)
Pathogenicity -related genes	/dsg, hrp genes	Anderson and Mills (1985)
	/gene expression	Mills et al., (1985)
	/gene distribution	Zhao & Mills (1989)
	/gene function	Mills et al., (1990)
LemA locus	lemA1 gene	Willis et al., (1990)
Hrp locus	hrpM gene expression	Mukhopadhyay et al., (1988)
	/gene expression	Huang et al., (1988)
	hrp/gene regulation	Lu and Hutchenson (1989)

REFERENCES

Anderson, D.M. and D. Mills. 1985. The use of transposon mutagenesis in the isolation of nutritional and virulence mutants in two pathovars of Pseudomonas syringae. Phytopathology 75:104-108.

Ankenbauer, R., S. Sriyosachati, and C.D. Cox. 1985. Effects of siderophores on the growth of Pseudomonas aeruginosa in human serum and transferrin. Infect. Immun. 49:132-140.

Atkinson, M.M., J.S. Huang, and J.A. Knopp. 1985. The hypersensitive reaction of tobacco to Pseudomonas syringae pv. pisi: Activation of a plasmalemma K^+/H^+ exchange mechanism. Plant Physiol. 79:843-847.

Atkinson, M.M., C.J. Baker, and A. Collmer. 1986. Transient activation of plasmalemma K^+ efflux and H^+ influx in tobacco by a pectate lyase isozyme from Erwinia chrysanthemi. Plant Physiol. 82:142-146.

Atkinson, M.M. and C.J. Baker. 1987. Alteration of plasmalemma sucrose transport in Phaseolus vulgaris by Pseudomonas syringae pv. syringae and its association with K^+/H^+ exchange. Phytopathology 77:1573-1578.

Baker, C.J., M.M. Atkinson, A. Collmer, and M. Roy. 1986. Inhibition of the hypersensitive response in tobacco by pectate lyase. Physiol. Mol. Plant Pathol. 29:217-225.

Bertoni, G. and D. Mills. 1987. A simple method to monitor growth of bacterial populations in leaf tissue. Phytopathology 77:832-835.

Chatterjee, A.K. and A. Vidaver. 1986. Genetics of pathogenicity factors: application to phytopathogenic bacteria. In: Ingram, D.S. and P.H. Williams (eds.) Advances in Plant Pathology. Academic Press, London. pp. 1-217.

Chatterjee, A.K., R.O. Nordeen, and G. Somlyai. 1989. Molecular cloning and expression of syringotoxin (ST) biosynthetic genes of Pseudomonas syringae pv. syringae. (Abst.) In: Pseudomonas '89: Biotransformations, Pathogenesis, and Evolving Biotechnology. American Society for Microbiology, Illinois, Chicago. Session III-10.

Clegg, S. and G. Gerlach. 1987. Enterobacterial fimbriae. J. Bacteriol. 169:943-948.

Cody, Y.S. and D.C. Gross. 1987. Outer membrane protein mediating iron uptake via pyoverdin$_{pss}$, the fluorescent siderophore produced by Pseudomonas syringae pv. syringae. J. Bacteriol. 169:2207-2214.

Coplin, D.L. 1989. Plasmids and their role in the evolution of plant pathogenic bacteria. Ann. Rev. Phytopathol. 27:187-270.

Cox, C.D. 1982. Effect of pyochelin on the virulence of Pseudomonas aeruginosa. Infect. Immun. 36:17-23.

Cox, C.D. and P.I. Adams. 1985. Siderophore activity of pyoverdin for Pseudomonas aeruginosa. Infect. Immun. 48:130-138.

Crosa, J.H. 1984. The relationship of plasmid-mediated iron transport and bacterial virulence. Ann. Rev. Microbiol. 38:69-89.

Currier, T.C and M.K. Morgan. 1983. Plasmids of Pseudomonas syringae: no evidence of a role in toxin production or pathogenicity. Can. J. Microbiol. 29:84-89.

Daniels, M.J., J.M. Dow, and A.E. Osbourn. 1988. Molecular genetics of pathogenicity in phytopathogenic bacteria. Ann. Rev. Phytopathol. 26:285-312.

DeVay, J.E., C.F. Gonzalez, and R.J. Wakeman. 1978. Comparison of the biocidal activities of syringomycin and syringotoxin and the characterization of isolates of Pseudomonas syringae from citrus hosts. In: Proc. 4th Int. Conf. Plant. Path. Bacteriol. Angers, France. pp 643-650.

Durbin, R.D. 1981. Metabolism Biosynthesis In: Durbin, R.D. (ed.) Toxins in Plant Disease. Academic Press, Inc., New York. pp 443-448.

Ellingboe, A.H. 1979. Inheritance of specificity: The gene-for-gene hypothesis. In: Daly, J.M. and I. Uritani (eds.) Recognition and Specificity in Plant Host-Parasite Interactions. pp. 115-131.

Expert, D. and A. Toussaint. 1985. Bacteriocin-resistant mutants of Erwinia chrysanthemi: possible involvement of iron acquisition in phytopathogenicity. J. Bacteriol. 163:221-227.

Flor, H.H. 1942. Inheritance of pathogenicity in Melampsora lini. Phytopathology 32:653-669.

Flor, H.H. 1971. Current status of the gene-for-gene concept. Ann. Rev. Pathol. 9:275-296.

Garrett, C.M.E., J.E. Crosse, and A. Sletten. 1974. Relations between phage sensitivity and virulence in Pseudomonas morsprunorum. J. Gen. Microbiol. 80:475-483.

Gonzalez, C.F, J.E. DeVay, and R.J. Wakeman. 1981. Syringotoxin: a phytotoxin unique to citrus isolates of Pseudomonas syringae. Physiol. Plant Pathol. 18:41-50.

Gonzalez, C.F., S.K. Layher, A.K. Vidaver, and R.H. Olsen. 1984. Transfer, mapping, and cloning of Pseudomonas syringae pv. syringae plasmid pCG131 and assessment of its role in virulence. Phytopathology 74:1245-1250.

Grimm, C., L. Rahme, R. Frederick, M. Mindrinos, P.B. Lindgren, and N. Panopoulos. 1989. The common pathogenicity genes of Pseudomonas syringae pathovars. In: Collmer, et al. (eds.) Molecular Biology of Plant-Pathogen Interactions. UCLA Symp. Mol. Cell Biol., Alan R. Liss, Inc., New York, NY. pp. 49-55.

Grimm, C. and N. Panopoulos. 1989. The predicted protein product of a pathogenicity locus from Pseudomonas syringae pv. phaseolicola is homologous to a highly conserved domain of several procaryotic regulatory proteins. J. Bacteriol. 171:5031-5038.

Gross, D.C. and J.E. DeVay. 1977a. Production and purification of syringomycin, a phytotoxin produced by Pseudomonas syringae. Physiol. Plant Pathol. 11:13-28.

Gross, D.C. and J.E. DeVay. 1977b. Role of syringomycin in holcus spot of maize and systemic necrosis of cowpea caused by Pseudomonas syringae. Physiol. Plant Pathol. 11:1-11.

Gross, D.C. and J.E. DeVay. 1977c. Chemical properties of syringomycin and syringotoxin: toxigenic peptides produced by Pseudomonas syringae. J. Appl. Bacteriol. 43:453-463.

Gross, D.C., Y.S. Cody, E.L. Proebsting Jr., G.K. Tadamaker, and R.A. Spotts. 1984. Ecotypes and pathogenicity of ice nucleation active Pseudomonas

syringae isolated from deciduous fruit tree-orchards. Phytopathology 74:241-248.

Gross, D.C. 1985. Regulation of syringomycin synthesis in Pseudomonas syringae pv. syringae and defined conditions for its production. J. Appl. Bacteriol. 58:167-174.

Haefele, D.M. and S.E. Lindow. 1987. Flagellar motility confers epiphytic fitness advantages upon Pseudomonas syringae. Appl. Environ. Microbiol. 53:2528-2533.

Hattermann, D.R. and S.M. Ries. 1989. Motility of Pseudomonas syringae pv. glycinea and its role in infection. Phytopathology 79:284-289.

Hoitink, H.A.J., D.J. Hagedorn, and E. McCoy. 1968. Survival, transmission and taxonomy of Pseudomonas syringae Van Hall, the causal organism of bacterial brown spot of bean (Phaseolus vulgaris L.). Can. J. Microbiol. 14:437-441.

Huang, H.C., R. Schuurink, T.P. Denny, M.M. Atkinson, C.J. Baker, I. Yucel, S.W. Hucheson and A. Collmer. 1988. Molecular cloning of a Pseudomonas syringae pv. syringae gene cluster that enables Pseudomonas fluorescens to elicit the hypersensitive reaction in tobacco plants. J. Bacteriol. 170:4748-4756.

Hurlbert, R.E. and D.C. Gross. 1983. Isolation and partial characterization of the cell wall of Pseudomonas syringae pv. syringae HS191: comparison of outer membrane proteins of HS191 with those of two plasmidless derivatives. J. Gen. Microbiol. 129:2241-2250.

Isogai, A. N. Fukuchi, S. Yamashita and A. Suzuki. 1989. Syringostatin, novel phytotoxins produced by Pseudomonas syringae pv. syringae. Biol. Chem. 53(1):3117-3119.

Keen, N.T. and B. Staskawicz. 1988. Host range determinants in plant pathogens and symbionts. Ann. Rev. Microbiol. 42:421-440.

Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature 199:299-300.

Korhonen, T.K., K. Haahtela, M. Romantschuk, and D. Bamford. 1986. Role of fimbriae and pili in the attachment of Klebsiella, Enterobacter and Pseudomonas to plant surface. In: Lugtenberg, D. (ed.) Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions.

NATO ASI Series, Vol. H4. Springer-Verlag, Berlin Heidelberg. pp. 229-241.

Kotoujansky, A. 1987. Molecular genetics of pathogenesis by soft-rot Erwinia. Ann. Rev. Phytopathol. pp. 405-430.

Leben, C. and R.E. Whitmoyer. 1979. Adherence of bacteria to leaves. Can. J. Microbiol. 25:896-901.

Lenhoff, H.M. 1963. An inverse relationship of the effects of oxygen and iron on the production of fluorescin and cytochrome c by Pseudomonas fluorescens. Nature, London 199:601-602.

Lindemann, J., D.C. Arny, and C.D. Upper. 1984. Use of an apparent infection threshold population of Pseudomonas syringae to predict incidence and severity of brown spot of bean. Phytopathology 74:1334-1339.

Lindgren P.B. and N.J. Panopoulos. 1986. Pseudomonas syringae pathovars contain DNA sequences that are structurally and functionally homologous and control hypersensitivity and pathogenicity. (Abst.) In: 3rd Int. Sym. on "The Molecular Genetics of Plant-Microbe Interaction". McGill Univ., Montreal, Canada. 17-c.

Lindgren, P.B., C. Grimm, N.J. Panopoulos, B.J. Staskawicz, and D. Dahlbeck. 1988a. The genetic control of interactions between Pseudomonas and host plants. (Abst.) In: 5th Int. Congr. of Plant Pathology, Kyoto, Japan. StVII 4-1.

Lindgren P.B., N.J. Panopoulos, B.J. Staskawicz, and D. Dahlbeck. 1988b. Genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathovars of Pseudomonas syringae. Molec. Gen. Genet. 211:499-506.

Loper, J.E. and S.E. Lindow. 1987. Lack of evidence for in situ fluorescent pigment production by Pseudomonas syringae pv. syringae on bean leaf surfaces. Phytopathology 77:1449-1454.

Loper, J.E. and J.S. Buyer. 1991. Siderophores in microbial interactions on plant surfaces. MPMI. 4(1):5-13.

Lu, Y. and S.W. Hutchenson. 1989. Identification of a regulatory locus controlling the expression of hrp gene in Pseudomonas syringae pv. syringae. (Abst.) In: Proc. 1st CAASS Conf., Communications Service, Cornell Univ., Ithaca, N.Y., U.S.A. Section C35.

Mills, D. 1985. Transposon mutagenesis and its potential for studying virulence genes in plant pathogens. Ann. Rev. Phytopathol. 23:297-320.

Mills, D., F. Niepold, and M. Zuber. 1985. Cloned sequence controlling colony morphology and pathogenesis of Pseudomonas syringae. In: Sussex, I., A. Ellingboe, M. Crouch, and R. Malmberg (eds.) Plant Cell/Cell Interactions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp. 97-102.

Mills, D., F. Niepold, and G. Bertoni. 1987. Molecular analysis of pathogenicity of Pseudomonas syringae pathovars phaseolicola and syringae. In: Civerolo, E.L., A. Collmer, R.E. Davis, and A.G. Gillaspie (eds.) Plant Pathogenic Bacteria: Proc. 6th Int. Conf. on Plant Pathogenic Bacteria, Maryland. Martinus Nijhoff Publishers. pp. 404-411.

Mills, D. and F. Niepold. 1987. Molecular analysis of pathogenesis of Pseudomonas syringae pv. syringae. In: Nishimura, S., C.P. Vance, and N. Doke (eds) Molecular Determinant of Plant Disease. Japan Sci. Soc. Press, Tokyo/Springer-Verlag, Berlin. pp. 185-200.

Mills, D., P. Mukhopadhyay, Y. Zhao, and M. Romantschuk. 1988. Analysis of virulence in Pseudomonas syringae pathovars. (Abst.) In: 5th Int. Congr. of Plant Pathol, Kyoto, Japan. St.II. 4-4.

Mills, D., P. Mukhopadhyay, Y. Zhao, and M. Romantschuk 1991. Organization and function of pathogenicity genes of Pseudomonas syringae pathovars phaseolicola and syringae. In: S.S. Patil, D. Mills, and C. Vance (eds.): Molecular Strategies of Pathogens and Host Plants. Springer-Verlag Publisher, New York, Berlin, Heidelberg, London, Paris, Tokyo, Hong Kong, Barcelona. p69-82.

Morgan, M.K. and A.K. Chatterjee. 1985. Isolation and characterization of Tn5 insertion mutants of Pseudomonas syringae pv. syringae altered in the production of the peptide phytotoxin syringotoxin. J. Bacteriol. 164:14-18.

Morgan, M.K. and A.K. Chatterjee. 1988. Molecular genetics of syringotoxin production. In: Proc. 3rd Int. Working Group on Pseudomonas syringae pathovars, Lisbon.

Mukhopadhyay, P., M. Mukhopadhyay, J. Williams, Y. Zhao, and D. Mills. 1988. Pathogenicity genes of Pseudomonas syringae. In: Palacios, R. and D.P.S. Verma (eds.)

Molecular Genetics of Plant-Microbe Interactions. pp. 247-252.

Niepold, F., D. Anderson, and D. Mills. 1985. Cloning determinants of pathogenesis from Pseudomonas syringae pathovar syringae. Proc. Natl. Acad. Sci. USA 82:406-410.

Osborn, M.J. and H.C.P. Wu. 1980. Proteins of the outer membrane of gram-negative bacteria. Ann. Rev. Microbiol. 34:369-422.

Panopoulos, N.J. and M.N. Schroth. 1974. Role of flagellar motility in the invasion of bean leaves by Pseudomonas phaseolicola. Phytopathology 64:1389-1397.

Panopoulos, N.J. and R.C. Peet. 1985. The molecular genetics of plant pathogenic bacteria and their plasmids. Ann. Rev. Phytopathol. 23:381-419.

Paynter, V.A. and R. Alconero. 1979. A specific fluorescent antibody for detection of syringomycin, a toxin produced by Pseudomonas syringae in infected peach tree tissues. Phytopathology 69:493-496.

Persmark, M., D. Expert and J.B. Neilands. 1989. Isolation, characterization and synthesis of chrysobactin, a compound with siderophore activity for Erwinia chrysanthemi. J. Biol. Chem. 264:3187-3193.

Romantschuk, M. and D.H. Bamford. 1986. The causal agent of halo blight in bean, Pseudomonas syringae pv. phaseolicola, attaches to stomata via its pili. Microbial Pathogen. 1:139-148.

Romantschuk, M. 1987. Early steps in bacteriophage p6 infection: function of pili, membrane fusion, and nucleocapsid penetration. Ph.D Thesis. U. of Helsinki, Finland. pp. 59-61.

Sands, D.C., M.N. Schroth, and D.C. Hildebrand. 1980. Pseudomonas. In: Schaad, N.W. (ed.) Laboratory Guide for Identification of Plant Pathogenic Bacteria. American Phytopathological Society, St. Paul, MN. pp. 36-44.

Smidt, M.L. and A.K. Vidaver. 1982. Bacteriocin production by Pseudomonas syringae PsW-1 in plant tissue. Can. J. Microbiol. 24:600-604.

Smidt, M.L. and A.K. Vidaver. 1986. Isolation and characterization of syringacin W-1, a bacteriocin

produced by Pseudomonas syringae pv. syringae. Can. J. Microbiol. 32:231-236.

Stapp, C. 1961. Bacterial Plant Pathogens. London:Oxford University Press.

Staskawicz, B.J., D. Dahlbeck, and N.T. Keen. 1984. Cloned avirulence gene of Pseudomonas syringae pv. glycinea determines race-specific incompatibility on Glycine max (L.) Merr. Proc. Natl. Acad. Sci. USA 81:6024-6028.

Surico, G. and J.E. De-Vay. 1982. Effect of syringomycin and syringotoxin produced by Pseudomonas syringae pv. syringae on structure and function of mitochondria isolated from holcus spot resistant and susceptible maize lines. Physiol. Plant Pathol. 21:39-53.

Willis, D.K., E.M. Hrabak, J.J. Rich, T.M. Barta, S.E. Lindow, and N.J. Panopoulos. 1990. Isolation and characterization of a Pseudomonas syringae pv. syringae mutant deficient in lesion formation on bean. MPMI. 3:149-156.

Wills, D.K., J.J. Rich and E.M. Hrabak. 1991. hrp genes of phytopathogenic bacteria. MPMI. 4(2):132-138.

Vidaver, A.K. and S. Buckner. 1978. Typing of fluorescent phytopathogenic pseudomonads by bacteriocin production. Can. J. Microbiol. 24:14-18.

Vidaver, A.K. 1976. Prospects for control of phytopathogenic bacteria by bacteriophages and bacteriocins. Ann. Rev. Phytopathol. 14:451-465.

Xu, G.W. and D. Gross. 1988. Evaluation of the role of syringomycin in plant pathogenesis by using Tn5 mutants of Pseudomonas syringae pv. syringae defective in syringomycin production. Appl. Envir. Microbiol. 54:1345-1353.

Zhang, L. and J.Y. Takemoto. 1987. Effects of Pseudomonas syringae phytotoxin, syringomycin, on plasma membrane functions of Rhodotorula pilimanae. Phytopathology 77:297-303.

Zhao, Y. and D. Mills. 1989. Distribution of Pathogenicity-related DNA sequences from Pseudomonas syringae pv. syringae R32 among phytopathogenic bacteria. (Abst.) In: Proc. 1st CAASS Conf., Communications Service, Cornell Univ., Ithaca, N.Y., U.S.A. Section C36.

PHYSIOLOGICAL AND PATHOGENIC PROPERTIES OF THE PATHOGENICITY-ATTENUATED MUTANT OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE*

CHAPTER TWO

INTRODUCTION

Pathovars of *Pseudomonas syringae* bring about diseases that may result in severe yield losses among a broad range of economically important plant species (Gross *et al.*, 1984; Hoitink *et al.*, 1968 and Lindemann *et al.*, 1984). Brown spot disease of the common bean, *Phaseolus vulgaris*, caused by an ecotype of *Pseudomonas syringae* pv. *syringae*, was first reported in 1964 in Wisconsin (Patel *et al.*, 1964). It has been subsequently found in various areas of the United States.

The identification of pathogenicity-related genes using transposon tagging has been successfully applied to a variety of prokaryotic phytopathogens (Turner *et al.*, 1984; Anderson and Mills, 1985; Morgan

and Chatterjee, 1985). Suicide vectors, vectors that cannot replicate in the recipient bacterial strain, have been used to deliver transposable elements into a bacterium and, subsequently, to allow for selection of transposition. This technique has broadened the horizon for studying host-pathogen interactions and

streptomycin-resistant strain that otherwise is isogenic to the wild type strain, R32 (Anderson and Mills, 1985). Symptomology, growth, motility and total cellular protein profiles of the pathogenic strain R32, and the mutant strain, PS9024, were examined both *in vitro* and *in planta*. The distribution of DNA sequences that have homology with the Tn*5*-containing *Eco*RI DNA fragment was also examined in a variety of other phytopathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study, with the exception of strains used in the distribution of sequences homologous to the mutant locus of PS9024, are summarized in Table 2-1.

A spontaneous streptomycin-resistant strain, PS9020, which is otherwise isogenic to the original wild type strain, R32, was used in the Tn*5* mutagenesis experiment that allowed for recovery of Path$^-$ mutants, including PS9024. This strain has been described in studies by Anderson and Mills (1985) and Niepold and Mills (1987). Although no apparent growth deficiency

Table 2-1. Bacterial Strains and Plamids.

Strains	Characters	Source or References
***P. s.* pv. *syringae*:**		
R32	wild type, pathogenic	K. Rudolph
PS9020	wild type, Sm^r, pathogenic isogenic to R32	Anderson and Mills (1985)
PS9024	Tn*5*-induced mutant of PS9020, pathogenicity-attenuated, Sm^r, Km^r,	Anderson and Mills (1985)
PS9022	Tn*5*-induced mutant of PS9020, pathogenicity-attenuated Sm^r, Km^r,	Anderson and Mills (1985)
Plasmids:		
pOSU9024	Tn*5*::*Eco*RI fragment from PS9024	Mills and Niepold (1987) and this study

Sm = Streptomycin; Km = Kanamycin; ^{r} = resistant

was found *in planta* relative to the original wild type strain R32, PS9020 produces slightly reduced symptoms on the susceptible bean plants, which is not consistent with previous descriptions of the symptomology of PS9020, (Anderson and Mills, 1985). Hence, all experiments in this study were conducted using the wild type strain, R32, instead of PS9020, with the exception of the *in planta* growth test described in this chapter.

Media

The strains of *P. s.* pv. *syringae* were grown in LB medium (Maniatis *et al.*, 1982). MAS minimal medium and complete nutrient medium, MaNY, (Szabo and Mills, 1984) were used for growth kinetics studies of *P. s.* pv. *syringae* in culture. King's medium B, KB, (King *et al.*, 1954) was used to test for the diagnostic fluorescence property of *P. s.* pv. *syringae*.

Pseudomonads were grown at 28°C and *E. coli* strains were grown at 37°C, either in shaking broth culture or on 1.5% agar plates. When appropriate, media were supplemented with antibiotics from Sigma Chemical Company (St. Louis, MO) to the following final concentrations: Streptomycin (Sm), 50 μg/ml; Kanamycin (Km), 50 μg/ml; Ampicillin (Amp), 100 μg/ml and Tetracycline (Tc), 12.5 μg/ml.

Plant leaf inoculation and determination of bacterial growth

Methods for inoculating bean leaves and for determining bacterial population dynamics have been described by Bertoni and Mills (1987).

The doubling time (generation time) of bacterial growth was calculated according to equation described by Jawetz et al. (1982) as follows:

$$g = \frac{\log N - \log No}{\log 2}$$

$$\text{doubling time} = g/t$$

Where g = generation time; No is the number of cells at time zero and N is the number of cells at any later time, t.

Bean pod inoculation

Phaseolus vulgaris cv. "Eagle" was used in the bean pod bioassay. Prior to inoculation, the bean pods that had developed approximately two weeks (ca. 10-12 cm in length) were pierced with a sterile toothpick and approximately 50 µl of the bacterial suspension in K-buffer (pH 7.0) were injected into the pod tissue through the wound using a 1 ml needless syringe. The inoculated pods were then grown in a growth chamber at

28°C with 12 hours light/12 hours dark cycle. Development of disease symptoms was observed throughout the two week test period and scored one week after inoculation.

It should be noted, however, that the necrotic and shrunken lesions incited by the wild type strain of R32 in this pod bioassay are not typical field symptoms of brown spot disease of bean. However, this assay was judged to be valid in assessing pathogenicity since the difference in symptomology between the mutant strain PS9024, and R32 are completely correlated with the bean leaf bioassay.

Growth of bacteria in culture media

A 3 ml aliquot of minimal MAS broth and complete MaNY broth, plus appropriate antibiotics, in a 15 ml Falcon tube was inoculated with a single colony of each strain (PS9024 and R32) and grown overnight or for two days in MAS to stationary phase medium in a shaking incubator at 28°C, with 275 RPM. Twice the amount of PS9024 cells were normally inoculated into the MaNY broth to ensure that both cultures would reach stationary phase at approximately the same time. An aliquot of the stationary culture (OD600 = 1.0-1.2) was used to inoculate a second tube containing the same broth. Cultures were allowed to grow to mid-log phase, which produces an optical density of OD600 = 0.2 to 0.3,

and represents a cell density of approximately 10^8 CFU/ml. Both cultures were then adjusted to the same optical densities by direct spectrophotometric measurement, and were further diluted into 250 ml flasks to the cell density of 10^3 CFU/ml for the growth test. The cell density during the growth test was precisely measured by diluting and directly counting the cells plated onto an agar plate. The number of colony-forming units (CFU) per ml at various time intervals was determined using the micro-plating method described by Keen et al., (1981) and modified by Bertoni and Mills (1987).

In vitro cell motility analysis

To test for cell motility, normal concentrations of minimal MAS and complete nutrient MaNY media containing 1.5% agar were diluted twenty-fold with distilled water, autoclaved and poured into petri dishes. A 3 ml aliquot of mid-log phase culture of bacteria was suspended in the K-buffer (pH 7.0) and 3 μl of the bacteria suspension was spotted at the center of the petri dish and incubated at room temperature for one to two days. Undiluted MAS medium with twenty-fold diluted agar was also used in these tests.

DNA extraction and manipulation

Total DNA was isolated from strains of Pseudomonas by the procedure of Szabo and Mills (1984). Plasmid DNA

was isolated from E. coli following a modified rapid mini-scale preparation procedure (Zhou et al., 1990).

Restriction endonucleases were purchased from either Bethesda Research Laboratories, Inc. (Gaithersburg, MD), or New England Biolabs, Inc. (Beverly, MA), and were used according to the recommendations of the suppliers.

Southern transfer of DNA and hybridizations using Genetran filters (Plasco Inc. Catalog# 4525 RG) were carried out using the following procedure. DNA in the 0.7% agarose gel was denatured with a solution containing 1.5 M NaCl and 0.5 M NaOH and neutralized with 0.5 M Tris-HCl, pH 7.5 containing 3 M NaCl. A solution of 6 x SSC (1 x SSC is 0.5 M NaCl, 0.05 M Sodium citrate, pH 7.0) was used for Southern transfer of DNA (Southern, 1975) from agarose gels to the filters. DNA on the filter was exposed to 320 nm of UV light for 5 minutes and then baked at 80°C for 2 hours. Hybridizations were carried out at 42°C following the method of Maniatis et al., (1982) using 50% formamide. Filter washes were performed at 60°C using 0.1% SDS and 0.1% SSC with active agitation.

Preparation of total cellular protein samples and SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

Total cellular protein was extracted from Pseudomonas using the procedure described by Klement et al., (1990) for Gram-negative bacteria. SDS-PAGE was performed with 0.1% SDS according to Laemmli (1970). A stacking gel with 5% acrylamide was layered on top of a 16% acrylamide gel in a Hoeffer SE600 slab gel apparatus to reveal proteins in the size range of 15 to 200 kD. Ten to 30 μg of total protein was applied to each sample well. A range of either low or high molecular weight protein standards (Bio-Rad Laboratories, Richmond, CA) were run in each slab gel. Electrophoresis was initiated with a 5 mA current until the tracking dye migrated beyond the stacking gel; the current then was increased to a constant 10 mA for approximately 24 hours. The gels were then fixed for 60 minutes in 30% methanol with 7% acetic acid and stained with 1% Coomassie Brilliant Blue G overnight. Destaining was performed in fixing solution with gentle agitation until the best visible resolution was achieved.

RESULTS

Symptomology of PS9024

The Tn5 transposon-tagged, Path$^-$ mutant strain, PS9024 was obtained and subsequently partially characterized by Anderson and Mills (1985). The mutation in PS9024 appears to occur within a disease specific gene locus (dsg locus; Lindgren and Panopoulos, 1986) since it affects only the ability of the pathogen to cause disease symptoms in the host bean plant, Phaseolus vulgaris L. PS9024 retains the ability to induce the hypersensitive reaction (HR) in the non-host tobacco plant (Mills and Mukhopadhyay, 1990; this study). This Tn5-derived mutantion appears to decrease the aggressiveness of the pathogen, which has become attenuated for symptom development in susceptible host bean plants. Approximately 5 x 10^7 CFU per ml injected into the bean leaf are required to produce disease symptoms in the host plant, and the symptoms appear less severe than those produced by 5 x 10^5 CFU/ml of the parental wild type strain (Anderson and Mills, 1985). The attenuation of PS9024 is shown in Figure 2-1.

Inoculation of PS9024 and R32 into bean pods of Phaseolus vulgaris cv. "Eagle" showed that a similar level of inoculum was required for the development of

Figure 2-1. Bean leaf bioassay for pathogenesis of P. s. pv. syringae. Representative symptoms of wild type R32 and the Tn5-induced mutant PS9024 on a primary bean leaf of Phaseolus vulgaris cv. "Eagle". Inoculum concentration of R32 ranges from 10^8 to 10^6 CFU/ml from the top of the leaf to the bottom; 10^7 to 10^5 CFU/ml of PS9024 was infiltrated into the leaf tissue in the same order. The leaf was photographed 7 days after inoculation.

disease symptoms. The bean pods inoculated with R32 at the level of approximately 10^6 CFU/ml showed a severe necrosis at the injection site (Figure 2-2) and appeared shrunken. Although chlorosis of the pods was observed when PS9024 was inoculated at 10^6 CFU/ml, no necrotic, shrunken symptoms were observed even when the bacterial inoculum density was as high as 10^8 CFU/ml (data not shown).

A summary of host reactions (disease symptom) and non-host reactions (HR) to R32 and PS9024 is presented in Table 2-2. All cultivars of Phaseolus vulgaris tested produced disease symptoms when inoculated with 10^6 CFU/ml of the parental strain, whereas no symptoms were induced by PS9024. The non-host plants, tobacco (Nicotina tabacum) and Chinese cabbage (Brassica sp.), produced a hypersensitive reaction (HR) when inoculated with either R32 or PS9024.

The mutant locus

The Tn5-containing EcoRI fragment (ca. 10.5 kb) from the mutant strain, PS9024, was cloned into pBR322 using a shot-gun cloning method and the resulting plasmid was designated pOSU9024 (Mills and Niepold, 1987). The preliminary restriction map of this fragment (Mills and Niepold, 1987) has been expanded and corrected in this study (Figure 2-3). An additional

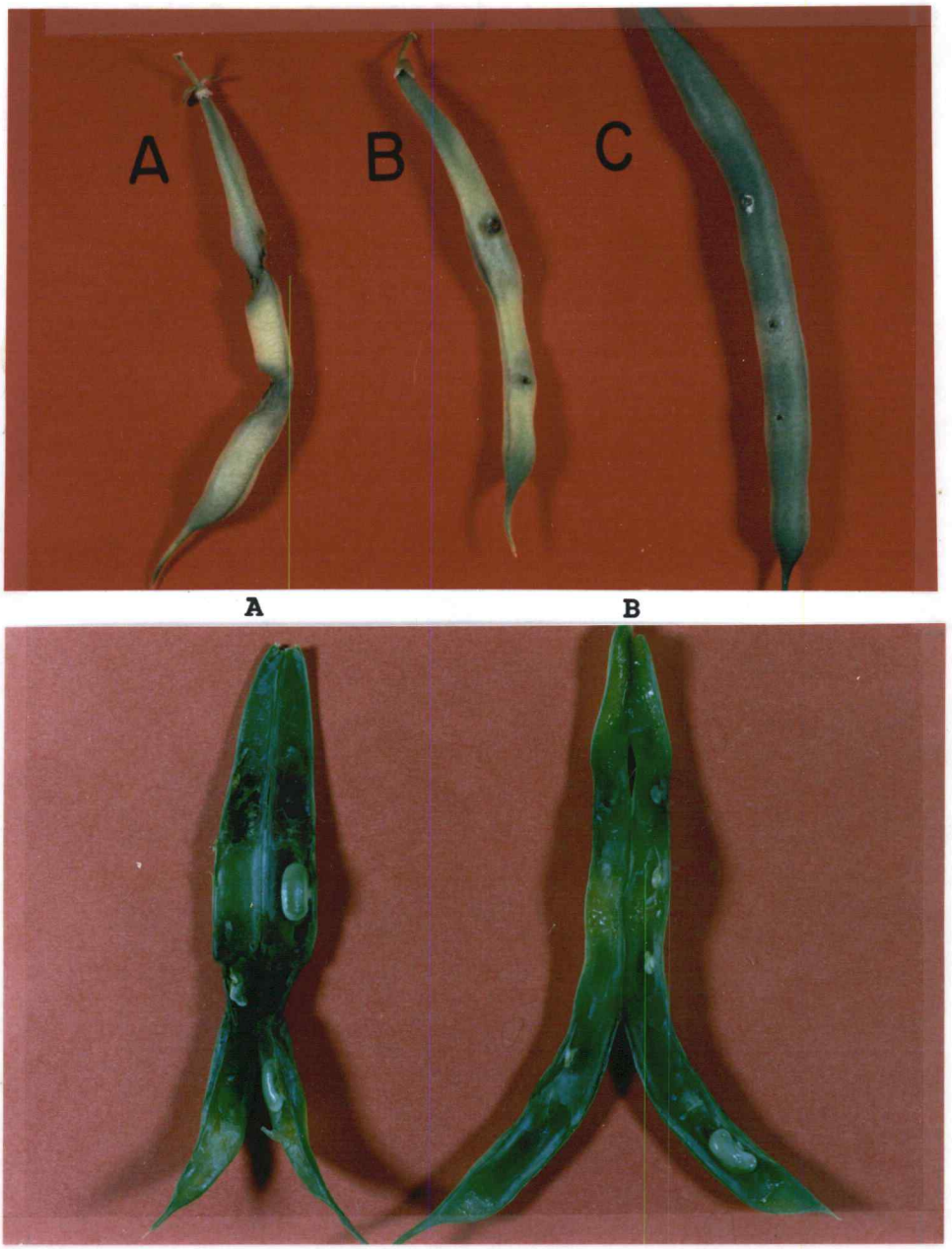


Figure 2-2. Bean pod bioassay for pathogenesis of P. s. pv. syringae. Representative symptoms on bean pods produced by the wild type strain, R32, and Tn5-induced mutant, PS9024 on a bean pod of Phaseolus vulgaris cv. "Eagle". Inoculum concentration of 10^6 CFU/ml was used in this experiment. **A** and **B**, symptoms induced by strain R32 and PS9024, respectively; **C**, bean pod infiltrated with K-buffer (pH 7.0). The top panel represents the outside view of the inoculated pods; whereas the bottom panel is the inner view of the inoculated pods. The photograph was taken 7 days after inoculation.

Table 2-2. Summary of host and non-host reactions to strains R32 and PS9024 of *Pseudomonas syringae* pv. *syringae*.

	Source	Host plant Reactions R32	PS9024
Phaseolus vulgaris cv.:			
Eagle (Snap bean)	Asgrow Seed Co.	+	-
Red Mexican UI-36	Unknown	+	-
Charlevoix (Dark red kidney)	Michigan St. Univ.	+	-
P1 150414	Nebraska plant selection	+	-
BBL290	same as above	+	-
NEBR1 #1, sel. 27	same as above	+	-
Broker's choice	Asgrow Seed Co.	+	-
Non-host Plants			
Nicotina tabacum cv.: (Tobacco)			
Samsun NN	Oregon St. Univ.	HR	HR
Brassica sp. cv.: (Chinese cabbage)			
Roundup	Cornell University	HR	HR

Designation: +, disease symptom appeared; -, no disease symptoms observed when inoculated under 10^6 cell CFU/ml; HR, hypersensitive reaction.

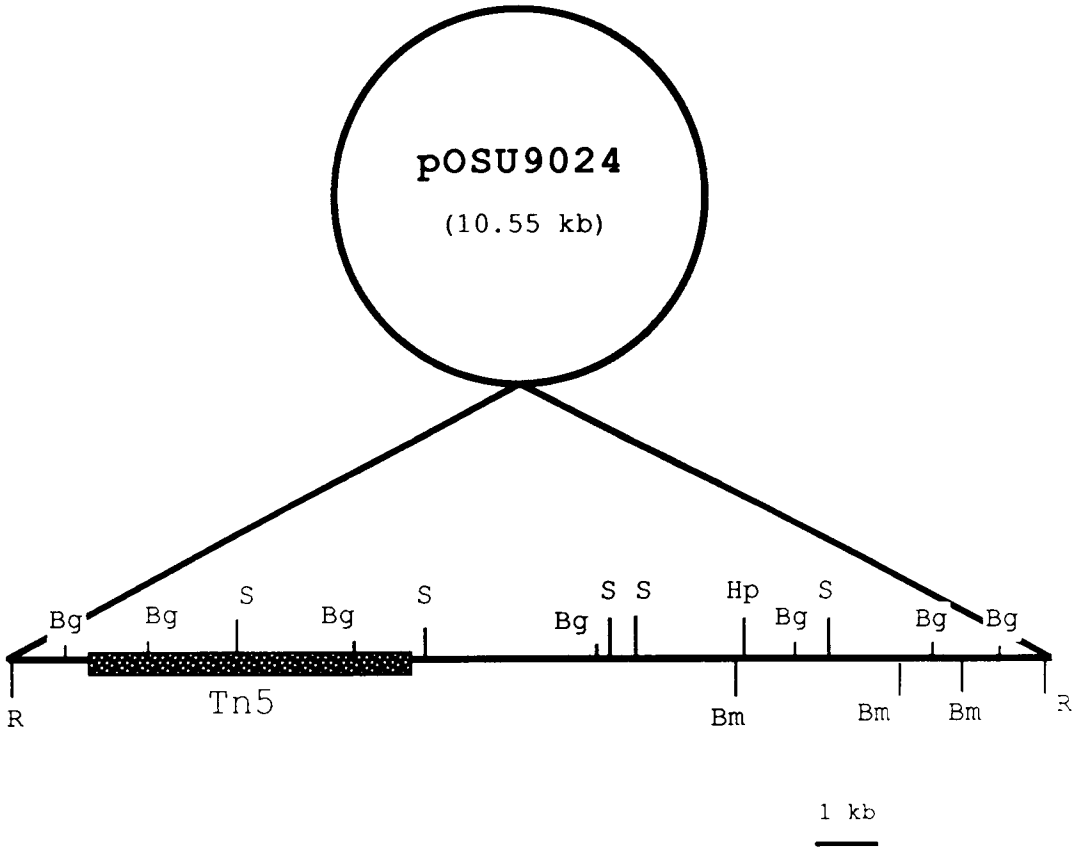


Figure 2-3. Restriction map of pOSU9024. The Tn5-containing EcoRI fragment from PS9024 was cloned from an EcoRI digest into EcoRI site of pBR322 and the resulting plasmid was designated pOSU9024. The thick bar represents the sequence of Tn5. Abbreviations of restriction enzymes: Bm, BamHI; Bg, BglII; Hp, HpaI; R, EcoRI and S, SalI.

BamHI site has been identified within the 3.0 kb SalI/EcoRI fragment at the right end. An EcoRI restriction site was thought to be present near the left end in the original construct. Subsequent Southern blot analysis in this study using a probe made from a fragment that spans the left EcoRI site failed to detect a small (ca. 300 bp in size) EcoRI fragment (data not shown).

To test whether the mutant phenotype of PS9024 was caused by a single Tn5 insertion in the genome, a ^{32}P-labeled DNA probe was made from the EcoRI::Tn5 fragment of pOSU9024 and hybridized with the EcoRI-digested total genomic DNA from R32 and PS9024 (Figure 2-4A). The results of these hybridizations indicated that the parental strain R32, contains two EcoRI fragments with homology to the probe. One corresponds in size to a fragment predicted from the physical map of the insert in pOSU9024 (ca. 10.5 kb excluding Tn5) and another, which weakly hybridizes with the probe is approximately 8.5 kb in size. Subsequent Southern blot hybridization with a DNA probe made from a different Tn5-containing EcoRI fragment from strain PS9022, which hybridizes to an 8.1 kb fragment confirmed that Tn5 was inserted into the 10.5 kb EcoRI fragment and ruled out the possibility that the 8.5 kb EcoRI fragment has any homology with Tn5 (Figure 2-4B).

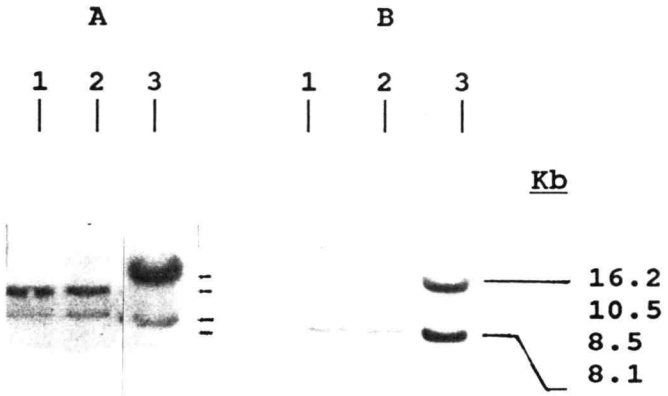


Figure 2-4. Southern hybridization analysis of total EcoRI-digested genomic DNA. Lane 1, R32; Lane 2, PS9020; Lane 3, PS9024. **A**, the ^{32}P labelled probe was made from the Tn5-containing EcoRI insert of pOSU9024. **B**, the probe was made from a Tn5-containing EcoRI fragment from strain PS9022. The size of the Tn5-containing EcoRI fragment in pOSU9024 is 16.2 kb; The EcoRI fragments homologous to the insert of pOSU9024 in R32 and PS9020 are 10.5 kb and 8.5 kb, respectively. The size of the wild type EcoRI fragment from PS9022 is 8.1 kb.

Growth kinetics

In planta growth kinetics

Preliminary *in planta* growth studies of PS9024 and the wild type strain, PS9020, were described by Bertoni and Mills (1987). While these data indicated that PS9024 and PS9020 had very similar growth kinetics during the first three days after inoculation, the mutant appeared to decline in both growth and viability for several days thereafter. Because PS9024 will produce symptoms if the inoculum density is sufficiently high, it was of interest to determine whether PS9024 could recover and produce disease symptoms over a longer period of time. Therefore, the growth kinetics of PS9020 and PS9024 were repeated and measured over a two week period.

During the first three days after inoculation, the population size of PS9024 increased approximately three logs *in planta* (Figure 2-5). The doubling time for PS9024 during the first three days was 6.28 hours per generation with a standard deviation of 1.27 hours, whereas the doubling time for PS9020 was 4.67 hours per generation with a standard deviation of 0.43 hour. At day three, PS9020 reached a maximum cell density of approximately 10^7 CFU/leaf disk sample, a density approximately 100-fold greater than that attained by the

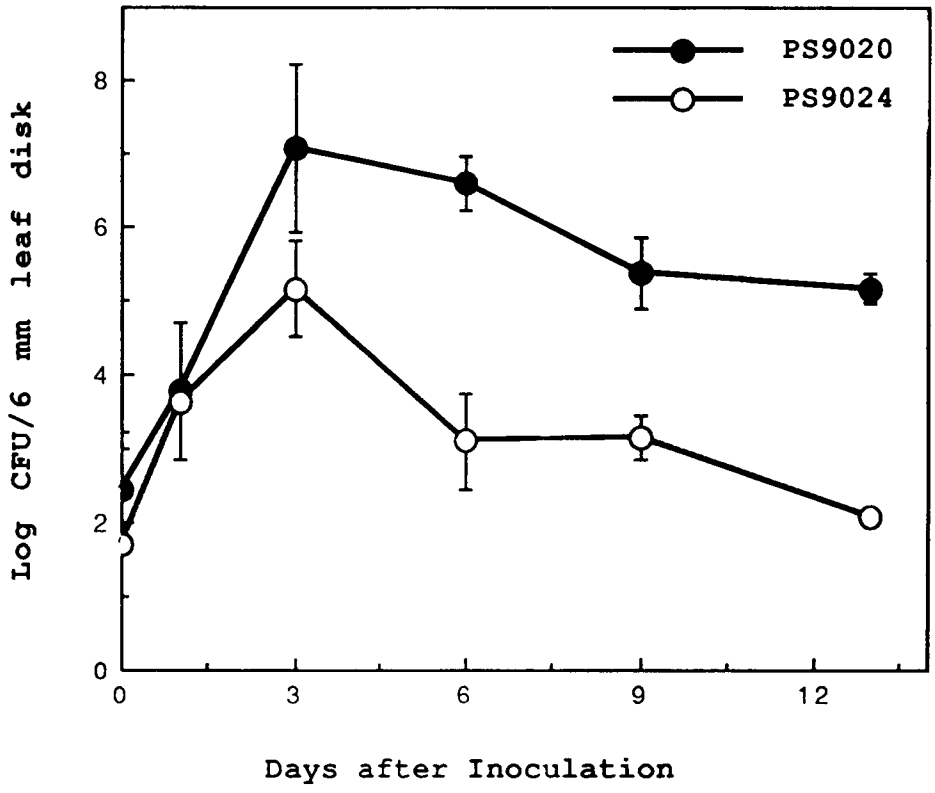


Figure 2-5. In planta growth kinetics of P. s. pv. syringae in a susceptible bean plant, Phaseolus vulgaris cv. "Eagle". Cells were harvested from mid-log phase culture and adjusted to equal optical density. 10^3 CFU/ml bacterial cells were infiltrated into the bean leaves according the procedure of Bertoni and Mills (1987). Each point represents an average of the CFU/ml from four 5 μl droplets in triplicate experiments.

mutant strain PS9024. After day three, PS9024 showed a rapid decline in population size, and by 14 days post-inoculation, the viable population of PS9024 declined to approximately 100 CFU/leaf disk. The population size of PS9020 also declined from days 3 onward but at 14 days post-inoculation, the population size was at least 10^5CFU/leaf disk, or a cell density approximately three logs higher than the initial inoculum and the final density of PS9024.

Growth kinetics in culture

The extent and kinetics of growth of PS9024 and of the parental strain R32 in minimal medium (MAS) appear to be similar (Figure 2-6A). R32 and PS9024 had similar doubling time of 5.76 hours, and 4.31 hours, respectively, and the two strains reached a maximum cell density of 10^8 CFU/ml within 52 hours. In contrast, however, a prolonged lag phase was observed for PS9024 in the complete nutrient MaNY medium as compared with R32 (Figure 2-6B). Consequently, R32 reaches its maximum density within 24 hours, whereas 48 hours were required for PS9024.

Bacterial motility

Bacteria can monitor their surroundings and respond to ambient change, which provides a mechanism whereby

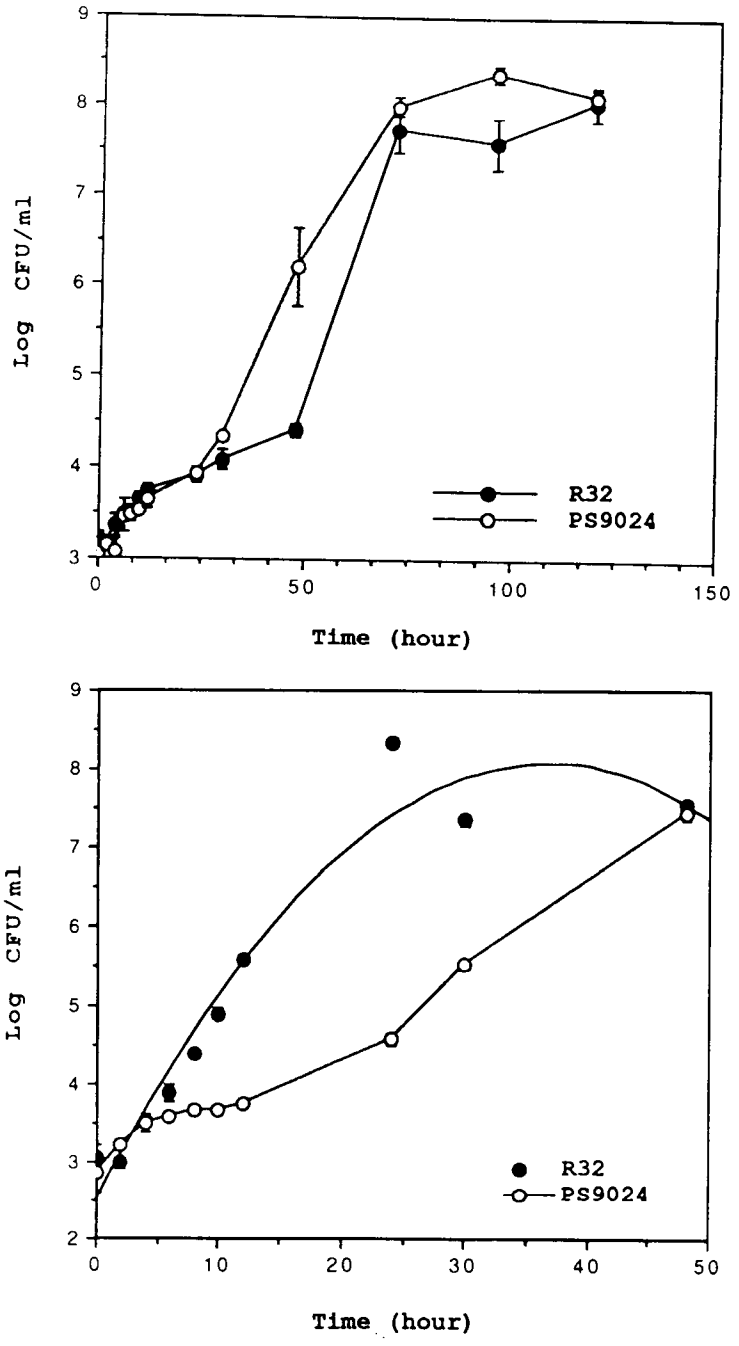


Figure 2-6. Growth kinetics of R32 and PS9024 in culture medium. Cells were harvested from mid-log phase culture and adjusted to an equal optical density. The culture was further diluted to approximately 10^3 CFU/ml for the growth test. Each sample point represents an average of the CFU/ml from four 5 μl droplets in triplicate experiments. **A.** Cells were pregrown and tested in minimal MAS medium; **B.** Cells were pregrown and tested in complete nutrient MaNY medium. Data from the experiment with R32 were fit to a 2 order regression curve.

they actively seek out favorable environments and avoid unfavorable ones (Parkinson, 1990). Reduced bacterial growth under poor nutrient conditions may be avoided by active migration of bacteria to a new environment. Nutrient limitation can provide a rapid *in vitro* test for potential mutations in genes essential for cell growth or motility. The inability of PS9024 to sustain growth *in planta* suggested that this strain could have a mutation within a gene controlling motility. Therefore, experiments were performed to monitor the motility of PS9024. The motility of PS9024 and R32 were measured using 20-fold diluted MaNY medium and undiluted MAS medium with twenty-fold diluted agar (Figure 2-7A-B). In comparison to R32, PS9024 showed a much reduced rate of migration on MaNY medium, although it eventually occupies the entire plate if incubated a few additional days. However, PS9024 and R32 migrate at nearly identical rates on MAS medium which contains twenty-fold diluted agar. In contrast, neither R32 nor PS9024 will grow nor migrate on 20-fold diluted MAS medium, apparently because of the low concentration of nutrients.

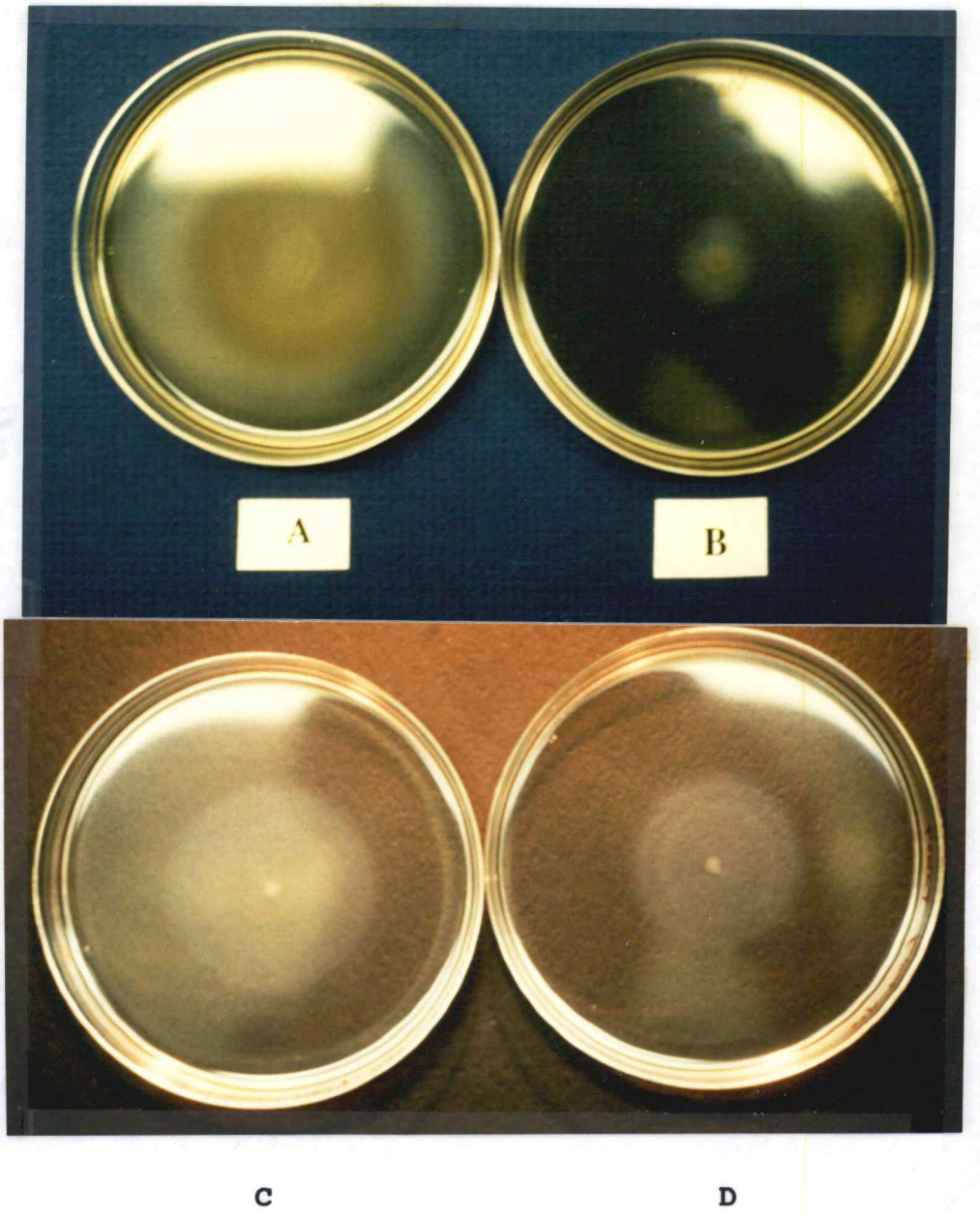


Figure 2-7. Bacterial motility test of R32 and PS9024. **A** and **B** are R32 and PS9024 grown on 20-fold diluted MaNY medium; **C** and **D** are R32 and PS9024 grown on on 20-fold diluted agar of undiluted MAS medium. Photographs were taken 2 days after inoculation of the plates.

Comparison of total cellular protein profiles of PS9024 and R32

Total cellular protein profiles have been used to identify pathovars of both Pseudomonas and Xanthomonas species (Dristig and Dianese, 1990; Minsavage and Schaad, 1983) and to detect mutational changes (Ielpi et al., 1990). The SDS-PAGE method was used in this study to determine whether the Tn5 insertion in PS9024 interrupted any gene whose product was absent from cell lysates of PS9024 but present in lysates of R32. A protein band, which migrated slower than β-galactosidase of E. coli (116.25 kD) but faster than myosin of rabbit skeletal muscle (200 kD), was detected by Coomassie Brilliant Blue staining in the lysates of strain R32 but not from PS9024 (Figure 2-8). Two new bands observed in protein extracts of PS9024, ca. 55 kD and 65 kD in size, correspond to the size of the transposase and neomycin phosphotransferase II gene products coded by transposon Tn5. All other protein bands appeared to be unchanged in PS9024.

DNA sequence distribution of the Tn5-containing insert of pOSU9024 among other phytopathogenic bacteria

The distribution of the presumptive pathogenicity-related Tn5-containing EcoRI fragment from PS9024 was

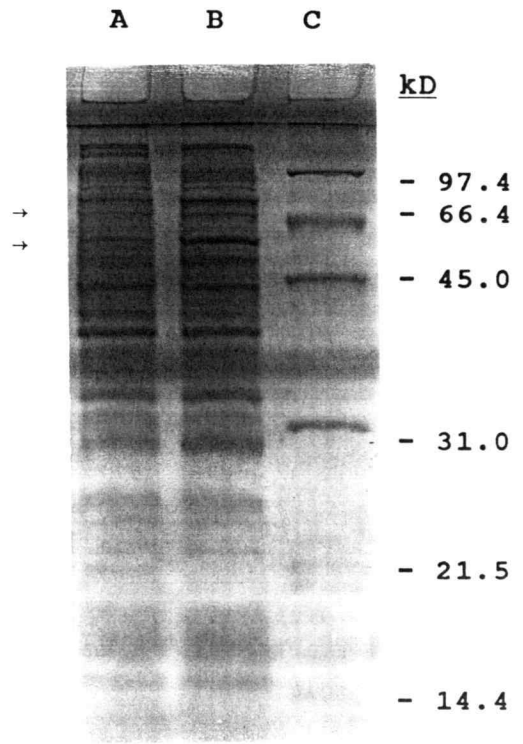


Figure 2-8. Comparison of total cellular protein profiles of R32 and PS9024 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane A, wild type strain R32. Lane B, mutant strain PS9024. Lane C, protein size markers (Bio-Rad Laboratories, Richmond, CA). Open arrow indicates the 150 kD protein band and the solid arrows from top to bottom indicate the possible 65 kD and 55 kD proteins of the neomycin transferase II and transposase of Tn5, respectively.

examined with respect to a variety of other phytopathogenic bacteria. Radiolabeled probe made from pOSU9024 was hybridized with Southern-blotted genomic DNA derived from strains of various genera, including Pseudomonas, Xanthomonas, Erwinia, Agrobacterium and the basidiomycete fungus, Tilletia spp. The results (Table 2-3) indicated that sequence homology was limited to certain strains of P. syringae, and was not detected in strains of Xanthomonas, Erwinia and Agrobacterium. Surprisingly, the DNA probe showed either very weak or no homology with some strains of P. syringae that cause brown spot disease. In a more limited study, similar results were obtained by Mills and Niepold (1987).

DISCUSSION

Transposon mutagenesis has proven to be a very powerful method for identifying pathogenicity-related genes in a variety of phytopathogens (Anderson and Mills, 1985; Turner et al., 1984; Morgan and Chatterjee, 1985; Peet et al., 1986). Frequently, the challenge remains to identify the function of the gene of interest in specific host-pathogen interactions. Unfortunately, the functions of most pathogenicity-related genes cloned

Table 2-3. Distribution of Sequences homologous to the Tn*5*-containing *Eco*RI fragment from PS9024 among Phytopathogenic Bacteria and Fungi

Strains		Host	Homology to pOSU9024
***Pseudomonas syringae* pv.**			
syringae	R32	Bean	+
syringae	J900	Bean	-
syringae	Y30	Bean	+/-
syringae	11/81	Bean	+
syringae	J634	Bean	+
syringae	84-43	Bean	+/-
syringae		Wheat	+
phaseolicola[a]		Bean	-
glycinea		Soybean	+/-
adzakicola		Adzaki Bean	-
tomato	8	Tomato	-
tomato	5796-36	Tomato	+/-
pisi		Peas	-
tabaci		Tobacco	+
coronafaciens		Oats	+/-
striafaciens		Oats	+/-
philadelphii B		Mock Orange	+
philadelphii D		Mock Orange	-
morsprunorum	LR920	Stone Fruits	-
Pseudomonas			
marginalis	LR900	Lettuce	+
marginata	LR980	Gladiolus	-
***Xanthomonas campestris* pv.**			
translucens[b]		Cereals	-
Erwinia			
carotovora	501	Potato	-
atroseptica		Potato	-
Agrobacterium			
tumefaciens	B6	Woody Plants	-
***Tillietia*:**			
caries	417	Wheat	-
controversa	628D	Wheat	-

[a] Nine strains belonging to various races were examined.

[b] Eleven strains belonging to various races were examined.

"+" = homology; "-", no homology; '+/-' = barely detectable homology.

by this approach remain unknown. Studies of alterations to physiologic and pathogenic processes that occur in pathogenesis-deficient mutants may provide insight into the genetic defects that block or substantially reduce pathogenicity.

The ability of a pathogen to proliferate and colonize bean leaves, as measured by the minimum effective dose of P. s. pv. syringae in the field, has been used to predict the disease development in the bean plant (Lindemann et al., 1984). Disease severity was observed to correlate positively with an increase of the epiphytic population size. It is conceivable, therefore, that virulence of the pathogen can be increased by increasing the inoculum density on the susceptible host plant. It has been demonstrated previously (Anderson and Mills, 1985), as well as in this study (Figure 2-5), that the wild-type parental strain, PS9020, grows to a cell density that is at least 100-fold greater than PS9024 in the susceptible bean plants. The results of this study further demonstrate that PS9024 cannot recover over longer periods of time nor induce disease symptoms throughout a two-week period. Inoculum as low as 5×10^5 CFU/ml of the wild type strain induces symptoms within a week when injected into leaves of Phaseolus vulgaris, whereas the mutant strain PS9024 requires about 100-fold greater inoculum

densities to induce any disease symptoms and these symptoms were less pronounced. These results indicate that the mutation in PS9024 has reduced, but not totally abolished, its aggressiveness and ability to cause disease in susceptible host plants.

PS9024 and R32 also showed marked differences in the development of disease symptoms in bean pods. As with the bean leaf bioassay, approximately 10^6 CFU/ml when inoculated with R32 caused a severe, shrunken necrosis at the inoculation site probably due to the cellular death and collapse of the inner cellular matrix. However, neither shrunken nor necrotic symptoms were shown on the susceptible bean pods when PS9024 was inoculated at 10^6 CFU/ml (Figure 2-2). The bean pod bioassay has been used successfully to breed Phaseolus vulgaris for field resistance to brown spot (Daub and Hagedorn, 1979). The non-typical symptoms shown by a pathogenic strain, B728a of P. s. pv. syringae, which also causes brown spot disease of bean was recently reported by Willis et al. (1990). However, B728a and R32 do not produce similar atypical symptoms on susceptible bean pods. This variability may result from the variability in the genetic background of these two strains or the method of inoculating the pods. In the study conducted by Wills et al., (1990), bacterial

colonies were picked and stabbed under the epidermis of the pods. The inoculated pods were detached from the plant and incubated individually in Whirl-Pak bags (Nasco, Chelsea, MA) in a growth chamber at ca. 24°C. In this study, an actively growing bacteria cell suspension was used to inject into the intercellular matrix of the bean pod. The inoculated pods were retained on the plant which was incubated at ca. 28°C.

A dsg DNA region controlling aggressiveness of P. solanacearum, which causes a lethal wilt disease on more than 200 different plant species world-wide, was reported recently (Arlat and Boucher, 1991). Insertions by transposons Tn5lac or MuDIIlac in an approximately 15 kb region resulted in reduced aggressiveness (hypoaggressiveness) of a wild type strain, GMI1000, to cause disease symptoms on tomato plants. Although no growth alteration was observed in minimal MM and rich B media in five randomly chosen insertion mutants, reduced growth rates were detected in two of the deletion mutants, MD34 and MD82. Sensitivity to acidic pH in MM medium was observed in all of the Dsg$^-$ mutants.

Decreased growth and viability of the mutant strain, PS9024, beginning the third day after inoculation into bean leaves possibly could have resulted from nutrient starvation, although the leaf environment appeared to provide a suitable source of

nutrients for the wild type strain. The possibility that the rapid decline in growth and viability of PS9024 *in planta* was due to the inability of the mutant to migrate, thereby limiting the nutrient availability surrounding the immediate tissue that was investigated. PS9024 was shown to be as motile as the wild type strain in culture motility tests. In a previous study by Bertoni (1987), the population of bacteria located 3 mm to 6 mm outside the points of inoculation on a leaf surface were measured over a period of 7 days. The migration of PS9024 appeared not to be affected *in planta*, and after a delay, presumably to allow for migration of bacteria, the growth kinetics of PS9024 were similar to the pattern at the inoculation site. Consequently, the decline in growth and viability of PS9024 *in planta* is not the result of nutrient limitation in the plant leaves.

Alternative hypotheses are required to explain the inability of PS9024 to grow *in planta*. It is possible that a toxic product produced either by the host or by the mutated pathogen may accumulate and eventually result in the subsequent death of the bacterial cells. Such a toxic product might be a by-product of catabolism, or simply a nutrient factor. An extended lag in growth of the mutant in complete MaNY medium might be taken to suggest that nutrient factors are

involved in such toxic inhibition. This hypothesis, however, does not explain why bacterial death was not observed in culture. Instead, the mutant bacteria grow less vigorously than the wild type strain either in complete MaNY broth or on the 20-fold diluted MaNY plates. Alternatively, the growth pattern of PS9024 both *in planta* and in MaNY medium may be explained if the mutated gene is used only as an alternative mechanism *in planta* to take up or assimilate a certain nutrient. A different uptake or assimilation pathway could be involved in the complete nutrient medium.

A protein band of approximately 150 kD in size found in the wild type strain, R32, was absent from PS9024, presumably because Tn*5* inserted into the gene that encodes this protein. Evidence for this insertion comes from the lack of this protein band in the mutant and the presence of two new bands, corresponding in size to the transposase and neomycin phosphotransferase II of Tn*5* (Lewin, 1987).

Of particular interest was the demonstration that the Tn*5*::*Eco*RI fragment of PS9024 has no homology with serveral strains of *Pseudomonas syringae*, including strains that cause brown spot disease (Table 2-3). This feature has been more extensively characterized in subsequent studies, as described in Chapter Four.

SUMMARY

1. A DNA region interrupted by a single Tn*5* insertion in the wild-type chromosome of strain R32 affects the aggressiveness of the pathogen on its host plant, *Phaseolus vulgaris*. This prototrophic mutant strain is designated PS9024.

2. The Tn*5* insertion in PS9024 identified a disease specific locus (*dsg* locus): *i.e.*, it affects only the development of symptoms on the host plant but does not affect the non-host HR reaction.

3. PS9024 is motile but exhibits an altered pattern of growth both *in planta* and in complete nutrient MaNY medium.

4. SDS-PAGE analysis of total cellular proteins revealed a protein band (*ca*. ~150 kD) that is absent from PS9024, as compared with the wild-type strain R32.

5. The probe made from the Tn*5*-containing *Eco*RI fragment of PS9024 showed no homology with phytopathogenic bacteria other than the phytopathogenic pseudomonads. Surprisingly, the probe shares either little or no homology with some strains of *Pseudomonas syringae*, including some that cause brown spot disease.

REFERENCES

Arlat, M and C. Boucher. 1991. Identification of a dsp DNA region controlling aggressiveness of Pseudomonas solanacearum. MPMI. 4(2):211-213.

Anderson D.M. and D. Mills. 1985. The use of transposon mutagenesis in the isolation of nutritional and virulence mutants in two pathovars of Pseudomonas syringae. Phytopathology 75:104-108.

Bertoni, G. and D. Mills. 1987. A simple method to monitor growth of bacteria populations in leaf tissue. Phytopathology. 77:832-835.

Bertoni, G. 1987. M.S. Thesis, Oregon State University

Daniels, M.J., J.M. Dow and A.E. Osbourn. 1988. Molecular genetics of pathogenicity in phytopathogenic bacteria. Ann. Rev. Phytopathol. 26:285-312.

Daub, M.E. and D. Hagedorn. 1979. Resistance of Phaseolus line WBR 133 to Pseudomonas syringae. Phytopathology. 69:946-951.

Dristig, M.C.G. and J.C. Dianese. 1990. Characterization of Pseudomonas solanacearum biovars based on Membrane protein patterns. Phytopathology. 80:641-646.

Grimm, C., L. Rahme, R. Frederick, M. Mindrinos, P.B. Lindgren, and N. Panopoulos. 1989. The common pathogenicity genes of Pseudomonas syringae pathovars. Molecular Biology of Plant-Pathogen Interactions. p49-55.

Grimm, C. and N. Panopoulos. 1989. The predicted protein product of a pathogenicity locus from Pseudomonas syringae pv. phaseolicola is homologous to a highly conserved domain of several procaryotic regulatory proteins. J. Bacteriol. 171:5031-5038.

Gross, D.C., Y.S. Cody, E.L. Proebsting Jr., G.K. Tadamaker and R.A. Spotts. 1984. Ecotypes and pathogenicity of ice nucleation-orchards. Phytopathology 74:241-248.

Haefele, D.M. and S.E. Lindow. 1987. Flagellar motility confers epiphytic fitness advantages upon Pseudomonas syringae. 53(10):2528-2533.

Hoitink, H.A.J., D.J. Hagedorn, and E. McCoy. 1968. Survival, transmission and taxonomy of Pseudomonas syringae van Hall, the causal organism of bacterial brownspot of bean (Phaseolus vulgaris L.). Can. J. Microbiol. 14:437-441.

Ielpi, L., T. Dylan, G.S. Ditta, D.R. Helinski and S.W. Stanfield. 1990. The ndvB locus of Rhizobium meliloti encodes a 319-kDa protein involved in the production of β-(1-2)-glucan. J. Biol. Chem. 265(5):2843-2851.

Jawetz, E., J.L. Melnick and E.A. Adelberg. 1982. Review of medical microbiology. 15th edition. Lange Medical Publications, Los Altos, California. p87-96.

Keen, N.T., T. Ersek, M. Long, B. Bruegger and M. Holliday. 1981. Inhibition f the hypersensitive reaction of soybean leaves to incompatible Pseudomonas spp. by blasticidin S, streptomycin, or elevated temperature. Physiol. Plant Pathol. 18:325-337.

King, E.O., M.K. Ward and D.E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Med. 44:301-307.

Klement, Z. K. Rudolph and D.C. Sands. 1990. Methods in Phytobacteriology. Akademiai Kiado, Budapest. p191-198.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Lindemann, J., D.C. Arny, and C.D. Upper. 1984. Use of an apparent infection threshold population of Pseudomonas syringae to predict incidence and severity of brown spot of bean. Phytopathology 74:1334-1339.

Lindgren P.B., R.C. Peet and N.J. Panopoulos. 1986. A gene cluster of Pseudomonas syringae pv. phaseolicola controls pathogenicity on bean and hypersensitivity on non-host plants. J. Bacteriol. 168:512.

Lindgren P.B., N.J. Panopoulos, B.J. Staskawicz and D. Dahlbeck. 1988a. Genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathovars of Pseudomonas syringae. Molec. Gen. Genet. 211:499.

Lindgren, P.B., C. Grimm, N.J. Panopoulos, B.J. Staskawicz, D. Dahlbeck. 1988b. The genetic control of interactions between Pseudomonas and host plants.

(Abstract) In: 5th Int. Congr. of Plant Pathology. StVII 4-1. p261.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982 Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory. p387-389.

Mills, D. 1985. Transposon mutagenesis and its potential for studying virulence genes in plant pathogens. Ann. Rev. Phytopathol. 23:297-320.

Mills, D., F. Niepold and M. Zuber. 1985 Cloned sequence controlling colony morphology and pathogenesis of Pseudomonas syringae. In: I. Sussex, A. Ellingboe, M. Crouch, and R. Malmberg (eds.): Plant Cell/cell Interactions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. p97-102.

Mills D. and F. Niepold. 1987. Molecular Analysis of pathogenesis of Pseudomonas syringae pv. syringae. In: S. Nishimura et al. (eds.) Molecular Determinants of Plant Disease. Japan Sci. Soc. Press. Tokyo/Springer-Verlag, Berlin, p185-200.

Mills. D., P. Mukhopadhyay, Y. Zhao and M. Romantschuk. 1990. Organization and function of pathogenicity genes of Pseudomonas syringae pathovars phaseolicola and syringae. In: S.S. Patil, S. Ouchi, D. Mills, and C. Vance (eds.) Molecular Strategies of Pathogens and Host Plants. Springer-Verlag, London, (In press).

Minsavage, G.V., and N.W. Schaad. 1983. Characterization of Membrane proteins of Xanthomonas campestris pv. campestris. Phytopathology 73:747-754.

Niepold, F., D. Anderson, and D. Mills. 1985. Cloning determinants of pathogenesis from Pseudomonas syringae pathovar syringae. Proc. Natl. Acad. Sci. USA 82:406-410.

Panopoulos, N.J. and M.N. Schroth. 1974. Role of flagellar motility in the invasion of bean leaves by Pseudomonas phaseolicola. Phytopathology 64:1389-1397.

Parkinson, J. 1990. Communication modules in bacterial signaling proteins: Do prokaryotes conduct their business affairs with cellular phones? ASM News. 56(4):197-201.

Patel, P.N., J.C. Walker, D.J. Hagedorn, C.D. Garcia and M. Teliz-Ortiz. 1964. Bacterial brown spot of bean in central Wisconsin. Plant Disease Rep. 48:335-337.

Peet, R.C., P.B. Lindgren, D.K. Willis and N.J. Panopoulos. 1986. Identification and cloning of genes involved in phaseolotoxin production by Pseudomonas syringae pv. phaseolicola. J. Bacteriol. 166(3):1096-1105.

Simon, R., Priefer U., and Puhler, A. 1983. A broad range mobilization system for in vitro genetic engineering: Transposon mutagenesis in Gram-negative bacteria. Biotechnology 1:784-791.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Staskawicz, B.J., D. Dahlbeck, and N.T. Keen. 1984. Cloned avirulence gene of Pseudomonas syringae pv. glycinea determines race-specific incompatibility on Glycine max (L.) Merr. Pro. Natl. Acad. Sci. USA 81:6024-6028.

Szabo, L.J. and D. Mills. 1984 Integration and excision of pMC7105 in Pseudomonas syringae pv. phaseolicola: Involvement of repetitive sequences. J. Bacteriol. 157:821-827.

Turner, P., C. Barber and M. Daniels. 1984. Behavior of the transposons Tn5 and Tn7 in Xanthomonas campestris pv. campestris Mol. Gen. Genet. 195:101-107.

Willis, D.K., E.M. Hrabak, S.E., Lindow and N.J. Panopoulos. 1988. Construction and characterization of Pseudomonas syringae recA mutant strains. Mol. Plant-Microbe Interact. 1:80-86.

Willis, D.K., E.M. Hrabak, J.J. Rich, T.M. Barta, S.E., Lindow and N.J. Panopoulos. 1990. Isolation and characterization of a P. s. pv. syringae mutant deficient in lesion formation on bean. Mol. Plant-Microbe Interact. 3(3):149-156.

Zhou, C., Y. Yang and A.Y. Jong. 1990. Mini-prep in ten minutes. BioFeedback. 8(2):172-173.

MOLECULAR CLONING AND COMPLEMENTATION OF THE PATHOGENICITY-ATTENUATED MUTANT PS9024 OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE*

CHAPTER THREE

INTRODUCTION

An understanding of the genetic mechanism(s) underlying the deficiency of the mutant strain PS9024 that results in its inability to incite brown spot disease symptoms in susceptible beans, as well as its altered growth properties *in planta* and in culture, ultimately relies on knowledge of the function of the gene involved. The wild type DNA (or the gene locus) for this particular mutation can be defined by molecular cloning of the DNA of the parental wild type chromosome homologous to the DNA that contains the known Tn*5* insertion and characterizing sequences of the flanking region. Physical mapping by either mapping endonuclease restriction sites or Southern hybridization analysis can furnish the physical identity of the specific cloned wild type DNA sequence.

The gene can be functionally identified by complementation. The question asked is whether the cloned wild type DNA sequence will complement the mutant defective gene(s) to restore the wild type phenotype when introduced into mutant cells in the *trans* configuration. The results frequently are unambiguous because the wild type and the mutant genes replicate on independent replicons. However, the limitations of using this method are that it is dependent on the stability of the vector and that *cis*-acting factors, such as enhancer elements, which may reside far from the gene of interest in the bacterial chromosome, cannot be identified. Furthermore, the size of the insert carried by the vector could also preclude complete complementation. If the gene of interest is transcribed from a large operon, it may be difficult to obtain a clone that includes the entire operon. For instance, at least 30 kb is required for phytotoxin coronatine synthesis in *P. s.* pv. *tomato* (Okabe), the causal agent of bacterial speck of tomato (Ma *et al.*, 1991).

A more desirable way to demonstrate the ability of the wild type DNA sequence to complement the mutant phenotype is the method of marker exchange, also known as "homogenotization", "transplacement" or "gene replacement" (Klement *et al.*, 1990). This is achieved

by replacing the mutated gene with the wild type gene copy. However, a common difficulty of this approach is the lack of proper genetic selection, such as selection for antibiotics resistance, to recover the recombination event. Furthermore, the imprecise exchange in the process of homologous recombination may complicate the results.

The marker exchange method has been used alternatively as a way to map functional domains of the gene of interest and to correlate a mutated locus with a particular phenotype. The strategy has been used to reconstruct the mutant by replacing the wild type gene with a copy which has been altered *in vitro*.

The objectives of this chapter are to describe the molecular cloning of the wild type DNA sequences that share homology with the Tn*5*-containing *Eco*RI fragment cloned from the mutant strain PS9024. Physical characterization of these sequences and complementation of the mutant strain PS9024 using these wild type sequences are also described. Finally, reconstruction of the mutations in the same locus using a different transposon, Tn*3*-HoHo1, and subsequent testing for pathogenicity of these mutants are demonstrated.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids were previously described in the Materials and Methods section of Chapter Two. Additional bacterial strains and plasmids used in the study of this chapter are summarized in Table 3-1.

Transformation of P. s. pv. syringae

Stationary phase cultures of R32 and PS9024 were diluted 100-fold in 250 ml LB broth and grown to A_{600} = 0.4-0.5. The bacterial cells were pelleted by low-speed centrifugation and washed with an equal volume of 0.1 M 3-[N-Morpholino]propane sulfonic acid (MOPS), pH 6.5. The bacteria were then resuspended in 100 ml of 0.1 M MOPS containing 50 mM $CaCl_2$ and incubated on ice for 2-16 hours. The cells were then pelleted again by low-speed centrifugation, resuspended in 8.4 ml of the same solution, and mixed with 1.6 ml of sterile glycerol. Following incubation on ice for another 30 minutes, the competent cells were divided into 400 μl aliquots that were rapidly frozen in liquid nitrogen and stored at -70°C. The frozen cells were thawed on ice and 200 μl of

Table 3-1. Bacterial Strains and Plamids.

Strains	Characters	Source or References
P. s. pv. syringae:		
J900	wild type, pathogenic It contains indigenous plasmid	J. Lindemann
PS9086	Tn3-HoHo1-inserted mutant, Amp^r pathogenicity-attenuated	This study
PS9066	Tn3-HoHo1-inserted mutant, Amp^r pathogenicity-attenuated	same as above
E. coli:		
HB101	hadS20, recA, lacY1, rpsL20	Maniatis et al., (1982)
C2110	polA, Nar^r	Stachel et al., (1985)
DH5a	F^-, endA1, hsdR17, supE44, recA1, gyrA96, lacZDM15	BRL, Inc.
Plasmids:		
pOSU226	A stable shuttle vector, Pss ori and ColE1, Amp^r	This study
pLAFR3	Broad host range vector, RK1, cos^+, IncP, rlx^+, Tc^r	Staskawicz et al., 1987
pHoHo1	Tn3-HoHo1 donar, ColE1, $tnpA^+$, Cm^r	Stachel et al., 1985
pSShe	Transposon helper plasmid, ColE1, $tnpA^+$, Cm^r	same as above
pRK2013	Conjugation helper plasmid, ColE1, tra^+, Km^r	Roberts et al., 1988
pUC19	ColE1, Amp^r	Yanisch et al., 1985

(Continue) **Table 3-1.** Bacterial Strains and Plamids.

Strains	Characters	Source or References
pOSU2401-2418	wild type cosmid clones homologous to insert of pOSU9024	This study
pOSU2401	wild type cosmid clone used in complementation test; ca. 16 kb contigous insert in pLAFR3	same as above
pOSU4201	wild type plasmid clone used in complementation test; 7.7 kb insert cloned in pOSU226	same as above
pOSU241	10.5 kb EcoRI fragment in pLAFR3 used in in planta complementation	same as above
pOSU242	6.5 kb EcoRI fragment in pLAFR3 used in in planta complementation	same as above
pOSU145	1.45 kb SalI fragment in pOSU226 used in in planta complementation	same as above
pOSU225	2.25 kb SalI fragment in pOSU226 used in in planta complementation	same as above
pOSU235	2.35 kb SalI fragment in pOSU226 used in in planta complementation	same as above
pOSU270	2.70 kb SalI fragment in pOSU226 used in in planta complementation	same as above

Amp = Ampicilin; Sm = Streptomycin; Km_r = Kanamycin; Tc = Tetracycline; Cm = chloraphenicol; Nal = Nalidixic acid; ^{r} = resistant

these cells were mixed with 100-200 ng of the plasmid (or ligated) DNA for each transformation. The cell-DNA mixture was incubated on ice for 30 minutes, subjected to heat-shock at 42°C for 2 minutes, and incubated with 1 ml of LB broth at 28°C for 90 minutes with shaking. The transformants were selected on King's medium B agar plates with appropriate antibiotics.

Tn3-HoHo1 mutagenesis

The procedure of Tn3-HoHo1 mutagenesis was followed according to the method of Stachel et al., (1985). Strain HB101 containing plasmids pSShe and pHoHo1 was transformed (Maniatis et al., 1982) with pOSU2401. E. coli transformants were selected on LB agar containing chloramphenicol, ampicillin, and tetracycline and then mated with strain C2110 by triparental conjugation with the helper strain HB101 (pRK2013) (Ditta et al., 1980). C2110 transconjugants were selected on LB agar containing nalidixic acid, ampicillin and tetracycline and further tested on chloramphenicol-containing agar medium for sensitivity. Individual colonies containing pOSU2401 with a Tn3-HoHo1 insertion were then transformed into P. s. pv. syringae strain R32. The orientation of the Tn3-HoHo1 insertion was determined by EcoRI and SalI restriction digestions, respectively.

Marker exchange mutagenesis of P. s. pv. syringae R32

Marker exchange mutagenesis was performed using transformants of strain R32 that contained a single Tn3-HoHo1 insertion within the insert of pOSU2401. The plasmid pOSU2401 encodes tetracycline resistance whereas ampicilin resistance is encoded by Tn3-HoHo1. Twenty two transformants of pOSU2401::Tn3-HoHo1 were chosen and verified by mini-scale DNA isolation analysis prior to marker exchange. A stationary culture of each transformant grown in LB medium containing ampicillin and tetracycline was transferred with 100-fold dilution into new broth without antibiotics. Cultures were incubated at 28°C with active agitation (275 RPM) and transferred into new broth every other day. After the fifth day of incubation, the culture was diluted and streaked onto LB agar plates containing ampicillin and incubated at 28°C. Colonies were then replicated onto tetracycline-containing agar medium. Colonies that were tetracycline-sensitive and ampicillin-resistant were further tested for loss of the cosmid by using a modified mini-scale plasmid DNA preparation procedure (Birnboine and Doly, 1979).

Chromosomal insertion of Tn3-HoHo1 resulting from marker exchange was verified by Southern blot analysis of EcoRI digested total genomic DNA using a ^{32}P labeled probe made from a subfragment within the EcoRI region

and from the lac gene sequence, which is part of Tn*3*-HoHo1.

Plant bioassay for functional complementation

The procedure for inoculating bean plants has been described by Bertoni and Mills (1987) and the bean pod inoculation was described in Materials and Methods of Chapter Two.

RESULTS

Molecular cloning of the wild-type DNA sequences from the R32 genomic library

Construction of the wild type genomic library of *P. s.* pv. *syringae* R32 using the cosmid pLAFR3

A genomic library of the parental wild type strain R32 was constructed using the cosmid pLAFR3 (Staskawicz *et al.*, 1987). The general strategy for the construction is schematized in Figure 3-1. To ensure the randomness of the inserts in the constructed library, size-fractionated, partially digested *Sau*3A fragments were used. The size distribution and randomness of the inserts in the genome library were confirmed by mini-scale DNA isolation (Figure 3-2). Out

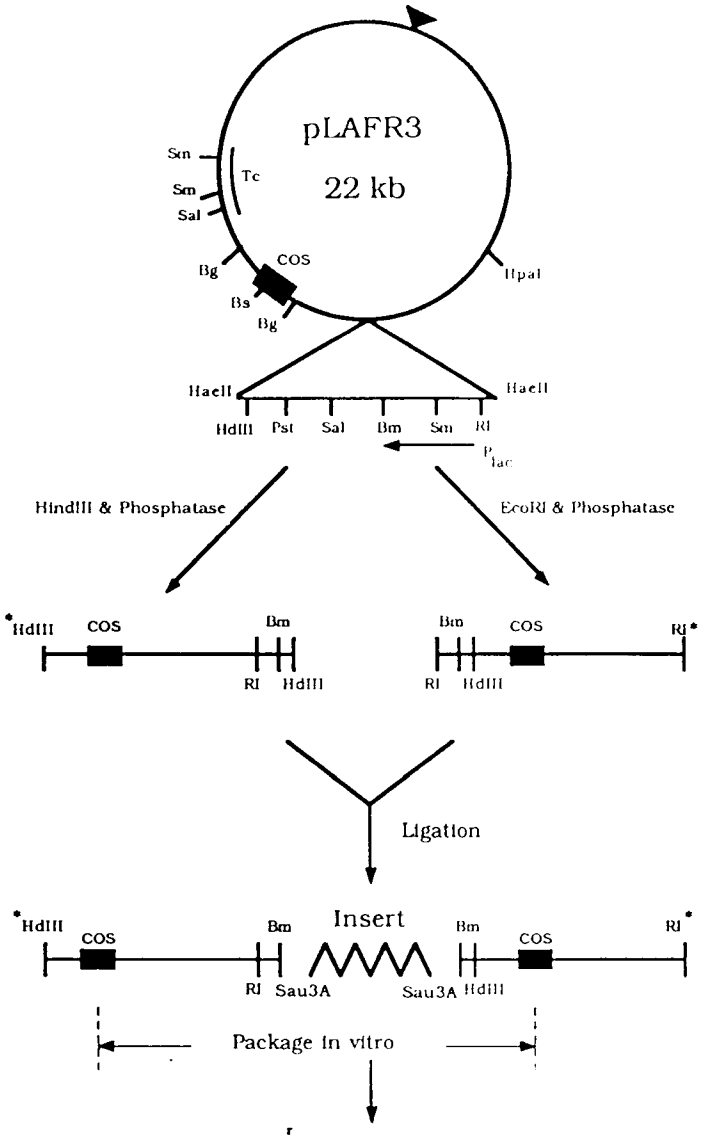


Figure 3-1. Construction of the R32 genomic library using cosmid pLAFR3. The strategy and procedure were described by Staskawicz et al., (1987). Briefly, Sau3A partially-digested R32 total genomic DNA was size fractionated (20-30 kb) using sucrose gradient centrifugation and ligated with the BamHI site of pLAFR3. The ligated molecules were pakaged in vitro and transfected into E. coli HB101 following manufacturer's recommendation. E. coli cells that contain inserts in pLAFR3 were selected for tetracycline resistance and lac$^-$ on X-gal plates (adapted from Staskawicz et al., 1987).

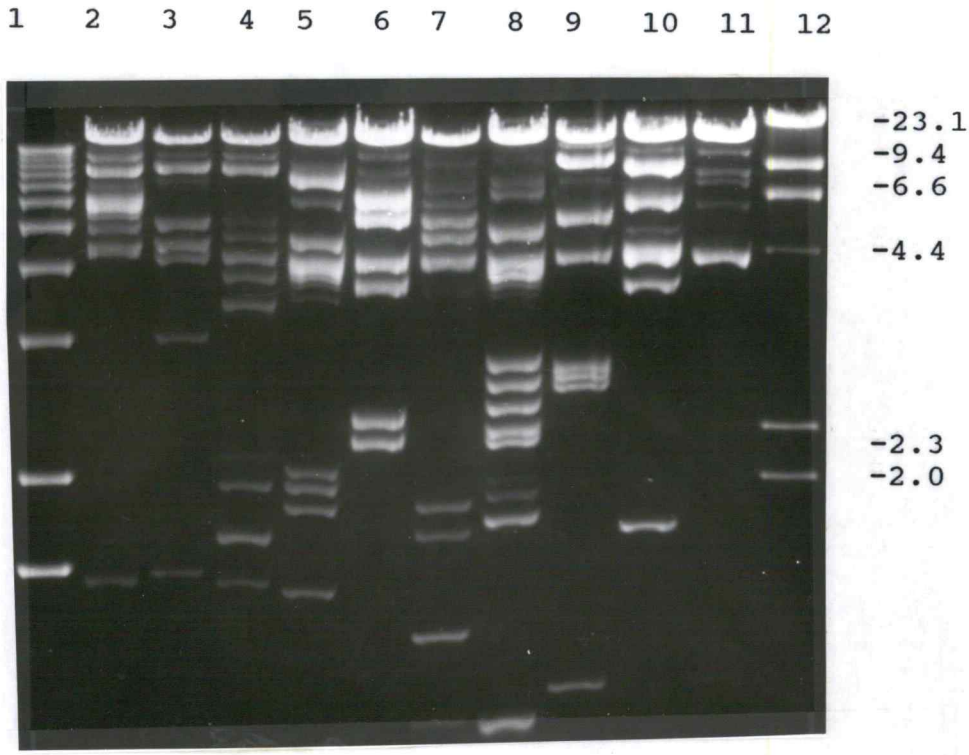


Figure 3-2. Agrose gel electrophoresis of EcoRI and SalI restriction digests of randomly selected clones from the cosmid library. Lanes 1 and 12 contain HindIII digested lambda DNA and a 1 kb DNA size marker, respectively. Lanes 2-11 contain cosmid library clones.

of 36 randomly picked colones, 35 colones (97.2%) contained inserts with an average size of 21.7 kb. The size of the inserts range from 15.8 kb to 30 kb. It was noticed, however, that 20% of the colonies lost their inserts after more than three month storage in -80°C freezer. Rearrangements and decreases in sizes of the inserts were also observed.

Recovery of the wild type sequences homologous to the Tn5-containing EcoRI fragment from PS9024

In situ hybridization (as described by Mantiatis, 1982) was utilized to screen the library for sequences homologous to the insert of pOSU9024. A ^{32}P-labelled DNA probe made of pOSU9024 identified 18 colonies from the library. An example of the in situ hybridization using this probe is shown in Figure 3-3. The plasmid DNA of these colonies were isolated and digested with endonuclease to determine the restriction profiles of these plasmids. These plasmids were digested with EcoRI/SalI, immobilized on Genetran filters (Maniatis et al., 1982) and probed with pOSU9024. The results of these hybridizations are shown in Figure 3-4. The complete wild type 10.5 kb EcoRI fragment was identified in plasmids pOSU2401, pOSU2403, pOSU2405, pOSU2407 and pOSU2415.

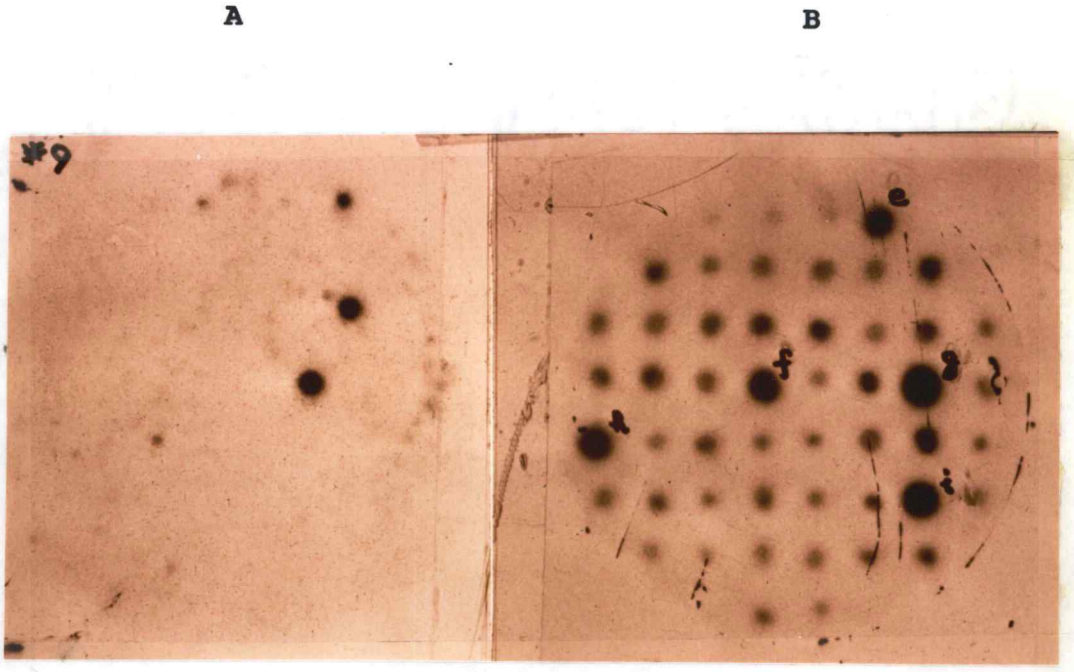


Figure 3-3. Identification of wild type sequences homologous to the insert of pOSU9024. **A.** Colony hybridization of random colonies from the genomic library. **B.** An example of the hybridization of colonies that showed a positive signal in the initial screening to the pOSU9024 probe.

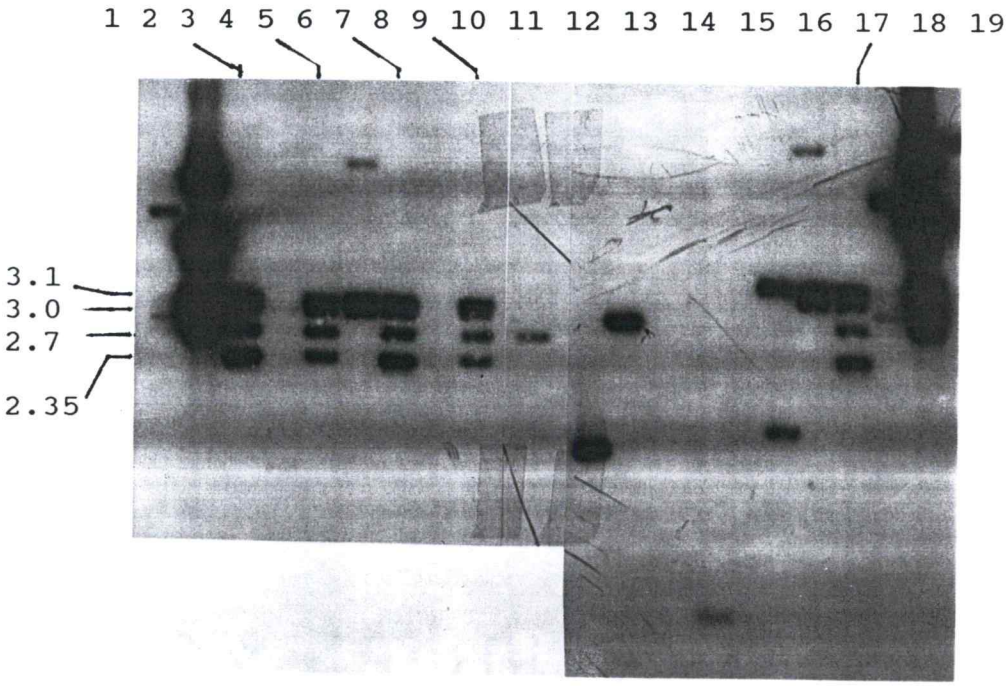


Figure 3-4. Identification of the wild type cosmid clones homologous to the insert of pOSU9024. The cosmid clones (lanes 3-10 and 12-17) and PS9024 total genomic DNA (lanes 1 and 19) were digested with SalI; pOSU9024 (lanes 2 and 18) was digested with SalI and EcoRI; The size marker (lane 11) is 1 kb ladder purchased from BRL Co.; This is the result of Southern hybridization analysis using the insert of pOSU9024 as the probe. The SalI fragment comprise the entire 10.5 kb EcoRI fragment homologous to the probe in lanes 3 (pOSU2401), 5 (pOSU2403), 7 (pOSU2405), 9 (pOSU2407) and 17 (pOSU2415), These SalI fragments are represented by 3.1 kb, 3.0 kb, 2.7 kb and 2.35 kb.

Plasmid pOSU2401, which has the insert of ca. 23 kb and a 10.5 kb EcoRI fragment, which is the expected size of the genomic fragment into which Tn5 inserted, was chosen for subsequent studies.

Restriction mapping and analysis of genomic continuity of the insert in pOSU2401

A restriction map of pOSU2401 is presented on Figure 3-5. Restriction sites within the EcoRI fragment correspond to sites mapped within pOSU9024.

To test for genomic continuity, total genomic DNA and pOSU2401 were digested with various enzymes, the fragments were Southern blotted and probed with the 2.25 kb and 2.35 kb SalI fragments of pOSU2401 (Figure 3-6A-B). Two EcoRI fragments from both pOSU2401 and R32 showed a positive signal with the probe. However, only one fragment, in which Tn5 inserted (the top 10.5 kb fragment) is identical in size, and is consistent with the size predicted from the Tn5-containing EcoRI region. The second 6.6 kb EcoRI fragment in the plasmid is smaller in size than the wild type EcoRI fragment (ca. 8.5 kb). Continuity within the entire 10.5 kb EcoRI fragment in pOSU2401 was also confirmed by SalI/EcoRI digestion (lanes 9 and 10). Although under these

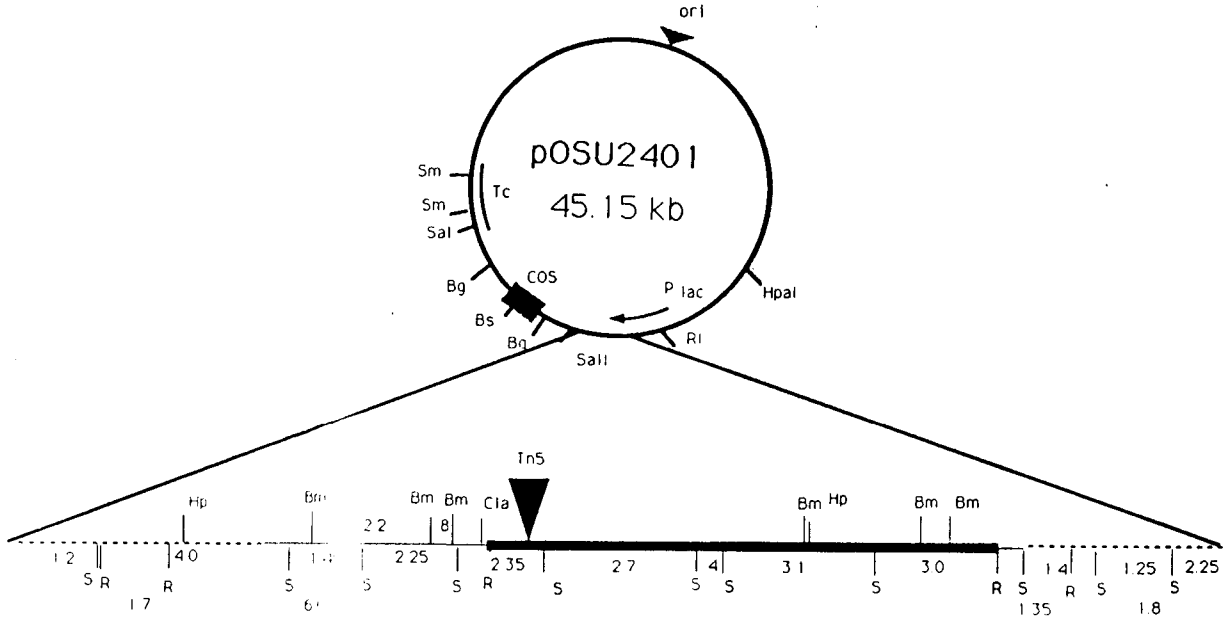


Figure 3-5. Endonuclease restriction map of pOSU2401. EcoRI into which Tn5 inserted is represented by the thick bar. The solid thin line indicates contigous sqeuence and the dashed line indicates noncontiguous seqences with the genomic DNA. The restriction sites are abbreviated as follows: R, EcoRI. S, SalI. Bm, BamHI. Cla, ClaI. Hp, HpaI. Bg, BglII. Numbers refer to distance measured in kb between sites.

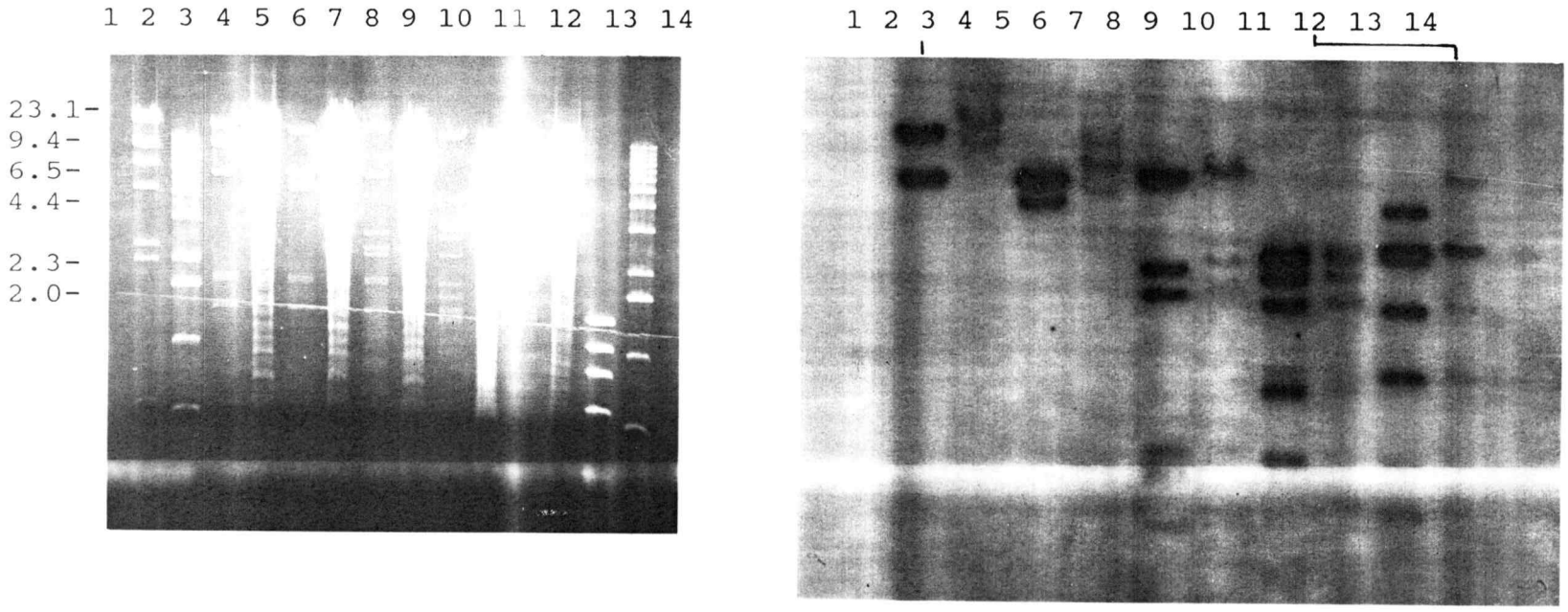


Figure 3-6. Comparison of R32 total genomic DNA with the cosmid clone pOSU2401 for DNA sequence continuity. **A.** Agrose gel electrophoresis of R32 and pOSU2401 DNA digested with various restriction enzymes. Lane 1 is lamda HindIII size marker; Lanes 2 and 14 are 1 kb ladder; Lane 13, .174; Lanes 3, 5, 7, 9 and 11 contain pOSU2401; Lanes 4, 6, 8, 10 and 12 contain R32 total genomic DNA. Lanes 3 and 4 digested by EcoRI; Lanes 5 and 6 double digested by EcoRI/HpaI; Lanes 7 and 8, digested by EcoRI/BamHI; Lanes 9 and 10, digested by EcoRI/SalI and Lanes 11 and 12 digested by EcoRI/BglII, **B.** The result of Southern hybridization of the same gel. The probe consisted of ^{32}P labelled 2.25 kb and 2.35 SalI fragments, which is adjacent to and spanning the left side of the 10.5 kb EcoRI fragment of pOSU2401 (Consult Figure 3-5).

conditions, the smallest SalI fragment (ca. 0.45 kb) had run off the gel, but was confirmed in a different blot. SalI/EcoRI mapping of pOSU2401 indicated that a 2.35 kb SalI fragment spans the left end of the EcoRI fragment, into which Tn5 inserted, and an adjacent EcoRI fragment both in pOSU2401 and in the genomic DNA. These results indicate continuity of these two EcoRI fragments in this region. Continuity within the 2.35 kb SalI fragment was further confirmed by SalI digestion (data not shown). Two additional SalI fragments with the sizes of 1.45 kb and 2.25 kb from the 6.6 kb EcoRI fragment appeared to be adjacent to each other as shown by BamHI/EcoRI and BglII/EcoRI digestions. BamHI/EcoRI and BglII/EcoRI mapping of pOSU2401 also indicated the linkage of the 2.25 kb and 2.35 kb SalI fragments. Plasmid DNA appeared not to be continuous at the left side of 3.2 kb BamHI fragment. It is not known how far the contiguous sequences extend leftward from the 1.45 kb SalI fragment. On the right side of the 10.5 kb EcoRI fragment, continuity extends to the SalI site, which is located approximately 200 bp outside the right EcoRI site. A BamHI site, which is approximately 3 kb away from the right side of the 10.5 kb EcoRI site, was shown to be not contiguous. A summary of the contiguous region of the insert in pOSU2401 is depicted in Figure 3-5. It demonstrates that at least 16 kb of the insert

is contiguous and that it extends at least 5.8 kb, as measured by SalI digestion, to the left of the Tn5 insertion site and at least 9.6 kb to the right of the Tn5 insertion.

Complementation of the mutant PS9024 using pOSU2401

Attempts to complement the Tn5 mutation of PS9024 were conducted by transforming PS9024 with pOSU2401 and other subclones using the procedure described in Materials and Methods. The tetracycline-resistant transformants were subsequently tested on the host plants. Because an inoculum density of approx. 5×10^7 CFU/ml is normally required to induce a visible symptom when PS9024 is inoculated into susceptible bean plant (Anderson and Mills, 1985; and this study), all of the complementation bioassays were conducted at an inoculum density level of 10^5-10^6 CFU/ml. Symptom development was followed throughout a two week interval from the day of inoculation. The bean pod inoculation bioassay was also used in the complementation analysis.

The complementation of PS9024 by pOSU2401 resulted only in partially restored disease symptoms and in a phenotype that was intermediate between wild type R32 and the mutant PS9024 (Figure 3-7). Careful observation of the inoculated plants indicated that neither PS9024

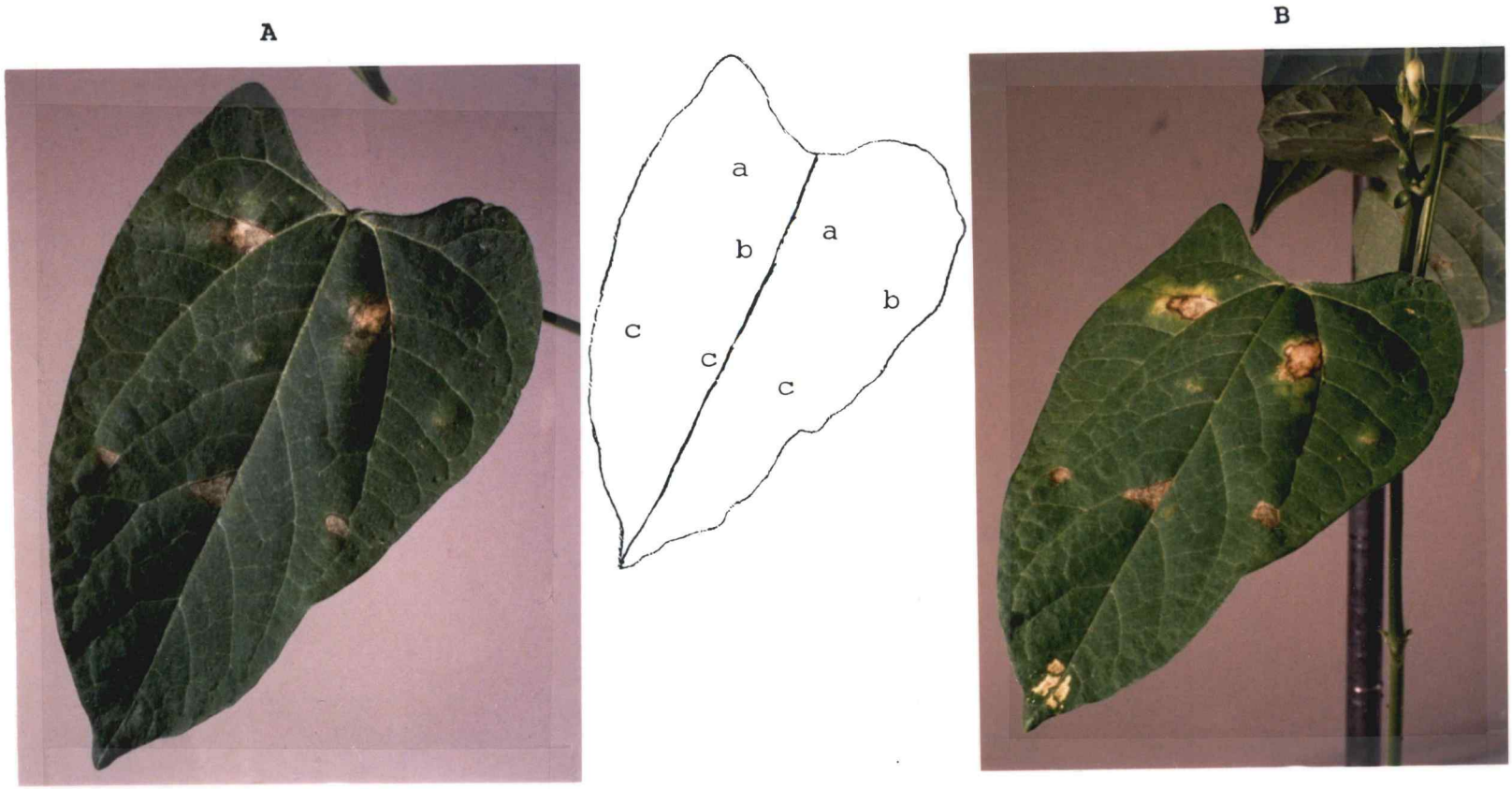


Figure 3-7. In planta complementation of PS9024 using pOSU2401 in bean leaf bioassays. Primary leaves of Phaseolus vulgaris cv. "Eagle" were inoculated using bacterial cells, which were resuspended in K-buffer (pH 7.0) and adjusted to 10^6 CFU/ml. The sites of inoculation are diagramed: **a**, phenotypes of R32(pLAFR3), **b**, phenotype of PS9024(pLAFR3) and **c**, phenotype of PS9024(pOSU2401). The reaction shown on the tip of the leaf was caused by ink of the marker pen. Plant reactions were scored at **A.**, one week after inoculation; **B.**, two week after inoculation.

nor PS9024(pOSU2401) produced any disease symptoms during the first three days after inoculation. However, noticeable brown, necrotic lesions were observed at the inoculation sites of PS9024(pOSU2401) beginning from one week after inoculation (Figure 3-7A). These lesions were more explicit two weeks after inoculation (Figure 3-7B). No restoration of disease symptoms was observed when either of the two EcoRI fragments of pOSU2401 (pOSU241 and pOSU242) were transformed into PS9024.

Partial restoration of the disease symptoms in PS9024(pOSU2401) were also observed in bean pod inoculation (Figure 3-8). A small necrotic lesion was observed within one week when pods were inoculated with PS9024(pOSU2401) but no lesion was detected when PS9024 containing the cloning vector, pLAFR3, was inoculated.

Construction of pOSU226, a stable shuttle vector for complementation studies in P. s. pv. syringae

In planta complementation studies of mutant strains with fragments cloned into vectors that replicate from the origin of replication sequence of plasmid RK2 (e.g., pLAFR3) have produced variable results (Thomas, 1983; Niepold et al., 1985). This variability is due, in part, to the instability of the vector in the absence of

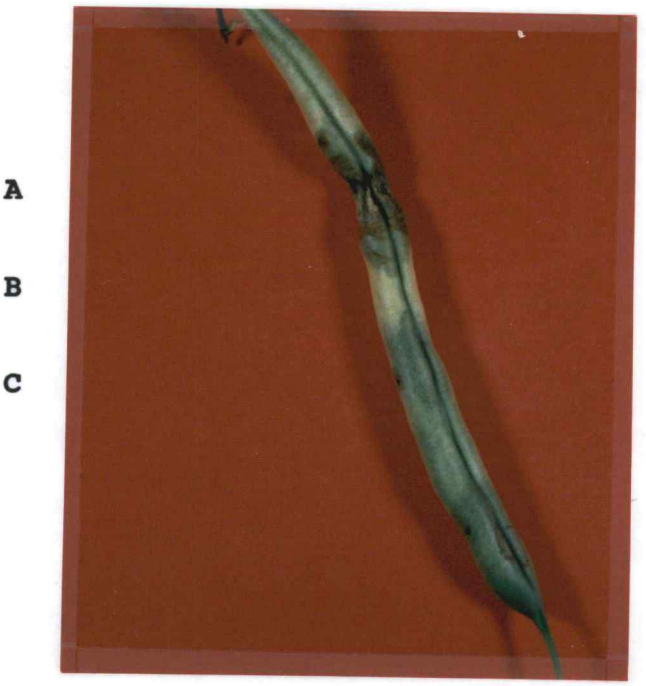


Figure 3-8. In planta complementation of PS9024 using pOSU2401 in the bean pod. Bacterial cells, suspended in K-buffer (pH 7.0), were adjusted to 10^6 CFU/ml and injected into the cavity of bean pods of Phaseolus vulgaris cv. "Eagle". **A**, R32(pLAFR3); **B**, PS9024(pLAFR3) and **C**, PS9024(pOSU2401). Reactions were scored at one week after inoculation.

antibiotic selection. Vectors pVK102 and pRK415 are known to be unstable *in planta* (Ehrenshaft and Mills, 1985; Niepold and Mills, 1985; Mukhopadhyay *et al.*, unpublished data). Approximately 75% of the *P. s.* pv. *phaseolicola* cells have been shown to lose pVK102 (an RK2 derivative) after four days of culturing in the absence of antibiotic selection (Ehrenshaft and Mills, 1985). Consequently, cosmids that are unstable in *Pseudomonas* in the absence of antibiotic selection may preclude any long-term *in planta* bioassays, including *in planta* complementation. An accurate measurement of *in planta* complementation requires the use of a vector that is stably maintained in *Pseudomonas* without selection.

A new shuttle vector, pOSU226, which is a derivative of pUC19 and contains an *ori* sequence from an indigenous plasmid in *P. s.* pv. *syringae* strain J900 (Table 3-1), was constructed in this study. The construction of pOSU226 is schematically shown in Figure 3-10 and is patterned after a similar strategy used to construct pOSU221 (Mukhopadhyay *et al.*, 1990). pOSUΔ22, a high copy number plasmid, which contains only a part of the pUC18 polylinker and is lacking *Kpn*I and *Sst*I restriction sites (Mukhopadhyay *et al.*, 1990), was used as the precursor plasmid. pOSUΔ22 was partially digested with *Eco*RI to release the 5.5 kb *ori*-containing DNA

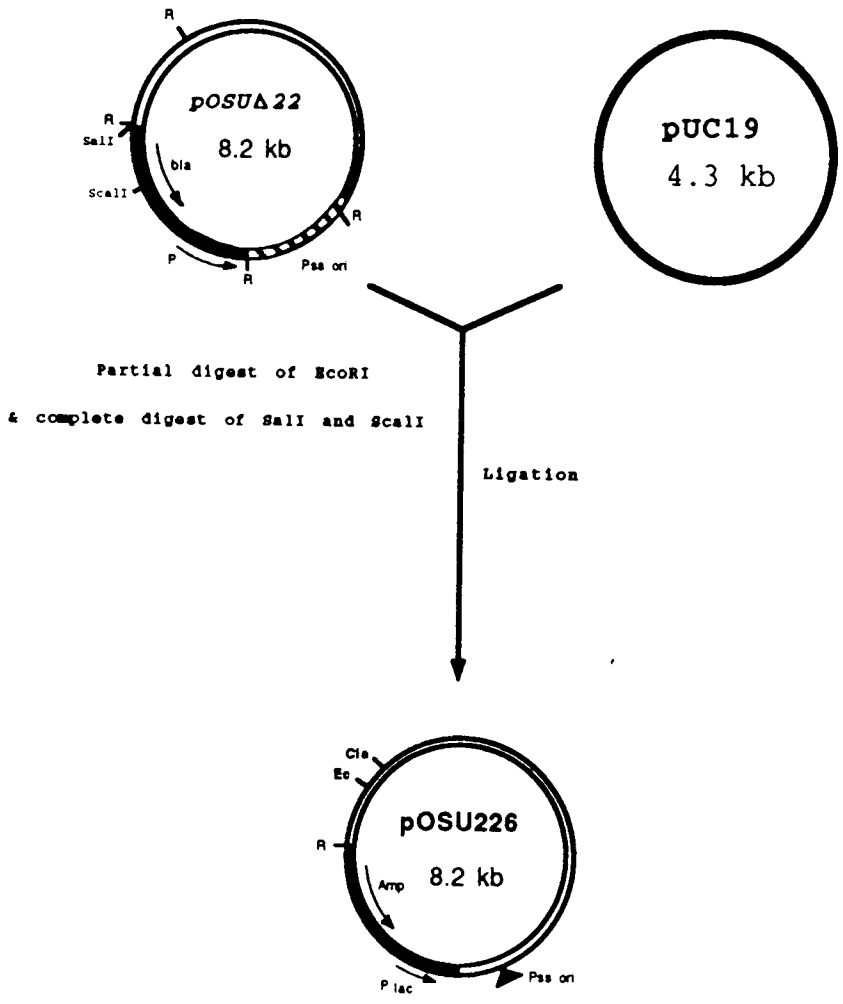


Figure 3-9. Construction of the stable shuttle vector pOSU226. This vector was constructed using an origin of replication sequence (Pss ori) from pOSU900, an indigenous plasmid of Pseudomonas. syringae. pv. syringae J900 (Mukhopadhyad et al., 1990), and ligated with pUC19 plasmid, which contains the ColE1 origin of replication. The solid bar represents pUC19 sequences, whereas the open bar represents Pseudomonas sequences. The Pss ori sequence is represented by the shaded bar. The lac promoter (P_{lac}) and bla (ampicilin resistant gene) in pUC19 are shown. Abbreviations: R, EcoRI; Sc, ScaI; B, BamHI; S, SalI and H, HindIII.

fragment. A complete digestion was followed with SalI and ScaI to fragment pUC18 to avoid regeneration of pOSUΔ22 during ligation. The digests were then ligated to EcoRI-digested pUC19 and the ligation mixture was used to transform both E. coli and P. s. pv. syringae R32. The authenticity of the newly-constructed vector was then verified by identifying the pUC19 portion of the plasmid which contains a 5.5 kb EcoRI fragment. KpnI and SstI digestion were also used to verfity the presence of the intact polylinker. This vector was designated pOSU226 and its physical map is presented in Figure 3-10.

Plasmid copy number

The copy number of pOSU226 was estimated in relation to that for pLAFR3 in Pseudomonas and that for pUC19 in E. coli. The plasmid pUC19 is a derivative of the plasmid ColE1 which has a plasmid copy number ranging from 10 to 15 copies per cell (Old and Primrose, 1989). pLAFR3 is a derivative of the broad host range plasmid RK2 and belongs to the IncP1 group of plasmids, which has approximately 5-7 plasmid copies per cell (Friedman et al., 1982; Ditta et al., 1980; Figurski et al., 1979; Figurski and Helinski, 1979). Equal numbers of E. coli cells containing pUC19 and pOSU226 or Pseudomonas cells containing pLAFR3 and pOSU226 were

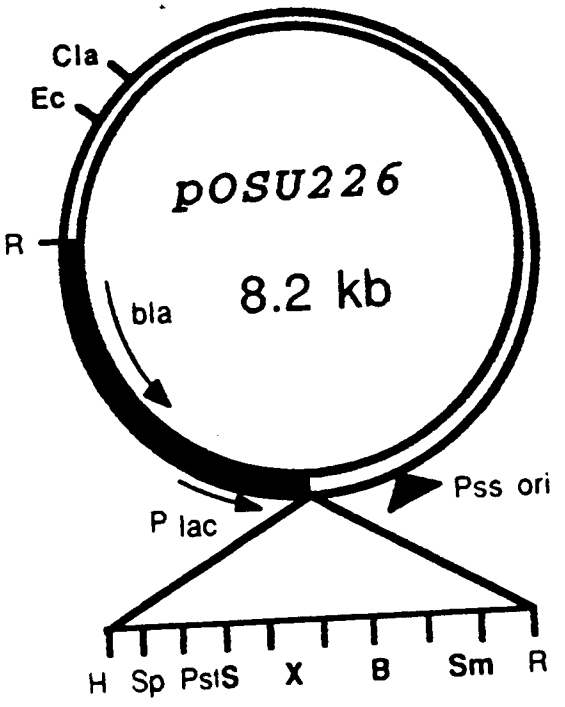


Figure 3-10. Physical map of the stable shuttle vector pOSU226 for E. coli-P. s. pv. syringae. The solid bar represents pUC19 sequences, whereas the open bar represents Pseudomonas sequences. The triangle indicates the Pss ori sequence; the lac promoter (P_{lac}) and bla (ampicilin resistance) gene in pUC19 are also shown. Abbreviations: R, EcoRI; St, SstI;, K, KpnI; Sm, SmaI; B, BamHI; X, XbaI; S, SalI; Pst, PstI; Sp, SphI; H, HindIII; Ec, EcoRV and Cla, Cla I. Unique restriction sites for cloning DNA are shown in boldface letters.

harvested during stationary phase (OD600 = 1.2) and plasmid DNA was isolated using a mini-scale procedure of DNA isolation (Zhou *et al.*, 1990). *Sal*I was used to linearize pUC19 and pOSU226, but pLAFR3 was linearlized with *Eco*RI. The linearlized plasmids were then electrophoresed in 0.7% agar and subjected to laser scanning analysis. The relative copy number of pOSU226 was measured by band intensity relative to pLAFR3 and pUC19 and adjusted according to the molecular size of each plasmid. It is estimated to have approximately 2.5 times lower copy number per cell than pUC19 in *E. coli* and 6.9 fold higher copy number per cell than pLAFR3 in *Pseudomonas*.

Stability of pOSU226 *in planta*

The full expression of disease symptoms under greenhouse conditions by various strains of *P. syringae* pathovars normally requires one to two weeks (Anderson and Mills, 1985; Bertoni and Mills, 1987; this study). Hence it is crucial that any newly constructed vector remain highly stable in *Pseudomonas* under these greenhouse conditions. To test the *in planta* stability of pOSU226, a logarithmically growing culture of R32 containing pOSU226 was used to inoculate the leaves of *Phaseolus vulgaris* cv. "Eagle". Leaf disk samples were collected at the inoculation site and spotted onto

King's medium B agar plates with or without ampicillin selection (Figure 3-11). Over a two week period, approximately 90% of the cells contained pOSU226. Variability in the number of cells that expressed amp^r thereafter suggested that the plasmid may be less stably maintained.

Distribution of the *Pseudomonas ori* sequence in pOSU226

The *P. s.* pv. *syringae ori* sequence was originally isolated from an indigenous plasmid of *P. s.* pv. *syringae* strain J900 (Mukhopadhyay *et al.*, 1990), which causes brown spot disease in bean. It has been shown to share homology with various indigenous plasmids of other pathovars, including the *ori* region of pMMC7105 of *P. s.* pv. *phaseolicola* and *P. s.* pv. *tomato* (Mukhopadhyay *et al.*, unpublished data). Because plasmids that replicate from the same *ori* may belong to a common plasmid incompatibility group and, therefore, would not be expected to co-habitate, it was of particular interest to obtain information about the distribution of this *ori* among other phytopathogenic bacteria. Total DNA of various phytopathogenic bacteria was therefore Southern-blotted and hybridized with a probe of a 2.7 kb fragment that carries the *P. s.* pv. *syringae ori* sequence. This sequence shares homology exclusively within the *P. syringae* pathovars, suggesting the existence of a

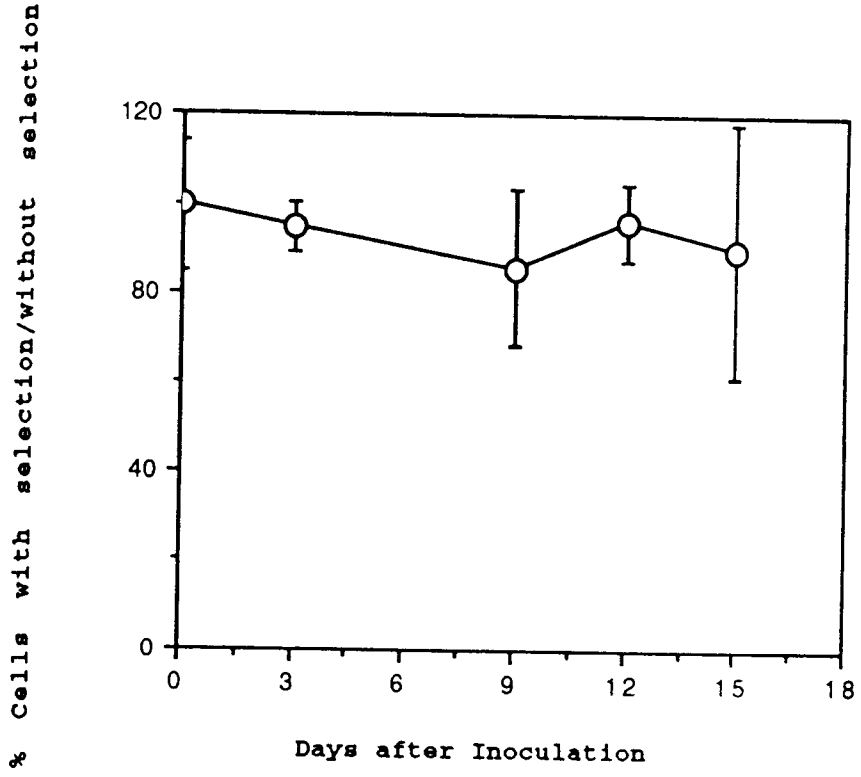


Figure 3-11. Stability of the shuttle vector pOSU226 in P. s. pv. syringae strain R32 growing in planta. Population size of R32(pOSU226) was determined by measuring the number of cells recovered from the plants with and without ampicilin selection on agar plates, at three day intervals.

similar origin of replication on indigenous plasmid in these pathovars (Table 3-2). Furthermore, this P. s. pv. syringae replication origin was previously demonstrated to be non-functional in E. coli, Rhizobium meliloti and Agrobacterium tumefaciens (Mukhopadhyay et al., 1990).

Complementation of the mutant PS9024 using pOSU4201, a construct made with the shuttle vector, pOSU226

The newly constructed vector, pOSU226, was used in subsequent complementation tests of the mutant PS9024. Since no appropriate restriction site is available for cloning the entire intact insert of pOSU2401, partial digestion of the insert with SalI enzyme was used to obtain a 7.75 kb SalI fragment cloned into SalI site of pOSU226 (Figure 3-12). This construct, designated pOSU4201, contains an insert that extends 4.3 kb to the left and 3.4 kb to the right of the site of Tn5 insertion. PS9024 was transformed with pOSU4201 and a transformant was used for complementation of PS9024. The level of complementation, as measured by restoration of disease symptoms, was similar to PS9024(pOSU4201), see Figure 3-13. To ascertain whether expression of disease symptoms was simply delayed, inoculated plants

Table 3-2. Distribution of Origin of Replication Sequence from P. syringae pv. syringae J900 Indigenous Plasmid among Phytopathogenic Bacteria

Strains		Host	Homology to *ori*
Pseudomonas syringae pv.			
syringae	R32	Bean	-
syringae	J900	Bean	+
syringae	Y30	Bean	-
syringae	11/81	Bean	-
syringae	84-43	Bean	-
syringae		Wheat	-
phaseolicola	LR700	Bean	+
phaseolicola	PP601	Bean	+
phaseolicola	PP631	Bean	+
phaseolicola	PP652	Bean	+
phaseolicola	1375A	Bean	+
phaseolicola	1299A	Bean	+
phaseolicola	1301A	Bean	+
phaseolicola	1383	Bean	+
phaseolicola	9	Bean	+
glycinea		Soybean	+
adzakicola		Adzaki Bean	+
tomato	8	Tomato	-
tomato	5796-36	Tomato	+
tabaci		Tobacco	+
coronafaciens		Oats	+
striafaciens		Oats	+
philadelphii B		Mock Orange	-
philadelphii D		Mock Orange	+
lacrymans		NA	+
Pseudomonas			
marginalis	LR900	Lettuce	-
cepacia	POPS	NA	-
putida		NA	-
fluorescens		NA	-

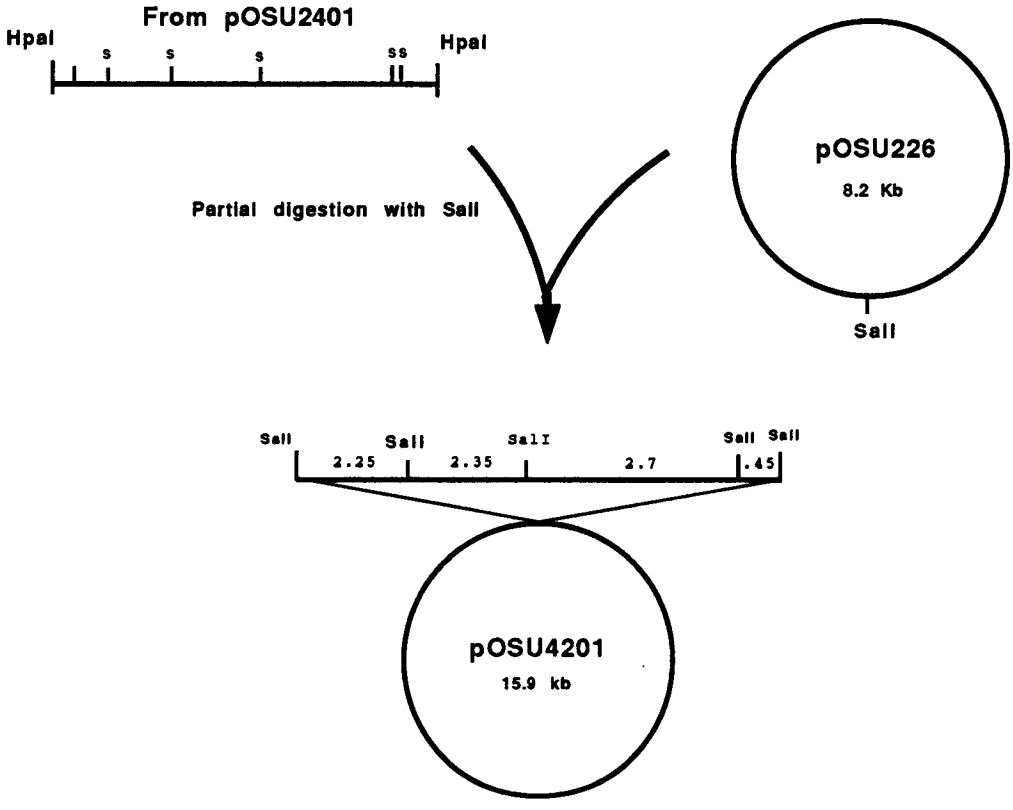


Figure 3-12. Construction of pOSU4201. A 11.5 kb HpaI fragement of pOSU2401 was partially digested with SalI and the incomplete digest was ligated with the SalI site of the shuttle vector, pOSU226. The resulting plasmid was designated pOSU4201, which contains a 7.7 kb SalI insert. The site of Tn5 insertion in PS9024 is represented by the triangle on the shaded bar. Abbreviations: R, EcoRI; S, SalI; Hp, HpaI and H, HindIII.

Figure 3-13. In planta complementation of PS9024 using pOSU4201 in bean leaf bioassays. Primary leaves of Phaseolus vulgaris cv. "Eagle" were inoculated using bacterial cells which were grown in LB medium, suspended in K-buffer (pH 7.0) and adjusted to 10^6 CFU/ml. The sites of inoculation is diagramed. a, R32(pOSU226), b, PS9024(pOSU226) and c. PS9024(pOSU4201). Reactions were scored at **A.**, one week after inoculation; **B.**, two weeks after inoculation. **C.**, three weeks after inoculation.

were also assayed at week three (Figure 3-13C). Again, only partial restoration of the disease symptom was observed. Comparable results were shown in complementation tests of inoculated bean pods (Figure 3-14).

Various other subfragments of pOSU2401 surrounding the site of Tn5 insertion have been cloned into pOSU226 and tested on bean leaves and pods. Complete restoration of diseases symptom was not observed with any of the cloned fragments. Results of complementation tests in which various inserts were cloned both in pLAFR3 and pOSU226 are summarized in Figure 3-15.

Growth kinetics of PS9024(pOSU4201) *in planta* and in growth medium

PS9024 cells transformed with pOSU4201 were partially complemented for increased pathogenicity. It was of interest, therefore, to ascertain whether pOSU4201 would confer a more robust growth *in planta* and in culture medium. Growth kinetics of the mutant PS9024(pOSU4201) were studied both *in planta* (Figure 3-16) and in MaNY medium (Figure 3-17). In both cases, growth of the mutant was partially restored. The *in planta* growth pattern of PS9024(pOSU4201) is similar to

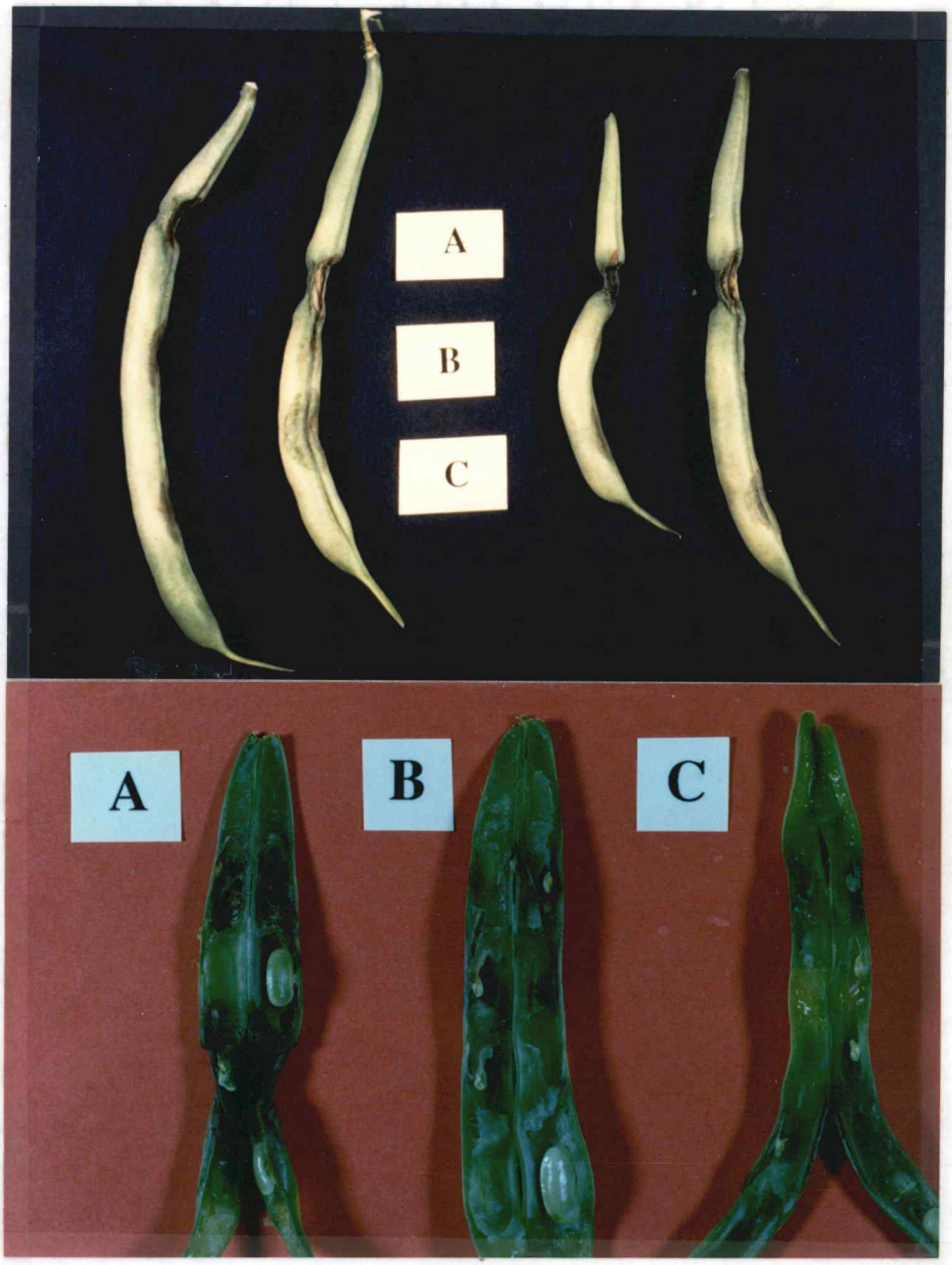


Figure 3-14. Complementation of PS9024 using pOSU4201 in the bean pod bioassay. Bacterial cells, suspended in K-buffer (pH 7.0), were adjusted to 10^6 CFU/ml and injected into the cavity of bean pods of Phaseolus vulgaris cv. "Eagle". **A**, R32(pOSU226); **B**, PS9024(pOSU226) and **C**, PS9024(pOSU4201). Reactions were scored at one week after inoculation.

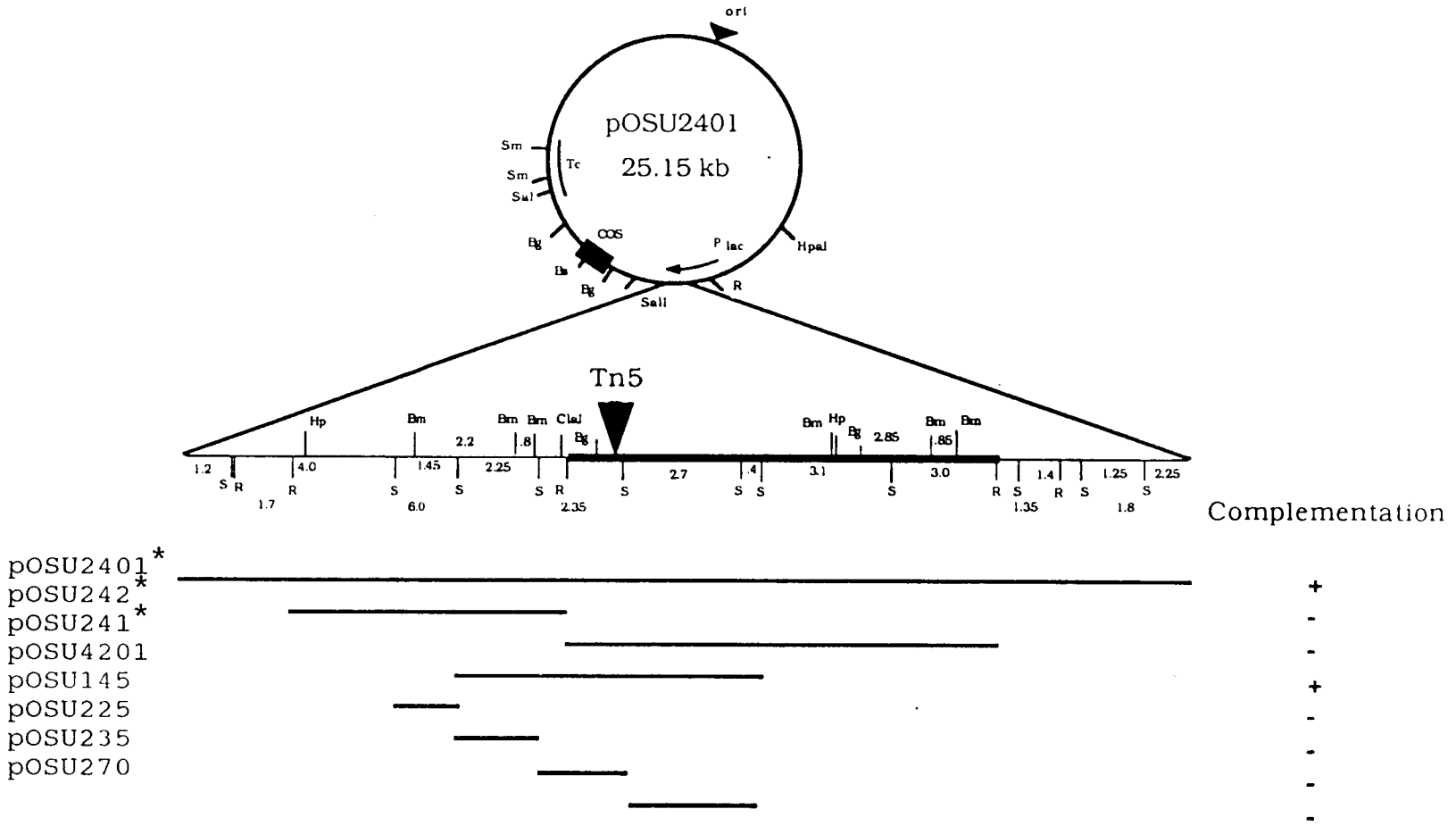


Figure 3-15. Summary of <u>in planta</u> complementations tests of PS9024 using various wild type sequences cloned in either pLAFR3 or pOSU226. The size of the inserts and location relative to Tn<u>5</u> insertion in PS9024 are represented in solid lines underneath the restriction map of pOSU2401. Partial restoration of disease symptoms in PS9024 was scored as "+", whereas "-" indicates no restoration of symptoms were observed. The symptoms incited by the parental wild type strain R32 would rated +++. The asterisk (*) indicates that the insert was cloned into pLAFR3. All others were cloned into pOSU226.

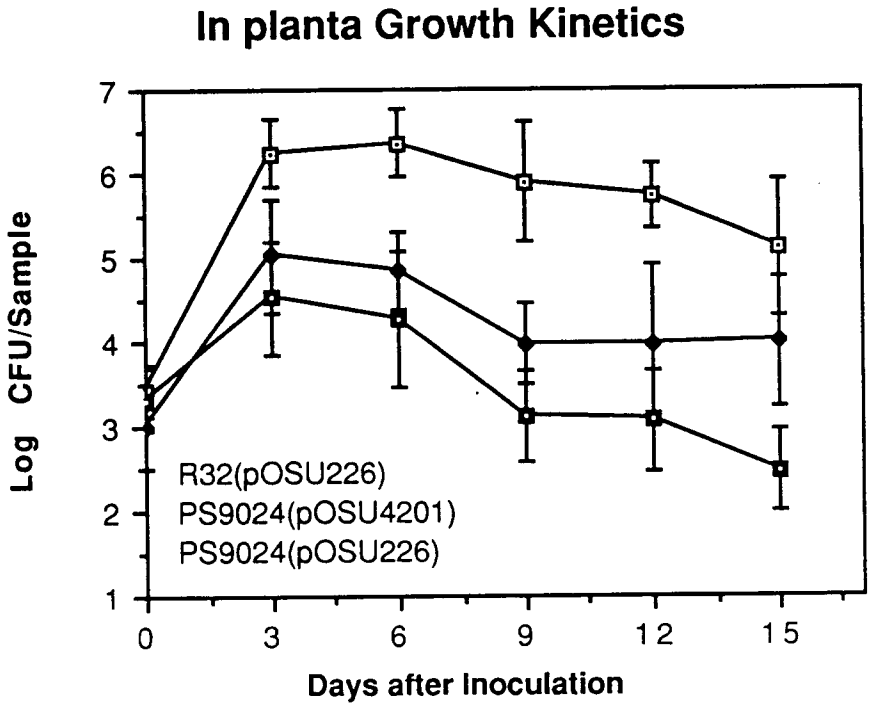


Figure 3-16. Growth kinetics of PS9024(pOSU4201) in planta. Cells were harvested from mid-log phase culture pregrown in minimal MAS medium and adjusted to equal optical density. The culture was further diluted to approximately 10^6 CFU/ml for the test. Each sampling point represents an average of counting four 5 μl droplets in triplicate experiments.

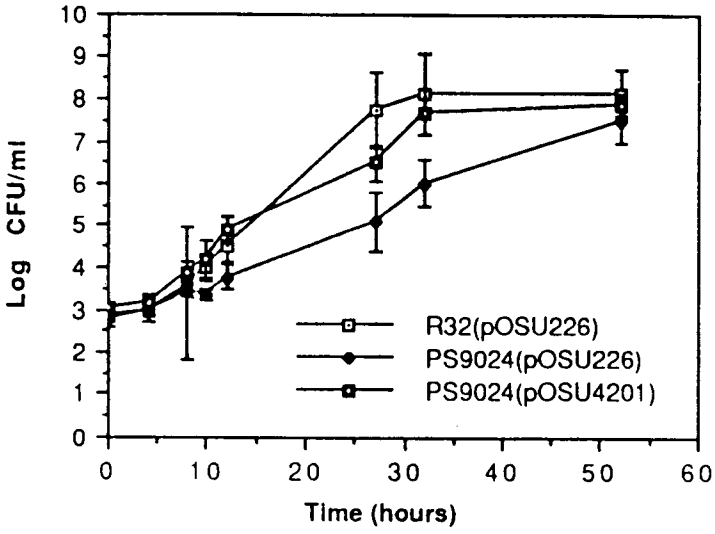


Figure 3-17. Growth kinetics of PS9024(pOSU4201) in MaNY medium. Cells were harvested from mid-log phase culture and adjusted to equal optical density to approximately 10^3 CFU/ml. Each sampling point represents an average of counting four 5 μl droplets in triplicate experiments.

the mutant PS9024. Although it has a slightly faster doubling time during the first three days after inoculation and its viability also decline thereafter. However, growth of PS9024(pOSU4201) is approximately 10 fold greater than PS9024 and about one log increase in growth was observed over the two week period. By week two, growth of PS9024(pOSU4201) differs from that of PS9024 by approximately two logs. In MaNY medium, the overall growth kinetics was almost indistinguishable from that of the wild type strain R32(pOSU226).

Total cellular protein analysis of the complementing strains using SDS-PAGE

An experiment was performed to determined whether pOSU4201 will restore the protein band that was shown to be absent from PS9024. Total cellular protein samples of the mutant strain PS9024 containing various subclones of the wild type sequences cloned into pOSU226 were analyzed by SDS-PAGE. The Coomassie brilliant blue staining method was used to reveal the protein banding patterns. The band absent from PS9024 was not restored by introducing any wild type sequence (Figure 3-18).

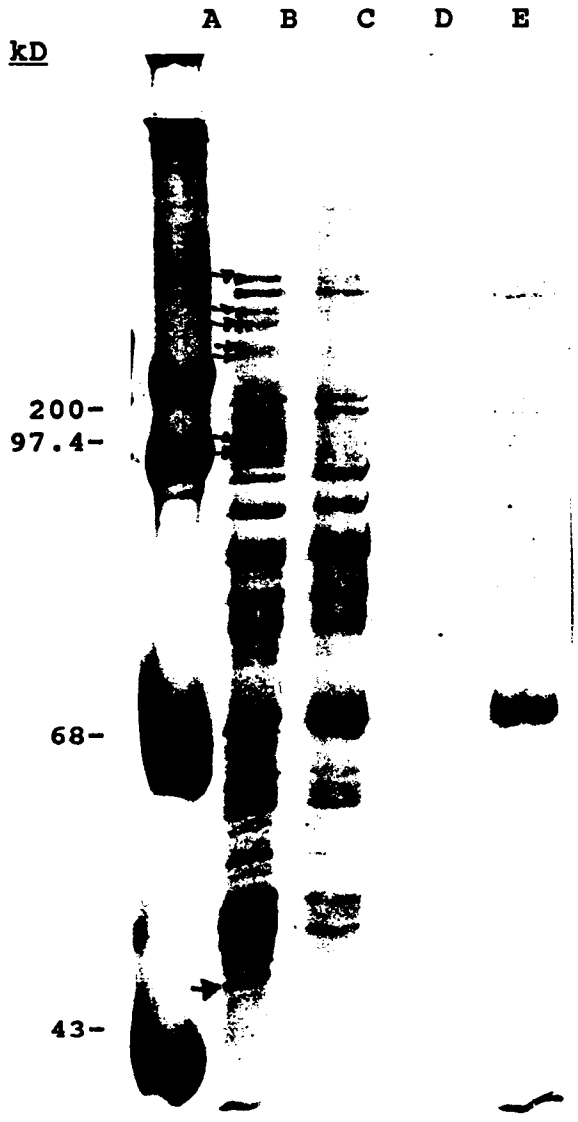


Figure 3-18. SDS-PAGE analysis of proteins made in the wild type strain, R32, PS9024 and PS9024(pOSU4201). Lane A, high range protein size marker (Bio-Rads Laboratories, Richmond, CA). Lane B, wild type strain R32; Lane C, mutant strain PS9024; Lane D, low range protein size marker (Bio-Rads Laboratories, Richmond, CA); Lane E, PS9024(pOSU4201).

Reconstruction of the mutant by the marker exchange method using Tn3-HoHo1 and complementation of the newly constructed mutants

One possible explanation for incomplete complementation of the pathogenicity mutation is the occurrence of a second-site mutation in PS9024 that may have arisen spontaneously and remained undetected. To test this hypothesis, numerous attempts were made to construct a mutant from the original parental strain, R32, by replacing the wild type gene with a homologous sequence containing a transposon. A series of Tn3-HoHo1 insertions were made throughout the entire insert of pOSU2401 (for details, see Chapter Four) and two mutant strains were constructed by marker exchange with two of those inserts. The locations of the two insertions are illustrated in Figure 3-19. The strain containing a Tn3-HoHo1 insertion close to the original site of the Tn5 insertion has been designated PS9086 and the other strain is designated as PS9066. A Southern blot of EcoRI-digested total DNA of these two mutant strains was probed with the 1.35 kb BglII/SalI wild type fragment, in which Tn5 was inserted. Two EcoRI fragments in PS9086 showed homology to this probe. One corresponds in size (8.5 kb) to a fragment, previously shown to share homology with this probe. The other, appears to

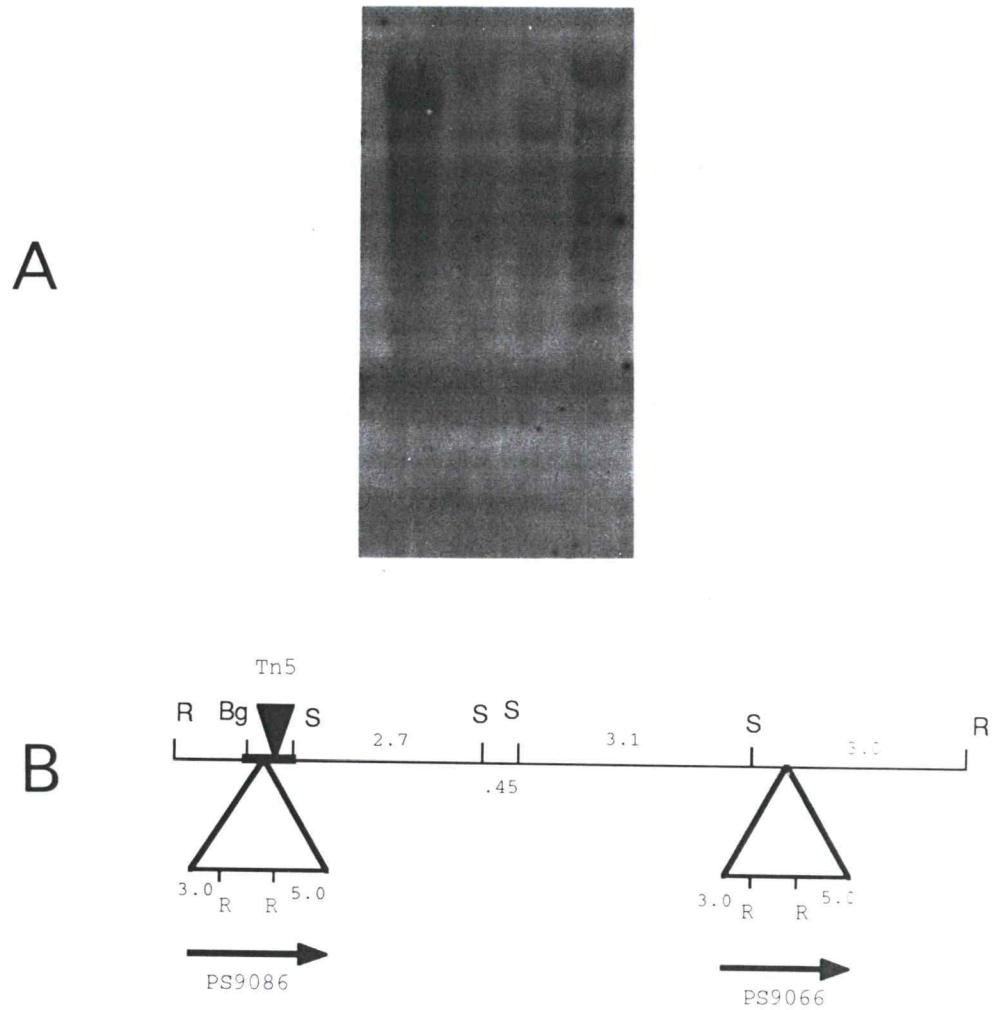


Figure 3-19. Confirmation of Tn3-HoHo1 insertion in the chromosome of R32. **A,** Southern hybridization analysis of the EcoRI-digested total genomic DNA. Lanes 1, HindIII digested Lamda DNA; 2, R32; 3, PS9086; 4, PS9066 and 5, PS9024. The probe was the ^{32}P-labelled wild type 580 bp BglII/SalI fragment from pOSU9024 and is indicated with thick bar in panel B. **B**, Location of Tn3-HoHo1 insertions in PS9086 and PS9066 are indicated on tThe 10.5 kb EcoRI-containing fragment of pOSU9024. Arrows indicate the direction of transcription of the lac operon in Tn3-HoHo1. Abbreviations: R, EcoRI; S, SalI and Bg, BglII.

be smaller in size than the 16.2 kb EcoRI fragment of PS9024 and larger than 10.5 kb EcoRI fragment of R32, and is predicted to be the 14.6 kb Tn3-HoHo1 containing fragment. The smaller 0.87 kb EcoRI fragment, as predicted from the physical map (Figure 3-19B), had either run off the gel under this condition or the signal was too weak. Two EcoRI fragments were also detected in PS9066. The size of one fragment is predicted to be 10.6 kb, which is almost identical to the 10.5 kb EcoRI fragement of R32. Another Tn3-HoHo1 containing fragment (8.0 kb) migrated with a rate similar to the 8.5 kb EcoRI fragment and formed a doublet bands. These results suggest that Tn3-HoHo1 gene replacement occurred as predicted (Figure 3-19). Tn3-HoHo1 insertions in the chromosome were further verified by a probe made from the lac gene, which is part of Tn3-HoHo1 (data not shown).

The newly constructed Tn3-HoHo1-derived mutants PS9086 and PS9066 were tested on bean leaves and pods for their ability to cause disease symptoms. The mutant PS9086 produced disease symptoms that were similar to those produced by PS9024 both in bean leaves (Figure 3-20) and pods (Figure 3-21). This is consistent with the fact that the physical location of the Tn3-HoHo1 insertion in PS9086 is very close to the original Tn5 insertion in PS9024. Interestingly, PS9066 produces a

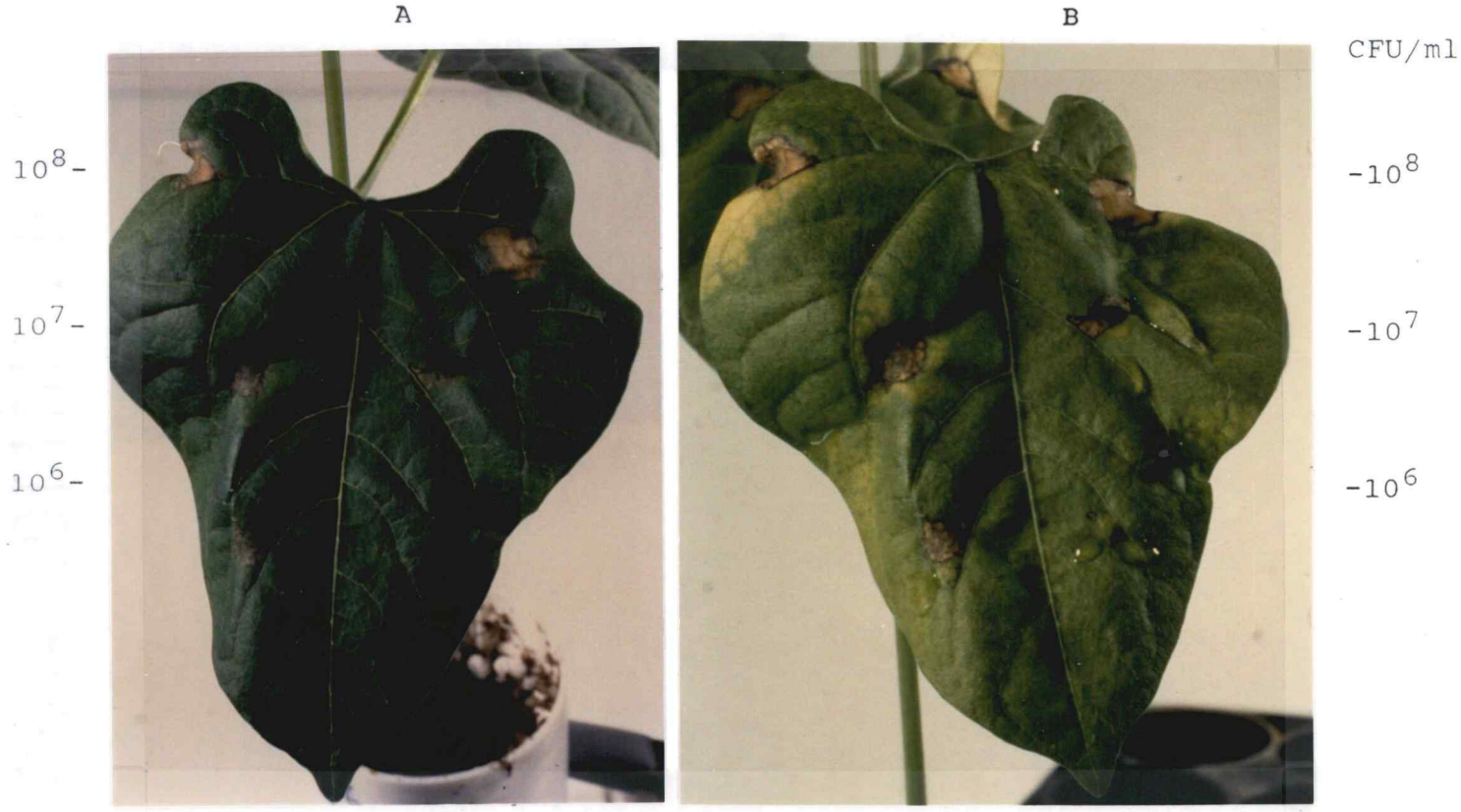


Figure 3-20. Reactions of the primary leaves of Phaseolus vulgaris cv. "Eagle" to inoculation with PS9086. Inoculum concentration from the top of the leaf to the bottom is 10^8 to 10^6 CFU/ml. R32 was inoculated on the left side of the leaf; whereas PS9086 on the right. The photograph was taken at **A**, one week after inoculation; **B**, two weeks after inoculation.

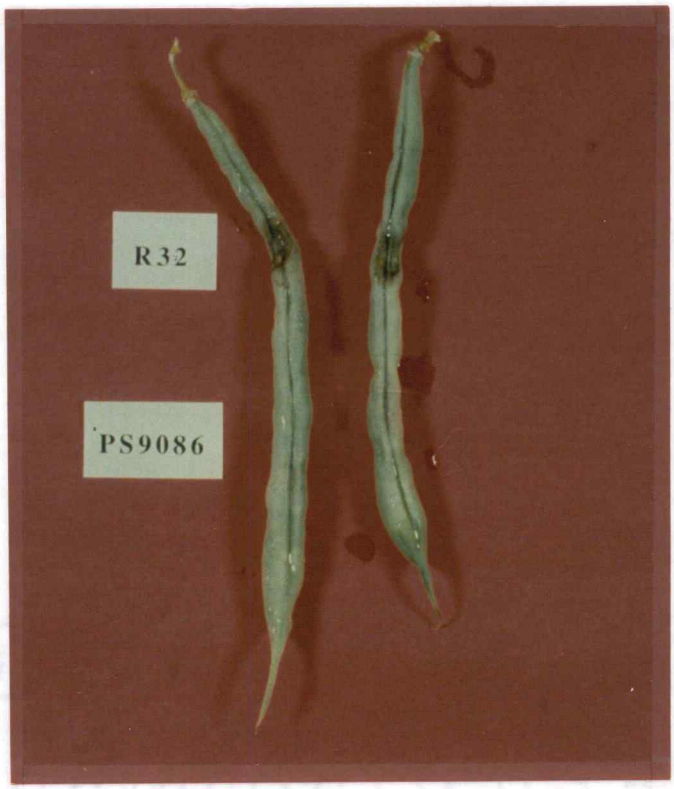


Figure 3-21. Reactions of bean pods of Phaseolus vulgaris cv. "Eagle" to inoculation with PS9086. Approximately 10^6 CFU/ml of a bacterial suspension was inoculated. The symptoms were scored at one week after inoculation.

more attenuated disease symptom than either PS9024 or PS9086 on bean leaves (Figure 3-22). Much reduced symptoms were also observed when PS9066 was inoculated into bean pods (Figure 3-23).

PS9066 was transformed with pOSU2401 to test whether this mutant can be complemented *in planta*. No restoration of disease symptoms was observed (data not shown).

DISCUSSION

The strategy of using members from a cosmid library to complement transposon-induced mutations has been successful in studies of *P. s.* pv. *syringae* (Niepold and Mills, 1987; Wills *et al.*, 1990). Cosmid pOSU2401 from the pLAFR3 genomic library of *P. s.* pv. *syringae* strain R32 contains a contiguous wild type DNA sequence (*ca.* 16 kb). *In planta* complementation of the mutant PS9024::Tn*5* with this cosmid resulted in only incomplete restoration of the disease symptoms normally produced by the wild type parental strain, R32. One possible explanation for this phenomenon is the known instability of vectors constructed from the RK2 replicon (Thomas, 1983; Niepold and Mills, 1985). However, functional complementation was not enhanced when a newly

Figure 3-22. Reactions of the primary leaves of Phaseolus vulgaris cv. "Eagle" to inoculation with PS9066. Inoculum concentration from the top of the leaf to the bottom is 10^7 to 10^5 CFU/ml for R32 inoculated on the left side of the leaf; and 10^8 to 10^6 CFU/ml for PS9066 on the right. The photograph was taken at **A**, one week after inoculation; **B**, two weeks after inoculation.

Figure 3-23. Reactions of the bean pods of Phaseolus vulgaris cv. "Eagle" to inoculation with PS9066. Inoculum concentration for R32, from left to right of the bean pods, was 10^7 (two pods on the left), 10^6 CFU/ml (middle pod) and 10^5 CFU/ml (two pods on the right); Inoculum concentration for PS9066, from the left to the right of the bean pods, was 10^8 (two pods on the left), 10^7 CFU/ml (middle pod) and 10^6 CFU/ml (two pods on the right). The symptoms were scored at one week after inoculation.

constructed, stable shuttle vector, pOSU226, was used to clone wild type DNA. It is apparent that the lack of full disease expression is not the result of instability, because approximately 90% of the cells retain pOSU226 *in planta* over a two-week period (Figure 3-11). A similar plasmid constructed with this *ori* was equally stable *in planta* (Mukhopadhyay *et al.*, 1990).

Factors unrelated to plasmid instability could also contribute to the incomplete complementation of PS9024. One possibility is the occurrence of an undefined spontaneous second site mutation that is involved in the attenuation of disease symptom development. It is entirely possible that Tn*5* insertion in PS9024 may not be directly relevant to pathogenesis. Second site mutations may arise by base changes during replication or by gross alterations of genes. It is also known that IS50 present at the termini of Tn*5* will transpose to other sites in the *Pseudomonas syringae* pv. *syringae* genome (Anderson and Mills, 1985), thereby providing a mechanism for second site mutations to occur.

One method of analyzing the cause and effect relationship between an existing transposon-induced insertion and a corresponding phenotype is to construct a mutant from the parental wild type strain by insertional mutation at the site of the original insertion. The new mutant can be tested on the host

plants to observe its effect on development of the disease symptoms. The results of this type of analysis will support the direct relevancy of the locus to pathogenesis since the physical location of the new insertion can be precisely mapped, and the symptomology of susceptible host plants can be determined and compared with the original mutant (Ruvkun and Ausubel, 1981). Using an unrelated transposon, Tn3-HoHo1, a site less than 300 bp from the original site of Tn5 insertion in PS9024 was mutated and the mutant (PS9086) was essentially similar to PS9024. Another Tn3-HoHo1 insertion (PS9066) approximately 6.5 kb away from the original site of Tn5 insertion even more effectively reduces disease symptoms on the bean plants. These results clearly suggests that this region of the bacterial chromosome may encode multiple pathogenicity-related determinants. Gene clusters or gene operons involved in the process of pathogenesis have been previously described for pathovars of P. syringae (Mukhopadhyay and Mills, 1988; Ma et al., 1991). Given the relative large distance (6.5 kb) between mutations in PS9086 and PS9066, this region appears to have properties of either a large operon or a gene cluster.

The growth deficiency of the mutant PS9024 both in planta and in culture were partially restored with pOSU2401 and pOSU4201, providing additional evidence

that sequences from this locus contribute to viability and, therefore, disease expression by strain R32.

Incomplete complementation of mutations in other bacteria, as manifested by incomplete phenotypic restoration, have been numerously reported (Jouanin et al., 1987; Spena et al., 1987; Schmulling et al., 1988; Panouplous, 1990). One specific example of incomplete complementation comes from the study of genes involved in ornithine carbomyltransferase (OCTase) specific toxin (phyaseolotoxin) production in P. s. pv. phaseolicola, the causal agent of the halo blight of bean Phaseolus vulgaris (Peet et al., 1986). All Tn5-derived Tox^- mutants were restored by two or more of the wild type recombinant plasmids. Toxin production was, however, restored at various levels, ranging from only partial restoration in some mutant-plasmid combinations to greater than wild type levels in one case. Restoration of the ability to cause local and systemic chlorosis was also variable, depending upon the mutant-plasmid combination. Among forty-two different mutant-plasmid combinations, only three combinations appeared to produce systemic chlorosis and stunting symptoms as severe as the wild type strain. The remaining thirty-nine mutant-plasmid combinations produced less severe systemic chlorosis and stunting symptoms. Interestingly, two plasmids, pRCP4 and pRCP17 both

carrying a gene(s) that encodes OCTase, were shown to have the largest variability in the restoration of the toxin production, ranging from no restoration to a toxin production greater than wild type. A clustering of the genes was hypothesized as the explanation for the pattern of restoration of toxin production observed among the mutants containing various regions of the chromosome in the plasmids. Further study has revealed that the genes involved in phaseolotoxin production map to a single KpnI fragment of ca. 22 kb (Peet et al., 1986).

Incomplete complementation has also been reported in transgenic plants harboring a set of rol genes (rol A-C genes) derived from the transferable T-DNA of the Ri plasmid of Agrobacterium rhizogenes. Rol mutants show pathological abnormalities known as hr ("hairy-root") syndrome (Tepfer, 1984). Although each of the three rol genes are independently able to promote root formation in tobacco, the full hr syndrome is established only in transgenic plants expressing the combined activities of rol A, B and C loci of the T1-DNA of Ri plasmid A4 (Jouanin et al., 1987; Spena et al., 1987).

It is unclear why restoration of any of the missing protein bands in PS9024 were not restored in complemention tests. One possibility could be due to a low level of expression of these proteins from the

cloned fragment, and their subsequent inability to be detected when using the Coomassie blue staining method. Alternatively, this region may be composed of a cluster of genes or an operon whose expression is regulated. Expression of the genes may also be under the control of elements that are cis-acting and absent from the cloned DNA.

SUMMARY

1. In complementation tests of the mutant PS9024, wild-type DNA sequences cloned into the cosmid pLAFR3 and into the shuttle vector pOSU226 only partially restored disease symptoms in susceptible host bean plants.

2. Restoration of growth in the mutant strain, PS9024, was observed in both the complete nutrient MaNY medium and partially in planta when pOSU4201 is provided in trans.

3. Using the Coomassie staining method, the complementing strains showed no apparent restoration of the protein band that is absent from PS9024 or the production of new protein bands. This could be due to a low level of expression of this protein. Alternatively,

the expression of this protein may require transcription of a large polycistronic operon. Other factor such as *cis*-acting elements that control the expression of this protein, also can not be excluded.

4. Reconstruction of the mutant directly supports the relevance of the original Tn*5* mutation to pathogenicity. The more reduced disease symptoms resulting from inoculation of bean plants with PS9066 suggests that a gene cluster or an operon may be involved in the process of pathogenesis for this pathogen.

REFERENCES

Anderson D.M. and D. Mills. 1985. The use of transposon mutagenesis in the isolation of nutritional and virulence mutants in two pathovars of Pseudomonas syringae. Phytopathology 75:104-108.

Bertoni, G. and D. Mills. 1987. A simple method to monitor growth of bacteria populations in leaf tissue. Phytopathology. 77:832-835.

Birnboine, and Doly. 1979. Mini-scale plasmid DNA isolation. NAR 7(15):

Ditta, G., S. Stanfield, D. Corbin and D.R. Helinski. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of Rhizobium meliloti. Proc. Natl. Acad. Sci. USA. 77(12):7347-7351.

Ehrenshaft M. and D. Mills. 1985. Construction of a cosmid clone library of Pseudomonas syringae pv. phaseolicola and isolation of genes by functional complementation. Appl. Environ. Microbiol. 50:169-171.

Figurski, D.H., R.J. Meyer and D.R. Helinski. 1979. J. Mol. Biol. 133:295-318.

Figurski D.H. and D.R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA. 76(4):1648-1652.

Friedman, A.M., S.R. Long, S.E. Brown, W.J. Buikema and F.M Ausubel. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of Rhizobium mutants. Gene 18:289-296.

Klement, Z., D. Rodolph and D.C. Sands. 1990. Methods in phytobacteriology. Akademiai Kiado, Budapest. p191-198.

Ma, S.W., V.L. Morris and D.A. Cuppels. 1991. Characterization of a DNA region required for production of the phytotoxin coronatine by P. s. pv. tomato MPMI. 4(1):69-74.

Niepold, F. and D. Mills. 1985. Cosmid instability in Pseudomonas syringae pathovars: evidence for directed excision of a cloned sequence. In: E.L. Clverolo, A. Collmer, R.E. Davis, and A.G. Gillaspie (eds.): "Plant Pathogenic Bacteria", Proc. of the Sixth Int. Conf. on

Plant Pathogenic Bacteria, Maryland, Martinus Nijhhoff Publisher. p448-452.

Niepold, F., D. Anderson, and D. Mills. 1985. Cloning determinants of pathogenesis from Pseudomonas syringae pathovar syringae. Proc. Natl. Acad. Sci. USA 82:406-410.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982 Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory. p387-389.

Mukhopadhyay, P., M. Mukhopadhyay and D. Mills. 1990. Construction of a stable shuttle vector for high-frequency transformation in P. s. pv. syringae. J. Bacteriol. 172(1):477-480.

Old, R.W. and S.B. Primrose. 1981. Principles of gene manipulation: An introduction to genetic engineering. Second edition. Studies in microbiology volume 2. University of California Press. p29.

Peet, R.C., P.B. Lindgren, D.K. Willis and N.J. Panopoulos. 1986. Identification and cloning of genes involved in phaseolotoxin production by Pseudomonas syringae pv. phaseolicola. J. Bacteriol. 166(3):1096-1105.

Ruvkun, G.B. and F.M. Ausubel. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature. 289:85-88.

Stachel, S.E., G. An, C. Flores and E.W. Nester. 1985. A Tn3 lacZ transposon for the random generation of ß-galactosidase gene fusion: Application to the analysis of gene expression in Agrobacterium. EMBO J. 4:891-898.

Thomas, C.M. 1983. Instability of a high-copy-mutant of a miniplasmid derived from broad host range IncP plasmid RK2. Plasmid 10:184-195.

Jouanin, L., F. Vilaine, J. Tourneur, C. Tourneur, V. Pautot, J.F. Muller and M. Caboche. 1987. Plant Sci. 53:53-63.

Spena, A., T. Schmulling, C. Koncz and J. Schell. 1987. EMBO J. 6:3891-3899.

Staskawicz, B., D. Dahlbeck, N. Keen and C. Napoli. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of P. s. pv. glycinea. J. Bacteriol. 169:5789-5794.

Tepfer, D. 1984. Transformation of several species of higher plants by Agrobacterium rhizogenes: sexual transmission of the transformed genotype and phenotype. Cell. 37:959-967.

Zhou, C., Y. Yang and A.Y. Jong. 1990. Mini-prep in ten minutes. BioFeedback. 8(2):172-173.

ORGANIZATION AND CHARACTERIZATION OF THE WILD TYPE DNA SEQUENCE IN THE PATHOGENICITY-RELATED LOCUS OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE*

CHAPTER FOUR

INTRODUCTION

Complementation is a method used to study the cause and effect relationship between the existing transposon insertion in the bacterial chromosome and the phenotype of reduced pathogenicity Saturation mutagenesis, an approach of using a transposon to mutagenize cloned wild type sequences that subsequently can be marker exchanged into the wild type chromosome, provides information on the boundary of the functional domain of the complementation group. Furthermore, the cassette mutagenesis technique, which combines a transposon and a promoterless reporter gene, has advanced this approach by providing a readily scorable method for the detection of the mutation and the direction of transcription of the gene. Tn*3*-HoHo1 is a such cassette (Stachel *et al.*, 1985). It contains transposon Tn*3* and a promoter-less β-galactosidase reporter gene which can be used to develop

a functional map of a gene by the marker exchange method and identify potential indigenous promoter activities by activation of the β-galactosidase reporter gene.

Gene sequencing is another method commonly used to gain insight into the structure of genes. The sequence of a gene provides information to deduce open reading frame (ORF) sequences, which may provide clues as to the function of the gene. The sequences of approximately one million genes, many with known functions, have been entered into data banks, and these sequences provide the possibility of determining a putative function for a sequenced gene.

The task of this chapter is to characterize the wild type sequence cloned in pOSU2401. It has been shown to partially restore the ability of the mutant PS9024 to incite disease symptoms. First, using Tn*3*-HoHo1 mutagenesis, a series of mutations was made and possible indigenous promoters were tested for expression of β-galactosidase. Second, the organization and structural features of the region identified by Tn*5*-insertion in PS9024 were characterized by Southern analysis. Third, the distribution of homologous DNA sequences from this region among other closely related pathovars of *Pseudomonas* was studied. Finally, the region identified by Tn*5* insertion in PS9024 was partially sequenced, allowing speculation about the

possible function(s) of this locus based on information obtained from a search of the gene data bank. Finally the number and size of proteins whose synthesis was blocked by Tn5 insertion in PS9024 were studied.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

Bacterial strains, plasmids and media have been described in Materials and Methods section of Chapter Two. Additional bacterial strains and plasmid used in the studies described in this chapter are summarized in Table 4-1.

DNA isolation and manipulation

The procedures for isolation of both plasmid and total DNA from E. coli and Pseudomonas have been described in the Materials and Methods section of Chapter Two. Southern blot analysis was performed according to the procedure of Maniatis et al., (1982). The hexamer labelling method (Feinberg and Vogelstein, 1983) was used to make ^{32}P labelled DNA probes using a commercial oligolabelling kit (cat# 27-9250-01)

Table 4-1. Bacterial Strains and Plamids.

Strains	Characters	Source or References
***P. s.* pv. *syringae*:**		
Clone 19	pOSU2401::Tn*3*-HoHo1, lac$^+$ insert in 1.45 kb *Sal*I fragment	This study
Clones 114, 115, 75 and 83	pOSU2401::Tn*3*-HoHo1, lac$^+$ insert in 2.25 kb *Sal*I fragment	same as above
Clone 17, 86	pOSU2401::Tn*3*-HoHo1, lac$^+$ insert in 2.35 kb *Sal*I fragment	same as above
Clone 119	pOSU2401::Tn*3*-HoHo1, lac$^+$ insert in 2.7 kb *Sal*I fragment	same as above
Clone 53	pOSU2401::Tn*3*-HoHo1, lac$^-$ insert in 2.7 kb *Sal*I fragment	same as above
Clone 66, 45	pOSU2401::Tn*3*-HoHo1,lac$^+$ insert in 3.0 kb *Sal*I fragment	same as above
Clone 113, 100 and 104	pOSU2401::Tn*3*-HoHo1, lac$^+$ insert in 3.0 kb *Sal*I fragment lac activity expressed both in *Pseudomonas* and *E. coli*	same as above
Clone 62	pOSU2401::Tn*3*-HoHo1, lac$^-$ insert in 3.0 kb *Sal*I fragment	same as above

These are the clones used in marker exchange study.

purchased from Pharmacia LKB Biotechnology Co (Alameda, CA).

Tn3-HoHo1 mutagenesis of pOSU2401

The cosmid clone, pOSU2401, containing a 16 kb insert was subjected to Tn3-HoHo1 mutagenesis (Figure 4-1) according to the method of Stachel et al., (1985), which was described in detail in Materials and Methods section of Chapter Three.

Assays for expression of β-galactosidase in pOSU2401::Tn3-HoHo1 fusions and detection of promoter activities in E. coli and in Pseudomonas

Indigenous promoter activity was identified by detecting the expression of β-galactosidase by E. coli colonies on LB agar plates, and by Pseudomonas colonies on King's medium B agar plates containing appropriate antibiotics and 0.1% X-gal.

Isolation of total RNA from P. s. pv. syringae cells grown in culture medium and in bean pods

Total RNA was isolated from P. s. pv. syringae cells grown either in LB medium or in bean pods following a procedure for extraction from Gram-negative bacteria (Ausubel et al., 1989). The method of obtaining cells from infected bean pods was as follows: a bacterial suspension (750 μl) at 10^6 CFU/ml of wild type strain R32 and the pathogenicity-attenuated strain, PS9024, were injected from a hyperdermic needle into the

cavity of two-week old pods. The bacterial cells were recovered three days after inoculation by slicing the pods into sections to expose the cavities and actively agitating the sections in a 250 ml flask containing K-buffer (pH 7.0). The cells were collected by centrifugation at 8,000 rpm using a SW40 rotor.

SDS-PAGE analysis

An SDS-PAGE gel with 5% acrylamide stacking and 10% running gel was used in these experiments to resolve the proteins in the range of 60 to 250 kD using a mini-gel apparatus (Hoffer, model SE250). Approximately 5 μg protein of each sample was loaded in each lane and electrophoresed at 20 mA for 1 hour and 10 minutes.

DNA sequencing and databank searches using the IntelliGenetics programs

DNA sequencing was performed by using the Gene Sequencer (Applied Biosystems, Model 373A) available through the Central Service Lab of Center for Gene Research and Biotechnology at Oregon State University.

Computer analysis of the nucleotide sequence was performed with the IntelliGenetics Suite Programs (IntelliGenetics, Inc.). Homologous DNA and amino acid sequence searches of the databank, including EMBL25, GenBank66, N-Geneseq 2.0, N-PSDB, PIR26, A-Geneseq 2.0 and Swiss-Port15 were conducted using the FASTDB program for overall sequence homology and IFIND for highly

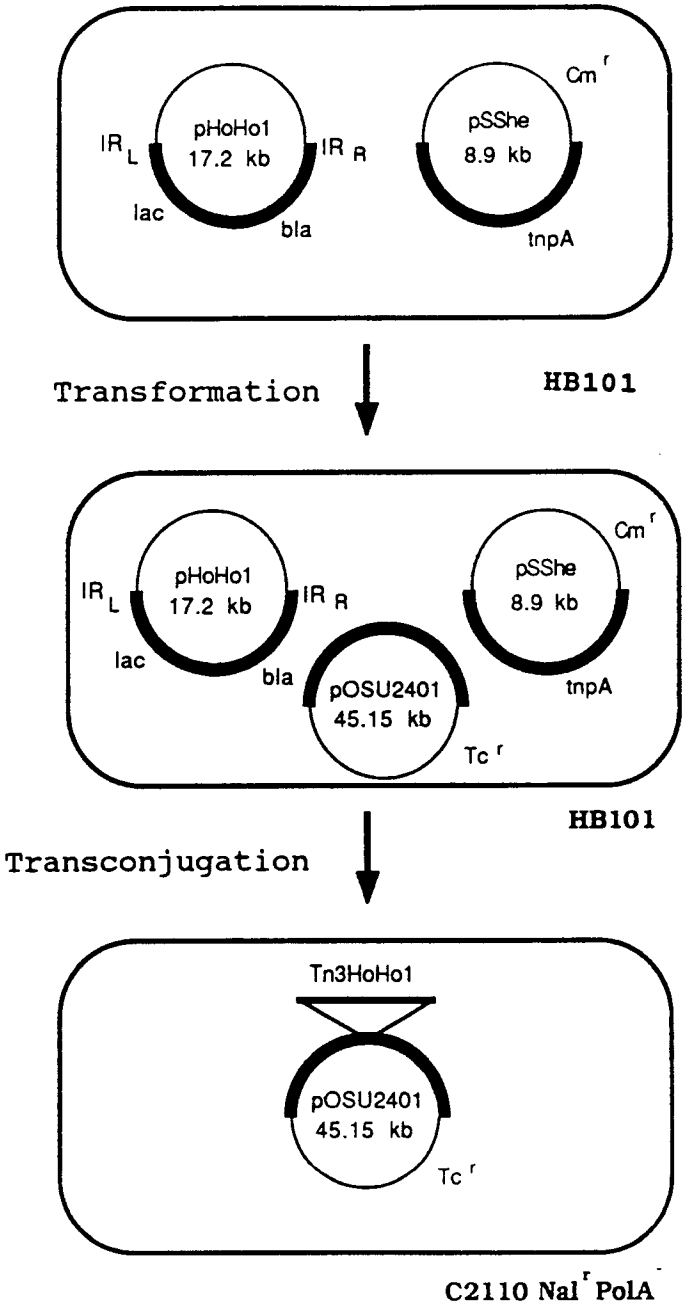


Figure 4-1. Tn3-HoHo1 mutagenesis. The detailed procedure of this method was described in Materials and Methods section of Chapter Three. Abbreviations: IR, inverted repeat; bla, β-lactamase for ampicilin resistance; Tc, tetracycline; Amp, ampicilin; Nal, Nalidixic acid; Cm, chloramphenicol; tnpA, transposase. ^{r}, resistant; ^{s}, sensitive.

conserved domains. Repeated DNA sequences within the sequenced region were searched using the SEQANA program. General sequence analysis and editing for DNA alignment and identification of restriction sites were performed using GENEALN and GENED programs, respectively.

RESULTS

Isolation and characterization of Tn*3*-HoHo1 insertions in pOSU2401

The cosmid pOSU2401, which contains a 16 kb contiguous sequence of genomic DNA from the wild type strain, R32, was subjected to Tn*3*-HoHo1 mutagenesis in *E. coli* HB101 to generate transcriptional fusions of a promoterless *lac* gene and the pathogenicity-related locus. Approximately 120 insertions were obtained, and sixty independent Tn*3*-HoHo1 insertions have been mapped within the insert of pOSU2401 (Figure 4-2). The location of each insertion was determined by restriction endonuclease mapping. Indigenous promoter activities, as measured by the *lac*$^+$ phenotype conferred upon pOSU2401::Tn*3*-HoHo1 fusions, were assayed both in the parental *Pseudomonas* strain, R32, and in *E. coli* strain

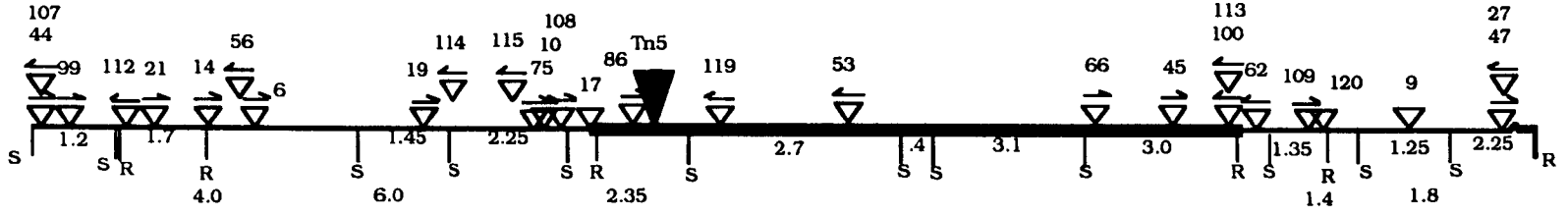


Figure 4-2. Map of Tn3-HoHo1 insertions and orientation of pOSU2401. The 10.5 kb EcoRI-containing fragment is presented by the thick bar. The site of Tn5 insertion is indicated by the triangle. The vertical lines represent the site of Tn3-HoHo1 insertion, and the corresponding clones are indicated by numbers. Direction of transcription of the lac operon, which is resided within Tn3-HoHo1 and driven by the indigenous promoter, is demonstrated using arrows.

Promoter Activities:

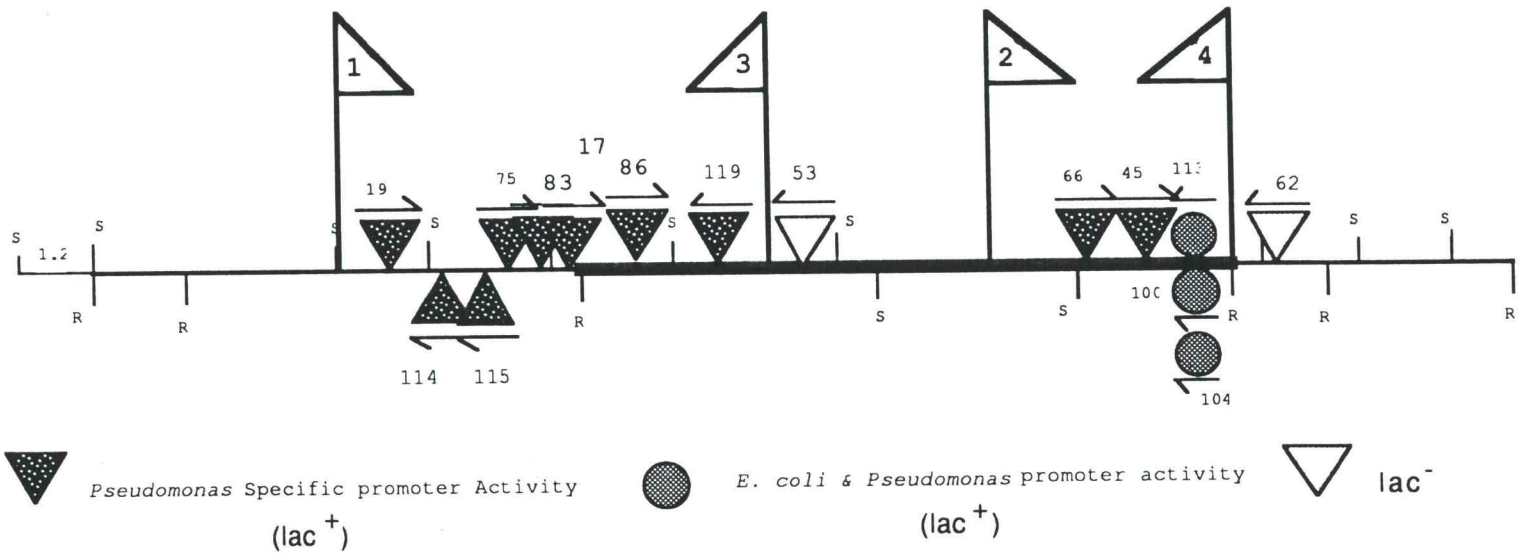


Figure 4-3. Dectection of indigenous promoter activities in the insert of pOSU2401. Tn3-HoHo1 contains a promoter-less lac operon. Indigenous promoter activity was measured by expression of b-galactosidase in the pOSU2401::Tn3-HoHo1 fusions. The 10.5 kb EcoRI-containing fragment is presented by the thick bar. The triangles represent the site of Tn3-HoHo1 insertion, and the corresponding clones are indicated by numbers. Direction of the promoters, i.e., transcription of the lac operon, is demonstrated using arrows. The possible location and direction of transcription of the indigenous promoters are indicated by the flags.

DH5α. Of eighteen unique insertions tested, two kinds of *lac*$^+$ phenotypes have been identified (Figure 4-3). One *lac*$^+$ phenotype was expressed only in *Pseudomonas* (*e.g.*, clone 86). The other *lac*$^+$ phenotype was detected both in *Pseudomonas* and in *E. coli* (*e.g.*, clones 100, 104 and 113). To test whether the *lac*$^+$ phenotype observed in clones 100, 104 and 113 was expressed from the *lac* promoter located within pLAFR3 sequences immediately upstream of Tn*3*-HoHo1 in these three fusions, clones in which Tn*3*-HoHo1 insertion was in the same orientation with the *lac* promoter were tested both in R32 and DH5α. Four clones of this type (53, 62, 114 and 115) had the *lac*$^-$ phenotype in DH5α. However, clones 114 and 115 showed the *lac*$^+$ phenotype in R32. The possibility that the lac promoter within the pLAFR3 vector might be active in R32 and PS9024 was also tested. As a control, the lac phenotype was also tested in *E. coli* strain, DH5α(pLAFR3). As expected, the *lac*$^+$ phenotype was observed. However, the *lac*$^+$ phenotype was not detected in either R32(pLAFR3) or PS9024(pLAFR3) (data not shown).

At least four regions identified by pOSU2401::Tn*3*-HoHo1 fusions showed promoter activities when scored by the expression of β-galactosidase (see Figure 4-3). One promoter, designated promoter-1, appears to map to the left of the insert of clone 19, and is responsible for

rightward transcription. This promoter may confer the *lac*$^+$ phenotypes to clones 19, 75, 83, 17 and 86. However, since this region spans more than 5 kb, it is possible other promoters may also exist within this region which transcribe in the rightward direction. In the same orientation as the putative promoter-1, a second promoter, promoter-2, maps to the left of the insertion in clone 66 and appears to confer the *lac*$^+$ phenotype to clones 66, 45 and 109. Expression of β-galactosidase driven by the same promoter in these three clones may also be indicated by the gradual decrease in intensity of the blue color shown on the X-gal-containing KB medium as a function of their relative distance from the promoter (Figure 4-4). Conversely, the presence of additional promoters in this region whose promoter activaties vary cannot be discounted. Two possible promoters oriented in the opposite direction were also detected by Tn*3*-HoHo1 insertions. One of them, promoter-3, maps between clones 119 and 53 since no β-galactosidase expression was detected in clone 53. The fourth promoter, promoter-4, which is expressed both in *Pseudomonas* and *E. coli*, maps to the right of clones of 113, 100 and 104 since no β-galactosidase activity was detected in clone 62 which is approximately 200 to 300 bp away from clone 104.

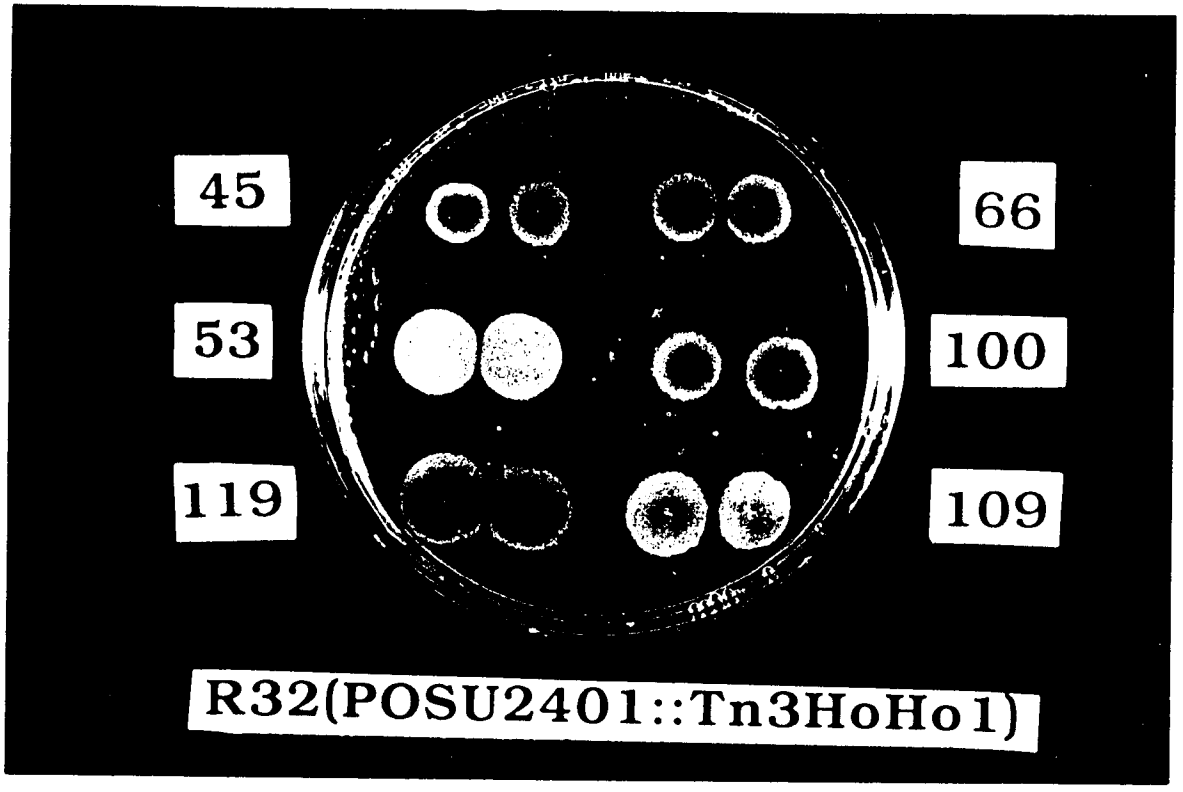


Figure 4-4. Bioassays for expression of β-galactosidase of the pOSU2401::Tn3-HoHo1 fusions in P. s. pv. syringae R32. The colones are R32 transformants containing pOSU2401::Tn3-HoHo1. The lac phenotype of these colonies was tested using King's medium B agar plate containing 1% Xgal. The lac^+ phenotype is scored by the blue color.

Southern hybridization analysis and detection of repetitive sequences in pOSU2401 and pOSU9024

It was previously shown by Southern hybridization analysis, using a radiolabelled DNA probe from the insert of pOSU9024, that sequences from this insert are absent from some P. s. pv. syringae strains which cause brown spot disease (Mills and Niepold, 1987; Zhao and Mills, 1989). Studies were, therefore, performed to further characterize the distribution of DNA from within this region. Subfragments of the Tn5-containing insert of pOSU9024 (16.2 kb) were cloned into pUC19 and probes constructed of these subfragments were used to hybridize to various digestions of pOSU9024 and pOSU2401. Unexpectedly, the 3.0 kb SalI fragment (probe 1) was shown to hybridize to 5 of 6 fragments produced by digesting pOSU9024 DNA with SalI/EcoRI (Figure 4-5A). No homology was detected with the Tn5-containing 2.8 kb SalI fragment, which contains a portion of Tn5 and about 280 bp of Pseudomonas sequence located adjacent to Tn5 on the right side. In a reciprocal hybridization experiment, probe 2, a 630 bp XhoI/SalI fragment from the 2.8 kb SalI fragment which also contains the 280 bp Pseudomonas DNA, confirmed the first probing experiment and was shown to have no homology with probe 1 (Figure 4-5B). However, probe 2 shares homology with two other

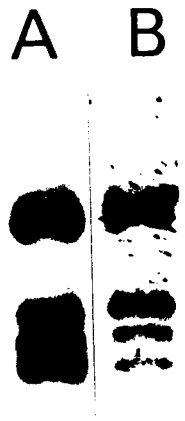


Figure 4-5. Demonstration of repetitive sequences within the insert of pOSU9024. DNA in both lanes was pOSU9024 digested with EcoRI/SalI. The ^{32}P-labelled DNA used to probe lane A is the pOSU9024 insert and for lane B, the 3.0 kb SalI/EcoRI subclone from pOSU9024 (probe 1).

SalI fragments within the Tn5-containing EcoRI fragment (see Figure 4-6). Homology with this probe was further defined and shown to reside within a BglII/SalI fragment (ca. 700 bp) of the 3.1 kb SalI fragment and with an adjacent 450 bp SalI fragment. These probes showed consistent hybridization profiles with the insert of pOSU9024 and the wild type EcoRI fragment in cosmid pOSU2401. No homology was detected beyond the Tn5-containing EcoRI region when using probe 2 to hybridize the wild type cosmid pOSU2401. Homology with an adjacent 8.5 kb EcoRI fragment was, however, observed when using probe 3, made from the 1.35 kb EcoRI/SalI fragment (Figure 4-7). Homology with this 8.5 kb EcoRI fragment was subsequently determined to be with sequences within the 2.25 kb SalI fragment. The results of Southern hybridization to pOSU9024 and pOSU2401 are summarized in Figure 4-6 and Figure 4-7.

Distribution of sequences from the Tn5-containing EcoRI fragment of PS9024 among other strains of Pseudomonas

DNA sequence distribution of the pathogenicity-related Tn5-containing EcoRI fragment from PS9024 was shown to be limited only to the pathovars of Pseudomonas as described in Chapter Two. DNA homology with related strains of Pseudomonas pathovars was further

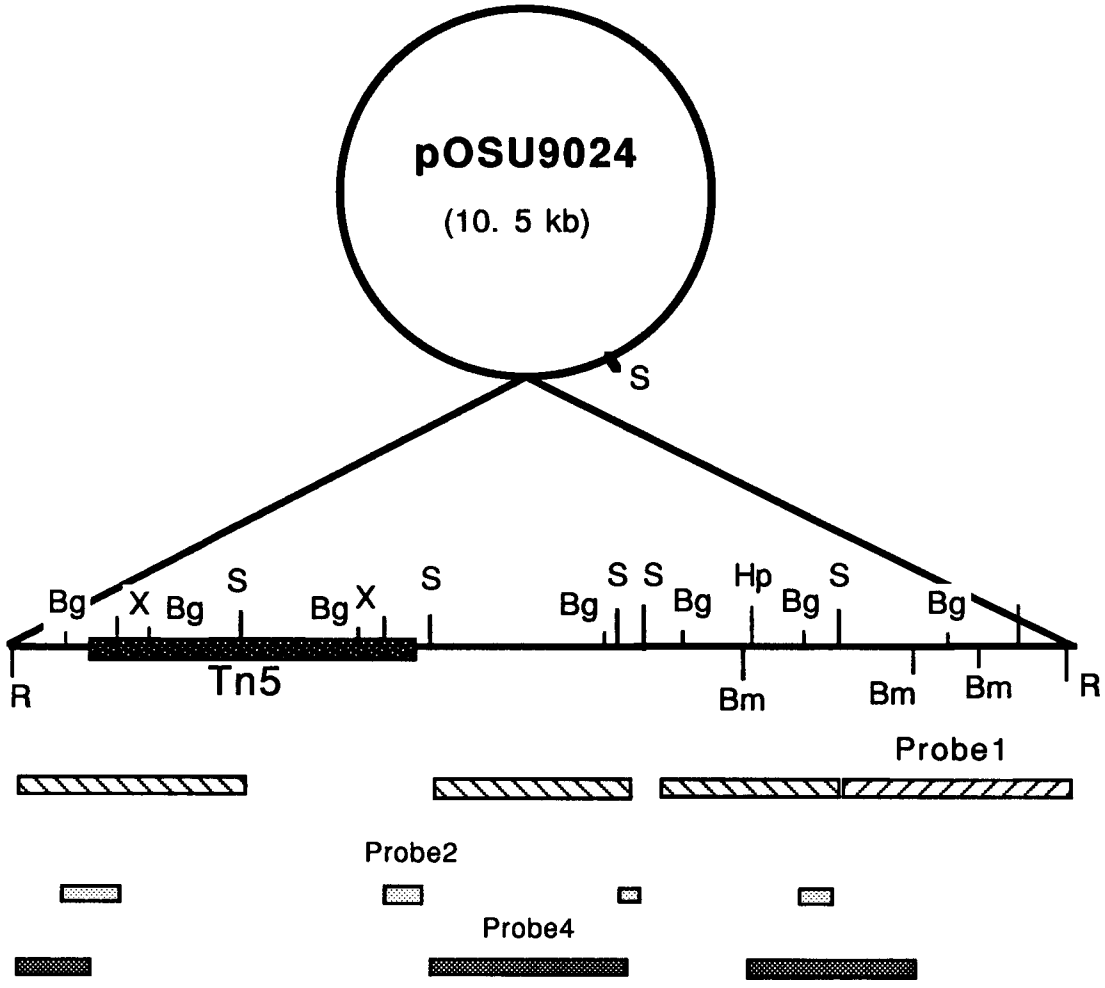


Figure 4-6. Summary of the distribution of repetitive sequences within pOSU9024. Fragments used to make probes are identified as probe 1, 2 and 4. The additional regions that share homology with these probes are identified by bars of similar type.

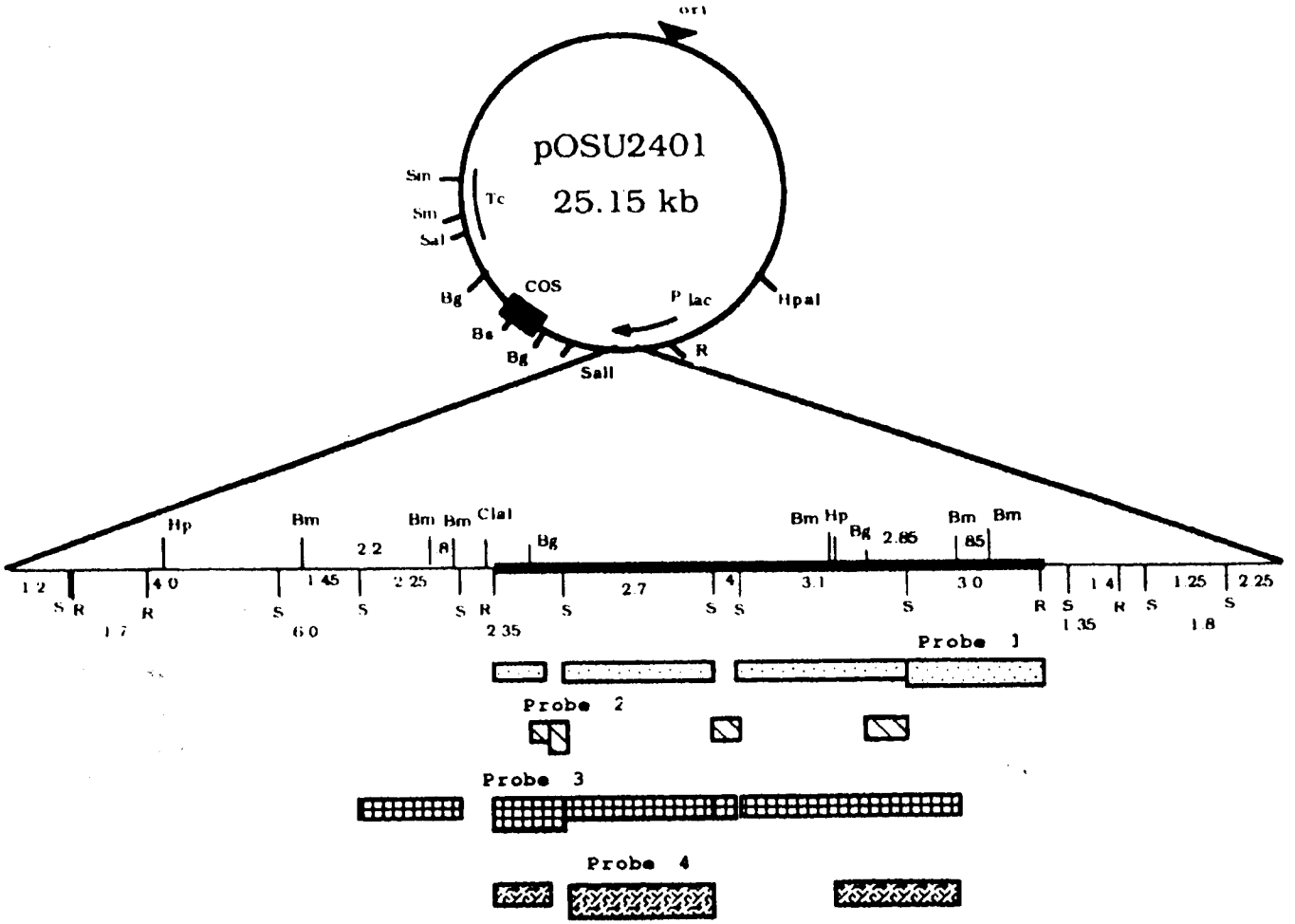
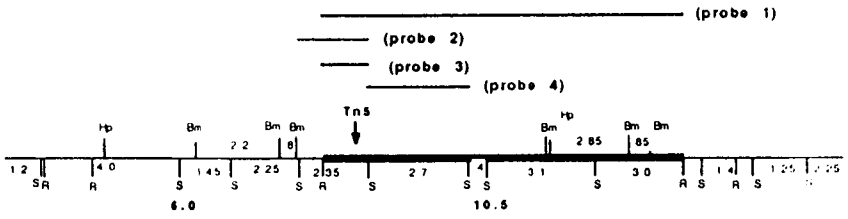


Figure 4-7 Summary of the distribution of repetitive sequences within pOSU2401. Fragments used to make probes are identified by the bars shown as probe 1, 2, 3 and 4. The additional regions that share homology with these probes are identified by bars of similar type.

characterized using probes constructed of subcloned regions of the Tn5-containing EcoRI fragment of PS9024. The results of Southern hybridizations using these probes are summarized in Table 4-2. A probe made from the Tn5-containing EcoRI fragment of pOSU9024 (probe 1) was shown to hybridize to the parental wild type strain, R32, as expected, and to an isolate of bean, 11/81, and a wheat isolate of pathovars of P. syringae. However, the probe had no homology with strain J900, a pathogenic strain of bean which causes brown spot disease. Barely detectable homology was observed with strain Y30, although no homology was detected using the same probe in a previous study (Mills and Niepold, 1987). When using a 2.35 kb SalI fragment that contains the site where Tn5 is inserted in PS9024 as the probe (probe 2), a similar pattern of DNA homology was observed with all strains except strain J900 of pathovar syringae and LR700 of pathovar phaseolicola, which showed homology with this probe. Strong homology was observed between probe 3 (1.35 kb EcoRI/SalI fragment) and DNA from all of the syringae pathovars. This probe is constructed from an internal fragment from probe 2 and contains the site of Tn5 insertion (Figure 4-8). Of particular interest was the observation that probe 4, made from the 2.7 kb fragment adjacent to the site of Tn5 insertion, showed homology only with the parental strain R32 and



P. syringae Pathovars / Probes	syringae					phaseolicola	philadelphia		marginalis	tomato
	R32	J900	Y30	11/81	"Wheat"	LR700	Isolate B	Isolate D	LR900	9796-36
pOSU 9024 (10.5 Kb EcoRI) (probe 1)	+	—	+/—	+	+	—	+	—	+	+/—
2.35 Kb SalI (probe 2)	+	+	+/—	+	+	+	+	—	+/—	+
1.35 Kb EcoRI (probe 3)	+	+	+	+	+	+/—	+	—	+/—	—
2.7 Kb SalI (Probe 4)	+	—	—	+	—	—	—	—	—	—

Table 4-2. DNA sequence distribution of regions from the 10.5 kb EcoRI fragment from pOSU9024 and pOSU2401 among P. syringae strains. Symbles "+" indicates positive signal to the probe; "-", no homology and "+/-", barely detectable homology.

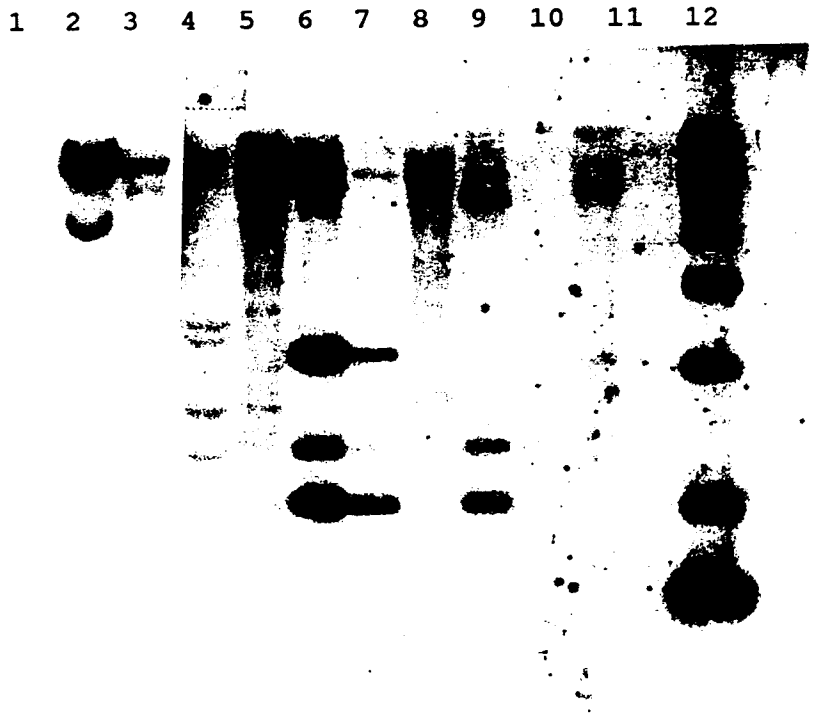


Figure 4-8 Demonstration of DNA sequences homologous to other strains of Pseudomonas syringae. The probe used was a ^{32}P-labelled 1.35 kb EcoRI/SalI fragment where Tn5 is inserted in PS9024. All DNAs were digested with EcoRI. Lane 1 contains pOSU2401; Lanes 2-6 are pathovars syringae strains. These are in the order: R32, J900, Y30, 11/81 and wheat isolate. Lanes 7 is strain LR700 of pathovar phaseolicola; Lanes 8 and 9 are isolates B and D of pathovar philadelphia. Lane 10, strain LR900 of pathovar marginalis and lane 11 is strain 9796-36 of pathovar tomato. Lane 12 is 1 kb ladder. Blot of pOSU2401 and R32 were exposed for two hour and the other lanes were exposed for 20 hours.

strain 11/81 (Figure 4-9). No homology was detected with other pathovars including three strains of pathovar syringae.

Nucleotide sequence analysis of the region identified by Tn5 insertion

The strategy and methods used for sequencing the region of Tn5 insertion in PS9024 are illustrated in Figure 4-10. The nucleotide sequence of a 580 bp BglII/SalI fragment, which contains the site of Tn5 insertion in PS9024, is presented in Figure 4-11. A search for homologous gene sequences was conducted with both strands of this region as query sequences using one of the IntelliGenetics Suite Program, FASTDB. All the up-to-date entries in the in EMBL25, GenBank66, N-Geneseq 2.0 and N-PSDB data banks were searched. The ten most homologous sequences, which were ranked by optimized scores, are summarized in Table 4-3. All of the ten gene sequences are from prokaryotes and the nucleotide identity ranges from 57.1% to 64.2%. Genes homologous to this sequence can be either chromosomal (e.g., arcB gene) or plasmid (e.g., aadB gene) in origin. In addition, the gene on Tn4000 that encodes 2"-aminoglycoside-nucleotidyl-transferease (aadB) is found both in the chromosome of Klebsiella pneumoniae

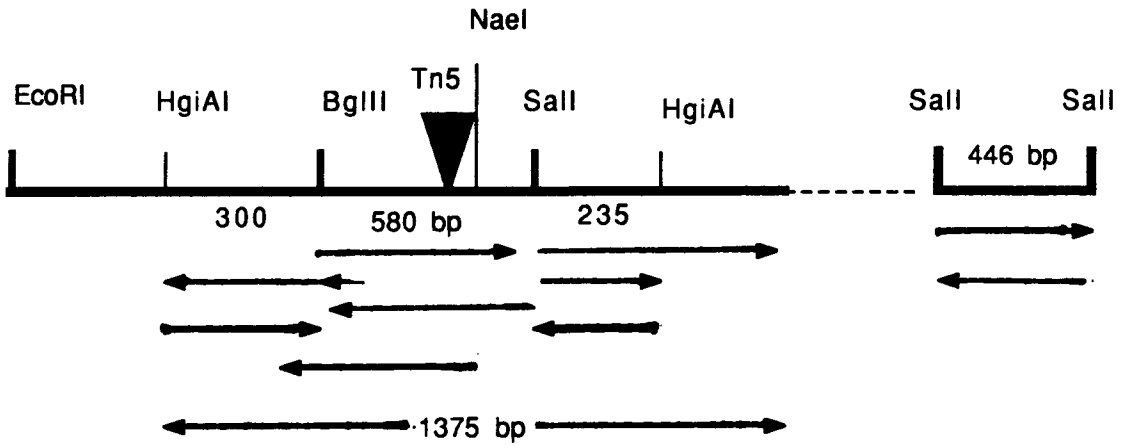


Figure 4-10. Strategy for sequencing the DNA region where Tn5 inserted. DNA sequencing was performed using the Gene Sequencer (Applied Biosystems, Model 373A). Two regions were sequenced. One is the region of Tn5 insertion and the other is a 446 bp region that was shown to share homology with this region. The site of Tn5 insertion is indicated by triangle. Arrows indicate the direction of sequencing. All subclones used in sequencing were cloned in pUC19.

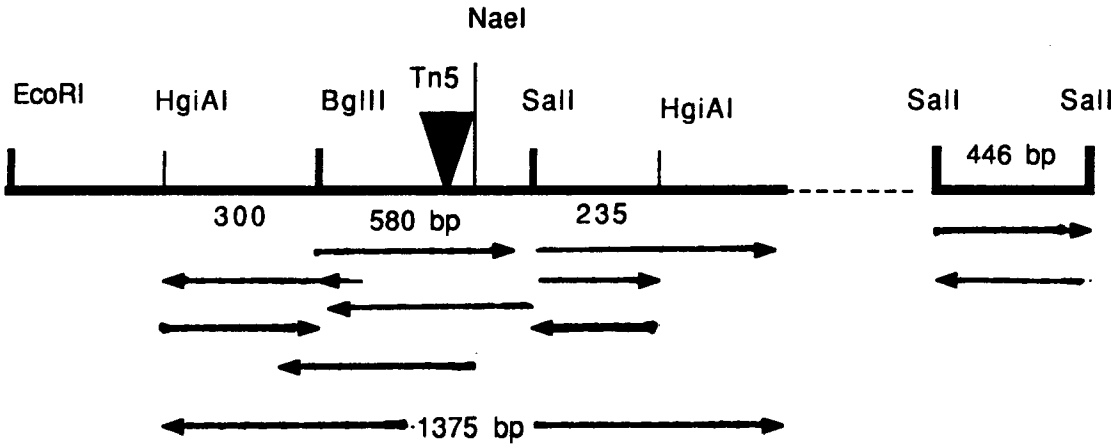


Figure 4-10. Strategy for sequencing the DNA region where Tn5 inserted. DNA sequencing was performed using the Gene Sequencer (Applied Biosystems, Model 373A). Two regions were sequenced. One is the region of Tn5 insertion and the other is a 446 bp region that was shown to share homology with this region. The site of Tn5 insertion is indicated by triangle. Arrows indicate the direction of sequencing. All subclones used in sequencing were cloned in pUC19.

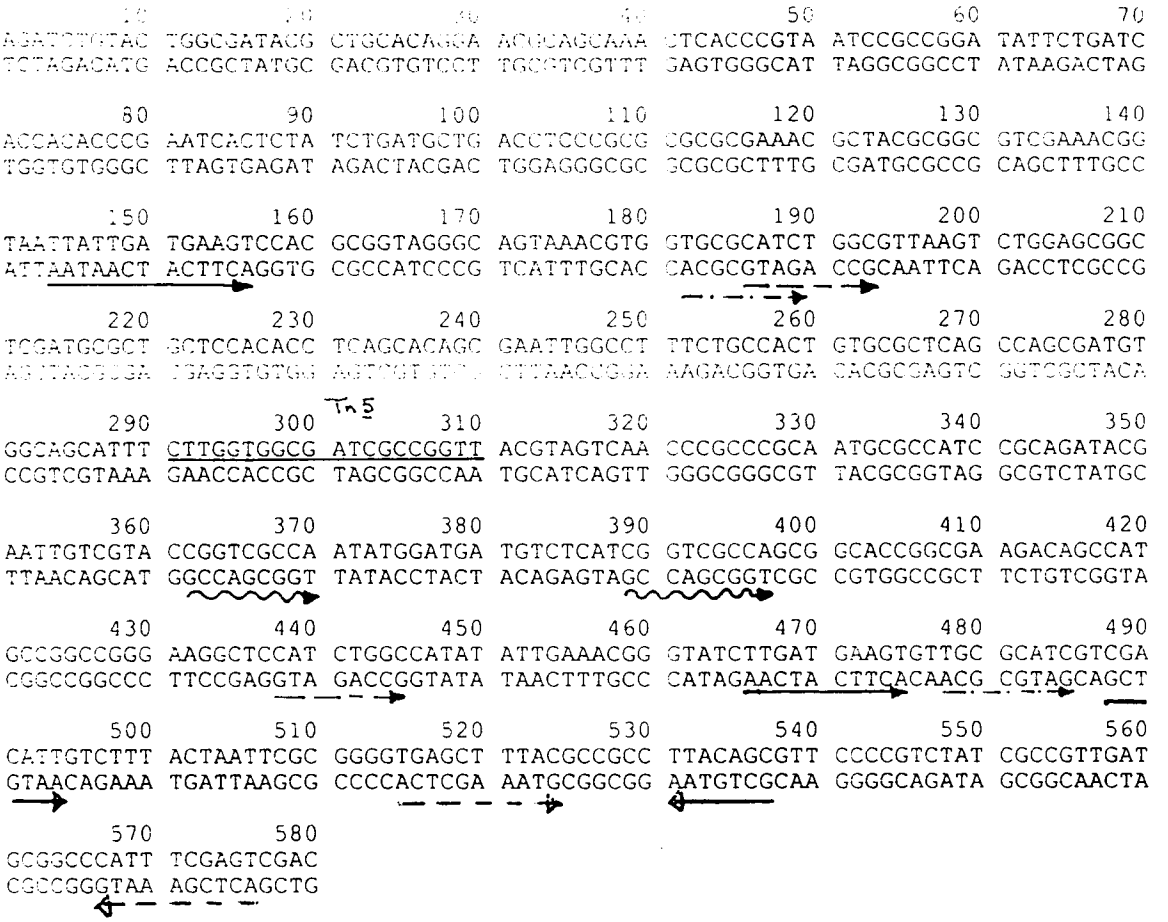


Figure 4-11. Nucleotide sequence of the Tn5-containing BglII/SalI fragement. The total length of this sequence is 580 bp. The cloning sites are underlined as Bg, BglII; S, SalI. The site of Tn5 insertion is also indicated based on the Southern hybridization data. The repeats with 100% match within this region are demonstrated using closed arrows for direct repeats and open arrows for inverted repeats. The GC content of this sequence is 63%.

Table 4-3. The ten most homologous genes to the 580 bp SalI/BglII fragment.

Genes	Optimal Scores	(%)Homology	Functions	Features
OCTase (P. aeruginosa)	275	59.3	Catabolism	Homologous to arg operon
ilvA (E. coli K-12)	274	59.82		Homologous ilv operon
curA (S. curacoi)	274	60.51	Polyketide synthesis	cur operon
aadB (K. pneumoniae)	273	57.07		Encoded by Tn4000
aadB (pDG0100)	273	57.07	aminoglycoside	On pDG0100, related to Tn21-like family
dnaZX (E. coli)	272	64.17	Replication gene	
phnM (E. coli)	272	60.59	Alkylphosphonate uptake	psiD locus, HisM-like integral membrane protein
dnaZX (E. coli)	272	64.17	Subunits of DNA polymerse III	
130 K ORF (Caulobacter crescentus)	272	59.71	Major surface excreted protein	Transmembrane protein temporally regulated
nifD (Thiobacillus ferrooxidans)	271	63.84	Nitrogen fixation	nif operon

(Schmidt et al., 1988) and on a plasmid (Cameron, et al., 1986). Although this 580 bp DNA sequence is shown to have homology primarily with the internal portion of the respective ORFs, the regions of homology within the ORFs of the different genes varies. For example, homology to the curA gene of Streptomyces curacoi (57%) was shown to be near the 5′ end of the ORF, whereas homology with the aadB gene (57%) in plasmid pDG0100 is near the 3′ end of the ORF.

Ranked by the optimized scores, which was determined by both the percent of nucleotide identity and the overall structural similarity between the two aligned DNA sequences, the ornithine carbamoyltransferase gene (arcB gene, also known as OCTase) of P. aeruginosa is listed as the gene sequence with the highest optimized score. A nucleotide identity of 59.3% was identified for this sequence (Figure 4-12). Interestingly, 57.8% nucleotide identity was observed with carbamate kinase gene (arcC gene), which is in the same operon as the arcB gene of P. aeruginosa when using the complementary strand as the query sequence (Figure 4-13).

A protein data bank search was conducted with all six possible reading frames (RF) using both the FASTDB and IFIND programs. The FASTDB program was set to search overall amino acid homology with the query

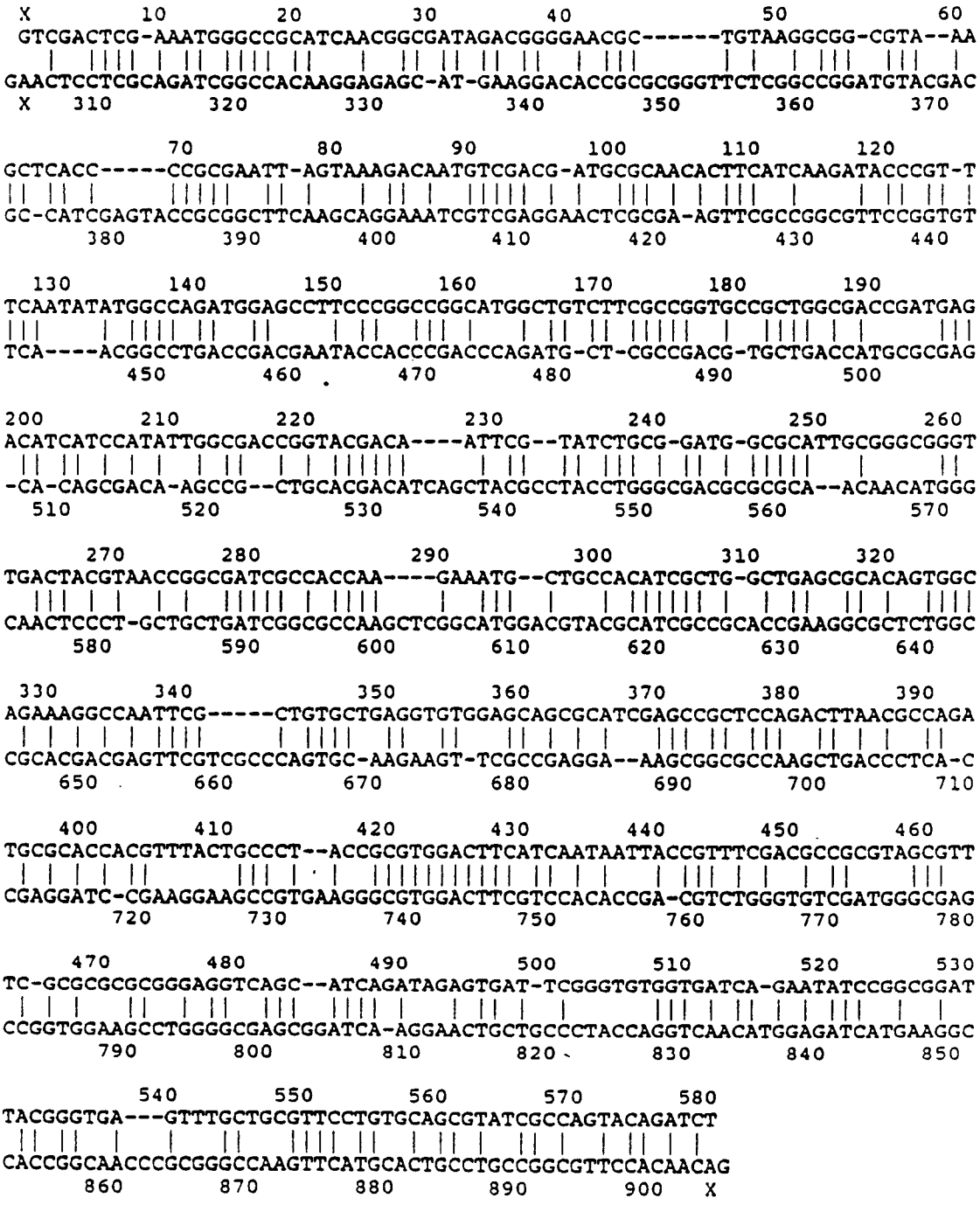


Figure 4-12. DNA sequence aligment of Ornithine cabarmoyltransferase gene (OCTase or arcB) of P. aeruginosa with the 580 bp SalI/BglII fragment. The total nueleotide identity between these two sequences is 59.3%.

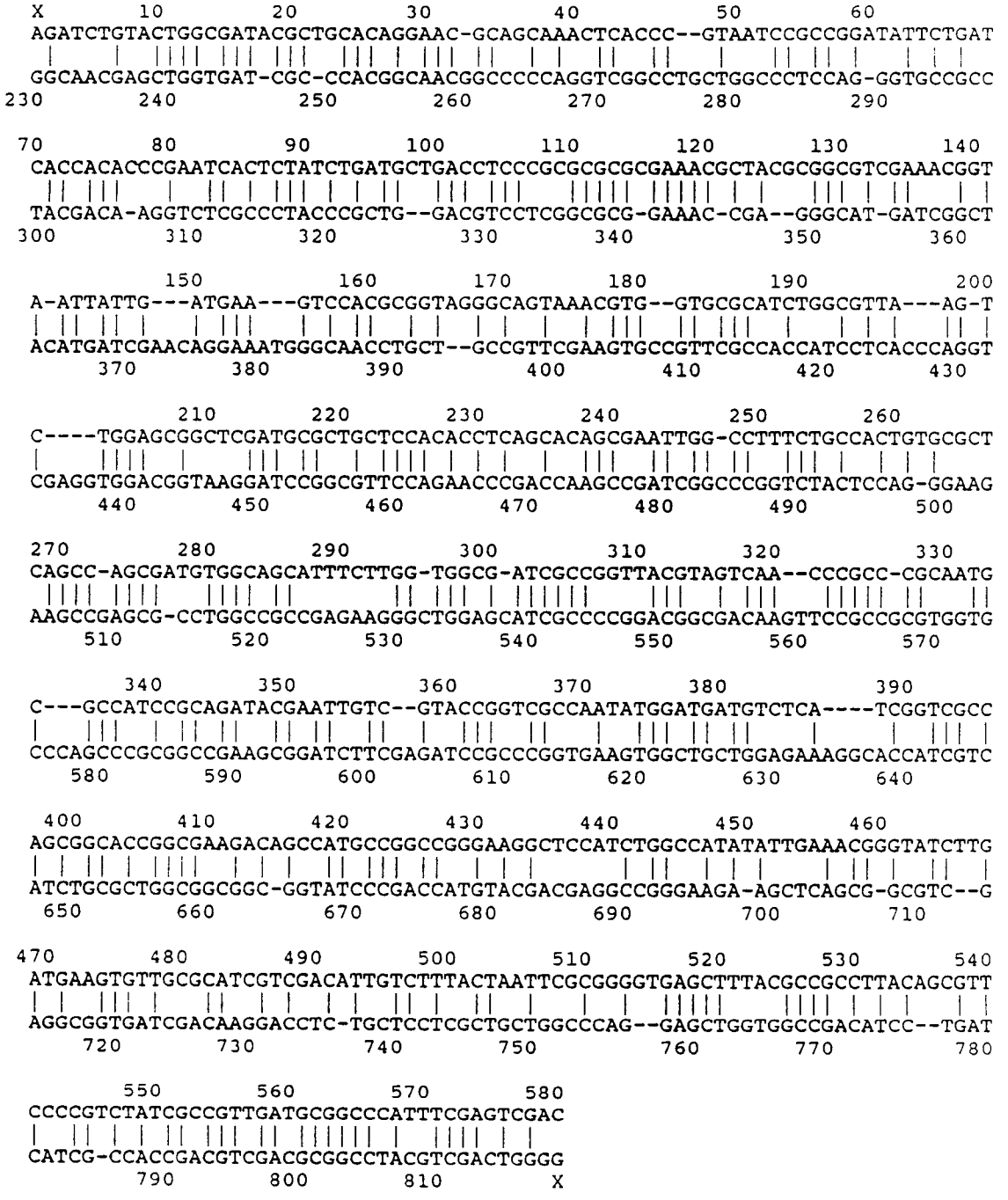


Figure 4-13. DNA sequence aligment of Carbamate kinase gene (arcC) of P. aeruginosa with the 580 bp BglII/SalI fragment. The total nueleotide identity between these two sequences is 57.8%.

sequence, whereas IFIND was used to locate specific domains that have high percent homology with the query sequence. The protein sequences with the highest scores identified by the FASTDB program are summarized in Table 4-4. A variety of prokaryotic genes showed amino acid homology at a range of from 30 to 38% identity. This sequence has 33.3% a. a. homology with mouse ornithine decarboxylase, a precursor of OCTase (Katz and Kahana, 1988), when using RF2 as the query sequence. Interestingly, a concensus amino acid sequence motif was found within OCTase precursor proteins of two fungi, Aspergillus nidulans and A. niger (Figure 4-14), when using RF1 as the query sequence. This sequence motif is also present within the nodD gene of Rhizobium leguminasarum and the nodD2 gene of R. meliloti.

Protein structural analysis of the putative RFs:

Biochemical features of the 580 bp sequence, including codon usage and hydrophobicity of the putative reading frames, were studied using a SEQANA program. The distribution of the codon usage indicated that a putative arginine-rich polypeptide is encoded by RF1 (11.8%) and the opposite strand of RF4 (12.8%).

The hydrophobicity profile of RF1 shows an alternative hydrophobic and hydrophilic pattern along

Table 4-4. Overall amino acid sequence homology of the deduced sequences of the 580 bp fragment to other proteins using FASTDB Program

Organisms	**Genes**	**% a.a.homology (% DNA homology)**
Reading frame 1:		
P. putida	*trp*C (indoleglycerol phosphate synthetase)	31.6
Reading frame 2:		
Mouse	Ornithine decarboxylase gene	33.3
Reading frame 3:		
P. aeruginosa	Part of *algQ* (transcriptional activator of *algD*)	36.14
E. coli	PsiD locus(*phn*M gene) (HisM-like integral membrance protein)	33.92(60.59)
P. aeruginosa	ETA gene(exotoxin A structural gene)	31.21(52.38)

Note: Reading frames were arbitrarily defined by using the first three nucleotides of the sequence as the first amino acid of the deduced sequence.

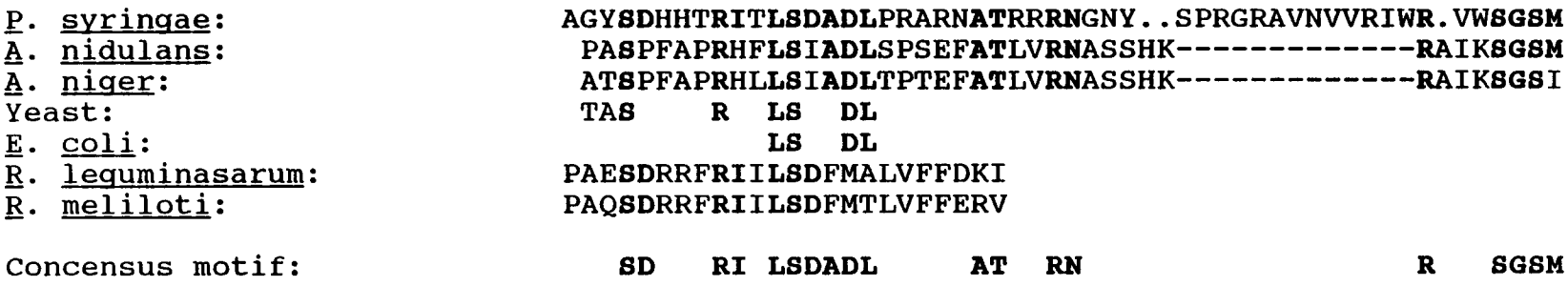


Figure 4-14. Identification of an amino acid motif between sequences of the 580 bp SalI/BglII fragment of P. s. pv. syringae and other genes of various organisms. P. syringae is the deduced amino acid sequence derived from the first nueleotide of the DNA sequence (Reading frame). Protein sequences homologous to this sequence identified are OCTase of A. nidulans, A. niger, yeast and E. coli; and, the nodD gene of Rhizobium leguminasarum and the nodD2 gene of R. meliloti.

with these sequences (Figure 4-15), a characteristic of inter-membrane proteins.

Nucleotide sequence analysis of another DNA region in the Tn5-containing EcoRI fragment

The 446 bp SalI DNA fragment, which was shown by Southern hybridization analysis to share homology with the 580 bp Tn5-containing BglII/SalI fragment (see Figure 4-7), was also sequenced (Figure 4-16). The nucleotide sequences were analyzed for regions of homology. Since the wash stringency used in the Southern hybridization experiments revealed DNA homology at approximately the 70% homology level, the DNA sequences in these fragments were aligned using a SEQANA program at the 70% match level. Thirty-five matches with an average size of 25 base pairs for each alignment were identified as the regions of potential hybridization between these two sequences (data not shown). The homology between these two sequences, however, is discrete and consists of numerous inverted repeats. This 446 bp region has noticeable DNA sequence homology with other genes including the rat ornithine decarboxylase gene exon (66.6% homology) and the transposase gene (tnpA) of Tn501 at 62.90% nucleotide identity.

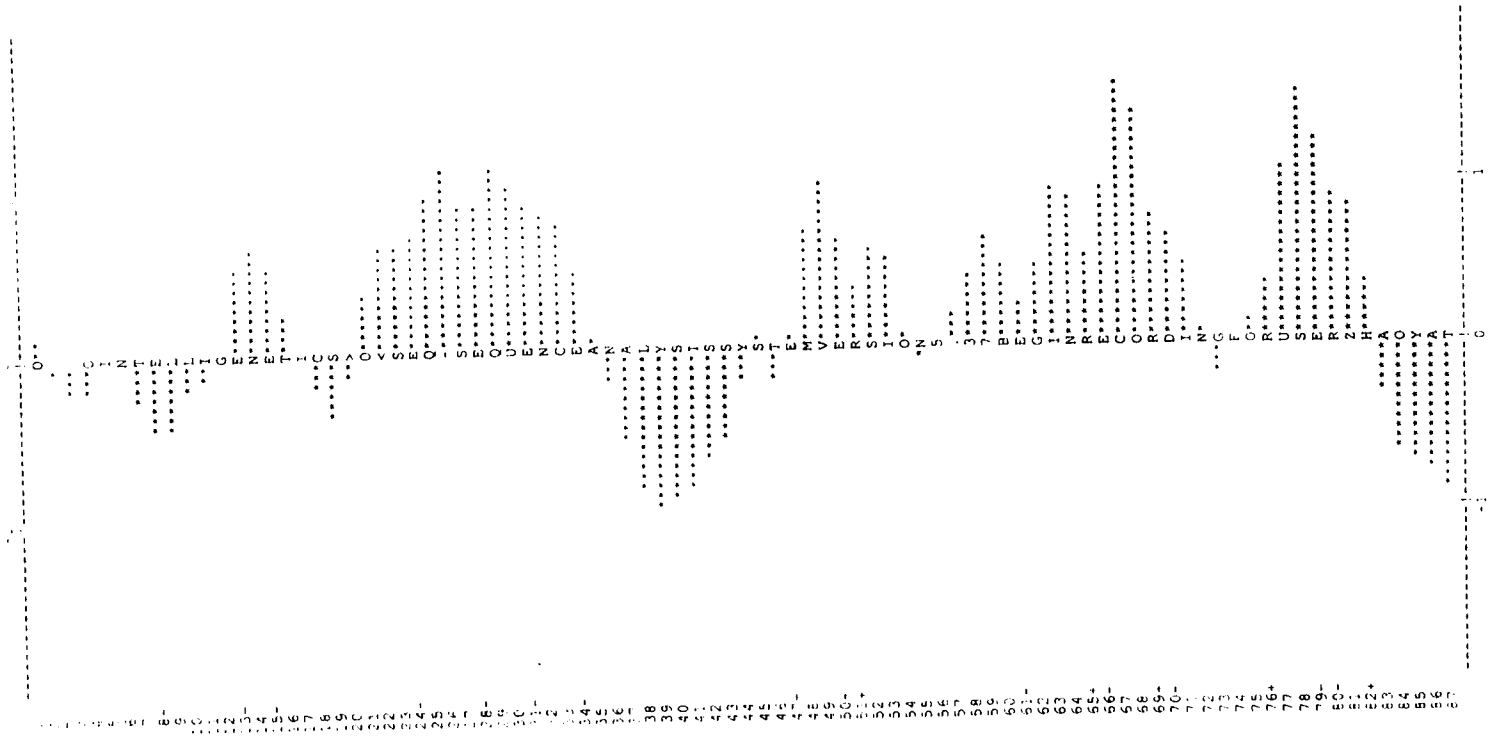


Figure 4-15. Hydrophobicity profile of the deduced amino acid sequence. The hydrophobicity was determined using a SEQUENA program (Intelligent Genetics, Co.).

```
        10          20          30          40          50          60          70
TCGACCAACC GTCCGAAACG ATATGGTGCT GCGTCACCAG CAGGTTGTGC TCCGTCTCCG ACAGTTGCAC
AGCTGGTTGG CAGGCTTTGC TATACCACGA CGCAGTGGTC GTCCAACACG AGGCAGAGGC TGTCAACGTG

        80          90          100         110         120         130         140
AAGCCGCCCG CGAATCAGCG GACCGCTCGA CAAGTCGAAC GGCGCCACGC CTCCTCGCCA GCGAGCCGCT
TTCGGCGGGC GCTTAGTCGC CTGGCGAGCT GTTCAGCTTG CCGCGGTGCG GAGGAGCGGT CGCTCGGCGA

        150         160         170         180         190         200         210
GCACCGCCTC GTGTTGCGCT GCGCTGCCCA GAGCGCGCAG GTCATGAGTG ACCAGGGCGA AACCATGGGT
CGTGGCGGAG CACAACGCGA CGCGACGGGT CTCGCGCGTC CAGTACTCAC TGGTCCCGCT TTGGTACCCA

        220         230         240         250         260         270         280
CTCCGGCGCG ATCACCTGGA TCGCCTGGCC CTCGTGCAAG GCAAAGTGGG TGCGCAGTGC TTCGTGGCGC
GAGGCCGCGC TAGTGGACCT AGCGGACCGG GAGCACGTTC CGTTTCACCC ACGCGTCACG AAGCACCGCG

        290         300         310         320         330         340         350
GCAACGATCC GGTCCAGCGT GGCCTGCAAG GCTCGGCTGT CCAATCGGCC GCGTAAGCGC AGTCCCGCCG
CGTTGCTAGG CCAGGTCGCA CCGGACGTTC CGAGCCGACA GGTTAGCCGG CGCATTCGCG TCAGGGCGGC

        360         370         380         390         400         410         420
GGATGTGATA TGTCGGCTCC GGCCGACGGG TCCAGTTGAT CGAGGAACCA ACATACGCTT GCGCCCACGA
CCTACACTAT ACAGCCGAGG CCGGCTGCCC AGGTCAACTA GCTCCTTGGT TGTATGCGAA CGCGGGTGCT

        430         440
CAGCGGCAAT GGCAGATCGC GGTCGA
GTCGCCGTTA CCGTCTAGCG CCAGCT
```

The sequence contains 446 nucleotides

81.50	(18.3)	ADENINE
151.50	(34.0)	CYTOSINE
143.50	(32.2)	GUANINE
69.50	(15.6)	THYMINE
.00	(.0)	OTHER

The molecular weight of the sequence: 139579.37

Figure 4-16. Nucleotide sequence of the SalI fragment that shares homology with the Tn*5*-containing fragment. The total length of this sequence is 446 bp. The cloning sites are underlined. The repeats with 100% match within this region are identified by closed arrows for direct repeats and open arrows for inverted repeats. The GC content of this region is 66.2%.

Transcriptional analysis of RNA synthesized from the region of Tn5-insertion in PS9024

Results of complementation tests, Tn3-HoHo1 mutagenesis and the gene data bank search of sequences homologous to the DNA in the region of Tn5 insertion suggested the possible involvement of a large polycistronic operon. To test this possibility, a Northern blot of total RNA from the wild type strain, R32, and the mutant, PS9024, was made using RNA isolated from cells that were grown in both complete LB medium and in susceptible bean pods (see Materials and Methods for details). The Northern blot was probed with the 1.35 kb EcoRI/SalI fragment which contains the site where Tn5 inserted. This region appears to be expressed only in bacteria growing *in planta* but not in cells growing in LB medium (Figure 4-17). Furthermore, the probe hybridized to a single mRNA that appears to be much larger than 9.4 kb, as indicated by the size marker.

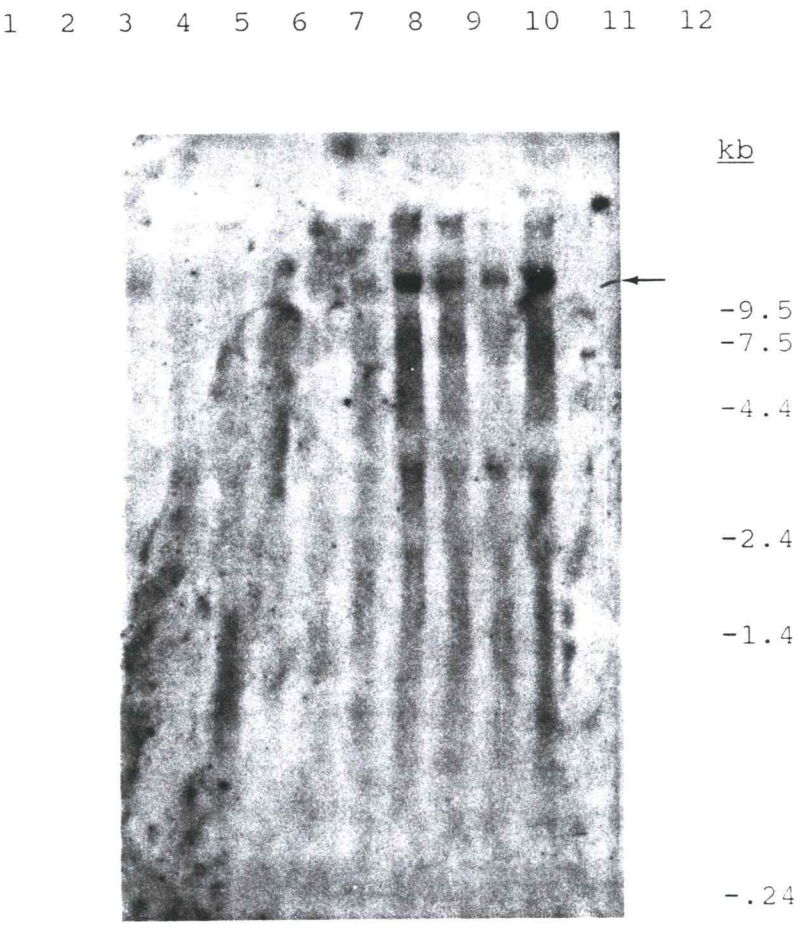


Figure 4-17. Northern transcription analysis of messenger RNA. Total RNA was isolated from cells grown either in LB medium or in bean pods. The DNA probe was made from the 1.35 kb EcoRI/SalI fragment where Tn5 is inserted in PS9024. Lanes 1 and 6 are RNA of R32(pOSU226); Lanes 2 and 7, PS9024(pOSU226); Lanes 3 and 8, PS9024(pOSU4201); Lanes 4 and 9, PS9066 and Lanes 5 and 10 are PS9086; Lanes 1 to 5 contain RNA isolated from cells grown in LB medium; Lanes 6 to 10 contain RNA isolated from cells recovered from infected bean pods. Lane 11 is theh K-buffer (pH 7.0) control, which was used to inject bean pod. RNA size marker is in lane 12.

SDS-PAGE analysis of the 150 kD protein band that was absent from PS9024

Comparison of the total protein profile between PS9024 and R32 in the early course of this study revealed that a band corresponding to a protein of ca. 150 kD was absent from PS9024. In light of the size of the mRNA transcribed from this locus, it was of interest to see whether this band represent one or multiple protein. Further separation of proteins was accomplished using a 10% acrylamide mini-gel and revealed three and possibly four bands within this size range (97 - 200 kD; Figure 4-18) that are present in R32 but absent from PS9024. Interestingly, there are five additional protein bands larger than 200 kD and one smaller protein band of ca. 45 kD which were synthesized by R32 but not by PS9024. These were not identified previously when using a 15% acrylamide gel.

DISCUSSION

Promoters can often be identified by transcriptional fusion to a reporter gene that gives an assayable gene product. In this study, a transposon

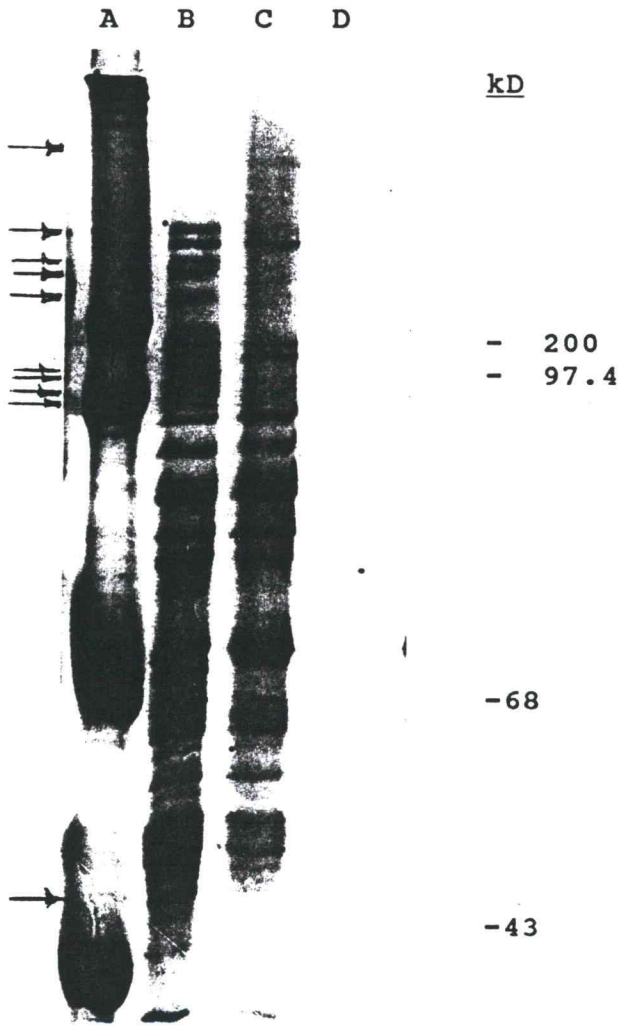


Figure 4-18. Comparison of total cellular protein profiles of R32 and PS9024 using SDS-PAGE. Lane A, high range protein size marker; Lane B, wild type strain R32. Lane C, mutant strain PS9024 and lane D, low range protein size markers (Bio-Rad Laboratories, Richmond, CA). Arrows indicate the protein bands that are present in R32 and absent from PS9024.

cartridge containing Tn*3* and the promoter-less *lac* reporter gene was used to identify potential indigenous promoter activities by identifying the expression of β-galactosidase (*lac*$^+$) phenotype. Two kinds of indigenous promoter activities were identified in the wild type sequence of pOSU2401. At least four possible promoters were identified in either orientation, with three promoters expressed only in *Pseudomonas* and one expressed both in *Pseudomonas* and in *E. coli*. Although these promoter activities were detected following growth of *Pseudomonas* cells in LB medium, no messenger RNA appeared to be transcribed from this region. This suggests that the genes at this locus are plant-inducible or possibly inducible by depletion of the carbon source.

A plant inducible *hrp*D locus of *P. s.* pv. *phaseolicola* was described by Lindgren *et al.*, (1989). This locus was expressed only in the infected plant but not during growth in a nutritionally complex medium. Nutritional downshift of cultures that had been pregrown on complex medium leads to rapid induction of *hrp*D and other Hrp loci, including *hrp*AB and *hrp*C of *P. s.* pv. *phaseolicola* (Mindrinos *et al.*, 1990). The level of expression is modulated by the type of carbon source present in the medium.

Plant-inducible genes also have been found in Agrobacterium and Rhizobium (Stachel et al., 1985; Shearman et al., 1986), and it is known that some plant cell wall degrading enzymes of pathogens are inducible by degradation products (Cooper, 1983). Promoters that function both in planta and in culture were also reported from Xanthomonas campestris pv. campestris, the causal agent of crucifer black rot (Osbourn et al., 1987). Of the nineteen promoters identified by expression of a promoter-less chloramphenicol resistance gene in plasmid pIJ3100, five were expressed in bacteria grown both in turnip seedlings and on agar plates. However, the remaining fourteen promoters were expressed in bacteria grown only in planta.

Numerous direct and inverted repeats were detected in the region of Tn5 insertion and in the adjacent 446 bp SalI fragment. No functional role has been assigned to these repetitive sequences. Repetitive sequences are widely distributed among many bacteria species (Kleckner, 1981), and their occurrence among phytopathogenic pseudomonads has been demonstrated (Szabo and Mills, 1984). Repetitive sequences (RS) were reported to serve as sites for homologous recombination in the process of integration and excision of the P. s. pv. phaseolicola indigenous plasmid pMMC7105 (Szabo and

Mills, 1984). One of these repetitive sequences was recently shown to be a novel insertion sequence element designated IS801 (Romantschuck *et al.*, 1991).
Repetitive sequences were shown to be involved in the process of plasmid-chromosome recombination (Szabo and Mills, 1984), replicative transposition (Grindley and Reed, 1985), and in the control of gene expression (Stalker *et al.*, 1981; Kline, 1985; Chattoraj *et al.*, 1984). Multiple repeats were also reported to be important for the function of the *algQ*-*algP* regulatory region in maintaining mucoidy of *P. aeruginosa* cells (Konyecsni and Deretic, 1990). In that study, it was noticed that the transition from the mucoid to the nonmucoid phenotype was due to DNA rearrangement within the *algP* repeated structure. Although the putative protein sequence encoded from the 580 bp region has 36% amino acid homology to the *algQ* gene, no direct relevancy was found with regard to the involvement of repetitive sequences in regulating gene function.

Although no evidence was obtained in this study to suggest that the repetitive sequences in the region of *Tn5* insertion play a role in recombination, the fact that multiple copies of DNA in this region are presented in other pathovars suggests such possibility cannot be excluded.

Closely related, rather than identical, direct or inverted repeats with an average length of between 15 and 25 bp are typical features of the termini of insertion sequence (IS) elements and transposons (Lewin, 1987). A unique 2.7 kb SalI fragment, which is present only in the parental wild type strain, R32, and one other strain, 11/81, is flanked by both direct and inverted repeats. This structure may resemble a possible composite transposon. Other evidence that this region may contain a transponsable element is supported by the finding that the 446 bp SalI fragment has 55% homology with the transposase gene of transposon Tn501 of P. aeruginosa.

Although a variety of genes showed homology with the DNA sequence from P. s. pv. syringae, the genes that appear to show significant homology at the DNA and protein level are genes involved in the ornithine anabolic or catabolic pathways (Table 4-5). One strand of the P. s. pv. syringae sequence has 59.3% nucleotide identity with OCTase (also known as arcB) of P. aeruginosa, whereas the other strand has 57.8% homology with carbamate kinase (arcC), another catabolic enzyme in ornithine synthetic pathway. However, the protein data bank did not reveal significant homology between the arcB or arcC genes of P. aeruginosa. A short sequence motif of RF1 spanning over 50 amino acids

Table 4-5. Homology to Genes in Ornithine Catabolic Biosynthetic Pathway

Organism	Gene	Function	%Homology	ORF	Reference
P.aeruginosa	arcB (OCTase)	Catabolic Ornithine Carbomyl transferase	59.31 (DNA)		Baur et al., 1987
P. aeruginosa	arcC	Carbamate Kinase	57.76 (DNA)		Baur et al., 1989
Mouse		OTCase	33.3 (a.a.)	ORF2	Katz & Kahana, 1988
Aspergillus nidulans	OCTase		motif	ORF1	Upshall et al., 1986
Aspergillus niger	OCTase	OTCase precusor	motif	ORF1	Buxton et al., 1987
Rat (Rattus norvegicus)		Ornithine decarboxylase gene (exon)	66.6 (DNA)		Van Steeg et al., 1988

(see Figure 4-14) showed a conserved domain with the precursor of the OCTase gene in two fungal species, A. nidulans and A. niger. Furthermore, the overall amino acid homology was found to be 33.3% with the mouse ornithine decarboxylase gene when using RF2 as the query sequence. Interestingly, the adjacent 446 bp fragment region shares 66.6% nucleotide sequence identity with the ornithine decarboxylase gene (exon) of rat (Rattus noruegicus). These results suggest that some of the genes identified in the region of Tn5 insertion may have functions similar to OCTase genes.

Additional evidence to support the premise that this region encodes an OCTase function is suggested by an amino acid sequence motif that is shared by the fungal OCTase gene from A. nidulans. Considerable amino acid sequence homology with OCTase of A. nidulans was detected in two other OCTase isoenzymes of E. coli, which are products of arcF and arcI (Upshall et al., 1986). Human mitochondrial OCTase and the OCTases in other eukaryotic organisms were, in turn, found to share significant sequence homology with arcF and arcI genes of E. coli (Horwich et al., 1984; Houghton et al., 1984; Lerner and Switzer, 1986). No significant homology was detected between this DNA sequence and those two genes. Nevertheless, it is possible that they are functionally similar. Antigenic differences have

been reported among OCTases from different organisms (Falmagne et al., 1985). The catabolic OCTase of P. putida shares antigenic determinants with the anabolic arcF OCTase of E. coli, but not with anabolic OCTase from several other bacterial species, including P. putida.

Some genes involved in ornithine biosynthesis in E. coli are known to be encoded from the same operon. The arcABC operon of P. aeruginosa is 17.6 kb in size (Baur et al., 1989). The homology between the P. s. pv. syringae sequence and the arcB and arcC genes may indicate the organization of a similar operon at this locus.

Association between symptom development and the inhibition of OCTase in bean leaves treated with phaseolotoxin has been observed in P. s. pv. syringae, the causal agent of halo blight disease of Phaseolus vulgaris L (Turner and Mitchell, 1985). It has been demonstrated that the chlorosis symptom that characterizes the halo blight disease of Phaseolus vulgaris L. is caused by phaseolotoxin produced by P. s. pv. phaseolicola. Phaseolotoxin is hydrolyzed by plant peptidases to N^{δ}(N'-sulpho-diaminophosphinyl)-L-ornithine (now designated octicidin) which also causes chlorosis and is reported to be an irreversible inhibitor of OCTase. Lack of chlorosis in the lesion

inoculated with the mutant strain PS9024 was noticed. This could be explained by the assumption that the mutation in a OCTase-like gene may result in the failure of blocking the activity of this enzyme and consequently chlorosis.

It was reported, however, that chlorosis can be reversed by a single application of arginine, the product of the pathway in which OCTase catalyze in the first step (Turner, 1986). To test whether a similar mutation occurs in the mutant PS9024, a single application of 200 picomolar arginine, glutamine, the end product of this pathway and glutamate was applied to the inoculation site of the wild type strain R32 and mutant PS9024, respectively, as suggested by Turner and Mitchell (1985). However, no regreening of the chlorosis caused by R32 and pathogenic changes induced by PS9024 were observed (data not shown). This may suggest that regardless the DNA and protein sequence similarity with OCTase, this gene locus may not encode the same enzyme but rather a similar toxin binding protein that shares the similar structural features.

The results of studies of the DNA sequence distribution of the pathogenicity locus of PS9024 by Southern hybridization, and the gene data bank searches suggest that this pathogenicity-related gene locus or part of the DNA sequence could have evolved from a

foreign source. This contention is supported by the fact that part of the DNA sequence present at this locus has homology only with some pathovars of P. syringae but not others. Indeed, it was shown that even among strains that cause brown spot disease, some do not have DNA that is homologous to this region of the genome (Niepold and Mills, 1987; Zhao and Mills, 1989; Table 4-2). It is apparent that the 2.7 kb SalI fragment adjacent to the region of Tn5 insertion is not a common region present in all pathovars of P. syringae.
The observation that probe made from 1.35 kb EcoRI/SalI fragment into which Tn5 inserted has homology to most of the pathovars of P. syringae is probably manifested by the presence of repetitive sequences in this region. There is precedent in other bacteria to suggest that the genes encoding enzymes in the arginine biosynthetic pathway may be mobilized on transposable elements.

Indirect evidence came from the fact that the arcB gene of P. aeruginosa shares strong homology (63% of nucleotide identity and 57% of the amino acids identity) with the argF gene of E. coli K-12 which codes for the anabolic ornithine carbamoyltransferase isoenzymes. Two direct repeated IS1 elements flank arcF, which gives the impression of a transposon-like structure (Hu and Deonier, 1981; York and Stodolsky, 1981). It was,

therefore, suggested that arcF was transmitted horizontally from P. aeruginosa to E. coli K-12 (Hu and Deonier, 1981; York and Stodolsky, 1981). Additional evidence supports the hypothesis that this DNA in E. coli is of foreign origin. This DNA sequence shares 57.1% nucleotide sequence identity with a unique region of the aadB gene of K. pneumoniae, which is present on the IncC plasmid pDG0100 (Cameron, et al., 1986) and may also be linked to the Tn21-like family of transposable elements (e.g., Tn4000). This transposon family is also present on IncFII plasmids and other plasmids present in gram-negative bacteria (Cameron, et al., 1986).

The internal homologous DNA region, in which direct and inverted repeats were found both in pOSU9024 and pOSU2401, spans a region of more than 10 kb. The unique 2.7 kb DNA region is bordered by repeats, suggesting an organization that may resemble a composite transposon. Although no direct evidence was obtained for transposition of this region, DNA sequence homology with several transposons and plasmid-borne genes was observed. For example, the aadB gene carried by Tn4000 has sequence homology with the left end of the 2.7 kb unique fragment, whereas the right end has homology to Tn501. Homology to a transposase (62.9% nucleotide identity) was also found in the 446 bp SalI fragment

which is about 3 kb from the site of Tn*5* insertion in PS9024.

Searches of gene data banks revealed DNA homology of this pathogenicity locus to numerous genes located in bacterial operons providing indirect evidence to support the previous hypothesis that this locus may comprise an operon. Operons that encode pathogenicity-related functions have been observed in numerous other bacteria (*e.g.*, coronatine operon, Ma *et al.*, 1991 and *vir* gene operon, Nester *et al.*). The size of these operons varies but may range from 17 kb (*arc* operon) up to 30 kb (Coronatine phytotoxin operon). The Northern blot analysis which revealed a transcript larger than 10 kb strongly suggested that an operon was identified at this pathogenicity-related locus. Furthermore, nine protein bands of at least 100 kD in size were absent from the mutant. This is presumably because of Tn*5* insertion into the gene that either encodes these proteins or regulates the expression of genes at other loci. Assuming the average molecular weight of an amino acid to be 120 daltons, a gene required to synthesize a 150 kD protein would be approximately 3 kb in length. If all of the proteins whose synthesis is blocked in PS9024 are transcribed from a large polycistronic operon, it would have an estimated size of at least 25 kb.

SUMMARY

1. Two kinds of indigenous promoter activities have been identified in pOSU2401; Pseudomonas specific promoters and promoters expressed both in Pseudomonas and E. coli.

2. At least three different repetitive sequences were identified within a region of the chromosome that encodes a pathogenicity determinant(s).

3. The results of the gene data bank searches suggested that one of the gene at this locus is functionally similar, but not identical to OCTase.

4. This pathogenicity-related locus appears to contain an operon which encodes an mRNA that is larger than 10 kb and is inducible in planta.

REFERENCES

Ausuel, F. M., et al.(eds.) 1989. Short Protocols in Molecular Biology. Greene Publishing Associates. p147.

Baur, H., V. Stalon, P. Falmagne, E. Luthi, and D. Haas. 1987. Eur. J. Biochem. 166:111-117.

Baur, H., E. Luethi, V. Stalon, A. Mercenier and D. Haas. 1989. Sequence analysis and expression of the arginine-deiminase and carbamate-kinase genes of P. aeruginosa. Eur. J. Biochem. 179:53-60.

Cameron, F.H., D.J.G. Obbink, V.P. Ackerman and R.M. Hall. Nucleotide sequence of the ADD(2'') aminoglycoside adenylyltransferase determinant aadB. Evolutionary relationship of this region with those surrounding aadA in R538-1 and dhfrII in R388. Nucleic Acids Res. 14:8625-8635.

Chattoraj D.K., K. Cordes and A. Abeles. 1984. Plasmid P1 replication: negative control by repeated DNA sequences. Proc. Natl. Acad. Sci. USA. 81:6456-6460.

Cooper, R.M. 1983. In J.A. Callow ed.), Biochemical Plant Pathology. Wiley, London, p101-135.

Ditta, G, S. Stanfield, D. Corbin and D.R. Helinski. 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Falmagne, P., D. Portetelle and V. Stalon. 1985. Immonological and structural relatedness of catabolic ornithine carbamoyltransferases and the anabolic enzymes of enterobacteria. J. Bacteriol. 161:714-719.

Feinberg, A.P. and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6.

Gay, P., D. Le Coq, M. Steinmetz, T. Berkelman and C.I. Kado. 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. J. Bacteriol. 164:918-921.

Grindley and Reed. 1985. Ann. Rev. Biochem. 54:863-896.

Haas, D., B.W. Holloway, A. Schambock and T. Leisinger. 1977. The genetic organization of arginine biosynthesis in P. aeruginosa. Mol. Gen. Genet. 154:7-22.

Houghton, J.E., D.A. Bencini, G.A. O'Donovan and J.R. Wild. 1984. Protein differentiation: A comparison of aspartate transcarbamoylase and ornithine transcarbamoylase from Escherichia coli K-12. Proc. Natl. acad. Sci. USA. 81:4864-4868.

Horwich, A.L., W.A. fenton, K.R. Williams, F. Kalousek, J.P. Kraus, R.F. Doolittle, W. Konigsberg and L.E. Rosenberg. 1984. Structure and expression of a complementary DNA for the nuclear precursor of human mitochondrial ornithine transcarbamylase. Science 224:1068-1074.

Katz, A. and C. Kahana. 1988. Isolation and characterization of the mouse orinithine decarboxylase gene. J. Biol. Chem. 263:7604-7609.

Kleckner, N. 1981. Transposable elements in prokaryotes. Ann. Rev. Genet. 15:341-404.

Kline, B.C. 1985. A review of mini-F maintenance. Plasmid. 14:1-16.

Konyecsni, W.M. and V. Deretic. 1990. DNA sequence and expression analysis of algP and algQ, components of the multigene sysmtem transcriptionally regulating mucoidy in P. aeruginosa. algP contains multiple direct repeats. J. Bacteriol. 172:2511-2520.

Lerner, C.G. and R.L. Switzer. 1986. Cloning and structure of the Bacillus subtilis aspartate transcarbamylase gene (pyrB). J. Biol. Chem. 261:11156-11165.

Lewin, B. 1987. Gene III. John Weley and Sons.

Lindgren, P.B., R. Frederick, N.J. Panopoulos, D. Dahlbeck, B.J. Staskawicz and S.E. Lindow. 1989. An ice nucleation reporter gene system: identification of an inducible pathogenicity gene in Pseudomonas syringae pv. phaseolicola. EMBO J. 8:2490-3001.

Ma, S.W., V.L. Morris and D.A. Cuppels. 1991. Characterization of a DNA region required for production of the phytotoxin coronatine by P. s. pv. tomato. MPMI. 4(1):69-74.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982 Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory. p387-389.

Miller, et al., 1985.

Mills, D. and F. Niepold. 1987. Molecular analysis of pathogenesis of Pseudomonas syringae pv. syringae. In: Nishimura, S., C.P. Vance, and N. Doke (eds) Molecular Determinant of Plant Disease. Japan Sci. Soc. Press, Tokyo/Springer-Verlag, Berlin. pp. 185-200.

Mindrinos, M.N., L.G. Rahme, R.D. Frederick, E. Hatziloukas, C. Crimm and N.J. Panopoulos. 1990. Structure, function, regulation, and evolution of genes involved in pathogenicity, the hypersensitive response, and phaseolotoxin immunity in the bean halo blight pathogen. In: S. Silver, A.M. Chakrabarty, B. Iglewski and S. Kaplan (eds.) "Pseudomonas: biotransformations, pathogenesis, and evoloving biotechnology. Ammrican Society for Microbiology. p74-81.

Osbourn, A.E., C.E. Barber and M.J. Daniels. 1987. Identification of plant-induced genes of the bacterial pathogen Xanthomonas campestris pv. campestris using a promoter-probe plasmid. The EMBO J. 6(1):23-28.

Quant R. and D. Mills. 1984. An integrative plasmid and multiple-sized plasmids of Pseudomonas syringae pv. phaseolicola have extensive homology. Mol. Gen. Genet. 193:459-466.

Romantschuk, M., Y. Zhao, K. McCluskey, J. Williams and D. Mills. Repeated sequences in Pseudomonas syringae pv. phaseolicola; Distribution and possible function as insertion sequences. Symbiosis. 8:21-31.

Schmidt, F.R.J., E.J. Nuecken and R.B. Henschke. 1988. Nucleotide sequence analysis of 2'-aminoglyoside nucleotide transferase ANT(2'') from Tn4000, its relationship to AAD(3'') impact on Tn2 evolution. Mol. Microbiol. 2:709-717.

Shearman, C.A., L. Rossen, A. Johnston and J.A. Downie. 1986. The Rhizobium leguminosarum nodulation gene nodF encodes a polypeptide similar to acryl-carrier protein and is regulated by nodD plus a factor in pea root exudate. EMBO J. 5:647-652.

Stachel, S.E., G. An, C. Flores and E.W. Nester. 1985. A Tn3 lacZ transposon for the random generation of ß-galactosidase gene fusion: Application to the analysis

of gene expression in Agrobacterium. EMBO J. 4:891-898.

Stalker, D.M., C.M. Thomas and D.R. Helinski. 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. 181:8-12.

Szabo, L. and D. Mills. 1984. Integration and excision of pMC7105 in Pseudomonas syringae pv. phaseolicola: Involvement of repetitive sequences. J. Bacteriol. 157(3):821-827.

Turner, J.G. and R.E. Mitchell. 1985. Association between symptom development and inhibition of ornithine carbamoyltransferase in bean leaves treated with phaseolotoxin. Plant Physiol. 79:468-473.

Turner, J.G. 1986. Effect of phaseolotoxin on the synthesis of arginine and protein. Plant Physiol. 80-760-765.

Upshall. 1986. Genetic and molecular characterization of $arg B^{+}$ transformants of Aspergillus. Curr. Genet. 10:593-599.

Wen, L., J.K. Huang and P.J. Blackshear. 1989. Rat ornithine decarboxylase gene: Nucleotide sequence, potential regulatory elements, and comparison to the mouse gene. J. Biol. Chem. 264:9016-9021.

Wright, J.M. and M.B. Stephen. 1982. Negative control of ornithine decarboxylase and arginine decarboxylase by adenosine-3′: 5′-cyclic monophosphate in E. coli. Mol. Gen. Genet. 186:482-487.

Zhao, Y. and D. Mills. 1989. Distribution of Pathogenicity-related DNA sequences from Pseudomonas syringae pv. syringae R32 among phytopathogenic bacteria. (Abst.) In: Proc. 1st CAASS Conf., Communications Service, Cornell Univ., Ithaca, N.Y., U.S.A. Section C36.

SUPPLEMENT

Chapter Five

INTRODUCTION

DNA sequencing of the pathogenicity locus was further extended after I distributed the final draft of my dissertation to the committee members. The results of gene bank searches using this newly sequenced nucleotide sequence revealed some potential features of this locus that suggest a possible role in the process of pathogenesis. Hence, this information is included as a supplementary chapter of this dissertation.

MATERIALS AND METHODS

The methods used in gene sequencing and in the data bank searches have been described previously in the Materials and Methods section of Chapter Four.

RESULTS

The strategy and methods used for sequencing the pathogenicity-related locus are illustrated in Figure 4-10. The nucleotide sequence of a 1375 bp *HgiA*I fragment, which extends approximately 800 bp to the left and approximately 570 bp to the right of the Tn*5* insertion in PS9024 was sequenced using the Gene Sequencer of the Central Service Laboratory of the Center for Gene Research and Biotechnology at Oregon State University. A search for homologous gene sequences was conducted using the methods described in the Materials and Methods section of Chapter Four. The distribution of the homologous DNA sequences at various levels is illustrated in Figure 5-1. A nucleotide identity of 93.3% (98% nucleotide identity with the promoter region and the ORF) was observed with 5′ end of the *app*A gene of *E. coli*, which encodes acid phosphatase of pH 2.5 (Dassa *et al.*, 1982), using both the IFIND and FASTDB programs (Figure 5-2). The nucleotide sequence of the total 1375 bp region with the structural features that are identical with *app*A gene are indicated in Figure 5-3.

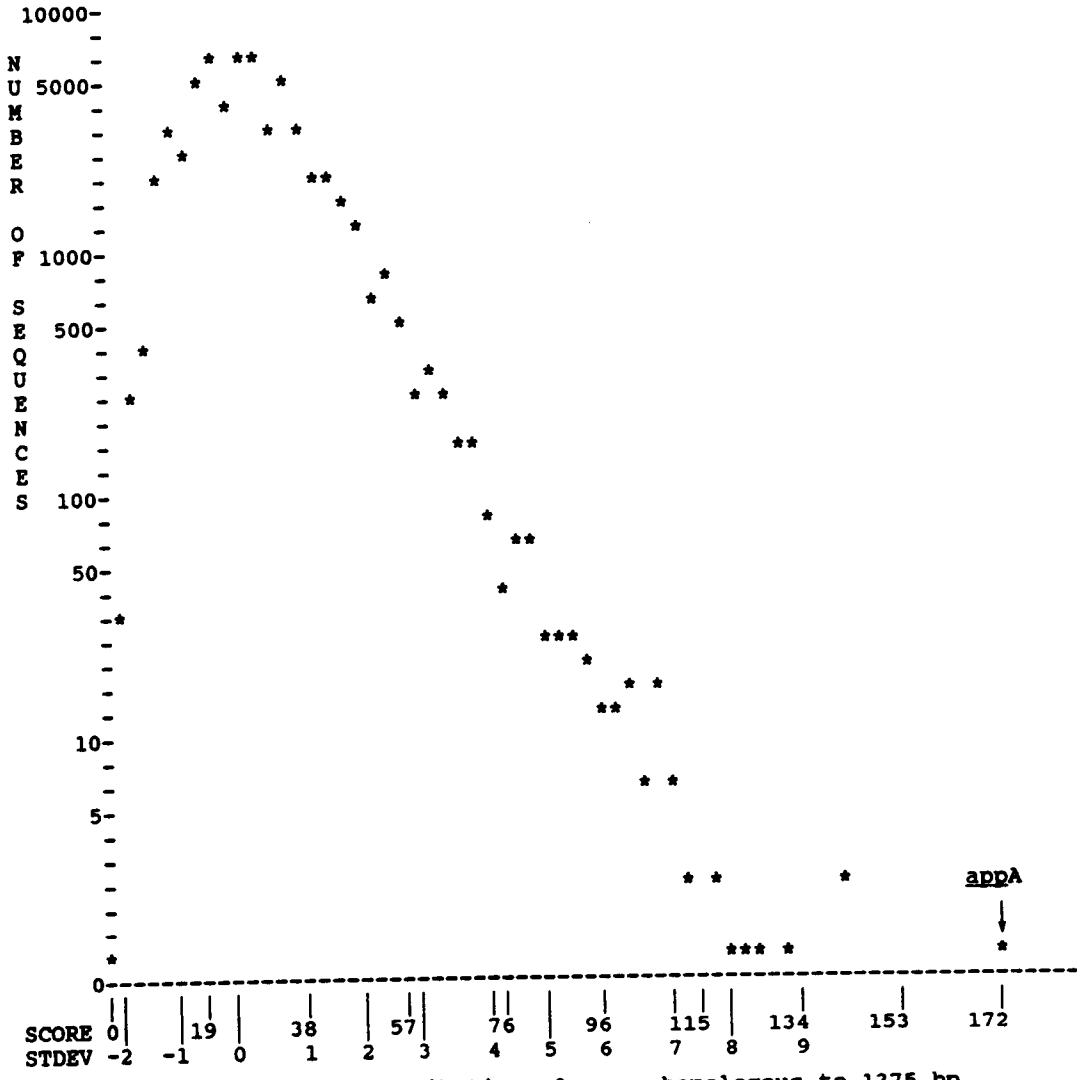


Figure 5-1. Distribution of genes homologous to 1375 bp HgiA fragment. Results of data bank searches using one of the IntelliGenetics Suite Program, FASTDB. Total 51610 different genes were compared from all the entries up-to-date in the data banks of EMBL 25, N-GeneSeq 2.0, UEMBL 25-66 and UGenBank 66-25. X-axis represents homologous scores and standard deviation from the average homology level. Y-axis indicates the number of sequences that showed homology at various homology levels. appA gene of E. coli is indicated by arrow.

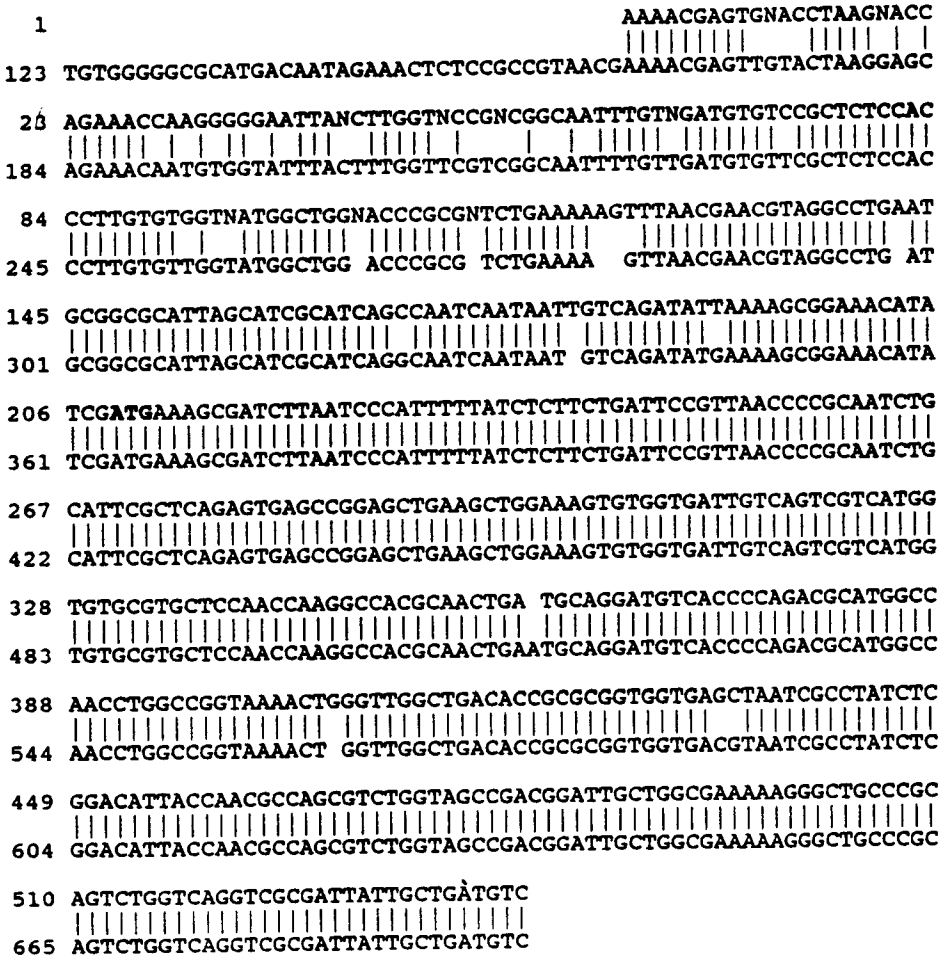


Figure 5-2. Nucleotide sequence alignment of dsg locus of P. s. pv. syringae with appA gene for pH 2.5 acid phosphatase N-terminus of E. coli. The top sequence is from P. s. pv. syringae and the bottom sequence is from E. coli. The putative start codon in ORF-1 is in boldface. The overall nucleotide identity is 93.3% and 98% nucleotide identity was found within the ORF and the promoter region.

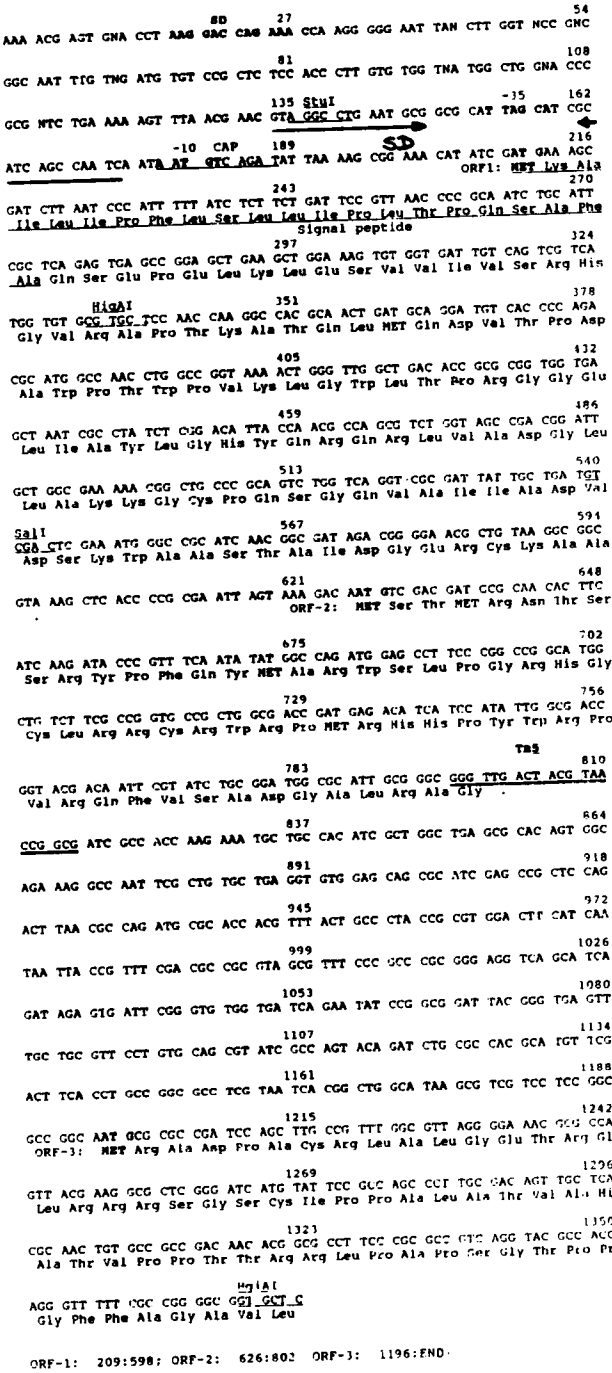


Figure 5-3. Nucleotide sequence of the 1375 bp HgiA fragment of P. s. pv. syringae strain R32. Promoter consensus bases (-35 and -10) are in boldfaces. The Shine and Dalgarno regions are indicated as SD. The putative CAP site is underlined and the palindromic units (p.u.) sequences are indicated by arrows. The amino acids sequence, correponding to the signal peptide of the acid phosphatase of E. coli is also underlined. The approximate site of Tn5 insertion is double-underlined.

Promoter:

A putative bacterial promoter which contains a "consensus -10 sequence" (TTAGCA, nucleotides 177-183) and a "-35 sequence" (TTAGCA, nucleotides 155-160) was located 11 nucleotides downstream of the StuI site. The spacing of 17 bp between the -10 and the -35 sequences indicates a defined optimal distance between these two sites (Stefano and Gralla, 1982). A palindromic sequence, nucleotides 135-148 and 161-174 (GTAGGCCTGATGCG, and GCATCAGGCAATC) was found overlapping the -35 region and it is homologous to the consensus sequences of palindromic units (p.u.) commonly found in extragenic regions (Gilson et al., 1984).

CAP Binding Site:

A catabolite activator protein (CAP) binding site at coordinates 180-189 was identified with a nucleotide sequence of AATGTCAGAT, which overlaps the -10 region.

ORFs:

Three ORFs were predicted in this DNA sequence: ORF-1, nucleotides 209-598; ORF-2, nucleotides 626-802 and ORF-3, nucleotides 1196-end. A typical Shine and Dalgarno (SD) sequence (Shine and Dalgarno, 1974) is located 7 nucleotides upstream of the translation initiation codon, ATG, of the first ORF. ORF-1 encodes

a putative polypeptide of ca. 15 kD and a signal peptide of 21 amino acids which are identical with the sequence of the appA gene.

Discrepancies of this sequence and appA gene:

Two mismatches were found at nucleotides 433 and 434 that differ from the appA gene. A base pair deletion was found at nucleotide 360, which causes a frame shift in ORF-1. This frame shift resulted in 15 amino acids that differ from the putative polypeptide encoded by E. coli. However, a base pair insertion at nucleotide 406 resumes the original reading frame, resulting in a sequence identical to acid phosphatase of E. coli.

DISCUSSION

A 98% nucleotide identity of this pathogenicity-related locus to the acid phosphatase gene (appA) of E. coli at the promoter region and the 5′ end of the ORF of this gene strongly suggests that a similar function may be encoded by P. s. pv. syringae. This gene or genes residing nearby are, therefore, implicated in the process of pathogenesis. It appears that a similar

control of gene expression exists at this locus, since a perfect match was found of the nucleotide sequence of this region and the appA gene.

A negative control of transcription of appA gene, which is mediated by cyclic AMP-CAP complex, has been reported (Dassa et al., 1982; Touati and Danchin, 1987). Using appA-lacZ protein fusions and deletion analysis within the promoter region revealed a CAP binding site, which plays a major role in the cyclic AMP-mediated negative control. The negative control of gene expression in E. coli and related bacteria by the cyclic AMP-CAP complex has been numerously reported in recent years. Genes negatively controlled include arginine decarboxylase (Wright and Boyle, 1982), ornithine decarboxylase (Wright and Boyle, 1982; Wright et al., 1986) and the adenylate cyclase gene (Aiba, 1985).

In addition to the cyclic AMP mediated control, expression of appA gene was also inducible when bacterial cultures reach stationary phase, and its synthesis is triggered when bacteria are starved for inorganic phosphate or shifted from aerobic to anaerobic growth conditions (Dassa et al., 1982; Touati et al., 1986).

The regulatory features of the appA gene may also imply a complex regulation for the pathogenicity locus of P. s. pv. syringae. Interestingly, an indigenous

promoter (promoter 3 as shown in Figure 4-3), which corresponds at the same location and orientation as for the putative appA gene, was identified previously at this locus using pOSU2401::Tn3-HoHo1 fusion. It was identified as a Pseudomonas-specific promoter, since expression of β-galactosidase was observed only in Pseudomonas and not in E. coli. However, this could be due to the fact that this promoter is only inducible in low phosphate medium, such as King's medium B, which was used to detect promoter activity in Pseudomonas; and the promoter was repressed during growth in the rich LB medium used to detect promoter activity in E. coli.

The acid phosphatase of E. coli has been purified and has an estimated molecular weight of 45 kD (Dassa et al., 1982). A DNA sequence of approximately 1.1 kb in size is required to synthesize a 45 kD protein, and an ORF of that size would extend beyond the region where Tn5 was inserted in PS9024. Using a SEQUENA program, a putative polypeptide of approximately 15 kD (129 amino acids) could be encoded by the gene at the pathogenicity-related locus of P. s. pv. syringae. A putative signal peptide of 21 amino acids could be encoded by the N-terminal region of ORF-1 that is identical to a similar peptide of appA. This suggests that a membrance-associated protein is encoded by the pathogenicity locus. The difference in the predicted

protein size between the acid phosphatase encoded by appA of E. coli and the putative protein encoded at this pathogenicity locus of P. s. pv. syringae may suggest: 1) a functionally similar protein of different size is synthesized in P. s. pv. syringae; 2) a truncated protein is synthesized from sequences derived from a foreign source. 3) it is also possible that the 45 kD enzyme is encoded by a different gene locus in E. coli, since the size estimation of acid phosphatase was based on the measurement of the purified protein extract in one study (Dassa et al., 1982) and only 700 bp was sequenced at the 5′ end of the appA gene by Touati and Danchin (1987) in another study; or 4) alternatively, a DNA sequencing error occurred in the ORF-1 that created an improper stop codon.

SUMMARY

The results of the gene data bank searches using 1375 bp HigAI fragment as the query sequence revealed 98% nucleotide identity of one region of this pathogenicity-related locus and the 5′ end of the acid phosphatase (appA) gene of E. coli. This may suggest a functionally similar protein of different size is encoded by P. s. pv. syringae.

REFERENCES

Aiba, H. 1985. Transcription of the Escherichia coli adenylate cyclase gene is negatively regulated by cAMP-cAMP receptor protein. J. Biol. Chem. 260:3063-3067.

Dassa, E., M. Cahu, B. Desjoyaux-Cherel and P.L. Boquet. 1982. The acid phosphatase with optimum pH of 2.5 of E. coli: physiological and biochemical study. J. of Biolo. Chem. 257(12):6669-6676.

Gilson, E., J.M. Clement, D. Brutlag and M. Hofnung. 1984. EMBO J. 3:1417-1421.

Stefano, J. and J. Gralla. 1982. Spacer mutations in the lac P^S promoter. Proc. Natl. Acad. Sci. USA. 79:1069-1072.

Touati, E. and A. Danchin. 1987. The structure of the promoter and amino terminal region of the pH 2.5 acid phosphatase structural gene (appA) of E. coli: a negative control of transcription mediated by cyclic AMP. Biochimie. 69:215-221.

Wright, J.M. and S.M. Boyle. 1982. Negative control of ornithine decarboxylase and arginine decarboxylase by adenosine-3':5'-cyclic monophosphate in Escherichia coli. Mol. Gen. Genet. 186:482-487.

Wright, J.M., C. Satishchandran and S.M. Boyle. 1986. Transcription of the speC (ornithine decarboxylase) gene of Escherichia coli is repressed by cyclic AMP and its receptor protein. Gene. 44:37-45.

GENERAL CONCLUSIONS

A pathogenicity locus of Pseudomonas syringae pv. syringae identified by Tn5 mutagenesis was investigated. The mutant strain PS9024 is attenuated for disease expression in its host, Phaseolus vulgaris, but produces the hypersensitive reaction (HR) in the nonhost, tobacco (Nicotina tabacum). A cosmid clone carrying 16 kilobases (kb) of contiguous genomic DNA partially complements this mutant. Altered growth of the mutant in planta was also partially restored. Marker exchange mutagenesis with Tn3-HoHo1 at two other sites within this locus results in mutants with attenuated and severely reduced pathogenicity. The locus is complex and contains repetitive DNA sequences. Northern analysis reveals that this locus is expressed in planta, but is not expressed in a rich growth medium, and the transcript is larger than 10 kb, suggesting that the locus is transcribed as a polycistronic mRNA. Comparison of total cellular protein profiles of R32 and PS9024 using SDS-PAGE analysis further reveals that at least nine protein bands ranging from approximately 100 kD or above in size are present in the wild type strain R32, but absent from the mutant. Additionally, a

protein of approximately 45 kD is absent from the mutant. The site of Tn*5* insertion has been partially sequenced. The initial search of the data banks suggested a gene or genes related to the ornithine biosynthetic pathway map to this locus. Most recent data, as provided in the supplementary chapter, strongly suggest a gene that encodes a membrance-associated protein and under the control of a promoter identical to *app*A gene promoter maps at this site and it is involved in the process of pathogenesis.

DIRECTIONS OF THE FUTURE RESEARCH

The ultimate goal of this type of research is to provide information on how phytopathogenic bacteria attack susceptible host plants. Understanding of the genetic mechanisms of bacterial disease symptoms incited by expression of pathogenicity-related genes could lead to the development of disease resistant plants. The studies of pathogenicity-related genes that employ molecular genetic approaches could provide such information and eventually prove beneficial to agriculture.

Although information has been gathered using this approach in the study of this specific pathogenicity-related locus of P. s. pv. syringae, a thorough understanding of this pathogenicity-related locus requires further efforts and experimentation. The following are suggestions for some of the experiments that could be done to resolve unanswered questions.

1. To test the possible function of the gene with high homology to appA gene of E. coli:

Since 98% nucleotide identity was identified with 5′ end of appA gene of E. coli, it is logical to hypothesize that a similar gene function might be

encoded by this pathogenicity-related locus. To test this hypothesis, one approach would be to determine whether the corresponding gene from E. coli will complement the mutant, PS9024, in planta. Alternatively, restoration of acid phosphatase activity could be measured in PS9024 using an appropriate bioassay. A similar experiment can also be conducted in E. coli using the Pseudomonas DNA sequence cloned in this study. If successful, these results would indicate that this gene is functional.

2. Control of gene expression:

Studies have indicated that the promoter for the appA gene is negatively controlled by a cyclic AMP-CAP complex but is inducible in a low phosphate environment and stationary phase cultures. To test whether a similar mechanism is present in Pseudomonas, experiments can be designed in a way that the promoter should be either repressed or induced under various conditions, e.g., compare the bacterial cultures in low phosphate medium vs. high phosphate medium; or bacterial cultures in mid-log phase vs. stationary phase cultures. The expression of the promoter activity can be detected by measuring the β-galactosidase activity in a construct such as pOSU2401::Tn3-HoHo1. Fusions in this region were already constructed by this study. Alternatively,

the promoter activity can be measured directly by using Northern hybridization analysis.

3. Gene operon vs. gene clusters:

Results shown in this study suggested a gene operon maps at the region of Tn5 insertion. However, it was also indicated that this locus may consist of more than one operon or consist of a gene cluster. To test this possibility, DNA probes made from various regions within the Tn5-containing EcoRI fragment can be hybridized to the Northern blot of RNA extracted from the wild type strain R32 and the mutant strain PS9024. The correspondence of the size of specific mRNA to a specific DNA probe will provide rudimentary information about borders of multiple operons, or of gene clusters, and their approximate size.

BIBLIOGRAPHY

Aiba, H. 1985. Transcription of the Escherichia coli adenylate cyclase gene is negatively regulated by cAMP-cAMP receptor protein. J. Biol. Chem. 260:3063-3067.

Anderson, D.M. and D. Mills. 1985. The use of transposon mutagenesis in the isolation of nutritional and virulence mutants in two pathovars of Pseudomonas syringae. Phytopathology 75:104-108.

Ankenbauer, R., S. Sriyosachati, and C.D. Cox. 1985. Effects of siderophores on the growth of Pseudomonas aeruginosa in human serum and transferrin. Infect. Immun. 49:132-140.

Arlat, M and C. Boucher. 1991. Identification of a dsp DNA region controlling aggressiveness of Pseudomonas solanacearum. MPMI. 4(2):211-213.

Atkinson, M.M., J.S. Huang, and J.A. Knopp. 1985. The hypersensitive reaction of tobacco to Pseudomonas syringae pv. pisi: Activation of a plasmalemma K^+/H^+ exchange mechanism. Plant Physiol. 79:843-847.

Atkinson, M.M., C.J. Baker, and A. Collmer. 1986. Transient activation of plasmalemma K^+ efflux and H^+ influx in tobacco by a pectate lyase isozyme from Erwinia chrysanthemi. Plant Physiol. 82:142-146.

Atkinson, M.M. and C.J. Baker. 1987. Alteration of plasmalemma sucrose transport in Phaseolus vulgaris by Pseudomonas syringae pv. syringae and its association with K^+/H^+ exchange. Phytopathology 77:1573-1578.

Ausuel, F. M., et al.(eds.) 1989. Short Protocols in Molecular Biology. Greene Publishing Associates. p147.

Baker, C.J., M.M. Atkinson, A. Collmer, and M. Roy. 1986. Inhibition of the hypersensitive response in tobacco by pectate lyase. Physiol. Mol. Plant Pathol. 29:217-225.

Baur, H., V. Stalon, P. Falmagne, E. Luthi, and D. Haas. 1987. Eur. J. Biochem. 166:111-117.

Baur, H., E. Luethi, V. Stalon, A. Mercenier and D. Haas. 1989. Sequence analysis and expression of the arginine-deiminase and carbamate-kinase genes of P. aeruginosa. Eur. J. Biochem. 179:53-60.

Bertoni, G. 1987. M.S. Thesis, Oregon State University

Bertoni, G. and D. Mills. 1987. A simple method to monitor growth of bacterial populations in leaf tissue. Phytopathology 77:832-835.

Birnboine, and Doly. 1979. Mini-scale plasmid DNA isolation. NAR 7(15):

Cameron, F.H., D.J.G. Obbink, V.P. Ackerman and R.M. Hall. 1986. Nucleotide sequence of the ADD(2'') aminoglycoside adenylyltransferase determinant aadB. Evolutionary relationship of this region with those surrounding aadA in R538-1 and dhfrII in R388. Nucleic Acids Res. 14:8625-8635.

Chattoraj D.K., K. Cordes and A. Abeles. 1984. Plasmid P1 replication: negative control by repeated DNA sequences. Proc. Natl. Acad. Sci. USA. 81:6456-6460.

Chatterjee, A.K. and A. Vidaver. 1986. Genetics of pathogenicity factors: application to phytopathogenic bacteria. In: Ingram, D.S. and P.H. Williams (eds.) Advances in Plant Pathology. Academic Press, London. pp. 1-217.

Chatterjee, A.K., R.O. Nordeen, and G. Somlyai. 1989. Molecular cloning and expression of syringotoxin (ST) biosynthetic genes of Pseudomonas syringae pv. syringae. (Abst.) In: Pseudomonas '89: Biotransformations, Pathogenesis, and Evolving Biotechnology. American Society for Microbiology, Illinois, Chicago. Session III-10.

Clegg, S. and G. Gerlach. 1987. Enterobacterial fimbriae. J. Bacteriol. 169:943-948.

Cody, Y.S. and D.C. Gross. 1987. Outer membrane protein mediating iron uptake via pyoverdin$_{pss}$, the fluorescent siderophore produced by Pseudomonas syringae pv. syringae. J. Bacteriol. 169:2207-2214.

Cooper, R.M. 1983. In J.A. Callow ed.), Biochemical Plant Pathology. Wiley, London, p101-135.

Coplin, D.L. 1989. Plasmids and their role in the evolution of plant pathogenic bacteria. Ann. Rev. Phytopathol. 27:187-270.

Cox, C.D. 1982. Effect of pyochelin on the virulence of Pseudomonas aeruginosa. Infect. Immun. 36:17-23.

Cox, C.D. and P.I. Adams. 1985. Siderophore activity of pyoverdin for Pseudomonas aeruginosa. Infect. Immun. 48:130-138.

Crosa, J.H. 1984. The relationship of plasmid-mediated iron transport and bacterial virulence. Ann. Rev. Microbiol. 38:69-89.

Currier, T.C and M.K. Morgan. 1983. Plasmids of Pseudomonas syringae: no evidence of a role in toxin production or pathogenicity. Can. J. Microbiol. 29:84-89.

Dassa, E., M. Cahu, B. Desjoyaux-Cherel and P.L. Boquet. 1982. The acid phosphatase with optimum pH of 2.5 of E. coli: physiological and biochemical study. J. of Biolo. Chem. 257(12):6669-6676.

Daniels, M.J., J.M. Dow, and A.E. Osbourn. 1988. Molecular genetics of pathogenicity in phytopathogenic bacteria. Ann. Rev. Phytopathol. 26:285-312.

Daub, M.E. and D. Hagedorn. 1979. Resistance of Phaseolus line WBR 133 to Pseudomonas syringae. Phytopathology. 69:946-951.

DeVay, J.E., C.F. Gonzalez, and R.J. Wakeman. 1978. Comparison of the biocidal activities of syringomycin and syringotoxin and the characterization of isolates of Pseudomonas syringae from citrus hosts. In: Proc. 4th Int. Conf. Plant. Path. Bacteriol. Angers, France. pp 643-650.

Ditta, G., S. Stanfield, D. Corbin and D.R. Helinski. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of Rhizobium meliloti. Proc. Natl. Acad. Sci. USA. 77(12):7347-7351.

Dristig, M.C.G. and J.C. Dianese. 1990. Characterization of Pseudomonas solanacearum biovars based on Membrane protein patterns. Phytopathology. 80:641-646.

Durbin, R.D. 1981. Metabolism Biosynthesis In: Durbin, R.D. (ed.) Toxins in Plant Disease. Academic Press, Inc., New York. pp 443-448.

Ehrenshaft M. and D. Mills. 1985. Construction of a cosmid clone library of Pseudomonas syringae pv. phaseolicola and isolation of genes by functional complementation. Appl. Environ. Microbiol. 50:169-171.

Ellingboe, A.H. 1979. Inheritance of specificity: The gene-for-gene hypothesis. In: Daly, J.M. and I. Uritani (eds.) Recognition and Specificity in Plant Host-Parasite Interactions. pp. 115-131.

Expert, D. and A. Toussaint. 1985. Bacteriocin-resistant mutants of Erwinia chrysanthemi: possible involvement of iron acquisition in phytopathogenicity. J. Bacteriol. 163:221-227.

Falmagne, P., D. Portetelle and V. Stalon. 1985. Immonological and structural relatedness of catabolic ornithine carbamoyltransferases and the anabolic enzymes of enterobacteria. J. Bacteriol. 161:714-719.

Feinberg, A.P. and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6.

Figurski, D.H., R.J. Meyer and D.R. Helinski. 1979. J. Mol. Biol. 133:295-318.

Figurski D.H. and D.R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA. 76(4):1648-1652.

Flor, H.H. 1942. Inheritance of pathogenicity in Melampsora lini. Phytopathology 32:653-669.

Flor, H.H. 1971. Current status of the gene-for-gene concept. Ann. Rev. Pathol. 9:275-296.

Friedman, A.M., S.R. Long, S.E. Brown, W.J. Buikema and F.M Ausubel. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of Rhizobium mutants. Gene 18:289-296.

Garrett, C.M.E., J.E. Crosse, and A. Sletten. 1974. Relations between phage sensitivity and virulence in Pseudomonas morsprunorum. J. Gen. Microbiol. 80:475-483.

Gay, P., D. Le Coq, M. Steinmetz, T. Berkelman and C.I. Kado. 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. J. Bacteriol. 164:918-921.

Gilson, E., J.M. Clement, D. Brutlag and M. Hofnung. 1984. EMBO J. 3:1417-1421.

Gonzalez, C.F, J.E. DeVay, and R.J. Wakeman. 1981. Syringotoxin: a phytotoxin unique to citrus isolates of Pseudomonas syringae. Physiol. Plant Pathol. 18:41-50.

Gonzalez, C.F., S.K. Layher, A.K. Vidaver, and R.H. Olsen. 1984. Transfer, mapping, and cloning of Pseudomonas syringae pv. syringae plasmid pCG131 and assessment of its role in virulence. Phytopathology 74:1245-1250.

Grimm, C., L. Rahme, R. Frederick, M. Mindrinos, P.B. Lindgren, and N. Panopoulos. 1989. The common pathogenicity genes of Pseudomonas syringae pathovars. In: Collmer, et al. (eds.) Molecular Biology of Plant-Pathogen Interactions. UCLA Symp. Mol. Cell Biol., Alan R. Liss, Inc., New York, NY. pp. 49-55.

Grimm, C. and N. Panopoulos. 1989. The predicted protein product of a pathogenicity locus from Pseudomonas syringae pv. phaseolicola is homologous to a highly conserved domain of several procaryotic regulatory proteins. J. Bacteriol. 171:5031-5038.

Grindley and Reed. 1985. Ann. Rev. Biochem. 54:863-896.

Gross, D.C. and J.E. DeVay. 1977a. Production and purification of syringomycin, a phytotoxin produced by Pseudomonas syringae. Physiol. Plant Pathol. 11:13-28.

Gross, D.C. and J.E. DeVay. 1977b. Role of syringomycin in holcus spot of maize and systemic necrosis of cowpea caused by Pseudomonas syringae. Physiol. Plant Pathol. 11:1-11.

Gross, D.C. and J.E. DeVay. 1977c. Chemical properties of syringomycin and syringotoxin: toxigenic peptides produced by Pseudomonas syringae. J. Appl. Bacteriol. 43:453-463.

Gross, D.C., Y.S. Cody, E.L. Proebsting Jr., G.K. Tadamaker, and R.A. Spotts. 1984. Ecotypes and pathogenicity of ice nucleation active Pseudomonas syringae isolated from deciduous fruit tree-orchards. Phytopathology 74:241-248.

Gross, D.C. 1985. Regulation of syringomycin synthesis in Pseudomonas syringae pv. syringae and defined conditions for its production. J. Appl. Bacteriol. 58:167-174.

Haas, D., B.W. Holloway, A. Schambock and T. Leisinger. 1977. The genetic organization of arginine biosynthesis in P. aeruginosa. Mol. Gen. Genet. 154:7-22.

Haefele, D.M. and S.E. Lindow. 1987. Flagellar motility confers epiphytic fitness advantages upon Pseudomonas syringae. Appl. Environ. Microbiol. 53:2528-2533.

Hattermann, D.R. and S.M. Ries. 1989. Motility of Pseudomonas syringae pv. glycinea and its role in infection. Phytopathology 79:284-289.

Hoitink, H.A.J., D.J. Hagedorn, and E. McCoy. 1968. Survival, transmission and taxonomy of Pseudomonas syringae Van Hall, the causal organism of bacterial brown spot of bean (Phaseolus vulgaris L.). Can. J. Microbiol. 14:437-441.

Horwich, A.L., W.A. fenton, K.R. Williams, F. Kalousek, J.P. Kraus, R.F. Doolittle, W. Konigsberg and L.E. Rosenberg. 1984. Structure and expression of a complementary DNA for the nuclear precursor of human mitochondrial ornithine transcarbamylase. Science 224:1068-1074.

Houghton, J.E., D.A. Bencini, G.A. O'Donovan and J.R. Wild. 1984. Protein differentiation: A comparison of aspartate transcarbamoylase and ornithine transcarbamoylase from Escherichia coli K-12. Proc. Natl. acad. Sci. USA. 81:4864-4868.

Huang, H.C., R. Schuurink, T.P. Denny, M.M. Atkinson, C.J. Baker, I. Yucel, S.W. Hucheson and A. Collmer. 1988. Molecular cloning of a Pseudomonas syringae pv. syringae gene cluster that enables Pseudomonas fluorescens to elicit the hypersensitive reaction in tobacco plants. J. Bacteriol. 170:4748-4756.

Hurlbert, R.E. and D.C. Gross. 1983. Isolation and partial characterization of the cell wall of Pseudomonas syringae pv. syringae HS191: comparison of outer membrane proteins of HS191 with those of two plasmidless derivatives. J. Gen. Microbiol. 129:2241-2250.

Ielpi, L., T. Dylan, G.S. Ditta, D.R. Helinski and S.W. Stanfield. 1990. The ndvB locus of Rhizobium meliloti encodes a 319-kDa protein involved in the production of β-(1-2)-glucan. J. Biol. Chem. 265(5):2843-2851.

Isogai, A. N. Fukuchi, S. Yamashita and A. Suzuki. 1989. Syringostatin, novel phytotoxins produced by Pseudomonas syringae pv. syringae. Biol. Chem. 53(1):3117-3119.

Jawetz, E., J.L. Melnick and E.A. Adelberg. 1982. Review of medical microbiology. 15th edition. Lange Medical Publications, Los Altos, California. p87-96.

Jouanin, L., F. Vilaine, J. Tourneur, C. Tourneur, V. Pautot, J.F. Muller and M. Caboche. 1987. Plant Sci. 53:53-63.

Katz, A. and C. Kahana. 1988. Isolation and characterization of the mouse orinithine decarboxylase gene. J. Biol. Chem. 263:7604-7609.

Keen, N.T., T. Ersek, M. Long, B. Bruegger and M. Holliday. 1981. Inhibition f the hypersensitive reaction of soybean leaves to incompatible Pseudomonas spp. by blasticidin S, streptomycin, or elevated temperature. Physiol. Plant Pathol. 18:325-337.

Keen, N.T. and B. Staskawicz. 1988. Host range determinants in plant pathogens and symbionts. Ann. Rev. Microbiol. 42:421-440.

King, E.O., M.K. Ward and D.E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Med. 44:301-307.

Kleckner, N. 1981. Transposable elements in prokaryotes. Ann. Rev. Genet. 15:341-404.

Klement, Z. 1963. Rapid detection of pathogenicity of phytopathogenic pseudomonads. Nature 199:299-300.

Klement, Z. K. Rudolph and D.C. Sands. 1990. Methods in Phytobacteriology. Akademiai Kiado, Budapest. p191-198.

Kline, B.C. 1985. A review of mini-F maintenance. Plasmid. 14:1-16.

Konyecsni, W.M. and V. Deretic. 1990. DNA sequence and expression analysis of algP and algQ, components of the multigene sysmtem transcriptionally regulating mucoidy in P. aeruginosa. algP contains multiple direct repeats. J. Bacteriol. 172:2511-2520.

Korhonen, T.K., K. Haahtela, M. Romantschuk, and D. Bamford. 1986. Role of fimbriae and pili in the attachment of Klebsiella, Enterobacter and Pseudomonas to plant surface. In: Lugtenberg, D. (ed.) Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions. NATO ASI Series, Vol. H4. Springer-Verlag, Berlin Heidelberg. pp. 229-241.

Kotoujansky, A. 1987. Molecular genetics of pathogenesis by soft-rot Erwinia. Ann. Rev. Phytopathol. pp. 405-430.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Leben, C. and R.E. Whitmoyer. 1979. Adherence of bacteria to leaves. Can. J. Microbiol. 25:896-901.

Lenhoff, H.M. 1963. An inverse relationship of the effects of oxygen and iron on the production of fluorescin and cytochrome c by Pseudomonas fluorescens. Nature, London 199:601-602.

Lerner, C.G. and R.L. Switzer. 1986. Cloning and structure of the Bacillus subtilis aspartate transcarbamylase gene (pyrB). J. Biol. Chem. 261:11156-11165.

Lewin, B. 1987. Gene III. John Weley and Sons.

Lindgren, P.B., R. Frederick, N.J. Panopoulos, D. Dahlbeck, B.J. Staskawicz and S.E. Lindow. 1989. An ice nucleation reporter gene system: identification of an inducible pathogenicity gene in Pseudomonas syringae pv. phaseolicola. EMBO J. 8:2490-3001.

Lindemann, J., D.C. Arny, and C.D. Upper. 1984. Use of an apparent infection threshold population of Pseudomonas syringae to predict incidence and severity of brown spot of bean. Phytopathology 74:1334-1339.

Lindgren P.B. and N.J. Panopoulos. 1986. Pseudomonas syringae pathovars contain DNA sequences that are structurally and functionally homologous and control hypersensitivity and pathogenicity. (Abst.) In: 3rd Int. Sym. on "The Molecular Genetics of Plant-Microbe Interaction". McGill Univ., Montreal, Canada. 17-c.

Lindgren P.B., R.C. Peet and N.J. Panopoulos. 1986. A gene cluster of Pseudomonas syringae pv. phaseolicola controls pathogenicity on bean and hypersensitivity on non-host plants. J. Bacteriol. 168:512.

Lindgren, P.B., C. Grimm, N.J. Panopoulos, B.J. Staskawicz, and D. Dahlbeck. 1988a. The genetic control of interactions between Pseudomonas and host plants. (Abst.) In: 5th Int. Congr. of Plant Pathology, Kyoto, Japan. StVII 4-1.

Lindgren P.B., N.J. Panopoulos, B.J. Staskawicz, and D. Dahlbeck. 1988b. Genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathovars of Pseudomonas syringae. Molec. Gen. Genet. 211:499-506.

Loper, J.E. and S.E. Lindow. 1987. Lack of evidence for in situ fluorescent pigment production by Pseudomonas syringae pv. syringae on bean leaf surfaces. Phytopathology 77:1449-1454.

Loper, J.E. and J.S. Buyer. 1991. Siderophores in microbial interactions on plant surfaces. MPMI. 4(1):5-13.

Lu, Y. and S.W. Hutchenson. 1989. Identification of a regulatory locus controlling the expression of hrp gene in Pseudomonas syringae pv. syringae. (Abst.) In: Proc. 1st CAASS Conf., Communications Service, Cornell Univ., Ithaca, N.Y., U.S.A. Section C35.

Ma, S.W., V.L. Morris and D.A. Cuppels. 1991. Characterization of a DNA region required for production of the phytotoxin coronatine by P. s. pv. tomato MPMI. 4(1):69-74.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982 Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory. p387-389.

Mills, D. 1985. Transposon mutagenesis and its potential for studying virulence genes in plant pathogens. Ann. Rev. Phytopathol. 23:297-320.

Mills, D., F. Niepold, and M. Zuber. 1985. Cloned sequence controlling colony morphology and pathogenesis of Pseudomonas syringae. In: Sussex, I., A. Ellingboe, M. Crouch, and R. Malmberg (eds.) Plant Cell/Cell Interactions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp. 97-102.

Mills, D., F. Niepold, and G. Bertoni. 1987. Molecular analysis of pathogenicity of Pseudomonas syringae pathovars phaseolicola and syringae. In: Civerolo, E.L., A. Collmer, R.E. Davis, and A.G. Gillaspie (eds.) Plant Pathogenic Bacteria: Proc. 6th Int. Conf. on Plant Pathogenic Bacteria, Maryland. Martinus Nijhoff Publishers. pp. 404-411.

Mills, D. and F. Niepold. 1987. Molecular analysis of pathogenesis of Pseudomonas syringae pv. syringae. In: Nishimura, S., C.P. Vance, and N. Doke (eds) Molecular Determinant of Plant Disease. Japan Sci. Soc. Press, Tokyo/Springer-Verlag, Berlin. pp. 185-200.

Mills, D., P. Mukhopadhyay, Y. Zhao, and M. Romantschuk. 1988. Analysis of virulence in Pseudomonas syringae pathovars. (Abst.) In: 5th Int. Congr. of Plant Pathol, Kyoto, Japan. St.II. 4-4.

Mills, D., P. Mukhopadhyay, Y. Zhao, and M. Romantschuk 1991. Organization and function of pathogenicity genes of Pseudomonas syringae pathovars phaseolicola and syringae. In: S.S. Patil, D. Mills, and C. Vance (eds.): Molecular Strategies of Pathogens and Host Plants. Springer-Verlag Publisher, New York, Berlin, Heidelberg, London, Paris, Tokyo, HongKong, Barcelona. p69-82.

Mindrinos, M.N., L.G. Rahme, R.D. Frederick, E. Hatziloukas, C. Crimm and N.J. Panopoulos. 1990. Structure, function, regulation, and evolution of genes involved in pathogenicity, the hypersensitive response, and phaseolotoxin immunity in the bean halo blight pathogen. In: S. Silver, A.M. Chakrabarty, B. Iglewski and S. Kaplan (eds.) "Pseudomonas: biotransformations, pathogenesis, and evoloving biotechnology. Ammrican Society for Microbiology. p74-81.

Minsavage, G.V., and N.W. Schaad. 1983. Characterization of Membrane proteins of Xanthomonas campestris pv. campestris. Phytopathology 73:747-754.

Morgan, M.K. and A.K. Chatterjee. 1985. Isolation and characterization of Tn5 insertion mutants of Pseudomonas syringae pv. syringae altered in the production of the peptide phytotoxin syringotoxin. J. Bacteriol. 164:14-18.

Morgan, M.K. and A.K. Chatterjee. 1988. Molecular genetics of syringotoxin production. In: Proc. 3rd Int. Working Group on Pseudomonas syringae pathovars, Lisbon.

Mukhopadhyay, P., M. Mukhopadhyay, J. Williams, Y. Zhao, and D. Mills. 1988. Pathogenicity genes of Pseudomonas syringae. In: Palacios, R. and D.P.S. Verma (eds.) Molecular Genetics of Plant-Microbe Interactions. pp. 247-252.

Mukhopadhyay, P., M. Mukhopadhyay and D. Mills. 1990. Construction of a stable shuttle vector for high-frequency transformation in P. s. pv. syringae. J. Bacteriol. 172(1):477-480.

Niepold, F. and D. Mills. 1985. Cosmid instability in Pseudomonas syringae pathovars: evidence for directed excision of a cloned sequence. In: E.L. Clverolo, A. Collmer, R.E. Davis, and A.G. Gillaspie (eds.): "Plant Pathogenic Bacteria", Proc. of the Sixth Int. Conf. on Plant Pathogenic Bacteria, Maryland, Martinus Nijhhoff Publisher. p448-452.

Niepold, F., D. Anderson, and D. Mills. 1985. Cloning determinants of pathogenesis from Pseudomonas syringae pathovar syringae. Proc. Natl. Acad. Sci. USA 82:406-410.

Old, R.W. and S.B. Primrose. 1981. Principles of gene manipulation: An introduction to genetic engineering. Second edition. Studies in microbiology volume 2. University of California Press. p29.

Osborn, M.J. and H.C.P. Wu. 1980. Proteins of the outer membrane of gram-negative bacteria. Ann. Rev. Microbiol. 34:369-422.

Osbourn, A.E., C.E. Barber and M.J. Daniels. 1987. Identification of plant-induced genes of the bacterial pathogen Xanthomonas campestris pv. campestris using a promoter-probe plasmid. The EMBO J. 6(1):23-28.

Panopoulos, N.J. and M.N. Schroth. 1974. Role of flagellar motility in the invasion of bean leaves by Pseudomonas phaseolicola. Phytopathology 64:1389-1397.

Panopoulos, N.J. and R.C. Peet. 1985. The molecular genetics of plant pathogenic bacteria and their plasmids. Ann. Rev. Phytopathol. 23:381-419.

Parkinson, J. 1990. Communication modules in bacterial signaling proteins: Do prokaryotes conduct their business affairs with cellular phones? ASM News. 56(4):197-201.

Patel, P.N., J.C. Walker, D.J. Hagedorn, C.D. Garcia and M. Teliz-Ortiz. 1964. Bacterial brown spot of bean in central Wisconsin. Plant Disease Rep. 48:335-337.

Paynter, V.A. and R. Alconero. 1979. A specific fluorescent antibody for detection of syringomycin, a toxin produced by Pseudomonas syringae in infected peach tree tissues. Phytopathology 69:493-496.

Peet, R.C., P.B. Lindgren, D.K. Willis and N.J. Panopoulos. 1986. Identification and cloning of genes involved in phaseolotoxin production by Pseudomonas syringae pv. phaseolicola. J. Bacteriol. 166(3):1096-1105.

Persmark, M., D. Expert and J.B. Neilands. 1989. Isolation, characterization and synthesis of chrysobactin, a compound with siderophore activity for Erwinia chrysanthemi. J. Biol. Chem. 264:3187-3193.

Quant R. and D. Mills. 1984. An integrative plasmid and multiple-sized plasmids of Pseudomonas syringae pv. phaseolicola have extensive homology. Mol. Gen. Genet. 193:459-466.

Romantschuk, M. and D.H. Bamford. 1986. The causal agent of halo blight in bean, Pseudomonas syringae pv. phaseolicola, attaches to stomata via its pili. Microbial Pathogen. 1:139-148.

Romantschuk, M. 1987. Early steps in bacteriophage p6 infection: function of pili, membrane fusion, and nucleocapsid penetration. Ph.D Thesis. U. of Helsinki, Finland. pp. 59-61.

Romantschuk, M., Y. Zhao, K. McCluskey, J. Williams and D. Mills. 1990. Repeated sequences in Pseudomonas syringae pv. phaseolicola; Distribution and possible function as insertion sequences. Symbiosis. 8:21-31.

Ruvkun, G.B. and F.M. Ausubel. 1981. A general method for site-directed mutagenesis in prokaryotes. Nature. 289:85-88.

Sands, D.C., M.N. Schroth, and D.C. Hildebrand. 1980. Pseudomonas. In: Schaad, N.W. (ed.) Laboratory Guide for Identification of Plant Pathogenic Bacteria. American Phytopathological Society, St. Paul, MN. pp. 36-44.

Schmidt, F.R.J., E.J. Nuecken and R.B. Henschke. 1988. Nucleotide sequence analysis of 2'-aminoglyoside nucleotide transferase ANT(2'') from Tn4000, its relationship to AAD(3'') impact on Tn2 evolution. Mol. Microbiol. 2:709-717.

Shearman, C.A., L. Rossen, A. Johnston and J.A. Downie. 1986. The Rhizobium leguminosarum nodulation gene nodF encodes a polypeptide similar to acryl-carrier protein and is regulated by nodD plus a factor in pea root exudate. EMBO J. 5:647-652.

Simon, R., Priefer U., and Puhler, A. 1983. A broad range mobilization system for in vitro genetic engineering: Transposon mutagenesis in Gram-negative bacteria. Biotechnology 1:784-791.

Smidt, M.L. and A.K. Vidaver. 1982. Bacteriocin production by Pseudomonas syringae PsW-1 in plant tissue. Can. J. Microbiol. 24:600-604.

Smidt, M.L. and A.K. Vidaver. 1986. Isolation and characterization of syringacin W-1, a bacteriocin produced by Pseudomonas syringae pv. syringae. Can. J. Microbiol. 32:231-236.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Spena, A., T. Schmulling, C. Koncz and J. Schell. 1987. EMBO J. 6:3891-3899.

Stachel, S.E., G. An, C. Flores and E.W. Nester. 1985. A Tn3 lacZ transposon for the random generation of β-galactosidase gene fusion: Application to the analysis of gene expression in Agrobacterium. EMBO J. 4:891-898.

Stalker, D.M., C.M. Thomas and D.R. Helinski. 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. 181:8-12.

Stapp, C. 1961. Bacterial Plant Pathogens. London:Oxford University Press.

Staskawicz, B.J., D. Dahlbeck, and N.T. Keen. 1984. Cloned avirulence gene of Pseudomonas syringae pv. glycinea determines race-specific incompatibility on Glycine max (L.) Merr. Proc. Natl. Acad. Sci. USA 81:6024-6028.

Staskawicz, B., D. Dahlbeck, N. Keen and C. Napoli. 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of P. s. pv. glycinea. J. Bacteriol. 169:5789-5794.

Stefano, J. and J. Gralla. 1982. Spacer mutations in the lac P^s promoter. Proc. Natl. Acad. Sci. USA. 79:1069-1072.

Surico, G. and J.E. De-Vay. 1982. Effect of syringomycin and syringotoxin produced by Pseudomonas syringae pv. syringae on structure and function of mitochondria isolated from holcus spot resistant and susceptible maize lines. Physiol. Plant Pathol. 21:39-53.

Szabo, L.J. and D. Mills. 1984 Integration and excision of pMC7105 in Pseudomonas syringae pv. phaseolicola: Involvement of repetitive sequences. J. Bacteriol. 157:821-827.

Tepfer, D. 1984. Transformation of several species of higher plants by Agrobacterium rhizogenes: sexual transmission of the transformed genotype and phenotype. Cell. 37:959-967.

Thomas, C.M. 1983. Instability of a high-copy-mutant of a miniplasmid derived from broad host range IncP plasmid RK2. Plasmid 10:184-195.

Touati, E. and A. Danchin. 1987. The structure of the promoter and amino terminal region of the pH 2.5 acid phosphatase structural gene (appA) of E. coli: a negative control of transcription mediated by cyclic AMP. Biochimie. 69:215-221.

Turner, P., C. Barber and M. Daniels. 1984. Behavior of the transposons Tn5 and Tn7 in Xanthomonas campestris pv. campestris Mol. Gen. Genet. 195:101-107.

Turner, J.G. and R.E. Mitchell. 1985. Association between symptom development and inhibition of ornithine carbamoyltransferase in bean leaves treated with phaseolotoxin. Plant Physiol. 79:468-473.

Turner, J.G. 1986. Effect of phaseolotoxin on the synthesis of arginine and protein. Plant Physiol. 80-760-765.

Upshall. 1986. Genetic and molecular characterization of $argB^{+}$ transformants of Aspergillus. Curr. Genet. 10:593-599.

Vidaver, A.K. 1976. Prospects for control of phytopathogenic bacteria by bacteriophages and bacteriocins. Ann. Rev. Phytopathol. 14:451-465.

Vidaver, A.K. and S. Buckner. 1978. Typing of fluorescent phytopathogenic pseudomonads by bacteriocin production. Can. J. Microbiol. 24:14-18.

Wen, L., J.K. Huang and P.J. Blackshear. 1989. Rat ornithine decarboxylase gene: Nucleotide sequence, potential regulatory elements, and comparison to the mouse gene. J. Biol. Chem. 264:9016-9021.

Willis, D.K., E.M. Hrabak, S.E., Lindow and N.J. Panopoulos. 1988. Construction and characterization of Pseudomonas syringae recA mutant strains. Mol. Plant-Microbe Interact. 1:80-86.

Willis, D.K., E.M. Hrabak, J.J. Rich, T.M. Barta, S.E. Lindow, and N.J. Panopoulos. 1990. Isolation and characterization of a Pseudomonas syringae pv. syringae mutant deficient in lesion formation on bean. MPMI. 3:149-156.

Wills, D.K., J.J. Rich and E.M. Hrabak. 1991. hrp genes of phytopathogenic bacteria. MPMI. 4(2):132-138.

Wright, J.M. and S.M. Boyle. 1982. Negative control of ornithine decarboxylase and arginine decarboxylase by adenosine-3':5'-cyclic monophosphate in Escherichia coli. Mol. Gen. Genet. 186:482-487.

Wright, J.M., C. Satishchandran and S.M. Boyle. 1986. Transcription of the speC (ornithine decarboxylase) gene of Escherichia coli is repressed by cyclic AMP and its receptor protein. Gene. 44:37-45.

Xu, G.W. and D. Gross. 1988. Evaluation of the role of syringomycin in plant pathogenesis by using Tn5 mutants of Pseudomonas syringae pv. syringae defective in syringomycin production. Appl. Envir. Microbiol. 54:1345-1353.

Zhang, L. and J.Y. Takemoto. 1987. Effects of Pseudomonas syringae phytotoxin, syringomycin, on plasma membrane functions of Rhodotorula pilimanae. Phytopathology 77:297-303.

Zhao, Y. and D. Mills. 1989. Distribution of Pathogenicity-related DNA sequences from Pseudomonas syringae pv. syringae R32 among phytopathogenic bacteria. (Abst.) In: Proc. 1st CAASS Conf., Communications Service, Cornell Univ., Ithaca, N.Y., U.S.A. Section C36.

Zhao, Y. and D. Mills. 1990. Molecular genetic approaches in the study of pathogenicity of P. s. pv. syringae. J. of CAASS. 1:97-108.

Zhao, Y. and D. Mills. 1990. Study of a pathogenicity locus of strain PS9024 in P. s. pv. syringae. In: Proc. 2nd CAASS Conf., Oregon State University, Corvallis, OR, USA. p90-92.

Zhou, C., Y. Yang and A.Y. Jong. 1990. Mini-prep in ten minutes. BioFeedback. 8(2):172-173.