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**Genetic organization of a cluster of genes involved in the
production of phaseolotoxin, a phytotoxin produced by
Pseudomonas syringae pv. phaseolicola and sequence analysis of
the *phtE* locus**

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University of Hawaii, 1994

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GENETIC ORGANIZATION OF A CLUSTER OF GENES INVOLVED IN THE
PRODUCTION OF PHASEOLOTOXIN, A PHYTOTOXIN PRODUCED BY
Pseudomonas syringae pv. phaseolicola AND SEQUENCE
ANALYSIS OF THE *phlE* LOCUS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
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ABSTRACT

Phaseolotoxin, N^δ(N'-sulfo-diaminophosphiny)ornithylalanylhomarginine, produced by *Pseudomonas syringae* pv. phaseolicola, the bean halo blight pathogen, is a potent inhibitor of ornithine carbamoyltransferase (OCT). Inhibition of OCT in infected plants leads to chlorosis and growth inhibition. A genomic cosmid clone, pHK120, containing a 25 kb fragment of DNA from a wild-type strain of *P. syringae* pv. phaseolicola restores toxin production to Tox⁻ mutants. Tn5 mutagenesis of pHK120 and marker exchange of pHK120::Tn5 plasmids in the wild-type strain resulted in the isolation of 39 chromosomal mutants that harbor Tn5 insertions at known positions. Toxin bioassays revealed that 28 of the mutants, with Tn5 insertions distributed throughout the insert of pHK120, were Tox⁻, indicating that a functional locus for toxin production in each mutant was inactivated. Complementation analysis was done by testing strains for toxin production that carried a genomic Tn5 at one location and a plasmid-borne Tn5 at another location (pair complementation). Pair complementation analysis of nine marker-exchange mutants and a random genomic Tn5 mutant revealed that there are a minimum of eight toxin loci (*phtA* through *phtH*) in pHK120. Mutants carrying Tn5 insertions in the *phtA*, *phtD* and *phtF* loci were complemented by deletion subclones containing fragments from pHK120; mutants carrying Tn5 insertions in the *phtC* locus were partially complemented by a subclone, and mutants carrying Tn5 insertions in the *phtB*, *phtE*, *phtG* and *phtH* loci were not complemented by any of the available subclones. A comparison of the insert from pHK120 with that from pRCP17, a clone reported previously (Peet et al, 1986, J. Bacteriol. 166:1096-1105) by another laboratory to contain some of the phaseolotoxin genes and the resistant-OCT gene, revealed that the inserts in these two cosmids overlap but differ in important respects.

The sequence of *phtE*, the largest locus identified in pHK120, revealed six putative open reading frames (ORFs), each preceded by a putative ribosomal binding site, and all oriented in the same direction. In order to decide whether all six ORFs are in an operon, RT-PCR was performed using RNA extracted from a transconjugant of 4612-1 containing pHK120. Expected fragments (with correct sizes and ability to hybridize to specific probes) were generated by RT-PCR using the RNA as the template. Each fragment overlapped the continuous, and the contig of them covered ORF1 through ORF6, indicating that all six ORFs are transcribed in a single message, i.e. *phtE* locus is an operon. A primer extension experiment demonstrated that the expression of this operon is not temperature regulated because the same primer-extended product was generated using RNA from the wild-type strain at both 18°C and 28°C. A comparison of the sequences of the putative ORFs with the sequences of known genes in the data bases revealed that ORF3, encoding a 395 aa protein, has about 55% homology with the acetylornithine aminotransferase gene from *E. coli* and ornithine aminotransferase genes from other organisms. These data suggest that ORF3 encodes a protein required for the biosynthesis of ornithine, a constituent of phaseolotoxin. ORF6, encoding a 359 aa protein, has 40% homology with the fatty acid desaturase gene, *desA*, of *Synechocystis* Pcc6803, suggesting that the gene product of ORF6 may be involved in the secretion of phaseolotoxin. The functions of the remaining ORFs are not known because computer searches did not discover significant homologous gene sequences for the rest of the ORFs in the data base. However, all of the deduced amino acid sequences of these ORFs contain one to several hydrophobic regions, suggesting that the products encoded by these ORFs may be membrane-associated.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	iii
ABSTRACT	iv
CHAPTER I. INTRODUCTION.....	1
A. THE PROBLEM, THE RESEARCH PROPOSAL.....	1
B. LITERATURE REVIEW	3
1. Coronatine.....	3
2. Rhizobitoxine	6
3. Syringomycin	7
4. Syringotoxin, syringostatin.....	10
5. Tabtoxin	11
6. Phaseolotoxin.....	14
a. Introduction.....	14
b. Structure of phaseolotoxin	14
c. Mode of action of phaseolotoxin	
<i>in vitro</i>	15
d. Mode of action of phaseolotoxin	
<i>in vivo</i>	16
e. Phaseolotoxin and symptom expression.....	17
f. Specificity of phaseolotoxin	18
g. Autoimmunity of <i>P. syringae</i> pv. phaseolicola	
to phaseolotoxin	18
h. Genetics of phaseolotoxin production	20
i. Thermoregulation and phaseolotoxin production	21
7. Organization of genes involved in production of	
antibiotics	22
a. Bialaphos	23
b. Gramicidin S	25
C. DEVELOPMENT OF HYPOTHESIS	27
LITERATURE CITED	30

CHAPTER II. GENETIC ORGANIZATION OF A CLUSTER OF GENES INVOLVED IN THE PRODUCTION OF PHASEOLOTOXIN, A TOXIN PRODUCED BY <i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>	43
A. ABSTRACT	43
B. INTRODUCTION	45
C. MATERIALS AND METHODS.....	47
1. Bacterial strains, plasmids and growth conditions	47
2. Tn5 mutagenesis	47
3. Determination of the location of Tn5 insertions in the plasmid	47
4. Triparental mating	48
5. Electroporation	49
6. Verification of plasmids in transconjugants.....	49
7. Marker exchange (Homogenotization).....	50
8. Toxin bioassay.....	50
9. DNA isolation and manipulation	51
D. RESULTS.....	52
1. Restriction map, Tn5 mutagenesis of pHK120, and marker exchange in the wild-type strain	52
2. Fine mapping of transcriptional loci involved in toxin production.....	53
a. The <i>phtA</i> locus	54
b. The <i>phtB</i> , <i>phtC</i> and <i>phtD</i> loci	54
c. The <i>phtE</i> , <i>phtF</i> and <i>phtG</i> loci.....	55
d. The <i>phtH</i> locus	55
3. Complementation of selected mutants by subclones of pHK120	56
4. A comparison of the genomic fragments from pHK120 and pRCP17.....	57
5. pHK120 and thermoregulation of phaseolotoxin production.....	58
E. DISCUSSION	65
LITERATURE CITED	71

CHAPTER III. NUCLEOTIDE SEQUENCE ANALYSIS OF THE <i>phtE</i> LOCUS INVOLVED IN THE PRODUCTION OF PHASEOLOTOXIN.....	76
A. ABSTRACT	76
B. INTRODUCTION	78
C. MATERIALS AND METHODS.....	80
1. Generation of a nested set of deletion subclones for sequencing	80
2. Sequencing of DNA	80
3. Isolation of total RNA.....	81
4. Preparation of probes.....	81
5. RT-PCR amplification of mRNA.....	82
6. Primer Extension.....	83
7. Computer-assisted analyses.....	83
D. RESULTS.....	84
1. The Sequence of the <i>phtE</i> locus	84
2. The <i>phtE</i> transcript	86
3. Primer extension	87
4. Product of ORF1	88
5. Product of ORF2.....	88
6. Product of ORF3.....	89
7. Product of ORF4.....	89
8. Product of ORF5.....	90
9. Product of ORF6.....	90
E. DISCUSSION	116
LITERATURE CITED	121
CHAPTER IV. CONCLUSIONS.....	127
LITERATURE CITED	130

LIST OF TABLES

Table	page
2.1 Bacterial strains and plasmids	62
2.2 Results of complementation and pair-complementation analysis	64
3.1 RT-PCR amplification of mRNA of the <i>phtE</i> locus	87

LIST OF FIGURES

Figure	page
1.1 Structure of coronatine	4
1.2 Structure of rhizobitoxine	6
1.3 Structure of syringomycin	8
1.4 Structures of syringotoxin and syringostatin.....	11
1.5 Structure of tabtoxin	11
1.6 Structure of phaseolotoxin	15
1.7 Pathway of chemical degradation of phaseolotoxin.....	17
1.8 Structure of bialaphos	23
1.9 Organization of genes involved in bialaphos production.....	23
1.10 Structure of gramicidin S.....	26
2.1 Restriction map of pHK120, phaseolotoxin loci and comparison of pHK120 with pRCP17	60
2.2 Toxin production in 4612-1 and 4612-1(pHK120).....	61
3.1 Open reading frames in the <i>phtE</i> locus	92
3.2 Nucleotide sequence of the <i>phtE</i> locus.....	94
3.3 Codon preference of the ORFs in the <i>phtE</i> locus as compared to that of other <i>Pseudomonas</i> genes.....	105
3.4 RT-PCR experiments	107
3.5 Transcriptional start site of the <i>phtE</i> locu	108
3.6 Hydropathy profiles of ORF1	109
3.7 Hydropathy profiles of ORF2	109
3.8 Alignment of ORF3 with ACOAT and OAT genes	110

3.9	Hydropathy profiles of ORF4	113
3.10	Hydropathy profiles of ORF5	113
3.11	Alignment of ORF6 with <i>desA</i>	114
3.12	Hydropathy profiles of <i>desA</i> and ORF6.....	115

CHAPTER I. INTRODUCTION

A. THE PROBLEM, THE RESEARCH PROPOSAL

Halo blight of beans caused by *Pseudomonas syringae* pv. phaseolicola continues to be a major threat to bean production in U. S., South America and Asia; no efficient control method is available. Research on this disease has been focused on two major areas: the elucidation of the genetics of pathogenesis and the elucidation of the virulence factor, phaseolotoxin, with the hope that either or both will lead to development of better control methods.

The research in the first area has resulted in the identification of the *hrp* gene cluster (19, 45) and its organization and regulation (78, 79). The ultimate goal of this research is to understand how the pathogen initiates infection and how it combats the resistance mechanism(s) of the host plant. The research in this area has also resulted in the understanding of the resistance mechanisms involved in gene-for-gene systems. Several workers (24, 31, 87) have identified *avr* genes in *P. syringae* pv. phaseolicola which induce resistance (hypersensitive reaction, HR) in appropriate incompatible bean cultivars carrying single, dominant complementary resistant genes. The understanding of the mechanism by which the *avr* genes induce resistance in the host will facilitate the search for the complementary resistance genes in bean cultivars. The cloning and characterization of the resistant genes will directly result in being able to engineer durable resistance in bean cultivars against *P. syringae* pv. phaseolicola.

The genetic research on phaseolotoxin is more recent. The ultimate goal of this research is to elucidate the biosynthetic pathway of phaseolotoxin and to determine how it is regulated. This knowledge will help to explore alternative methods of controlling this disease. The biosynthetic pathway is believed to be very complex, because among the three constituent amino acids, ornithine, alanine and homoarginine, ornithine and homoarginine

are not normally found in proteins. In addition, an inorganic group is present in the structure, which adds additional complexity to the biosynthetic pathway. Fortunately, with the advent of the tools of molecular biology, it has now become possible to dissect this complex process by isolating the genes involved in the various biosynthetic steps and individually analyzing them.

Prior to this study, work from another laboratory (73) suggested that the genes involved in phaseolotoxin production are clustered because a genomic clone complemented several independent Tn5 Tox⁻ mutants of *P. syringae* pv. phaseolicola. Our laboratory had also been involved in similar studies. Kamdar et al (32) isolated a genomic clone (pHK120) that complemented EMS, UV and Tn5 mutants, indicating that the pHK120 contained a cluster of structural and regulatory genes involved in phaseolotoxin biosynthesis. The objectives of this study were to determine the number of loci in pHK120 involved in toxin production and to determine the DNA sequence of the largest locus with a view of understanding the number of open reading frames (ORFs) in that locus.

B. LITERATURE REVIEW

Enzymes, phytotoxins and plant growth hormones have been shown to be the three main factors that are involved in pathogen virulence in plants. Phytotoxins, defined by Graniti (18) as "microbial metabolites that are harmful to plants at very low concentrations", represent the best chemically described class of compounds among the three classes of virulence factors. Their structures, modes of action in plants and biosyntheses are understood better than the other factors. Phytotoxins can be divided into two categories based on their involvement in pathogenesis: host-selective toxins, which are necessary for pathogenesis and are toxic only to susceptible hosts; non-host-selective toxins, which are dispensable in pathogenesis but important in virulence, and generally equally toxic to all hosts (not cultivar-specific). Toxins produced by phytopathogenic bacteria studied so far belong to the second category. The structures of bacterial phytotoxins are very diverse: some are peptides, some are phenolic compounds whereas others are low molecular weight carboxylic acids (53). Their modes of action are also quite diverse: some work on cytoplasmic enzymes, some on chloroplastic enzymes whereas others affect membrane function. The attention they have received from scientists is also varied: some are merely mentioned in the literature, while others have been studied extensively. Among the best-studied bacterial phytotoxins are coronatine, rhizobitoxine, syringomycin, syringotoxin, phaseolotoxin and tabtoxin. The following review focuses mainly on symptomatology, modes of action, biosyntheses and genetics of these toxins.

1. Coronatine

Coronatine, a non-specific toxin produced by several *Pseudomonas syringae* pathovars, viz. pv. glycinea, pv. tomato, pv. atropurpurea and pv. morsprunorum, is an

amide formed by coupling the polyketide coronafacic acid to the novel amino acid coronamic amid (Fig. 1.1) (60). In addition to coronatine, N-coronafacoyl-L-valine and some other minor products, all with a coronafacic acid moiety, are found in liquid cultures of *P. syringae* pathovars mentioned above (53).

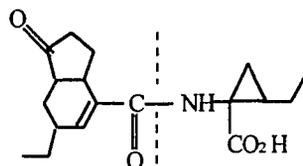


Fig. 1.1. Structure of coronatine

It has been postulated that the two chemical components of coronatine, coronafacic acid and coronamic amid, are synthesized by different pathways (67). The coronafacic acid moiety may be derived from two polyketides and a pyruvate, whereas the cyclopropyl moiety is derived from isoleucine. Final assembly of coronatine occurs when an amide bond is formed between these two components. Since coronafacoyl-isoleucine was found in the liquid cultures of *P. syringae* pv. *syringae*, it was suggested that the amide bond is formed before the cyclization of the 3-member ring.

Coronatine is not a necessary factor in pathogenesis because *Tox⁻* mutants still cause symptoms on plants (6). However, the lesions caused by these *Tox⁻* mutants were smaller than those caused by the wild-type strain and the populations of the mutated bacteria in the leaves were far less than the wild-type strain, indicating that coronatine participates in the disease development as a virulence factor. The typical symptoms caused by coronatine as well as the coronatine-producing strains are similar; both cause stunting and chlorosis. However, hypertrophy was found in diseased potato tubers (16). It was suggested that hypertrophy results from an increase in cell wall plasticity, alternation in the

plasmalemma or an increase in osmotic pressure (9); however, the mechanisms of chlorosis induction and stunting are still unknown. It is noteworthy that, unlike many other phytotoxins produced by bacteria, coronatine shows no antibiotic activity against other microorganisms (104).

In *P. syringae* pv. tomato pT23.2, an indigenous plasmid, pPT23A (101 kb), was implicated in the synthesis of coronatine (5) because Tn5 insertions in some positions of this plasmid prevented toxin production. Furthermore, introduction of this plasmid into *P. syringae* pv. *syringae* PS61, a strain that does not produce coronatine, resulted in toxin production in the transconjugants. A 30 kb sequence in this plasmid was found to be conserved in plasmids from four coronatine-producing pathovars despite their disparate origins (7). Tn5 mutagenesis of the 30 kb DNA in the insert (109) narrowed down the region necessary for coronatine production to 20.5 kb and that for coronafacic acid formation to 4.4 kb. However, another group (8, 60) reported a chromosomal location for genes involved in coronatine production by *P. syringae* pv. tomato. Southern hybridization analysis also demonstrated the presence of homologous sequences in the chromosomes of two other coronatine-producing strains, *P. syringae* pv. *atropurpurea* and *P. syringae* pv. *glycinea*. A cosmid clone (46) containing 30 kb of chromosomal DNA from *P. syringae* pv. tomato complemented all Tn5-generated Tox⁻ mutants. Tn3-*Spice* and Tn*phoA* mutagenesis of this region revealed six complementation groups involved in coronatine production. None of the Tn*phoA*-induced mutants showed alkaline phosphatase activity indicating that none of the genes in this region encoded membrane bound proteins. The expression of genes in one of the regions (CorII) was shown to be induced by tomato plant extracts (46).

2. Rhizobitoxine

Rhizobitoxine, 2-amino-4-alkoxy-but-3-enoic acid (Fig. 1.2), is produced by *Bradyrhizobium japonicum* and other species of *Bradyrhizobium* from diverse geographical areas. It is considered to be a phytotoxin because it is also produced by *Pseudomonas andropognis* and is involved in bacterial stripe disease of corn (55). One of its inactive derivatives, dihydrorhizobitoxine, was found in the liquid culture of *P. andropognis* as well as in nodules induced by *Bradyrhizobium japonicum*.

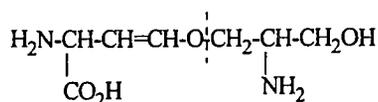


Fig. 1.2. Structure of rhizobitoxine.

Hydroxythreonine ($\text{CO}_2\text{H}-\text{CH}(\text{NH}_2)-\text{CH}=\text{CH}_2\text{OH}$) and serinol ($\text{HOCH}_2-\text{CH}(\text{NH}_2)-\text{CH}_2\text{OH}$) are probably two immediate intermediates in the rhizobitoxine biosynthetic pathway because they were detected in the liquid culture of *P. andropogonis* and in plant nodules respectively (53). Linkage of these two compounds by an ether bond results in the formation of rhizobitoxine. This process was confirmed in a ^{14}C feeding experiment in which 40% of ^{14}C -hydroxythreonine was incorporated into rhizobitoxine (52). These studies further showed that hydroxythreonine was derived from aspartic acid. The precursor of serinol is not known (52).

Since rhizobitoxine inhibits the activity of β -cystathionase in plants, its involvement in disease development is apparent although the actual mechanism of symptom formation remains undetermined. In addition to its toxic effect on plants, rhizobitoxine may help the producing organism to develop the disease by inhibiting ethylene formation in plants. Ethylene is thought to be able to trigger activation of plant disease resistance mechanisms,

therefore a decrease in ethylene concentration caused by rhizobitoxine may make plants more vulnerable to infection (53).

Unlike *P. andropogonis*, *Bradyrhizobium japonicum* generally associates with its hosts as a mutualistic symbiont. Therefore, it would be interesting to determine what role this phytotoxin plays in such a relationship. By studying several Tn5-generated Tox⁻ mutants of *Bradyrhizobium japonicum*, Ruan and Peters (82) showed that rhizobitoxine plays an apparent role in the nodulation of soybeans. Because ethylene is known to substantially reduce nodule formation (75) through nitrate inhibition of nodulation, and rhizobitoxine inhibits ethylene synthesis in plants, it was suggested that rhizobitoxine may contribute to nodulation through inhibition of ethylene. However, Ruan and Peters (82) demonstrated that the nodulation induced by the wild-type and Tox⁻ strains of *Bradyrhizobium japonicum* were equally suppressed by nitrate. Nevertheless, with its antibiotic ability, rhizobitoxine may help *B. japonicum* to colonize the roots of plants better than other microorganisms which are present in the rhizosphere. *Bradyrhizobium* infected plants show chlorosis indicating that rhizobitoxine also causes deleterious effects in infected plants.

3. Syringomycin

Syringomycin (Fig. 1.3), produced by most strains of *Pseudomonas syringae* pv. *syringae* that cause disease on stone fruit, pears and grass hosts (21), exists as a mixture of several distinct but related compounds. The main component is designated SR-E which consists of arginine, phenylalanine, serine, 3-hydroxyaspartic acid (HyAsp), 2,3-dehydrothreonine (DhThr), diaminobutyric acid (Dab) in a 1:1:1:1:1:2 molar ratio that are joined in a cyclic configuration (84).

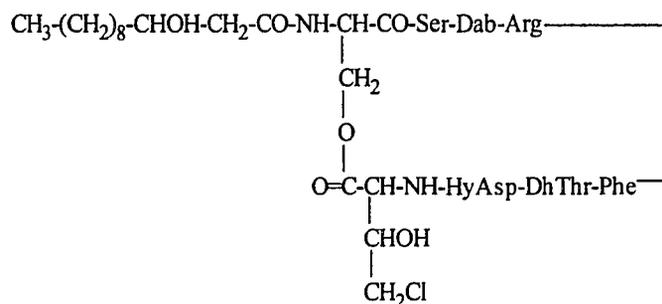


Fig. 1.3. Structure of syringomycin.

How syringomycin is involved in disease development is not clear. Xu and Gross (107) suggested that syringomycin contributes to pathogenicity of *P. syringae* pv. *syringae* because the Tn5 mutants they obtained were Tox^- as well as Path^- . However, Mitchell (53) argued that since the bioassay for toxin production (inhibition of growth of *Geotrichum candidum*) they used was not quantitative, some of the mutants may be scored erroneously as Tox^- . Nevertheless, syringomycin contributes significantly to virulence because the lesions caused by Tox^- mutants are approximately one third the size of those caused by Tox^+ strains.

Several lines of evidence from studies of plants and fungi (110) suggested that syringomycin affects ion transport processes rather than causing a general effect on membrane integrity. Hyperpolarization, rapid efflux of K^+ and stimulation of the proton pump ATPase occurred after application of syringomycin (93). Another effect of syringomycin on plants is stomatal closure similar to that caused by abscisic acid (ABA) (64). The two toxins may initiate this effect differently but they both activate K^+ efflux, which is consistent with the study of Takemoto et al (93) that showed that a H^+/K^+ antiport system was affected by syringomycin. It was proposed (20) that the cellular release of K^+ would raise the relatively acidic pH of intercellular fluids to near neutrality, an environment more conducive to bacterial multiplication.

Little is known about the pathway of syringomycin biosynthesis. A model involving multifunctional peptide synthetases similar to those involved in the biosynthesis of some antibiotics such as polymyxin has been proposed (20). The genes for syringomycin biosynthesis are believed to be located in the chromosome because a plasmid-free *P. syringae* pv. *syringae* strain retained its ability to produce the toxin (17). Two toxin genes, *syrA* and *syrB*, 2.5 kb and 2.7 kb long, respectively, were isolated from a toxin-producing strain of *P. syringae* pv. *syringae* (106). Marker-exchange mutants containing Tn5 in *syrA* or Tn3*HoHo1* in *syrB* failed to produce toxin and cause symptoms on plants. Such mutants also lost their ability to produce SR4 and SR5, two of the five cellular proteins that are associated with toxin production in the wild-type strain. A strain containing *syrB::lacZ* gene fusion exhibited galactosidase activity in response to plant extracts and increased ion concentration, indicating that *syrB* is plant inducible. Further studies (57, 58) showed that *syrB*-inducing substances are plant phenolic compounds, including arbutin, phenyl- β -D-glucopyranoside and salicin, and their *syrB*-inducing activity is enhanced by plant sugars, such as D-fructose, D-mannose and sucrose. Two other genes, *syrC* and *syrD*, were found to be clustered with *syrB* in a 7 kb DNA fragment (20). A mutant containing disrupted *syrD* was defective in the expression of *syrB* and *syrA* and in the production of the five proteins mentioned above, indicating that *syrD* is a regulatory gene that governs the expression of other toxin genes, including *syrB* and *syrA*. However, recent sequence data on *syrD* (77) has revealed that the *syrD* product is an ATP-binding membrane protein homologue, and that it may be involved in the secretion of syringomycin. The "regulatory" effect of *syrD* on other *syr* genes was actually the result of a feedback mechanism. Failure to export toxin in the *syrD* mutant apparently results in the accumulation of higher than the tolerable threshold of toxin which then shuts down toxin gene expression to prevent autotoxicity.

4. Syringotoxin (ST), syringostatin (SS)

Syringotoxin (ST) is produced only by the citrus isolate of *P. syringae* whereas syringostatin (SS) is produced only by the lilac isolate. Their structures (Fig. 1.4) are similar to that of syringomycin but contain different amino acid residues. ST differs from Syringomycin in that serine, arginine and phenylalanine substitute for homoserine, ornithine and threonine, respectively. Likewise, SS differs from ST in that 2,4-diaminobutyric acid in SS is replaced by glycine to form ST (Fig. 1.4). As syringomycin, both ST and SS inhibit the growth of fungi, such as *Geotrichum candidum* and *Rhodotorula pilimanae*, which provides a useful bioassay for toxin production (104).

By conducting Tn5-mutagenesis of a toxin-producing strain, *Pseudomonas syringae* pv. *syringae* (B457), Morgan and Chatterjee (61) obtained several ST⁻ mutants which had increased sensitivity to lysozyme-EDTA-induced lysis in addition to the loss of ST production, indicating that the toxin production is related to membrane integrity. Southern blot analysis of ST⁻ mutants showed that the Tn5 insertions were in three different but adjacent chromosomal *Eco*RI fragments, of 10.5, 17.8 and 19.3 kb, respectively. Two large proteins, 470 kd and 435 kd, respectively, were associated with ST production in the toxin-producing strain (62). Analysis of the truncated proteins in the Tn5 mutants and mapping of the Tn5 insertion indicated that the genes for these two proteins are in the same transcriptional unit. Expression of these two proteins was growth-dependent and induced by high concentration of iron.

biosynthetic pathway, and this was confirmed by Engst and Shaw (13) by showing that a gene product required for tabtoxin biosynthesis shares considerable homology to bacterial lysine biosynthetic enzymes: diaminopimelate decarboxylase and Δ^1 -piperidine-2,6-dicarboxylate succinyltransferase.

The typical symptom caused by tabtoxin is chlorosis surrounding necrotic centers. That fact that light is required for the development of chlorosis, and that methionine sulfoximine (MSO) inhibits glutamine synthetase (GS) indicates that T β L is an irreversible inhibitor of GS (98). The accumulation of ammonia is thought to be the major factor associated with the *in planta* chlorosis induced by this toxin.

Since GS from plants, fungi as well as bacteria are inhibited by T β L, it was of interest to determine how the tabtoxin-producing strains prevent autotoxicity. One possibility is that *P. syringae* pv. *tabaci* cells prevent autotoxicity by producing tabtoxin which does not inhibit GS. However, a Zn⁺-dependent dipeptide-specific aminopeptidase located in the plasmalemma of the toxin-producing bacteria (44) should be able to activate tabtoxin by converting it to T β L, which is inhibitory to GS. Thus, the toxin-producing bacteria must have additional mechanisms of protection from T β L. Knight et al (36) reported isolation of a GS from a Tox⁺ strain that is insensitive to T β L. In this case, the protection of GS from T β L was attributed to adenylation of the enzyme which made it insensitive to T β L. In addition, Tox⁺ strains contain β -lactamase which can inactivate T β L by destroying the ring structure in T β L (37). Recently, Anzai et al (2) and Yoneyama et al (108) isolated a gene from a tabtoxin-producing strain and demonstrated that its product provided resistance against T β L to tabtoxin-sensitive *Pseudomonas* strains or *E. coli*. The sequence of this gene revealed that it encodes a transacetylase (*ttr*), raising the possibility that the tabtoxin-producing strains use this enzyme to degrade the toxin to protect themselves (2). However, the *ttr* gene was shown to be present in Tox⁻ mutants, naturally

occurring non-producing strains of *P. syringae* pv. *tabaci* and some tabtoxin-sensitive strains of *P. syringae* (34). Therefore, the role of this gene in the self-protection against tabtoxin within tabtoxin-producing strains remains unclear.

Except in one case (34), production of tabtoxin is not necessary for pathogenicity but enhances virulence of the pathogen significantly (53). Kinscherf et al (34) generated 23 *Tox*⁻ mutants using Tn5 mutagenesis of the wild-type strain *P. syringae* BR2, which had lost the ability to cause symptoms in host plants. All of these mutants, except three, were sensitive to tabtoxin, indicating that the resistance gene is physically linked to the toxin-producing genes in a cluster. A cosmid clone, pRTBL823, containing ~31 kb DNA from the chromosome of the wild-type strain, restored toxin production, symptom-forming ability and tabtoxin resistance to all *Tox*⁻ mutants. In addition, it made a non-toxin-producing strain, *P. syringae* epiphyte Cit 7, *Tox*⁺ and tolerant to tabtoxin. These results suggested that pRTBL823 contains a complete set of genes for tabtoxin biosynthesis and the resistance gene.

Tn5-mutagenesis of another strain, *Pseudomonas syringae* pv. *coronafaciens*, led to the isolation of seven *Tox*⁻*Tox*^r mutants (3). Southern hybridization revealed that in all seven mutants, the Tn5 was inserted into *lemA*, a regulatory gene first identified in strain B728a of *Pseudomonas syringae* pv. *syringae*. *lemA* was conserved in all *Pseudomonas syringae* pathovars with different functions: in *P. syringae* pv. *syringae*, *lemA* is required for formation of lesion, production of protease and syringomycin (28, 105); in *P. syringae* pv. *phaseolicola*, mutation in *lemA* has no effect on lesion formation or phaseolotoxin production; in *P. syringae* pv. *coronafaciens*, *lemA* mutants lost the ability to produce tabtoxin and protease, but still cause disease in the oat plants. The DNA sequence of *lemA* revealed that it has homology with genes involved in the two-component signal transduction pathway of bacteria (28). It has also been shown (3) that the *lemA* product regulates at least

one of the tabtoxin genes at the transcriptional level. However, since the *lemA* mutants of *P. syringae* pv. *coronafaciens* are still tolerant to tabtoxin, *lemA* does not seem to regulate the genes involved in tabtoxin resistance.

6. Phaseolotoxin

a. Introduction

Pseudomonas syringae pv. *phaseolicola* (Burkh.) causes a disease on common bean (*Phaseolus vulgaris* L.), called halo blight. The infection of susceptible adult plants normally results in water-soaking followed by a chlorotic zone or halo around infection sites. Infection of young developing trifoliolate leaves leads to systemic chlorosis.

In addition to chlorosis, ornithine, a non-protein amino acid, accumulates in infected bean leaves (68, 69). Hoitink et al (25) showed that the sterile culture filtrates from the bacterial cultures induced chlorotic halos characteristic of the disease caused by the wild-type strain in bean leaves, and the substance responsible for symptom-formation is thermostable. The toxin was partially purified by Rudolph and Stahmann (83) using a method combining methanol extraction, charcoal absorption and thin layer chromatography and it induced chlorosis when injected into bean leaves. These studies clearly indicated that halo blight of bean is caused by a toxin produced by *Pseudomonas syringae* pv. *phaseolicola*.

b. Structure of phaseolotoxin

Mitchell (50) used the following steps to purify the toxin: absorption to activated charcoal, elution from the charcoal with methanol-chloroform-aqueous ammonia, QAE-sephadex chromatography, and sephadex LH 20 chromatography with methanol-chloroform-aqueous ammonia as a solvent. This procedure yielded a toxin about 90% pure.

Mitchell (49) showed that three amino acids: ornithine, alanine and homoarginine, were liberated after hydrolysis of the toxin with strong acids. End group analysis indicated that the tripeptide starts at the N-terminal of an ornithine and ends at the C-terminal of a homoarginine. The ornithine was found to be modified by a substitutive group containing P and S at the NH₂ position of ornithine. A detailed study (50) revealed that the substituting group is phosphosulphamic acid. Therefore, Mitchell (50) proposed the structure for the toxin as (N-phosphosulphamyl)-ornithyl-alanyl-homoarginine (Fig. 1.6) and gave it the trivial name phaseolotoxin. Moore et al (59) revised the structure to N^δ(N'-sulfodiamophosphinyl)-ornithyl-alanyl-homoarginine. Mild hydrolysis of phaseolo-toxin releases alanine, homoarginine and octicidin (Fig. 1.6).

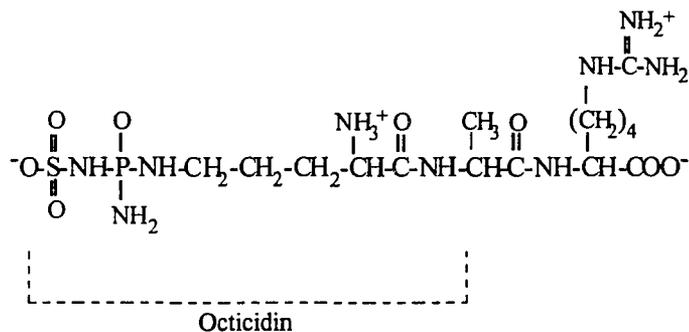


Fig. 1.6. Structure of phaseolotoxin.

c. Mode of action of phaseolotoxin *in vitro*

Accumulation of ornithine in chlorotic tissues of bean plants infected with *P. syringae* pv. *phaseolicola* or injected with phaseolotoxin (83) indicated that the toxin interferes with the metabolism of ornithine. Using partially purified toxin, Patil et al (70) demonstrated that ornithine carbamoyltransferase (OCTase), an enzyme involved in conversion of ornithine to citrulline, was inhibited by the toxin *in vitro*. This was supported by the fact that application of citrulline or arginine to plants prior to or after injection of

toxin could protect plants from chlorosis, presumably because application of citrulline or arginine relieved the deficiency of arginine that results from inhibition of OCTase by the toxin (70). Further studies (71) with all the enzymes related to ornithine cycle in plants showed that only OCTase was inhibited by the toxin. Tam and Patil (94) further showed that the toxin binds to the carbamoylphosphate site of the enzyme competitively. Kwok et al (42) showed that the peptidase degradation product of the toxin bound to the enzyme irreversibly and that the phosphate ion protected the active site of the enzyme from binding to the toxin. Thus, phaseolotoxin binds to the carbamoylphosphate site of the enzyme in a competitive fashion whereas the peptidase degradation product of the toxin binds the same site in an irreversible manner.

d. Mode of action of phaseolotoxin *in vivo*

Several lines of evidence indicated that phaseolotoxin also inhibits OCTase *in vivo* (54, 72). However, when the toxin was applied to susceptible plants, the dominant component recovered from the leaves was a degradation product, N-phospho-sulfamyl-ornithine (PSorn, 54), now called octicidin (59). Synthetic octicidin (56) was able to cause chlorosis and accumulation of ornithine in plants, indicating that octicidin is the main toxic component of phaseolotoxin in diseased plants. An *in vitro* enzymatic assay (42) indicated that octicidin has about 20 fold higher inhibitory activity to OCTase as compared to phaseolotoxin. However, it shows very low inhibitory activity to *E. coli*, probably due to the fact that octicidin can not be efficiently taken up by *E. coli*. Enzymatic assay (42) demonstrated that octicidin is a potent irreversible inhibitor of OCTase. It may compete for the carbomylphosphate binding site of the enzyme because its inhibitory activity is prevented by phosphate. In infected plants, octicidin is released from phaseolotoxin by plant peptidase (43) and continuously broken down to sulfate by some other enzymes (54).

However, *in vitro* hydrolysis of phaseolotoxin yields a tripeptide and a phosphosulfate (100). The different pathways of phaseolotoxin degradation (Fig. 1.7) indicate that the peptidase-catalyzed hydrolysis of phaseolotoxin *in vivo* (Fig. 1.7a) dominates a slower chemical hydrolysis (Fig. 1.7b) of the P-N bond (100) that normally takes place *in vitro*.

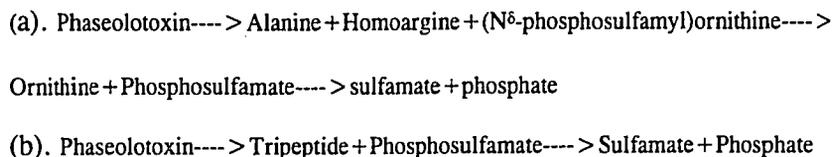


Fig. 1.7. Degradation pathway of phaseolotoxin.

Phaseolotoxin is transported in plants through the phloem (54). When ³⁵S-labeled phaseolotoxin was applied to bean leaves, the greatest amount of radioactivity accumulated within the minor veins. When the stem higher than the petiole of the treated leaf was examined, marked accumulation of labeled phaseolotoxin was found in the phloem, but little in xylem, and nothing in parenchyma cells. Chilling the petiole of the treated leaf and application of arsenate on the spot where toxin was applied (both treatments prevent transportation of solute through the phloem) significantly reduced the movement of ³⁵S-labeled phaseolotoxin to other parts of the plant, confirming that the movement of phaseolotoxin in plants is through the phloem. The speed with which it was transported was estimated to be greater than 3 cm/h.

e. Phaseolotoxin and symptom expression

Smith and Rubery (90) found that symptom formation in primary leaves of beans after application of partially purified toxin depended on the age of the leaves and illumination. No symptoms were formed in leaves 14 days old or young leaves kept in the dark. A common feature of old leaves and young leaves kept in the dark is that no

chlorophyll is synthesized in these leaves. Thus phaseolotoxin affects leaves in which active chlorophyll synthesis is taking place by inhibiting chlorophyll synthesis.

Turner and Mitchell (100) compared OCTase content of leaf discs from the toxin-induced chlorotic zones to the controls. The toxin-affected tissues had only 20% of OCTase of the control tissues. However, inhibition of OCTase alone does not lead to symptom formation, because the OCTase activity was also inhibited in old leaves which did not show symptoms. OCTase inhibition in plants leads to a deficiency of arginine and lack of protein synthesis. If this happens in growing leaves, it decreases chlorophyll synthesis and causes chlorosis in affected tissues.

f. Specificity of phaseolotoxin

Production of phaseolotoxin is closely correlated with chlorosis-inducing ability of *P. syringae* pv. *phaseolicola* because only chlorosis-inducing isolates produce the toxin (51). However, because it is a non-selective toxin, phaseolotoxin causes similar symptoms in leaves of resistant, susceptible or even non-host plants and inhibits the isolated OCTases from these plants (14, 15). Phaseolotoxin also inhibits OCTase from *E. coli* and some other bacteria (92, 96). Based on this finding, Staskawicz and Panopoulos (91) developed a convenient microbiological bioassay using *E. coli* K12 as an indicator strain.

g. Autoimmunity of *P. syringae* pv. *phaseolicola* to phaseolotoxin

As mentioned above, phaseolotoxin inhibits OCTase from all sources (15). Therefore, *P. syringae* pv. *phaseolicola* must have a system to protect itself from its own toxin. The common strategies of antibiotic or toxin-producing bacteria to protect themselves include one or several of the following: production of a toxin degrading enzyme, production of an insensitive form of the target enzyme, overproduction of the

sensitive target enzyme, fast secretion of the toxic substance, or production of an inactive protoxin (10, 86). It was suggested (92) that *P. syringae* pv. phaseolicola is immune to its own toxin because two forms of OCTase are present in a toxin-producing strain, one sensitive and the other resistant to phaseolotoxin. The activity of the resistant OCTase (ROCT) could only be detected when the bacterium was grown at 18°C, the optimum temperature for toxin production, but not at 30°C, at which no toxin is produced. However, the toxin-sensitive OCTase was detected in the same strain at both temperatures. In the non-producing strains, only the sensitive form of OCTase was detected, suggesting that *P. syringae* pv. phaseolicola produces the ROCT only when it is necessary. The two different OCTases were separated on a DEAE-Sephadex A-2 column by eluting with buffers of different ionic strengths (29). Kinetic studies (97) showed that the ROCT has lower affinity for carbamoylphosphate and a slower binding rate to ornithine as compared to the sensitive OCTase. This finding may explain why ROCT is insensitive to phaseolotoxin, because inhibition of the sensitive OCTase by phaseolotoxin relies on rapid binding of the toxin to the ornithine and carbomylphosphate binding sites of the enzyme.

Peet and Panopoulos (74) isolated the gene encoding the ROCT from a cosmid clone that complemented Tox⁻ROCT⁻ mutants of *P. s.* pv. phaseolicola. They also isolated the gene encoding toxin-sensitive OCTase by screening the genomic library of *P. syringae* pv. phaseolicola using cloned OCTase gene from *E. coli* or *Pseudomonas aeruginosa* as a probe. The ROCT and the sensitive OCTase genes are located in different parts of the bacterial chromosome: the ROCT gene is clustered with genes involved in toxin production, whereas the sensitive OCTase gene is at a distant unidentified location. The ROCT gene did not hybridize with the sensitive OCTase genes from *P. syringae* pv. phaseolicola, *E. coli*, or *Pseudomonas aeruginosa*, indicating that it is distantly related to the sensitive OCTase genes (23). Mosqueda et al (63) isolated and sequenced the ROCT

gene from a Mexican isolate of *P. syringae* pv. phaseolicola and showed that the deduced amino acid sequence showed only 56% homology to the OCTase gene from *E. coli* or *Pseudomonas aeruginosa*. Among the three domains that are important for enzymatic function, two are strictly conserved in both enzymes, but the third domain, which is involved in carbamoylphosphate binding, differs substantially. The deduced amino acid sequence of the ROCT gene (*argK*) obtained by Hatziloukas and Panopoulos (23) also showed a distant relationship (30 to 45% homology) to 13 other OCTase genes (8 bacterial, 2 fungal and 3 mammalian). Expression of *argK* in *E. coli* was not affected by temperature, but its expression in *P. syringae* pv. phaseolicola is inhibited at 28°C (23), indicating that a repressor produced by *P. syringae* pv. phaseolicola at 28°C may be involved in the regulation of *argK* (81). *argK* was successfully introduced into the chloroplasts of *N. tabacum* (23) by transforming a ROCT-*rbcS* gene fusion into the plant using *Agrobacterium*. Both *in vitro* and *in vivo* tests demonstrated that the presence of the ROCT gene in plants rendered the transgenic plants resistant to phaseolotoxin.

h. Genetics of phaseolotoxin production

Little is known about the biosynthetic pathway of phaseolotoxin in *P. syringae* pv. phaseolicola. Markisch and Reuter (47) proposed that two precursors of phaseolotoxin, ornithine and homoarginine, may be generated by the transfer of an amidino group from arginine to lysine catalyzed by an amidinotransferase. An amidinotransferase was detected only in toxin-producing strains and the amidinotransferase activity found in the toxin-producing strains grown at 30°C was only 50% of that found at 18°C.

Application of molecular techniques has greatly speeded up the studies of genetics of phaseolotoxin production. The genes for phaseolotoxin biosynthesis were found to be located on the chromosome, and not on plasmids (30, 76). Using Tn5 mutagenesis, Peet et

al (73) generated 6 independent *Tox*⁻ mutants of *P. syringae* pv. *phaseolicola*; each of the mutants contained a *Tn5* insertion in one of the five different *EcoRI* fragments. Southern blot analysis revealed that the *EcoRI* fragments were located in a 28 kb *KpnI* fragment, indicating that the genes involved in phaseolotoxin production are clustered. Using an *EcoRI* fragment containing *Tn5* insertions as probes, two cosmid clones were isolated from the genomic library of *P. syringae* pv. *phaseolicola*, which complemented *Tox*⁻ mutants and protected the ROCT-deficient *Pseudomonas* mutants and *E. coli* cells from the toxicity of phaseolotoxin. This indicated that the ROCT gene is clustered along with genes involved in toxin production.

Independently, Kamdar et al (32) also generated 1 UV-, 89 EMS- and 5 *Tn5*-induced *Tox*⁻ mutants. Screening of the genomic library of the wild-type strain for complementation of *Tox*⁻ mutants led to the identification of 18 clones that suppressed all the UV- and EMS-induced mutants. The clones were classified into three groups based on restriction enzyme digestion patterns and southern blot analysis: clones in each group had the similar restriction pattern; clones in each group showed homology to each other, but not to clones from the other groups. One clone, pHK120, complemented all the *Tox*⁻ mutants, including 5 *Tn5* mutants. Since the *Tn5* insertions in these *Tn5* mutants are at different locations in the chromosome, it was suggested that pHK120 contained a cluster of structural and/or regulatory genes involved in phaseolotoxin production.

i. Thermoregulation of phaseolotoxin production

As discussed above, production of phaseolotoxin in *P. s.* pv. *phaseolicola* is temperature regulated (25, 66); it is produced at 18°C but not at 28°C. A cosmid clone, pDC938 (32), restored toxin production to G50-1UV (a UV-induced *Tox*⁻ mutant), and G50-1 (wild-type strain) at 28°C. Several *Tn5* insertions close to the unique *Bam*HI site in

the insert of pDC938 inactivated its complementing ability, but the corresponding marker-exchange mutants of the wild-type strain still produced toxin, suggesting that the original copy of the pDC938 insert in the chromosome is not directly involved in toxin production (32). Sequence of the region around the unique *Bam*HI site of pDC938 did not reveal the presence of an ORF, and the cloning of pDC938 in an expression vector failed to show a protein product. However, the DNA sequence on either side of the unique *Bam*HI site revealed several motifs characteristics of DNA binding sites. This led to the hypothesis that pDC938 complements G50-1UV because the DNA binding sites in pDC938 titrate the repressor produced by G50-1 at 28°C, or G50-1UV at both 18°C and 28°C (G50-1UV was proposed to be a constitutive mutant that expresses the repressor at both temperatures) (81). A gel-retardation assay showed that a 400 bp fragment from the pDC938 insert containing the *Bam*HI site was retarded by the protein extracts from G50-1UV and G50-1 grown at 28 °C (81). It was also shown that the same protein extracts bound to a fragment in the promoter region of *argK*, suggesting that the repressor is a global regulator (Rowley et al, unpublished data).

7. Organization of Genes Involved in the Production of Antibiotics

Production of antibiotics has been under extensive study because of their great practical value in medicine, food industry and agriculture. Antibiotic biosynthetic pathways as well as the genes involved in them have been well documented in several cases. Many phytotoxins and antibiotics are secondary metabolites produced by microorganisms, and many of them share structural similarities. Therefore, the understanding of the genetics of antibiotic production might offer clues to the genetics of phytotoxin production. Two antibiotics, bialaphos and gramicidin, are chosen for review here because their biosynthetic pathways and genetic organization are well understood.

a. Bialaphos

Bialaphos (BA) is produced by *Streptomyces hygroscopicus* and *S. viridochromogenes*. Its structure (Fig. 1.8) consists of two L-alanine residues and phosphinothricin, a compound with a unique C-P-C bond (85) and inhibitory activity against glutamine synthetase. It is noteworthy that the intact tripeptide (BA) shows no inhibitory activity against glutamine synthetase (41), and this may be why the producing organism can protect itself from BA.

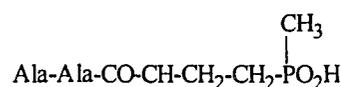


Fig. 1.8. Structure of bialaphos.

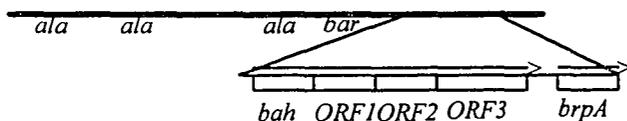


Fig. 1.9. Organization of genes involved in bialaphos production (Raibaud et al, 1991)

By examining various mutants that were blocked at various steps in the biosynthetic pathway, Murakami et al, 1986 proposed a 13 step pathway for bialaphos biosynthesis. A 16 kb genomic fragment from a BA-producing strain, *S. hygroscopicus*, restored bialaphos production to all BA⁻ mutants, indicating that the genes for bialaphos production (*bap* genes) are physically linked (65). Localization of individual genes corresponding to steps 1, 3, 4, 5, 6, 10, 12 and 13 was accomplished by complementing various mutants with deletion subclones. As expected, a BA-resistant gene (*bar*) was identified in this 16 kb region. However, the product of the *bar* gene (acetyl transferase) is used not only for self-

protection, but also in the biosynthesis of bialaphos (41). Inactivation of this gene resulted in the loss of BA resistance as expected, but it also led to the production of a low level of BA, and to considerable accumulation of an intermediate, demethyl-phosphinothricin (DMPT). When the plasmid containing the 16 kb genomic DNA was mobilized into various BA⁻ mutants, only low levels of BA were found in the transformants, suggesting that some additional information not included in the 16 kb fragment (probably a regulatory factor) is required for the full expression of *bap* genes (65). In fact, three genes isolated by Hara et al (22) that carry out the alanylation steps in the biosynthesis of BA hybridized to a contiguous region of the 16 kb *bap* cluster, thus extending the size of the DNA region involved in BA production to 35 kb. In addition, a regulatory gene, *brpA*, is located adjacent to the 16 kb region. This gene was found by Anzai et al (1) using a subclone containing a 1.3 kb fragment adjacent to the *bap* gene cluster to complement a pleiotropic mutant, NP57. Sequencing of the 5 kb DNA spanning the region from *bar* to *brpA* (Fig. 1.9; 80) revealed 5 ORFs in the following order: *bah*, ORF1, ORF2, ORF3 and *brpA*. Four structural genes, *bah*, ORF1, ORF2 and ORF3 were believed to be in one operon and were translationally coupled because the ribosomal binding sites of ORF1, ORF2 and ORF3 are located in each case in the 3'-coding region of the preceding gene. *bapA* is transcribed in the same direction, but separately from this operon. *bah* may encode an acetyl-hydrolase since it contains a conserved sequence common to lipases, and since the cloned *bah* gene restored BA production to a mutant in which N-acetyl bialaphos accumulates. Data base searches also revealed that ORF1 and ORF2 are homologous to *grsT*, a gene associated with gramicidin S synthesis in *Bacillus brevis* and one that is co-transcribed with gramicidin S synthetase I gene, *grsB*. Further analysis showed that ORF1, ORF2 and *grsT* are homologous to the vertebrate thioesterase, implying that the proteins encoded by ORF1 and ORF2 are involved in the cleavage of thioester bonds, a necessary

step in the synthesis of fatty acids as well as some peptides (80). The amino acid sequence of ORF3 contained a motif characteristic of many membrane proteins that transport solutes, such as citrate in *E. coli* and glucose in humans, raising the possibility that the ORF3 gene product is responsible for the uptake of carbon sources and the export of bialaphos. Most interestingly, the *brpA* sequence shows homology to 10 genes from the two-component regulatory family. Although an N-terminal domain common to most receivers of the two-component systems was not found, a C-terminal helix-turn-helix motif was found in *brpA*. Three potential promoters and their corresponding transcripts, *brpA1*, *brpA2* and *brpA3*, for *brpA*, were found using the S1 nuclease protection assay. The expression of all three transcripts was very low up to 10 h after inoculation, but it increased substantially after 14 h. At 20 h, the level of *brpA3* continued to increase while *brpA1* and *brpA2* levels remained constant. This result indicated that a transition in *brpA* expression results during the biosynthesis of BA.

Another BA-producing strain, *S. viridochromogenes*, also contains the counterpart of *brpA*, *bar*, and other structural genes in the same order as that in *S. hygroscopicus* (22). Subclones containing the various genes were able to complement mutants from *S. hygroscopicus*, further confirming that these two strains employ the same mechanism to synthesize BA. Surprisingly, a *bar*-like gene was also isolated from a BA non-producing strain, *S. coelicolor* A3(2) (4). The gene showed 30-35% identity to the *bar* genes of two BA-producing strains. The reason for the presence of a *bar*-like gene in this strain is not known.

b. Gramicidin S

Gramicidin S, a cyclic decapeptide antibiotic (Fig. 1.10), is produced by *Bacillus brevis* in the transition phase from vegetative to stationary growth. A multienzyme system,

consisting of two multifunctional enzymes, synthetase I and synthetase II, are involved in its biosynthesis in a non-ribosomal fashion (40). Synthetase I, with a molecular weight of 100,000 dalton, activates and racemizes the first amino acid, phenylalanine, while synthetase II, with the molecular weight of 280,000 dalton, catalyzes activation and condensation of other constitutive amino acids in the order of Pro, Val, Orn and Leu (39). Biochemical evidence showed that both enzymes are composed of more than one functional domain. One domain of the isolated synthetase I was involved in activating phenylalanine whereas the second domain was involved in forming a thioester bond between the enzyme and the substrate (33). Limited proteolysis of synthetase II led to the recovery of three different fragments, each being able to activate Pro, Leu or Val, respectively (88, 89). Two research groups (26, 39, 40) independently cloned the gene for synthetase I (*grsA*). The DNA sequences they obtained are almost identical to each other and both share considerable homology to the synthetase I gene involved in the synthesis of tyrocidine in *Bacillus brevis*. These two groups (27, 39, 40) also obtained a partial sequence for the synthetase II gene (*grsB*) and the DNA fragment obtained by the second group (27) was shown to encode the pro-activating domain (*grsB-pro*). However, a third gene designated as *grsT*, was found by Kratzschmar et al (38) in the same fragment in which *grsA* and *grsB* are located. Furthermore, S1 nuclease protection analysis indicated that the three genes are in a single transcriptional unit in the order of *grsT*, *grsA* and *grsB*. Data base searches showed that *grsT* is similar to the genes encoding the fatty acid thioesterase and the vertebrate lipase, suggesting that its product plays a role in acyl group transfer (38).

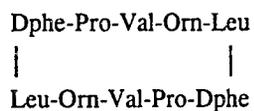


Fig. 1.10. Structure of gramicidin S.

C. DEVELOPMENT OF HYPOTHESIS

The biosynthetic pathway of phaseolotoxin, which contains a tripeptide (Ornithyl-alanyl-homoarginine) and a phosphosulfamyl moiety attached to the δ -amino group of ornithine, has not been elucidated. It is important to determine its biosynthetic pathway not only for scientific reasons but also for devising appropriate strategies to minimize its effects on plants exposed to it. There are two strategies that can be employed to determine the steps involved in the biosynthesis of phaseolotoxin: one, a chemical-biochemical approach which would involve use of labeled precursors, isolation of enzymes and a stepwise construction of the pathway in *in vitro* experiments, as was done in the elucidation of the pathway of bialaphos biosynthesis (65); and two, a genetic approach combined with biochemical experiments which involves obtaining transposon tagged *Tox*⁻ mutants blocked at specific steps and their complementation to obtain individual genes and gene products. I propose to follow the later approach to begin to elucidate the pathway of phaseolotoxin biosynthesis.

In bacteria, the biosynthesis of antibiotics or secondary metabolites have been shown to be complex processes involving numerous steps. Researchers have shown that often the genes involved in the biosynthetic schemes of these compounds are clustered such that they are coordinately regulated to produce gene products that are balanced in terms of their molar ratios for the efficient production of these metabolites (48, 86). Kamdar et al (32) and Peet et al (73) previously suggested that the genes involved in phaseolotoxin production may also be clustered.

As mentioned above, phaseolotoxin contains a tripeptide. Two possible ways by which small peptides can be synthesized have been proposed (35): a) the peptide is synthesized by a glutathione-type mechanism by which a glutathione synthetase activates the starting amino acid as aminoacyl phosphate, which then undergoes nucleophilic attack

by the amino group of the succeeding amino acid in the sequence; b) the peptide is assembled by a thiotemplate mechanism in which a multifunctional peptide synthetase acts as a template in directing the synthesis of the peptide and as an activating enzyme. One distinguishable feature of the second mechanism is that the enzyme forms a thioester bond with the amino acid.

In addition to the tripeptide, phaseolotoxin contains an inorganic moiety. Except perhaps for bialaphos (65), little is known about how inorganic elements such as phosphorus and sulfur are incorporated in toxins. Isolation of genes involved in the incorporation of the phosphosulfamyl group of phaseolotoxin in the tripeptide would shed light on the type of biochemical reactions involved in such processes. An investigation on the genetics of phaseolotoxin production is also likely to shed light on regulatory genes such as genes involved in its thermoregulation and possibly others, such as genes involved in its secretion.

Kamdar et al (32) previously isolated a genomic cosmid clone pHK120 that complemented all the Tox^- mutants (EMS, UV and Tn5) in our possession. Since the Tn5 insertions in different Tn5 mutants are located in different sites of the chromosome, my hypothesis is that this cosmid clone harbors many genes involved in the biosynthesis of phaseolotoxin and these genes are clustered. Some of the genes within the pHK120 insert may have regulatory function because the production of phaseolotoxin in the wild-type strain is regulated by temperature. It is also possible that some genes within the pHK120 insert may encode proteins involved in secretion of phaseolotoxin because it is an extracellular toxin.

I will undertake the following to test this hypothesis:

1. Tn5 mutagenesis of the insert in pHK120;

2. Marker exchange of individual pHK120::Tn5 hybrid plasmids in the genome of wild-type strain;

3. Pair complementation analysis to fine map the boundaries of the loci involved in toxin production and determine the number of loci in pHK120;

4. Sequencing of the largest locus to determine if it contains a peptide synthetase-like gene;

5. Transcript analysis of the genes which are present in this locus and the effect of temperature on their expression.

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**CHAPTER II. GENETIC ORGANIZATION OF A CLUSTER OF GENES
INVOLVED IN THE PRODUCTION OF PHASEOLOTOXIN, A TOXIN
PRODUCED BY *PSEUDOMONAS SYRINGAE* PV. PHASEOLICOLA**

A. ABSTRACT

Phaseolotoxin, [N^δ(N'-sulfo-diaminophosphinyl)-ornithyl-alanyl-homoarginine] produced by *Pseudomonas syringae* pv. phaseolicola, the bean halo blight pathogen, is a potent inhibitor of ornithine carbamoyltransferase (OCT). Inhibition of OCT in infected plants leads to chlorosis and growth inhibition. A genomic cosmid clone, pHK120, containing a 25 kb fragment of DNA from a wild-type strain of *P. syringae* pv. phaseolicola restores toxin production in Tox⁻ mutants. Tn5 mutagenesis of pHK120 and marker exchange of pHK120::Tn5 plasmids in the wild-type strain resulted in the isolation of 39 chromosomal mutants that harbor Tn5 insertions at known positions. Toxin bioassays revealed that 28 of the mutants, with Tn5 insertions distributed throughout the insert of pHK120, were Tox⁻, indicating that a functional locus for toxin production in each mutant was inactivated. Complementation analysis was done by testing for toxin production strains that carried a genomic Tn5 at one location and a plasmid-borne Tn5 at another location (pair-complementation). Pair-complementation analysis of nine marker-exchange mutants and a random genomic Tn5 mutant revealed that there are a minimum of eight toxin loci (*phtA* through *phtH*) in pHK120. Mutants carrying Tn5 insertions in the *phtA*, *phtD* and *phtF* loci were complemented by deletion subclones containing fragments from pHK120; mutants carrying Tn5 insertions in the *phtC* locus were partially complemented by a subclone, and mutants carrying Tn5 insertions in the *phtB*, *phtE*, *phtG* and *phtH* loci were not complemented by any of the available subclones. A comparison of the insert from

pHK120 with that from pRCP17, a clone reported previously (31) by another laboratory to contain some of the phaseolotoxin genes and the phaseolotoxin resistant-OCT gene, revealed that the inserts in these two cosmids overlap but differ in important respects.

B. INTRODUCTION

Phaseolotoxin is an extracellular, nonspecific, chlorosis-inducing phytotoxin produced (at 18 to 22°C) by *Pseudomonas syringae* pv. phaseolicola, the causative agent of halo blight of beans (*Phaseolus vulgaris* L.). Phaseolotoxin, [N^δ(N'-sulfo-diaminophosphinyl)-ornithyl-alanyl-homoarginine] (22) and its non-peptide degradation product octicidin [N^δ(N'-sulfo-diaminophosphinyl)-ornithine] (21), inhibit a key enzyme, ornithine carbamoyltransferase (OCT) in the arginine biosynthetic pathway (21, 29) *in vitro* as well as in bean plants infected with toxigenic strains of *P. syringae* pv. phaseolicola (19). The inhibition of OCT in infected plants leads to chlorosis and growth inhibition.

Phytotoxins are produced by several bacterial pathogens, and although much is known about their chemical structures and their physiological and biochemical effects in diseased plants (20, 28), little is known about the genetics of their biosynthesis or their regulation (7). Kamdar et al (13) reported the isolation of three non-overlapping classes of genomic cosmid clones from a library of *P. syringae* pv. phaseolicola all of which complement 1 UV-induced and 80 ethyl methanesulfonate-induced Tox⁻ mutants. A representative clone from class III (pDC938) does not contain a gene(s) involved in phaseolotoxin production but restores the Tox⁺ phenotype to these mutants by nonallelic complementation. Subsequently, Rowley et al (32) demonstrated that a 485 bp sequence in the insert of pDC938 was involved in this complementation and that this sequence contains DNA binding sites which specifically bind to a protein that is produced by *P. syringae* pv. phaseolicola at 28°C. They proposed that this protein was a putative repressor that is titrated by the DNA binding sites in the 485 bp fragment from pDC938.

Unlike pDC938, pHK120 (a class I clone) complements five chromosomal Tn5 mutants in addition to the UV- and ethyl methanesulfonate-induced mutants (13). The

complementation of all five Tn5 mutants by pHK120 suggested that its insert harbored a cluster of genes involved in phaseolotoxin production, because the Tn5 insertions in the mutants are located in different chromosomal *EcoRI* fragments.

Peet et al (31) previously isolated a genomic fragment from *P. syringae* pv. phaseolicola that contained genes involved in phaseolotoxin production as well as a gene involved in the production of a phaseolotoxin-resistant OCT (ROCT) encoded by the *argK* gene (9). The *argK* gene is physically linked to and coordinately regulated with the phaseolotoxin genes by temperature (9, 26, 31).

Here I describe the genetic characterization of the pHK120 insert, which was performed by Tn5 mutagenesis of pHK120, marker exchange of pHK120::Tn5 hybrid plasmids in the chromosome of the wild-type strain, pair-complementation analysis, and complementation of marker-exchange mutants by subclones containing fragments from the pHK120 insert. We found that the insert from pHK120 harbors at least eight loci involved in the production of phaseolotoxin.

C. MATERIALS AND METHODS

1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2.1. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or M9 minimal medium (18) at 37°C. *P. syringae* pv. phaseolicola 4612-1 and G50-1 and their derivatives were cultured in Kings-B (KB) broth medium (14), M9 minimal medium or Nutrient-Yeast-Glycerol (NYG) medium (37) at 18°C or 28°C. When necessary, the following antibiotics were used: rifampicin (50µg/ml), tetracycline hydrochloride (20µg/ml), kanamycin (50µg/ml), ampicillin (50µg/ml), spectinomycin (50µg/ml), and chloramphenicol (15µg/ml).

2. Tn5 mutagenesis

Tn5 mutagenesis was carried out according to the method of de Bruijn and Lupski (4). *E. coli* Q1 harboring pHK120 was mixed with λ467 phage at a multiplicity of infection of 1:10 and incubated for 2 hours at 30°C. Aliquots (0.2 ml) were spread on LB agar plates containing kanamycin and tetracycline and incubated for 48 hours at 30°C. Colonies were pooled using 5 ml of a solution containing 20% sucrose, 10 mM Tris (pH 7.8), and 1 mM EDTA. Plasmid DNA was extracted from a culture containing the pooled mixture and transformed into *E. coli* HB101. Plasmid DNA from individual transformants was isolated and the location of the Tn5 in the insert was determined as described below.

3. Determination of the location of Tn5 in the plasmid

Tn5 contains two *Hind*III sites located 1.2 kb from either end, and a *Bam*HI site

located approximately in the middle of the transposon (3). Hybrid plasmids (plasmids containing a Tn5 insertion) were digested with *Hind*III, and their digestion patterns were compared to that of the original plasmids. Three new fragments were present in the profiles of a hybrid plasmid as compared to that of the corresponding original plasmids, and the fragment of 3.7 kb was an internal fragment of Tn5. The length of the smallest new fragment subtracting 1.2 kb represented the distance between the location Tn5 and the end of the Tn5-carrying *Hind*III fragment. The same plasmid was then digested with *Bam*HI (if the restriction map showed that no *Bam*HI site was available in the Tn5-carrying *Hind*III fragment, other enzymes, such as *Sma*I, *Eco*RI or *Pst*I were used to substitute for *Bam*HI if they were present in the fragment of interest), and compared with the *Bam*HI pattern of the original plasmid. The result was used to determine the location of Tn5 in the *Bam*HI fragment. The *Hind*III and *Bam*HI digestion data were analyzed to determine the location of the Tn5 insertion.

4. Triparental mating

Bacterial conjugation was carried out according to Kamdar et al (13) with *E. coli* (pRK2073) as an intermediate donor in triparental matings. Overnight cultures of *E. coli* and *P. syringae* pv. phaseolicola were diluted 1:10 and 1:5 in NYG medium respectively, and were shaken at the appropriate temperature for 6 h. The cells were collected by centrifugation, washed once with the same volume of NYG medium and resuspended in 3 ml of NYG. Concentration of the cells was determined by measuring the optical density at 600 nm. About 4×10^9 cells (0.5 O.D 600) of each component (donor, helper and recipient) were mixed together and brought to 100 μ l. The cell mixtures were transferred onto a NYG plate with 10 μ l per spot and kept at room temperature overnight. When the cells grew into visible lawns, they were transferred to a NYG plate containing appropriate antibiotics and

incubated at 28°C until individual colonies appeared on the plate. The colonies were transferred to another NYG plate containing the same antibiotics for the second time in order to eliminate any remaining *E. coli* cells.

5. Electroporation

Overnight cultures of *P. syringae* pv. phaseolicola and its derivatives were diluted 1:50 and shaken for 7 h at 28°C. The Cells were harvested (at 4°C) by centrifugation, and resuspended in the same volume of pre-cooled MOPS buffer. After holding on ice for 30 min, the cells were collected by centrifugation at 4°C and resuspended in pre-cooled MOPS 1/250 of the starting volume. Four µl of plasmid DNA (less than 1 µg) were mixed with 20 µl of MOPS-treated cells and electrically shocked in an electroporation chamber at the following settings: Voltage, 200 V; Resistance, 4 Ω; Capacity, 300 µF; Time, 6 msec. The electroporated cells were transferred into 1 ml of KB medium and shaken at 37°C for 2 h before they were spread onto KB plates containing appropriate antibiotics.

6. Verification of plasmids in transconjugants

Plasmid DNA was extracted from transconjugants obtained either by triparental mating or electroporation and transformed into *E. coli* DH5α. Transformants were selected on plates containing tetracycline to eliminate transformants containing only the *P. syringae* pv. phaseolicola plasmids. The plasmid DNA was then extracted from Tc resistant transformants and digested with *Hind*III or *Bam*HI. The plasmid profiles were compared to that of the original plasmids as described above.

7. Marker exchange (Homogenotization)

Marker exchange in transconjugants was forced by withdrawing tetracycline from the medium and by repeated subculturing of the transconjugants in the presence of kanamycin (8). Overnight cultures of transconjugants growing in media containing all necessary antibiotics were subcultured 1:100 in KB medium containing all antibiotics but tetracycline and shaken overnight. This process was repeated 6 times. 200 μ l of diluted culture (final concentration is 10^3 cells/ml) were spread on KB plates and incubated at 28°C until colonies appeared. The colonies (about 100 for each transconjugant) were transferred to a KB plate containing both tetracycline and kanamycin (plate I) and a duplicate plate containing only kanamycin (plate II). Colonies which appeared only in plate II but not in plate I (marker-exchange mutants or homogenotes) were saved. Southern hybridization analysis of *Hind*III or *Bam*HI-digested genomic DNA from marker-exchange mutants was performed using the 32 P-labeled internal fragment from Tn5 as a probe to verify the position of Tn5 in the homogenote. Appropriate pHK120::Tn5 plasmid DNA was used as the control in these analyses.

8. Toxin bioassay

Toxin bioassay was performed according to the method of Staskawicz and Panoupolos (36). *P. syringae* pv. phaseolicola or its derivatives were inoculated onto a M9 plate with toothpicks and incubated at 28°C for 4 h. The plates were shifted to 18°C (if toxin production at 28°C was to be assayed, the plate was kept at 28°C) and incubated for 12 h. Five ml of liquid M9 top agar (melted and cooled down to 55°C) were mixed with 200 μ l of overnight culture of *E. coli* K12 and overlaid on the M9 plate. The plates were incubated at 37°C for at least 12 h before examining for the inhibitory zones.

9. DNA isolation and manipulation

Plasmid DNA was extracted by the rapid boiling method or by the alkaline lysis method (2) and purified by CsCl-gradient centrifugation. Genomic DNA extraction and Southern hybridization were performed as previously described (13), except when noted otherwise. DNA fragments for subcloning were isolated from low-melting agarose after electrophoresis and purified using the USBioclean kit (USBiochemical, Cleveland, Ohio). Restriction enzyme digestion and ligation of DNA were carried out using standard protocols (2).

D. RESULTS

1. Restriction map, Tn5 mutagenesis of pHK120, and marker exchange in the wild-type strain

The restriction map of pHK120 was constructed and the *Hind*III, *Bam*HI, *Sma*I and relevant *Sst*I and *Pst*I sites are shown in Fig. 2.1A. The plasmid was mutagenized with Tn5 and of the 350 pHK120::Tn5 hybrid plasmids examined by digestion with *Hind*III, 200 contained a Tn5 within the insert. The position of the Tn5 insertion in each clone was mapped, and those mapped to a unique site within the insert of pHK120 are shown in Fig. 2.1B (represented by an arrowhead or an open circle; the symbol # represents the chromosomal Tn5 mutants, as described below). A total of 44 plasmids containing Tn5 insertions at unique sites were mobilized into *P. syringae* pv. phaseolicola 4612-1 (Tox⁺) by triparental mating. The flanking sequences and the Tn5 were marker exchanged into the chromosome of the transconjugant by double homologous recombination as described in Materials and Methods. Sixteen randomly chosen Tc^SKm^R colonies from a single mating for each marker exchange mutant (designated by the prefix HO followed by a number representing the position of the Tn5, as shown in Fig. 2.1) were tested for toxin production, and the genomic DNA prepared from two of these colonies was examined by Southern hybridization to verify the location of the Tn5 insertion. Twenty-eight marker exchange mutants (Fig. 2.1B, shaded arrowheads) failed to produce toxin, indicating that the Tn5 insertion in these mutants had interrupted genes necessary for toxin production. Eleven marker exchange mutants (Fig. 2.1B, open arrowheads) retained their ability to produce toxin, suggesting that the Tn5 insertions in these mutants were outside of regions essential for toxin production. No marker exchange mutants with an expected Tn5 insertion in the chromosome were recovered from the region containing Tn5 insertions at T63, AB2,

T13, B25 and C13 after two attempts (Fig. 2.1B, open circles). Although Ditta (5) reported that the frequency of secondary transposition of Tn5 in *Rhizobium* strains is low, Madrid et al (16) found that the frequency of secondary transposition within a multicopy plasmid in *E. coli* was approximately 10%. It is possible that the frequency of secondary transposition of Tn5 in *P. syringae* pv. phaseolicola is higher than that reported for *Rhizobium* strains and this may account for the lack of marker exchange of the five Tn5 insertions into the chromosome. In summary, the Tn5 insertions that affected toxin production were distributed across most of the 25 kb insert of pHK120 and fell into 4 regions separated by small gaps where Tn5 insertions did not affect toxin production.

Three Tn5 mutants, TNM5, TNM7 and TNM11, previously obtained by random Tn5 mutagenesis of the chromosome of the wild-type strain (13), were complemented by pHK120, implying that the Tn5 insertions were located within the chromosomal region represented by the insert in pHK120. Southern blots of the genomic DNA from the three Tn5 mutants were probed with ³²P-labeled pHK120 DNA, and a comparison of the size of the hybridizing genomic fragments from the three mutants to that of pHK120 revealed that the insertions were located within the pHK120 region at the positions denoted by the symbol # (Fig. 2.1B).

2. Fine mapping of transcriptional loci involved in toxin production

In order to fine map the transcriptional loci within the pHK120 insert, nine Tox⁻ marker exchange mutants (one to four per region; listed in Table 2.2 and indicated in Fig. 2.1B by the symbol ★), and one random Tn5 mutant (TNM7) (Table 2.2 and Fig. 2.1B), were chosen for complementation by pHK120::Tn5 hybrid plasmids. Since these experiments involved a pair of Tn5 insertions (one in the chromosome of the mutant and one in the hybrid plasmid) in the transconjugants, they are referred to as pair-

complementation experiments. If the resulting transconjugant produced toxin, we concluded that the location of the Tn5 in the hybrid plasmid was outside the insertionally inactivated transcriptional unit in the mutant. However, if a hybrid plasmid failed to complement the mutant, the location of Tn5 in the pHK120::Tn5 plasmid was assumed to be within the insertionally inactivated transcriptional unit. To ensure that recombination had not occurred in these transconjugants, the plasmids were retrieved from the transconjugants, transformed into *E. coli* DH5 α and examined by restriction enzyme digestion. The results of marker exchange mutagenesis and pair complementation showed that the insert in pHK120 harbors a minimum of eight transcriptional loci involved in toxin production (designated *phtA* through *phtH*; Fig. 2.1B).

a. The *phtA* locus

Since hybrid plasmids containing Tn5 insertions at AF4, A46, A41 and A13 (Fig. 2.1B) all failed to complement marker exchange mutants HOA41 and HOA46, these Tn5 insertions are within the same locus, designated *phtA*. The *phtA* locus encompasses a 1.3 kb region between B25 and A18, since hybrid plasmids containing Tn5 insertions outside this region complemented both HOA41 and HOA46.

b. The *phtB*, *phtC* and *phtD* loci

The second Tox⁻ region, encompassing a 6.7 kb region between C37 and D19, was divided into three transcriptional units on the basis of pair-complementation experiments (Table 2.2). HOA7, TNM7, HOE1 and HOE4 contained Tn5 insertions within the same locus, *phtB*, whereas HOE8, HOE27, HOE47 and HOA25 contained the Tn5 in another locus, *phtC*. HOA4 contained a Tn5 in a third locus, *phtD*. The left border of the *phtB* locus is delimited by a Tox⁺ mutant, HOC37. The boundary between the *phtB* locus and

the *phtC* locus is approximately placed between E4 and E8, two Tn5 insertions that are located at the far right side of the *phtB* locus and the far left of the *phtC* locus. The right boundary of the *phtC* locus could not be determined because of a lack of Tn5 insertions in that region. However, the fact that a subclone, pYZB302, complemented the Tox⁻ mutant HOA25 (see below) indicates that the right boundary of the *phtC* locus is to the left of the third *Bam*HI site. Similarly, the left boundary of the *phtD* locus is located at the right of the fourth *Sst*I site, whereas its right boundary is delimited by a Tox⁺ mutant, HOD19.

c. The *phtE*, *phtF* and *phtG* loci

Another Tox⁻ region, encompassing an 8.0 kb region between C7 and G4 (Fig. 2.1B), consists of three transcriptional units designated *phtE*, *phtF*, and *phtG*, respectively (Table 2.2). Nine marker-exchange mutants, HOA14, HOE17, HOG1, HOA21, HOD23, HOD13, HOC25, HOD18 and HOC28, and two random Tn5 mutants, TNM5 and TNM11, contained Tn5 insertions in the *phtE* locus. The size of this locus is ~6.4 kb. Two marker-exchange mutants, HOB36 and HOD9, mapped within the *phtF* locus and three marker-exchange mutants, HOA47, HOA31 and HOA5, mapped within the *phtG* locus. The boundaries between *phtE* and *phtF* as well as those between *phtF* and *phtG* are loosely assigned because no Tn5 insertions were available for localizing the boundaries around these regions more precisely.

d. The *phtH* locus

Two Tox⁻ marker-exchange mutants, HOA1 and HOH5, mapped within the last transcriptional unit, the *phtH* locus. HOTP7 and HOB1, two Tox⁺ marker exchange mutants on the left and right sides, respectively, delimited the boundaries of this locus. It was surprising that pHK120::Tn5 plasmids containing Tn5 insertions at positions A47, A31

and A5 that mapped within the *phtG* locus (Fig. 2.1B) failed to restore toxin production to marker-exchange mutant HOH5, whereas HOA5 containing pHK120::Tn5 plasmids with Tn5 insertions in the *phtH* locus (A1 and H5) did produce the toxin (Table 2.2). Hybrid plasmids with Tn5 insertions to the left of A47, i.e., in the *phtF* locus, complemented the mutants with the mutations in the *phtH* locus. These results suggest that the *phtG* locus is required for the *phtH* locus to be functional.

3. Complementation of selected mutants by subclones of pHK120

Selected *HindIII*, *BamHI*, *PstI*, and *SstI* fragments from pHK120 were cloned into the broad-host-range vectors pLAFR3, pRK404, or pLAFR376 and mobilized into one or more of the marker exchange mutants or the random chromosomal Tn5 mutants by electroporation or conjugation (Table 2.2 and Fig. 2.1C). The plasmid pYZB2102, containing the 4.9 kb *BamHI* (B21) fragment (Fig. 2.1A, 1C), restored toxin production to the marker exchange mutants HOA41 and HOA46, which contain mutations in the *phtA* locus. The *phtB* locus, represented by the mutant TNM7, was not complemented by either the plasmid pYZB2102 or plasmid pYZH302, containing the 4.9 kb *BamHI* (B21) or the 4.3 kb *HindIII* (H3) fragment, respectively (Fig. 2.1A and 1C). Therefore, the *phtB* locus must extend beyond the third *HindIII* site in pHK120 (Fig. 2.1A). The plasmid pYZB302, containing a 2.2 kb *BamHI* (B3) fragment (Fig. 2.1A, 1C), partially complemented HOA25 (Table 2.2), a mutant with a mutation in the *phtC* locus. Mutant HOA4 containing a mutation in the *phtD* locus, was partially complemented by the plasmid that contains the 4.6 kb *HindIII* (H2) fragment from pHK120, pYZH202 (Fig. 2.1A and 1C) and fully complemented (i.e. it produced toxin at levels equivalent to those produced by the wild-type strain) by the plasmid pYZST102, which contains the 8.6 kb *SstI* (St1) fragment from pHK120 (Fig. 2.1A and 2.1C). Surprisingly, the plasmid pYZB2202, which contains the

4.9 kb *Bam*HI (B22) fragment (Fig. 2.1A and 2.1C) failed to complement HOA4. The *Tox*⁻ mutants with mutations in the *phtE* locus, which spans a region of about 6.4 kb according to the pair complementation analysis, should have been complemented by the 8.6 kb *Sst*I (St1) fragment (Fig. 2.1A and 2.1C), which covers the putative *phtE* locus. However, mutants HOA14 and HOC25 were not complemented by pYZST102. Three other plasmids, pYZH102, containing the 7.0 kb *Hind*III (H1) fragment; pYZB102, containing the 7.8 kb *Bam*HI (B1) fragment (Fig. 2.1A and 2.1C); and pYZP102, containing the 8.2 kb *Pst*I (P1) fragment (Fig. 2.1A and 2.1C), also failed to complement HOC25. Both pYZB102 and pYZP102 complemented the marker exchange mutant HOB36, which contains a mutation in the *phtF* locus, whereas pYZH102 failed to do so, suggesting that the right boundary of the *phtF* locus extends beyond the sixth *Hind*III site. On the basis of the pair-complementation analysis, the *phtG* and the *phtH* loci are located within the B1 or P1 fragments of the pHK120 insert (Fig. 2.1A). However, the plasmids pYZB102 and pYZP102, containing the B1 and P1 fragments, respectively, failed to complement marker exchange mutants HOA31 and HOH5, two mutants with *Tn5* insertions in the *phtG* locus and the *phtH* locus, respectively (Fig. 2.1A and 2.1C).

4. A comparison of the genomic fragments from pHK120 and pRCP17

Previously, Peet et al (30) reported that pRCP17, a cosmid containing ~24 kb of genomic DNA from *P. syringae* pv. phaseolicola, harbors the ROCT gene and restores toxin production to several random *Tn5* *Tox*⁻ mutants obtained by them. Restriction analysis of the DNA from both plasmids and Southern blot hybridization using pRCP17 as the probe revealed that these two clones have overlapping inserts (Fig. 2.1D). pHK120 contains about 4.8 kb of DNA (Fig. 2.1D, H4 and part of H3) at the left end of its insert

which is not present in pRCP17, whereas pRCP17 harbors about 5 kb of DNA which is not present in the insert of pHK120.

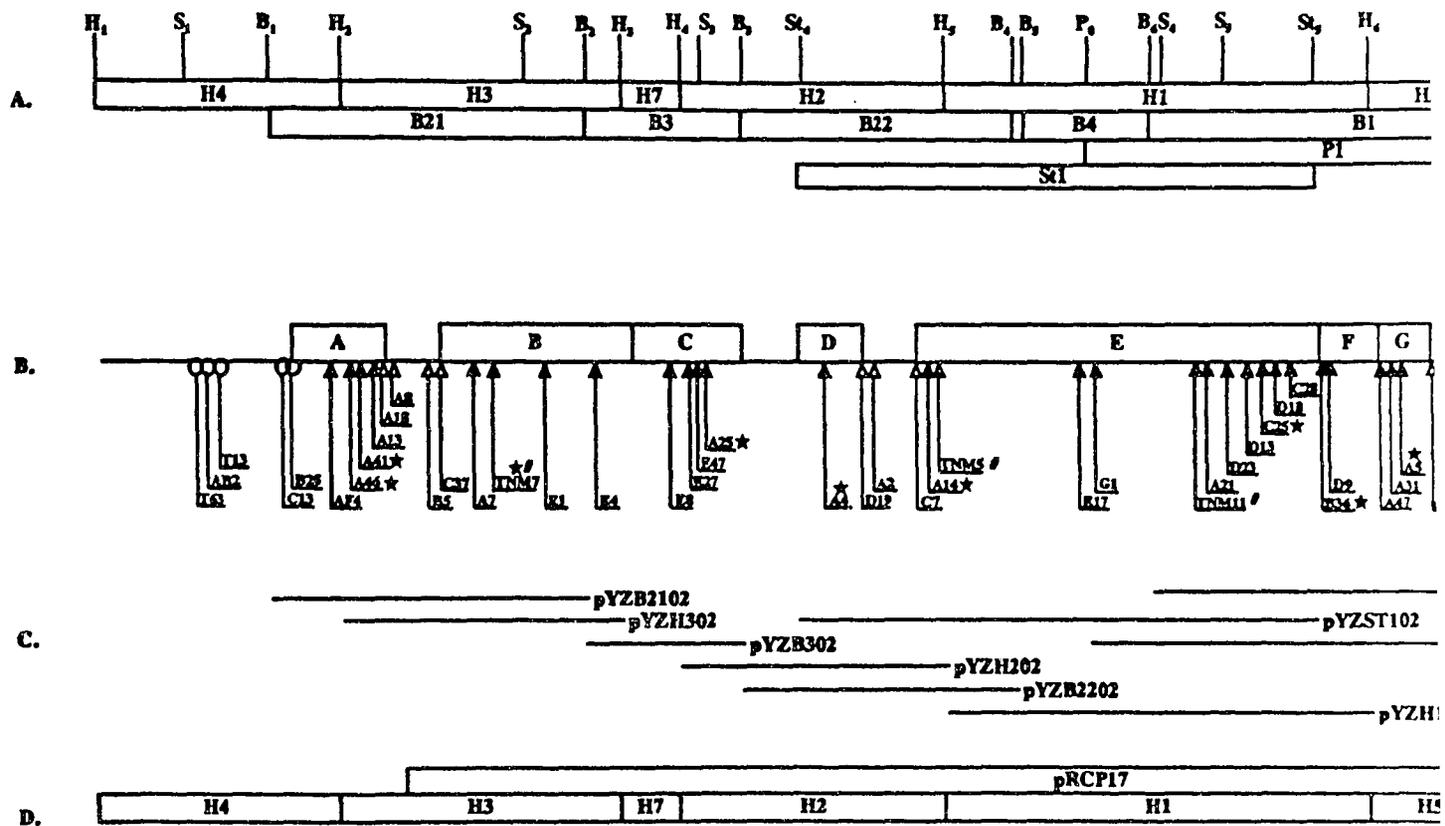
Because pRCP17 contains the ROCT gene (31), transformants of *E. coli* HB101 containing pRCP17 show resistance to phaseolotoxin. However, the transformants of *E. coli* HB101 containing pHK120 remained sensitive to the toxin (data not shown), indicating that this plasmid does not contain the ROCT gene. Thus, I conclude that the ROCT gene must be located within the 5 kb DNA fragment in pRCP17 that is not present in pHK120.

5. pHK120 and thermoregulation of phaseolotoxin production

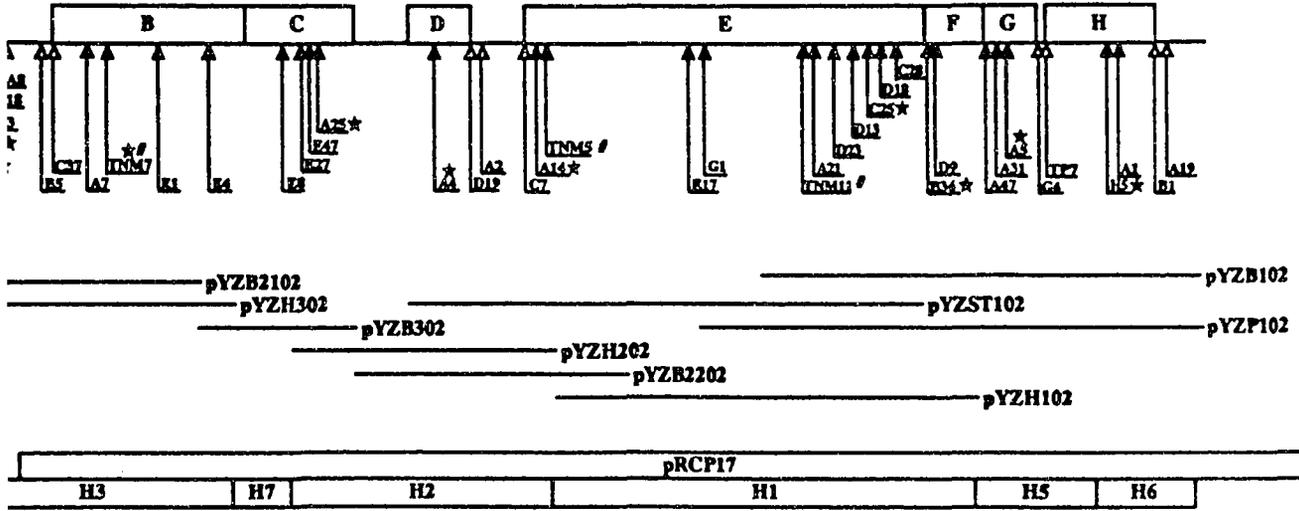
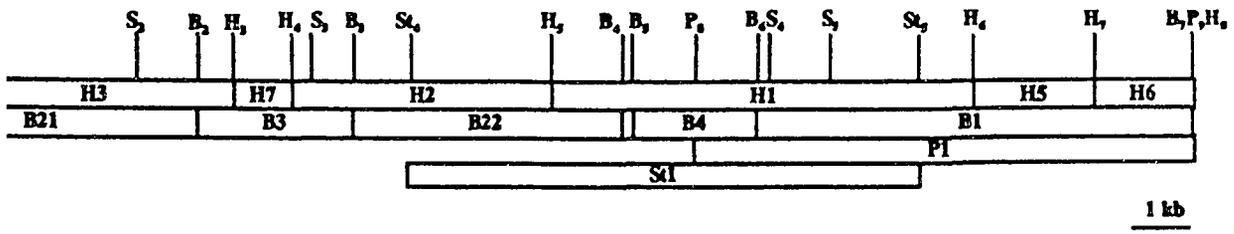
P. syringae pv. *phaseolicola* produces phaseolotoxin at 18°C, the optimum temperature for disease development, but not at 28°C, the optimum temperature for growth (11). Transconjugants of the wild-type strain G50-1 containing pHK120, however, produced toxin at the nonpermissive temperature (28°C) (data not shown). The level of toxin produced by the transconjugants 4612-1 (pHK120) at 28°C was equal to that produced by the wild-type strain at the permissive temperature (18°C), and the transconjugants grown at 18°C produced toxin roughly 3 times (Fig. 2.2) that produced by the controls. Transconjugants of the Tox⁻ mutant, G50-1UV, containing pHK120 also produced toxin at 28°C, although less than when grown at 18°C.

Fig. 2.1

- (A) Endonuclease restriction map of the pHK120 insert. Rectangular boxes represent the various restriction fragments. Only relevant sites and fragments for *SstI* and *PstI* are shown. **H:** *HindIII*, **S:** *SmaI*, **B:** *BamHI* **St:** *SstI*, **P:** *PstI*.
- (B) Map of Tn5 insertions in the 25 kb region of pHK120 and the phaseolotoxin loci. The positions of Tn5 insertions are shown by vertical lines. The number of the marker exchange mutation (a total of 44) is shown horizontally at the bottom of each line. Three random chromosomal mutants (TNM5, TNM7 and TNM11) which also mapped within the pHK120 region are shown and marked with the symbol #. The phaseolotoxin loci and their boundaries are shown by rectangular boxes labeled A through H. Symbols: ▲, *Tox*⁻; △, *Tox*⁺; ○, corresponding marker-exchange mutant could not be obtained; ★, mutants used in pair-complementation analysis.
- (C) Deletion subclones containing fragments from the insert of pHK120.
- (D) Comparison of the insert from pHK120 and pRCP17. A *HindIII* map of the insert from pHK120 is shown below pRCP17; no restriction map of pRCP17 is available. H, *HindIII*.



(Fig. 2.1)



(Fig. 2.1)

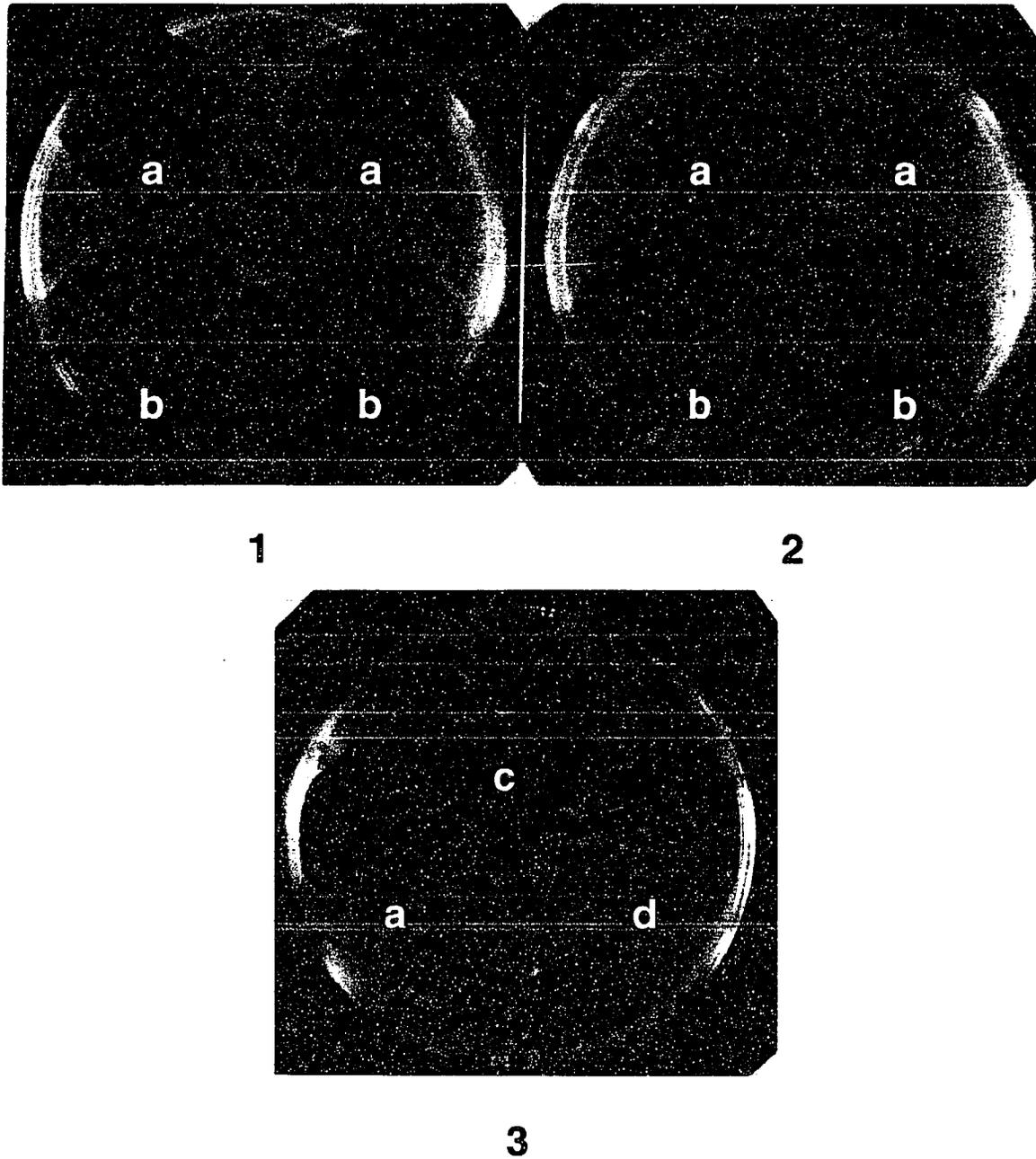


Fig. 2.2. Toxin bioassay. **1** and **3**. These two plates were incubated at 28°C for 4 h and then shifted to 18°C and incubated for 12 h before overlaying with *E. coli* K12. **2**. This plate was incubated at 28°C for 16 h before overlaying. a: 4612-1; b: 4612-1 containing pHK120; c: G50-1; d: G50-1UV(pHK120).

Table 2.1 Bacterial strains and plasmids

Strains and plasmids	Genotype, phenotype or descriptions	Reference and source
<i>E. coli</i>		
DH5 α	F' 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (<i>r_K</i> ⁻ , <i>m_K</i> ⁺) <i>supE44</i> λ ⁻ <i>thi-1gyrA relA1</i>	BRL
HB101	F' <i>mcrB mrr hsdS20</i> (<i>r_B</i> ⁻ , <i>m_B</i> ⁻) <i>recA13 supE44 ara14 galk2 lacY1 proA2 rpsL20</i> (<i>Sm^r</i>) <i>xyl5 leu mut1</i> λ ⁻	BRL
Q1	<i>thr-1 leu-6 thi-1 lacY supE44 tonA21 T₅^r ϕ80^r</i>	4
Bacteriophage		
λ 467	λ b221 <i>rex::Tn5 cI857 Oam29 Pam80</i>	4
<i>P. syringae</i> pv. <i>phaseolicola</i>		
4612-1	Rif ^r 4612, Tox ⁺	13
G50-1	Rif ^r G50, Tox ⁺	13
G50-UV	G50-1 UV-nduced, Tox ⁻	This study
HOAF4	4612-1::Tn5- <i>phtA</i> , Tox ⁻	This study
HOA46	4612-1::Tn5- <i>phtA</i> , Tox ⁻	This study
HOA41	4612-1::Tn5- <i>phtA</i> , Tox ⁻	This study
HOA13	4612-1::Tn5- <i>phtA</i> , Tox ⁻	This study
HOA18	4612-1::Tn5, Tox ⁺	This study
HOA8	4612-1::Tn5, Tox ⁺	This study
HOB5	4612-1::Tn5, Tox ⁺	This study
HOA7	4612-1::Tn5- <i>phtB</i> , Tox ⁻	This study
TNM7	G50-1::Tn5- <i>phtB</i> , Tox ⁻	This study
HOE1	4612-1::Tn5- <i>phtB</i> , Tox ⁻	This study
HOE4	4612-1::Tn5- <i>phtB</i> , Tox ⁻	This study
HOE8	4612-1::Tn5- <i>phtC</i> , Tox ⁻	This study
HOE27	4612-1::Tn5- <i>phtC</i> , Tox ⁻	This study
HOE47	4612-1::Tn5- <i>phtC</i> , Tox ⁻	This study
HOA25	4612-1::Tn5- <i>phtC</i> , Tox ⁻	This study
HOA4	4612-1::Tn5- <i>phtD</i> , Tox ⁻	This study
HOD19	4612-1::Tn5, Tox ⁺	This study
HOA2	4612-1::Tn5, Tox ⁺	This study
HOC7	4612-1::Tn5, Tox ⁺	This study
HOA14	4612-1::Tn5- <i>phtE</i> , Tox ⁻	This study
TNM5	G50-1::Tn5- <i>phtE</i> , Tox ⁻	This study
HOE17	4612-1::Tn5- <i>phtE</i> , Tox ⁻	This study
HOG1	4612-1::Tn5- <i>phtE</i> , Tox ⁻	This study
TNM11	G50-1::Tn5- <i>phtE</i> , Tox ⁻	This study
HOA21	4612-1::Tn5- <i>phtE</i> , Tox ⁻	This study
HOD23	4612-1::Tn5- <i>phtE</i> , Tox ⁻	This study
HOC25	4612-1::Tn5- <i>phtE</i> , Tox ⁻	This study
HOD18	4612-1::Tn5- <i>phtE</i> , Tox ⁻	This study
HOC28	4612-1::Tn5- <i>phtE</i> , Tox ⁻	This study
HOB36	4612-1::Tn5- <i>phtF</i> , Tox ⁻	This study
HOD9	4612-1::Tn5- <i>phtF</i> , Tox ⁻	This study
HOA47	4612-1::Tn5- <i>phtG</i> , Tox ⁻	This study
HOA31	4612-1::Tn5- <i>phtG</i> , Tox ⁻	This study

Table 2.1 Bacterial strains and plasmids (CONT'D)

Strains and plasmids	Genotype, phenotype or descriptions	Reference and source
HOA5	4612-1::Tn5- <i>phtG</i> , Tox ⁻	This study
HOG4	4612-1::Tn5, Tox ⁺	This study
HOTP7	4612-1::Tn5, Tox ⁺	This study
HOH5	4612-1::Tn5- <i>phtH</i> , Tox ⁻	This study
HOA1	4612-1::Tn5- <i>phtH</i> , Tox ⁻	This study
HOB1	4612-1::Tn5, Tox ⁺	This study
HOA19	4612-1::Tn5, Tox ⁺	This study
Plasmids		
pLAFR3	derivative of pLAFR1	35
pRK404	wide-host-range P1-group cloning vector	6
LAFR376	pLAFR3 with polylinker from pIBI76	N. J. Panopoulos
pRK2073	Tra ⁺ Mob ⁺ Sp ^r ColE1 replicon	13
pHK120	Contains 25 kb chromosomal DNA from G50-1 in pLAFR3	13
pYZB2102	4.9 kb <i>Bam</i> HI- <i>Bam</i> HI fragment in pLAFR3	This study
pYZH302	4.3 kb <i>Hind</i> III- <i>Hind</i> III fragment in pLAFR3	This study
pYZB302	2.2 kb <i>Bam</i> HI- <i>Bam</i> HI fragment in pLAFR3	This study
pYZH202	4.6 kb <i>Hind</i> III- <i>Hind</i> III fragment in pLAFR3	This study
pYZB2202	4.9 kb <i>Bam</i> HI- <i>Bam</i> HI fragment in pLAFR3	This study
pYZH102	7.0 kb <i>Hind</i> III- <i>Hind</i> III fragment in pLAFR3	This study
pYZB102	7.4 kb <i>Bam</i> HI- <i>Bam</i> HI fragment in pRK404	This study
pYZP102	7.8 kb <i>Pst</i> I- <i>Pst</i> I fragment in pRK404	This study
pYZST102	8.6 kb <i>Sst</i> I- <i>Sst</i> I fragment in pLAFR376	This study

Table 2.2. Results of pair-complementation and complementation using pHK120::Tn5hybrid plasmids and various subclones containing fragments from the insert of pHK120

Hybrid plasmid & subclones	SELECTED MARKER EXCHANGE MUTANTS OR RANDOM Tn5 MUTANS									
	HOA4 6	HOA4 1	TNM7	HOA2 5	HOA4	HOA1 4	HOC2 5	HOB3 6	HOA5	HOH5
pHK120::Tn5-B25	+ ^a	+	+	ND ^c	ND	ND	ND	ND	ND	ND
pHK120::Tn5-AF4	- ^b	-	+	ND	ND	ND	ND	ND	ND	ND
pHK120::Tn5-A46	-	-	+	ND	ND	ND	ND	ND	ND	ND
pHK120::Tn5-A41	-	-	+	ND	ND	ND	ND	ND	ND	ND
pHK120::Tn5-A13	-	-	+	ND	ND	ND	ND	ND	ND	ND
pHK120::Tn5-A18	+	+	+	ND	ND	ND	ND	ND	ND	ND
pHK120::Tn5-B5	+	+	+	ND	ND	ND	ND	ND	ND	ND
pHK120::Tn5-C37	+	+	+	+	ND	ND	ND	ND	ND	ND
pHK120::Tn5-A7	+	+	-	+	ND	ND	ND	ND	ND	ND
pHK120::Tn5-E1	+	+	-	+	ND	ND	ND	ND	ND	ND
pHK120::Tn5-E4	+	+	-	+	ND	ND	ND	ND	ND	ND
pHK120::Tn5-E8	ND	+	+	-	+	ND	ND	ND	ND	ND
pHK120::Tn5-E27	ND	+	+	-	+	ND	ND	ND	ND	ND
pHK120::Tn5-E47	ND	+	+	-	+	ND	ND	ND	ND	ND
pHK120::Tn5-A25	ND	+	+	-	+	+	ND	ND	ND	ND
pHK120::Tn5-A4	ND	ND	+	+	-	+	+	ND	ND	ND
pHK120::Tn5-D19	ND	ND	ND	+	+	+	ND	ND	ND	ND
pHK120::Tn5-A2	ND	ND	ND	+	+	+	ND	ND	ND	ND
pHK120::Tn5-C7	ND	ND	ND	+	+	+	+	ND	ND	ND
pHK120::Tn5-A14	ND	ND	ND	+	+	-	-	+	ND	ND
pHK120::Tn5-E17	ND	ND	ND	ND	+	-	-	+	ND	ND
pHK120::Tn5-G1	ND	ND	ND	ND	+	-	-	+	ND	ND
pHK120::Tn5-A21	ND	ND	ND	ND	+	-	-	+	+	ND
pHK120::Tn5-D23	ND	ND	ND	ND	+	-	-	+	+	ND
pHK120::Tn5-D13	ND	ND	ND	ND	+	-	-	+	+	ND
pHK120::Tn5-C25	ND	ND	ND	ND	+	-	-	+	+	ND
pHK120::Tn5-D18	ND	ND	ND	ND	+	-	-	+	+	ND
pHK120::Tn5-C28	ND	ND	ND	ND	+	-	-	+	+	ND
pHK120::Tn5-B36	ND	ND	ND	ND	ND	+	+	-	+	+
pHK120::Tn5-D9	ND	ND	ND	ND	ND	+	+	-	+	+
pHK120::Tn5-A47	ND	ND	ND	ND	ND	ND	+	+	-	-
pHK120::Tn5-A31	ND	ND	ND	ND	ND	ND	+	+	-	-
pHK120::Tn5-A5	ND	ND	ND	ND	ND	ND	+	+	-	-
pHK120::Tn5-G4	ND	ND	ND	ND	ND	ND	ND	+	+	+
pHK120::Tn5-TP7	ND	ND	ND	ND	ND	ND	ND	+	+	+
pHK120::Tn5-H5	ND	ND	ND	ND	ND	ND	ND	+	+	-
pHK120::Tn5-A1	ND	ND	ND	ND	ND	ND	ND	+	+	-
pHK120::Tn5-B1	ND	ND	ND	ND	ND	ND	ND	ND	+	+
pHK120::Tn5-A19	ND	ND	ND	ND	ND	ND	ND	ND	+	+
pYZB2102	+	+	-	ND	ND	ND	ND	ND	ND	ND
pYZH302	-	-	-	ND	ND	ND	ND	ND	ND	ND
pYZB302	ND	ND	ND	+/- ^d	ND	ND	ND	ND	ND	ND
pYZH201	ND	ND	ND	-	+/-	-	ND	ND	ND	ND
pYZB2202	ND	ND	ND	ND	-	-	ND	ND	ND	ND
pYZH102	ND	ND	ND	ND	ND	ND	-	-	ND	ND
pYZST102	ND	ND	ND	ND	+	-	-	ND	ND	ND
pYZB102	ND	ND	ND	ND	ND	ND	-	+	-	-
pYZP102	ND	ND	ND	ND	ND	ND	-	+	-	-

Note: +, toxin was detected in the transconjugant; -, toxin was not detected in the transconjugant;

+/-, limited amount of toxin was detected in the transconjugant; ND, test was not done.

E. DISCUSSION

We have shown that a *P. syringae* pv. phaseolicola genomic clone (pHK120) which complements all *Tox*⁻ mutants in our possession can be divided into at least eight complementation groups. Peet et al (30) previously reported that some of the genes involved in phaseolotoxin production are clustered. The comparison of pHK120 with one of their clones (pRCP17) in this study indicated that the two clones differ from each other only in two respects: unlike pRCP17, pHK120 does not harbor the ROCT gene and pHK120 contains a region of DNA (Fig. 2.1D, fragment H4 and part of H3) that is not shared by pRCP17. The genes for biosynthesis of many antibiotics, viz. bialaphos, are clustered (27). Genes involved in the production of phytotoxins, such as syringomycin and syringotoxin (7, 24, 25, 39) produced by *P. syringae* pv. *syringae*, coronatine (22, 40) produced by *P. syringae* pv. *tomato*, and tabtoxin (15, 38) produced by *P. syringae* pv. *tabaci*, have also been reported to be clustered. Martin and Liras (17) have suggested that clustering of genes involved in the biosynthesis of secondary metabolites may be related to the sequential formation of the enzymes involved in the biosynthesis of these metabolites.

No leaky mutants were identified in this study in contrast to the finding of Peet et al (30), who reported several randomly generated chromosomal Tn5 mutants that were leaky. This suggests that the products of the genes in the eight complementation groups are directly involved in toxin production. Toxin production was restored to the level produced by the wild-type strain in transconjugants of mutants HOA41 and HOA46 (with mutations in the *phtA* locus) containing pYZB2102 and in transconjugants of mutant HOB36 (with the mutation in the *phtF* locus) containing plasmids pYZB102 as well as pYZP102, indicating that sufficient information to fully express the gene(s) in the *phtA* and *phtF* loci is present in the respective subclones. Mutants with the mutations in the *phtC* locus were only partially complemented by the plasmid pYZB302, although this plasmid contained ~0.9 kb

of DNA to the left of Tn5 insertion E8 and ~0.4 kb of DNA to the right of Tn5 insertion A25 in the *phtC* locus. The simplest explanation for this result may be that the promoter for the gene(s) in this region is not present in the subclone and that the low level of expression of the gene(s) is the result of a read-through from the vector promoter. It is also possible that the transcriptional unit contains a single gene and that the 3'-end of the gene is not present in the subclone, which results in the production of a truncated product that is only partially active.

Partial complementation was also obtained in a *phtD* mutant, HOA4, when subclone pYZH202 was mobilized into this mutant. Surprisingly, this mutant was not complemented by pYZB2202 but was fully complemented by pYZST102, a plasmid containing less DNA overlapping with the insert in pYZH202 than pYZB2202 did but containing an additional region of DNA to the right of the B22 fragment (Fig. 2.1A). The test for complementation of HOA4 by these three subclones was repeated (data not shown), and the same results were obtained. Furthermore, restriction analysis of the plasmid constructs in the transconjugants showed that they were not altered. Although the reason for these unexpected complementation results is not known, the fact that pYZST102 fully restores toxin production in mutant HOA4 suggests that a *trans*-acting activator may be required for the *phtD* locus to function.

Although the original cosmid clone (pHK120) was able to complement mutants with mutations in the *phtB*, *phtE*, *phtG*, and *phtH* loci, none of the subclones shown in Fig. 2.1C complemented these mutants. I expected this with regard to the *phtB* locus because none of the subclones contained the entire predicted locus. However, on the basis of pair-complementation analysis, the insert in pYZST102 should have complemented the *phtE* mutants (HOA14 and HOC25). The fact that such complementation did not occur raises the possibility that pYZST102 does not contain the entire *phtE* locus. I have sequenced 6.7 kb

of DNA (41) encompassing this locus, and preliminary analysis shows that the fifth *Sst*I site (Fig. 2.1A) interrupts a putative open reading frame (ORF). If this ORF is part of the *phtE* locus, then this locus must be larger than the predicted one (Fig. 2.1B), and this may explain why pYZST102 does not complement the *phtE* mutants (41). A similar discrepancy between complementation analysis and sequencing data was reported by Jacobs et al (12) for nodulation genes in *Rhizobium meliloti*. I also found that there is a gap of 200 bp between the ORF mentioned above and the one adjacent to it, and a sequence showing homology with the *E. coli* canonical promoter (10) is located in this gap, making it possible that the ORF interrupted by the fifth *Sst*I site is part of the *phtF* locus. If this is the case, the reason for the failure of St1 fragment to complement the mutants with mutations in this locus may have to do with the lack of a trans-acting positive regulatory factor, which is located outside of this fragment. Alternatively, the increase in copy number of the genes in the subclone may lead to an accumulation of toxic intermediates in the transconjugant, which could down regulate toxin synthesis by a feed back mechanism.

The last two loci, *phtG* and *phtH*, are located in the B1 or the P1 fragment of pHK120 according to pair complementation analysis. However, neither pYZB102 nor pYZP102 complemented the mutants with mutations in these two loci, even though the inserts in both of these plasmids contain substantial regions of DNA on either side of these loci. Currently, I have no explanation for this unexpected result. Pair complementation analysis also indicated that the pHK120::Tn5 hybrid plasmids containing Tn5 insertions within the *phtG* locus failed to complement the *phtH* mutants, but pHK120::Tn5 hybrid plasmids containing Tn5 insertions within the *phtH* locus complemented the *phtG* mutant, indicating that the *phtG* locus is required for the function of the *phtH* locus. This is similar to the relationship between *syrD* and *syrB* (7), two genes involved in the production of syringomycin by *P. syringae* pv. *syringae*, in which *syrD* regulates the transcription of *syrB*

and the production of five high molecular weight proteins which have been shown to be involved in syringomycin production. It is also possible that the *phtG* and *phtH* loci constitute one locus and the two Tox⁺ mutants with mutations between the *phtG* and *phtH* loci are nonpolar mutants.

Although pHK120 contains most of the genes involved in the production of phaseolotoxin, I conclude that it does not contain all of them. This conclusion is based on the fact that the transformants of *E. coli* containing pHK120 grew on minimal media (data not shown). Since pHK120 does not contain the ROCT gene and *E. coli* is sensitive to phaseolotoxin, production of the toxin should have resulted in growth inhibition of the transformants of *E. coli* on minimal media. Another observation which supports the conclusion that the pHK120 does not contain all the toxin genes is that the transconjugants of *P. syringae* pv. *syringae* containing pHK120 did not produce toxin when tested in the *E. coli* toxin bioassay (data not shown).

Transconjugants of the wild-type *P. syringae* pv. *phaseolicola* (G50-1) containing pHK120 produced more toxin than the wild-type strain at 18°C, the permissive temperature. This increase in toxin production may imply that an activator gene is located in the pHK120 insert. Activators are involved in the biosynthesis of bialaphos (1, 27), tylosin, actinorhodin and streptomycin (17) produced by *Streptomyces hygroscopicus*, *Streptomyces fradiae*, *Streptomyces colicolor* and *Streptomyces griseus*, respectively. Transconjugants of the wild-type strain of *S. coelicolor* (actinorhodin positive) containing a cosmid clone that harbors an activator gene produced 30-40 fold more actinorhodin (17). It is also possible that the gene cluster in the pHK120 insert contains a gene encoding a rate-limiting product involved in the synthesis of phaseolotoxin. Multiple copies of such a gene would result in higher-than-normal levels of this product and lead to increased levels of toxin in the transconjugants.

The fact that transconjugants containing pHK120 produced toxin at 28°C indicated that the thermoregulation of toxin production was overridden by pHK120. As mentioned above, if the pHK120 insert contains an activator, over-production of this activator may abolish thermoregulation. However, a more likely explanation is that some or all of the phaseolotoxin genes present in pHK120 harbor DNA binding sites which titrate the putative repressor protein produced by *P. syringae* pv. phaseolicola at 28°C. A similar explanation for the production of phaseolotoxin by transconjugants of the wild-type strain containing pDC938, a class III genomic clone from *P. syringae* pv. phaseolicola, was previously proposed (32). This clone does not harbor a structural or regulatory gene involved in toxin production but contains DNA binding motifs similar to those involved in binding proteins in other systems (34). Rowley et al (32) showed that at 28°C the wild-type strain produces a protein (s) that specifically binds to a 485 bp fragment from the pDC938 insert that contains the DNA binding motifs. On the basis of these results, it was proposed that when present in multiple copies, the fragment harboring DNA binding sites titrates a repressor protein produced at 28°C, thereby allowing toxin biosynthesis to occur. Similar DNA binding motifs are found in the upstream region of the *argK* of *P. syringae* pv. phaseolicola (9); the *argK* gene is coordinately regulated with phaseolotoxin genes by temperature (9). Recent results from our laboratory (33) have shown that a ~490 bp fragment from the *argK* promoter region also specifically binds to a protein(s) produced by the wild-type strain at 28°C. I am currently examining the DNA sequence of the *phtE* locus to determine whether homologous sequences that bind to the putative repressor protein exist in the genes in this region.

The elucidation of the genetic organization of the cluster in pHK120 described above makes it possible to determine the number of genes in the cluster and their nucleotide sequences, to obtain their gene products, and to determine which of these genes are

thermoregulated. These studies are currently underway. In the longer term, the availability of site-directed mutants will allow us to elucidate the pathway of biosynthesis of phaseolotoxin by determining the biosynthetic steps that are blocked in the various marker-exchange mutants.

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CHAPTER III. NUCLEOTIDE SEQUENCE ANALYSIS OF THE *phtE* LOCUS INVOLVED IN THE PRODUCTION OF PHASEOLOTOXIN.

A. ABSTRACT

In chapter II, I reported that the insert in the cosmid clone, pHK120, contains eight loci (*phtA* through *phtH*) harboring genes involved in the production of phaseolotoxin, a phytotoxin produced by *Pseudomonas syringae* pv. phaseolicola. Both strands of the DNA encompassing the *phtE* locus were sequenced which revealed six putative open reading frames (ORFs), each preceded by a putative ribosomal binding site, and all oriented in the same direction. Using the RT-PCR technique I showed that the *phtE* locus is transcribed into a large transcript (~5 kb) indicating that the ORFs in this locus are arranged in a single operon. A primer extension experiment determined that the transcript starts with a T, located 31 bp upstream of the ATG codon of ORF1. The primer extension experiment also showed that the expression of this operon is not temperature regulated because the same primer-extension product resulted when RNA extracted from the wild-type strain grown at either 18°C or 28°C was used as the template. A comparison of the sequences of the putative ORFs with the sequences of known genes revealed that ORF3, encoding a protein containing 395 aa, has about 55% homology with the acetylornithine aminotransferase gene of *E. coli* and the ornithine aminotransferase genes of other organisms. A Lys residue which is a binding site for pyridoxal phosphate and an Arg residue which is a binding site for the α -carboxylate group of the substrate are conserved in ORF3. These data suggest that ORF3 encodes a protein required for the biosynthesis of ornithine, a constituent of phaseolotoxin. ORF6, encoding a protein containing 359 aa, has about 40% homology with the fatty acid desaturase gene, *desA*, of *Synechocystis* Pcc6803. Both genes show very similar hydropathy profiles and contain a copper binding signature. These results suggest

that the gene product of ORF6 may be involved in the secretion of phaseolotoxin, presumably by increasing membrane fluidity at the lower temperature at which the toxin is produced. Computer searches did not discover significant homologous gene sequences in the data base for the rest of the ORFs but the hydropathy analysis showed that all of them contain one to several hydrophobic domains, suggesting that the gene products of these ORFs are possibly membrane-associated.

B. INTRODUCTION

Phaseolotoxin is a phytotoxin produced by *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of halo blight of beans. It contains a tripeptide, consisting ornithine, alanine and homoarginine with an inorganic group attached to the N^δ position of ornithine (28). The toxin inhibits ornithine carbamoyltransferase (OCTase), a key enzyme involved in the biosynthesis of arginine. The inactivation of the OCTase in the toxin-exposed tissues of plants results in arginine deficiency, and chlorosis (29).

Even though there is extensive chemical and biochemical information about phaseolotoxin and its role in halo blight, little was known about the genetics of its production. In the first half of this dissertation (Chapter II) I have shown that there are 8 transcriptional units (indicating the presence of a minimum of eight genes) in the insert of the previously described genomic clone pHK120 which complements EMS-, UV- and Tn5-induced Tox⁻ mutants. The largest locus among the 8 transcriptional units is *phtE*, which spans a region of 6.4 kb as determined by pair complementation analysis. The rest of the loci are in the range of 1 to 2.5 kb. Phaseolotoxin contains a tripeptide, ornithyl-alanyl-homoarginine. Small peptides have not been found to be synthesized by the ribosomal mechanism. Instead, they are synthesized by large multienzyme complexes (60-600 kda) called peptide synthetases which act as templates in the activation of the amino acids and their assembly into peptides. I reasoned that the *phtE* locus, which is ~7 kb is large enough to harbor a peptide synthetase, and therefore decided to sequence it first.

Presented in this chapter is a complete nucleotide sequence of DNA in the *phtE* locus. Based on the sequence data, the transcript, and the primer extension analyses, I have concluded that the *phtE* locus contains six ORFs that are arranged in a single operon. I have also concluded that the expression of the *phtE* locus is not temperature regulated in *P. syringae* pv. *phaseolicola*. The computer analyses of the sequence indicated that the product

of ORF3 is probably involved in production of ornithine and the product of ORF6 may be involved in secretion of phaseolotoxin. No homologous sequences for any of the other ORFs were found in the data bases.

C. MATERIALS AND METHODS

1. Generation of a nested set of deletion subclones for sequencing

A nested set of deletion clones was generated according to the method described by Henikoff (21). Plasmids containing DNA fragments of interest in pT7/T3 α -18 (BRL, Gaithersburg, MD) were digested with two selected enzymes. Both enzymes cut between the sequencing primer binding site in the vector and the end of the insert. One enzyme that cut closest to the primer site created a 3'-overhang end and the other generated a 5'-overhang or a blunt end. Five μ g of linearized DNA was digested with 250 units of exonuclease III (Exo III, Promega, Wisconsin, WI) in a solution containing 60 mM Tris-HCl [pH 8.0] and 0.66 mM MgCl₂. Aliquots were removed from the tube at 30 second time intervals and treated with 60 units of S1 nuclease (Promega, Wisconsin, WI), which blunt-ended the plasmids. The plasmids were circularized with T4 DNA ligase (Promega, Wisconsin, WI) and transformed into *E. coli* HB101. The plasmid DNA of the transformants were extracted and the length of the insert of the plasmids was examined by digestion.

2. DNA Sequencing

DNA was sequenced by the dideoxy-nucleotide chain termination method (37), using a modified T7 DNA polymerase (Sequenase 2.0, Promega, Madison, WI). A nested set of deletion clones was sequenced with the T7 primer, and the rest of the clones were sequenced with synthetic primers (15-mers) which were synthesized on a 380B DNA synthesizer (Applied Biosystem, CA). Both strands of DNA were completely sequenced with dGTP and some regions were sequenced with dITP to resolve compressions created by the high G+C content of *Pseudomonas* DNA.

3. Isolation of total RNA

Total RNA was extracted from *P. syringae* pv. *phaseolicola* or its derivatives according to the method described by Innes et al (24). Overnight bacterial cultures in KB medium were subcultured to an optical density of 0.3 (600 nm) and shaken at the appropriate temperature for 4 h. Ten ml of bacterial cells were harvested by centrifugation and quick frozen in dry ice. The cell pellets were resuspended in 1 ml of hot lysis buffer (50 mM Tris-HCl [pH 9.0], 50 mM EDTA, 300 mM Sodium acetate, 0.625 % [Wt/Vol] SDS) and 1 ml hot phenol (65°C), mixed and then boiled for 5 min. After centrifuging at 700 rpm (bench top clinical centrifuge, International Equipment Co., MA) for 5 min, the aqueous phase was removed and extracted twice with 1 volume of phenol, twice with 1 volume of phenol:chloroform, and once with 1 volume of chloroform. The RNA was precipitated with 2 volumes of cold EtOH and dried under vacuum. The pellets were resuspended in 96 µl of DNase digestion buffer (20 mM Tris-HCl [pH 8.0], 10 mM MgCl₂) and digested with 4 µl (40 units) of RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN) at 37°C for 15 min. The DNase-treated RNA was extracted with phenol and chloroform, precipitated with EtOH, and dried as described above.

4. Preparation of probes

RNA probes were synthesized *in vitro* using T3 or T7 RNA polymerase. Plasmids containing fragments of interest in pT7/T3α-18 were digested with an enzyme that cut within the insert but not more than 2 kb from the T7 or T3 promoter site. One µg of the digested DNA was used as the template for the *in vitro* synthesis of ³²P-labeled RNA with 20 units of T7 or T3 RNA polymerase (Promega, Wisconsin, WI) in a solution containing 40 mM Tris-HCl [pH 7.5], 6 mM MgCl₂, 2 mM Spermidine, 10 mM NaCl, 10 mM DTT,

20 units of RNase inhibitor, 0.5 mM ATP, GTP UTP and α -³²P CTP (50 μ Ci). The reaction was terminated by adding 1 μ l of 0.5M EDTA and the DNA templates were digested by adding to the reaction mixture 40 units of RNase-free DNase I (Promega, Madison, WI) at 37°C for 15 min. Alternatively, DNA probes were made using the Random Priming Kit (BRL, Gaithersburg, MD). A hundred ng of DNA fragments isolated from the agarose gel using the USBioclean Kit (US Biochemicals, Cleveland, OH) was labeled with the klenow fragment (BRL, Gaithersburg, MD) in a solution containing 200 mM HEPES, 51 mM Tris-HCl, 5.1 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.4 mg/ml BSA, 0.45 μ g/ml hexamer, 0.1 mM dATP, dGTP, dTTP and α -³²P dCTP (50 μ Ci) at 25°C for 1 hr.

5. RT-PCR amplification of mRNA

One to 3 μ g of total RNA was used for the synthesis of cDNA by reverse transcriptase (Perkin Elmer Co., Nutley, NJ) using 2.5 μ moles of random hexamers as the primer. To eliminate DNA contamination (12), the RNA samples were treated with 40 units of RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN) in a solution containing 5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, 1 mM of dATP, dGTP, dTTP and dCTP at 37°C for 1 h. The reaction mixtures were immediately heated at 99°C for 5 min to inactivate the remaining DNase I and cooled to 4°C. One μ g of reverse transcriptase and RNase inhibitor were added to the reaction tubes which were incubated at 42°C for 30 min. Two specific primers (15-mer), one identical and the other complementary to the mRNA sequence, were used in the amplification of the randomly generated cDNA using 25 units of Taq polymerase (Perkin Elmer Co., Nutley, NJ) in a solution containing 2 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM dATP, dGTP, dCTP and dTTP in a final volume of 100 μ l. PCR was performed for 40 cycles with a setting of 1 min at 95°C, 1 min

at 55°C and 1 min at 72°C using the Perkin Elmer 9600 PCR Thermal Cycler (Perkin Elmer, Nutley, NJ). Twenty µl aliquot of each PCR product was analyzed to determine whether the expected fragment was present using a 2% agarose gel. Southern blot analysis was performed with specific probes to confirm the PCR results.

6. Primer Extension

One µl (about 100 ng) of primer was labeled with ³²P-dCTP using 10 units of T4 polynucleotide kinase in a solution containing 50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 5 mM DTT, 0.1 mM Spermidine at 37°C for 30 min. The labeled primer and 10 µg of total RNA were mixed together and incubated at 70°C for 10 min, and immediately chilled on ice. cDNA synthesis (primer extension) was carried out in a solution containing 50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM MgCl₂, 200 mM DTT and 200 units of SuperScript RT (GIBCO BRL, Gaithersburg, MD) at 42°C for 1 h. The reaction was terminated by adding 4 µl of 0.5 M EDTA. Ten µl of primer extension products were run in a 6% polyacrylamide gel along with a sequencing ladder which was generated using the same primer in a sequencing reaction according to the standard chain-termination method of Sanger et al (37).

7. Computer-assisted analyses

Computer analyses of DNA sequences was done using the GCG (Genetic Computer Group, Wisconsin) package. Codon preference was analyzed using a codon frequency table for *Pseudomonas* genes. Searches for homologous sequences (27) were conducted using GeneEMBL Data bases at both DNA and amino acid levels.

D. RESULTS

1. The Sequences of the *phtE* locus

Both strands of the DNA encompassing the *phtE* locus were sequenced and a 7084 bp long sequence was identified (Fig. 3.2). Six complete and two truncated ORFs, all with the same orientation (Fig. 3.1), are present in this sequence. A prokaryotic ρ -independent terminator-like sequence (19) (from nt 569 to nt 581, Fig. 3.2) was located between the first truncated ORF and ORF1. Search for the *E. coli*-like promoter led to the identification of a possible -10 sequence (TcTAcTcA, located 16 bp from the putative transcriptional start site (see below), but no -35 sequence was found. Another sequence, GGAACACttctactCACCA (from nt 684 to nt 701, Fig. 3.2), similar to the promoter sequence that is conserved in several *avr* and *hrp* genes (24, 39) was located 18 bp upstream of the transcriptional start site. Three possible initiation codons (at positions 728, 747 and 786, respectively) are located at the 5'-end of ORF1, but only the second one (located at position 747) is preceded by a possible ribosomal binding site, AGcGA. Therefore, this ATG was assigned as the start codon for ORF1. ORF2, starting with the ATG codon at position 1647, is preceded by a putative ribosomal binding site, AAGGAG. A 137 bp long untranslated gap (Fig. 3.2) exists between ORF2 and ORF1. ORF3 also has two possible initiation codons, located at position 2297 and 2306, respectively. The first ATG codon was assigned as the initiation site because a putative ribosomal binding site, AAcAGG, was found 5 nt upstream. ORF3 is also preceded by a 103 bp long untranslated region between ORF2 and ORF3. The initiation site (ATG) for ORF4 is located at position

3469 which is preceded by a ribosomal binding site, AAGGAA. The sequence of the first three amino acid residues of ORF4 overlaps with the sequence of the end of ORF3, as does the ribosomal binding site of ORF4. The initiation codon (ATG, located at position 3937) for ORF5 is only 3 bp downstream of the stop codon of ORF4, and the putative ribosomal binding site, GAGGCA (from nt 3925 to nt 3930), is located within the 5'-end of ORF4. Similarly, the initiation codon for ORF6 (ATG, located at nt 5079) is only 5 bp downstream of the stop codon for ORF5 and a ribosomal binding site, GAGGAG (from nt 5065 to nt 5070), was found within ORF5. Following ORF6 is a 549 bp long untranslated region in which a prokaryotic ρ -independent terminator-like sequence and an *E. coli* promoter-like sequence were found. The terminator-like sequence runs from nt 6303 to nt 6318, 176 bp downstream of the stop codon for ORF6. The promoter-like sequence, with TATAAT as the -10 region and TcGCA as the -35 region, starts at nt 6610 and ends at nt 6584, respectively. This putative promoter may serve as the RNA polymerase binding site for the down stream gene, starting with the GTG codon at nt 6675. The complete sequence of this ORF was not determined because this region was outside the *phtE* locus. Codon preference analysis using the codon usage table of published *Pseudomonas* genes revealed that ORF1 through ORF5 have a similar pattern of codon usage as most *Pseudomonas* genes (average codon preference >0.8). However, the pattern of codon usage for ORF6 is not characteristic of most *Pseudomonas* genes (average codon preference=0.5) (Fig. 3.3).

2. The *phtE* transcript

The *phtE* locus, as shown in Chapter II, spans a 6.4 kb region of DNA. To detect the message (s) transcribed by this locus, total RNA from the wild-type strain G50-1 (Tox⁺) grown at 18°C or 28°C was ³²P-labeled using polynucleotide kinase and used as a probe in the hybridization analysis of the *Hind*III-digested pHK120 DNA. The hybridization of the H1 fragment (Fig. 3.1) of pHK120 to the labeled probe (data not shown) indicated that a message(s) was transcribed from the *phtE* locus in the wild-type strain grown at 18°C as well as 28°C. To obtain more detailed information on the *phtE* transcript(s), RT-PCR was performed. Total RNA was isolated from the transconjugant of the wild-type strain 4612-1 containing the plasmid pHK120, on the assumption that multiple copies of pHK120 in the transconjugant would result in an increase in the copy number of the transcript(s) as demonstrated by the fact that this transconjugant produces about three times more toxin than the wild-type strain. Expected fragments (with the correct sizes and ability to hybridize to specific probes) were detected after PCR-amplification in the cases where the specific primers bound to sites within the ORFs (Table 3.1 and Fig. 3.2). Negative controls to which no RT was added showed no PCR products, indicating that all the PCR fragments shown in Table 3.1 were amplified from the mRNA-based cDNA, and not from contaminating genomic DNA in the RNA samples. Since the regions covered by these specific primers overlap (Fig. 3.2), these results suggest that a continuous transcript that covers all six ORFs is produced. PCR-amplified fragments were not generated in two cases in which one of the two specific primers binds to a site outside of the ORFs (Table 3.1; Fig. 3.2), indicating that the transcript of the *phtE* locus starts

between the St1uni and the St1rev primer binding sites and ends after the HB12uni primer binding site.

Table 3.1. RT-PCR amplification of mRNA of the *phtE* locus.

Primers	Fragments	Hybridization
St1uni, St1rev	- a	-
E72rev, HS27T7	+ b	+
E72rev1, HB37T7B	+	+
S6uni, HB37T7A	+	+
S6uniA, HB37T7	+	+
S6uniB, HE45T7	+	+
B4uni, HP46T7	+	+
HE25T7, B4T7A	+	+
HP22T7, B4T7	+	+
HB12T7, HB12uniB	+	+
HB12T7A, HB12uniA	+	+
HB12T7B, HB12uni	+	+
H2T7, H2T7Arev	-	-
H2T7A, H2T7Arev1	+	+

Note: **a**: PCR fragment was not detected on the agarose gel and hybridization to specific primers did not occur. **b**: PCR fragment was detected on agarose gel that hybridized to a specific probe.

3. Primer extension

To determine the transcriptional start site, total RNA from the wild-type strain G50-1 grown at 18°C or 28°C and from transconjugants of 4612-1 containing pHK120 was used as the template for primer extension. A synthetic oligo, St1rev (5'-CGGTGCTGACAGGCT-3'), was used as the primer. A 65 bp long fragment was generated (Fig. 3.5) regardless of the RNA sample used, indicating that the expression of the *phtE* locus is independent of temperature regulation. This transcript starts with

the nucleotide T, located 31 bp upstream of the ATG codon of ORF1 (Fig. 3.2). It is noteworthy that minor fragments with variable sizes detected in the primer extension experiment may have resulted from contamination of the primer by smaller or longer oligos during primer synthesis or reflects the existence of longer or shorter transcripts due to RNA processing.

4. The Product of ORF1

ORF1 encodes a protein containing 254 aa. A computer search did not reveal significant homology to any gene sequence in the GeneEMBL data base. Hydropathy analysis of the deduced amino acid sequence of ORF1 (Fig. 3.6) showed that there are five hydrophobic regions, each followed by a hydrophilic region, indicating that the product of ORF1 is a trans-membrane protein.

5. The Product of ORF2

The deduced amino acid sequence of ORF2 (182 aa) did not show significant homology to any gene in the data base, except that it showed a modest homology (17.6% identity and 40% homology over 142 amino acid residues) to the *outD* gene of *Erwinia carotovora* sub. *carotovora*. *outD* encodes a protein of 650 aa which is one of thirteen proteins involved in the secretion of pectinases and cellulases (34). The homologous sequence is located in the central portion of OutD. Hydropathy analysis revealed that the ORF2 product is highly hydrophobic and containing five such regions (Fig. 3.7), suggesting that this is also a membrane-spanning protein.

6. The Product of ORF3

The deduced amino acid sequence of ORF3 contains 395 aa, the largest gene product in the *phtE* locus. A GeneEMBL data base search revealed that the ORF3 amino acid sequence has significant homology (average of 35% identity, 55% homology over 300 aa residues) with that of acetylornithine aminotransferase (ACOAT) from *E. coli*; ornithine aminotransferase (OAT) from yeast, rat and human; glutamate-1-semialdehyde 2,1-aminotransferase from *Bacillus subtilis*, *Salmonella typhimurium* and *Nicotiana tabacum*, and glutamate:succinic semialdehyde transaminase from *E. coli*. When the amino acid sequences of these genes are aligned (Fig. 3.8), conserved amino acid residues were detected throughout most of the region, except at the N-terminal end. A Lys residue found in aspartate aminotransferase (AAT) (25) and in histidinol phosphate aminotransferase (HPAT) (22) to which the cofactor pyridoxal phosphate (PLP) is believed to bind is also conserved in ORF3 (Fig. 3.8). An Arg residue which is conserved in many aminotransferase genes and is believed to be involved in the binding of the α -carboxylate group of the substrate, is located at position 313 in ORF3. The N-terminal sequence of ORF3 is significantly different from the mammalian OATs, which is not surprising since the mammalian OATs are mitochondrial enzymes which contain a leader sequence that is responsible for mitochondrial targeting (23).

7. The Product of ORF4

The deduced amino acid sequence of ORF4, which contains 155 aa residues, showed some homology (21.2% identity in a 146 aa overlap) with the *unch* gene which encodes the ATP synthase delta subunit in *E. coli* (48). A protein containing 223 aa, the δ -subunit of ATP synthase of *E. coli* connects the subunit b and α , located in the

membrane and cytoplasmic regions, respectively. Hydropathy analysis (Fig. 3.9) indicated that there is only one hydrophobic region in the ORF4 gene product, suggesting that it may have only one of its ends associated with the membrane.

8. The Product of ORF5

With 379 aa residues, the product of ORF5 is the second largest protein product of the *phtE* locus. Its N-terminal end has some homology (23.3% identity in 90 aa overlap) with the N-terminal end of *crtE*, a gene involved in the synthesis of prephytoene in *Erwinia herbicola*. The C-terminal region of ORF5 has some homology (19.3% identity in the 140 aa overlap) with the N-terminal end of *dnaG* of *Streptomyces typhimurium* (15). *dnaG* encodes a 582 aa protein that functions as a primase. The hydropathy analysis showed that the N-terminal end of ORF5 is highly hydrophobic whereas the C-terminal end is highly hydrophilic (Fig. 3.10).

9. The Product of ORF6

A computer search in the GeneEMBL data base revealed that ORF6 has significant homology (22% identity and 48% homology over 284 aa residues) with the fatty acid desaturase gene, *desA*, of *Synechocystis* Pcc6803 (47). However, ORF6 has very little homology with fatty acid desaturase genes from mammals (38), plants (30) and yeast (41). Analysis of the hydropathy profiles showed that ORF6 bears striking resemblance to the *desA* gene (Fig. 3.12). Two membrane-spanning regions are perfectly aligned between these two genes, indicating that these regions are crucial for association with the membrane. The computer search also identified a copper-binding domain in ORF6 (nt 260 to nt 270; Fig. 11), similar to that in the cytochrome *c* subunit I (9). ORF6 contains the fifth *HindIII* site in the pHK120 insert (Fig. 3.1). In Chapter

II, I reported that the plasmid pYZH102 failed to complement the mutants with mutations in the *phtE* locus. The failure of pYZH102 to complement the mutants in the *phtE* locus can be attributed to the fact that the ORF6 product is truncated at the *HindIII* site, as the sequence data shows.

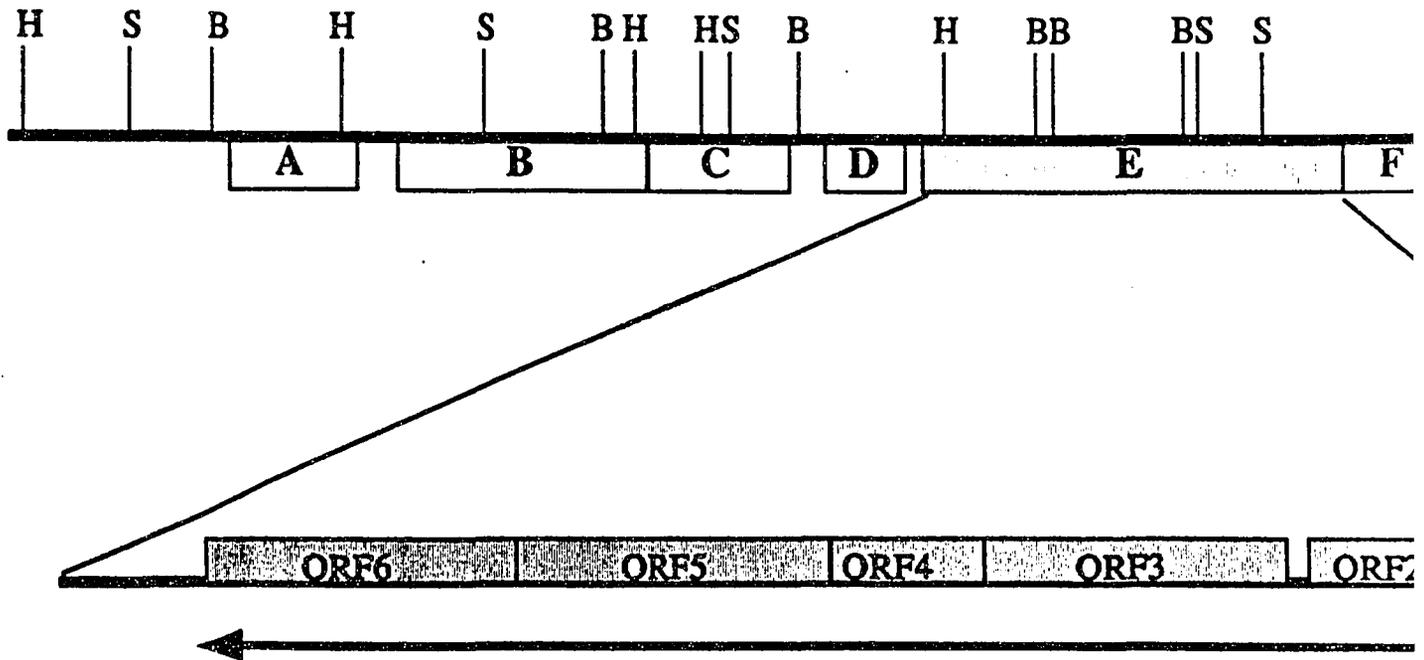
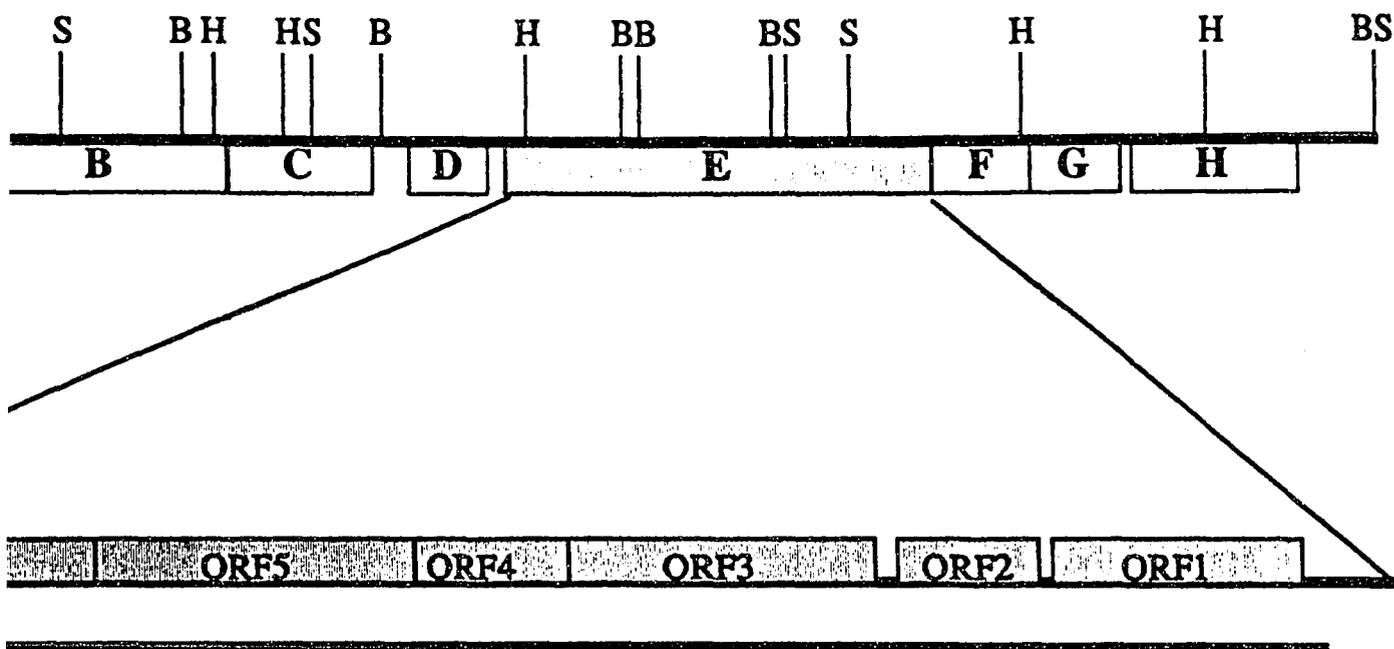


Fig. 3.1. Transcriptional units within pHK120 that are involved in the production of pl and open reading frames (ORFs) in the *ph*E locus. At the top is shown the restriction map of the pHK120 insert and eight transcriptional loci (rectangular boxes). H: *Hind*III, S: *Sma*I, B: *Bam*HI, BH: *Bam*HI-*Hind*III, HS: *Hind*III-*Sma*I, BB: *Bam*HI-*Bam*HI, BS: *Bam*HI-*Sma*I. Below are shown the ORFs in the *ph*E locus, their transcriptional orientation and the location of the pHK120 insert.



Transcriptional units within pHK120 that are involved in the production of phaseolotoxin reading frames (ORFs) in the *phtE* locus. At the top is shown the restriction map of the insert and eight transcriptional loci (rectangular boxes). H: *Hind*III, S: *Sma*I, B: *Bam*HI. e shown the ORFs in the *phtE* locus, their transcriptional orientation and the length of each

Fig. 3.2. Nucleotide sequence of the DNA encompassing the *phtE* locus. Amino acid sequences of ORFs are shown below the nucleotide sequence with a single letter abbreviation. The start codons and the putative ribosomal bindings sites are shown in bold, and the stop codons are represented by the symbol *. The promoter-like sequences are marked by the symbol ^ underneath, and the terminator-like sequences are shown with a dotted line on the top and ^ underneath. The transcriptional start site is marked by a an arrow (↓) on the top. Synthetic primers used for RT-PCR were marked using dotted lines on the top and arrows indicating orientations of the primers.


```

721 . . . . . 780
TTGCCACCATGGCCAGCGATATTGATATGTTGTGCGTGGTTGACGAGCCTGTCAGCACCG
      M L C V V D E P V S T E
      ORF1⇨ <----- St1rev

781 . . . . . 840
AGCAAATGGCAACCCAGATTCATCAGGAAGGTCTGCATCTCGAACTGTTGCTGTTCAATG
  Q M A T Q I H Q E G L H L E L L L F N A

841 . . . . . 900
CTGCCGATGTTCACTTGGCCCTTGGCGAACTCACCAGCCTGGCGCAAAGCCCAGCGCTGA
  A D V H L A L G E L T S L A Q S P A L I

901 . . . . . 960
TCTGCCTGCAAATATTCAAGATGGGATAAGCGCAACAAGGTTCCGCGCAAATATGTCG
  C L Q N Y S R W D K R N K V P R K Y V E

961 . . . . . 1020
AGCGCTTGGTCTATGGCGTGACCAATGATCTCACCCTCCTTATCTCTCGGCACCTTGGCG
  R L V Y G V T N D L T T P Y L S A L G D

1021 . . . . . 1080
ACCTGTGTGTCGTTGGTGTCTCGCAAAGACCTGGATGTTTTTCAGAGTGAATGTCGCCTGCT
  L C V V V S R K D L D V F R V N V A C S

1801 . . . . . 1140
CACGGCTGGCGCAACGGTCTGACGAGCCTTTTTCGCGCGATCGGCTATTTGATGAATGCAC
  R L A Q R S D E P F A A I G Y L M N A L

1141 . . . . . 1200
TGACCGCAGCCATGAACTCGCTGCTTTTCGATGTGCGGGTGGGTGTTGTCCAACAAGAAGT
  T A A M N S L L S M S G W V L S N K K W

1201 . . . . . 1260
GGACGCTGCGTGCCTGGAATAAAAGCCGCGACCTGGTTTTCTTGGATCTTTGCCCTACCA
  T L R R W N K S R D L V F L D L C P T T

1261 . . . . . 1320
      E72rev ----->
CCTGGACGGAGGTTTCAGCGCTCTGGGCAGACCTTGCGAAAGGTCCAGCGGTGTTGGACG
  W T E V S A L W A D L A K G P A V L D E

1321 . . . . . 1380
AAGCCCTGTCACGCATCGAGACGTTATTGCAGCAAATGACAGGCAGGCTGGCTCCGGAAC
  A L S R I E T L L Q Q M T G R L A P E L

1381 . . . . . 1440
TCCTTCGTGATGAACCATTTACCGGGTTGCAGGGAAATGTTGCGCGGTATCCGTTGCACG
  L R D E P F T G L Q G N V A R Y P L H A

```

Fig 3.2 (CONT'D)

1441 1500
 CCAAGCCGCTCTGGTCTGCGCTGGCAACGGTTATGTACGACTGGCCGAGAACGTGCCGGC
 K P L W S A L A T V M Y D W P R T C R P

1501 1560
 CGGCGTAACGCTGGCGCAGTGTTCGCCGATCTGTGCCAGTTGCCGCCGCTCTCTGCCCT
 A *

1561 1620
 CGAACACCTGCAAGCGATCCGCGCAGGGACCTGGAGTCCATCATTGCAACGCCAGTCCGG

1621 1680
E72rev1----->
 GGCCAGCGCCAGCTAAGGAGCACAGCATGTTTGAAGCAGACAAGACTCAACCTTGCCTT
 M F E A R Q D S T L R W
 ORF2⇨

1681 1740
 GGTTCCTCGGCTGACCGGAGGCGTGGGGGTGGAGGGCAATAGCATGGCCCAGCCATCG
 F P R L T G G V G V E G N S M A R A I V

1741 1800
 TCAGCGCGCATGGCTGGTGTATGCCGAACGTATGCCTACCTCGAAGATCTGGAAGGCG
 S A A W L V M S E L Y A Y L E D L E G A
 <----- **HS27T7**

1801 1860
 CCATGGATGCTCCGGATGCATCGGTGTTGATTAAAGTGAAGATTGCTGAACTGCTGGTGC
 M D A P D A S V L I K V K I A E L L V Q

1861 1920
 AAATCGACTGCACGTTGGGGCGCACGGCGGTGCTCGACGAAGAGCACAGATTGCCGTGGT
 I D C T L G R T A V L D E E H R L P W L

1921 1980
 TACTTGAGTACGGCTTGTGCGAGGTGATCAATTTACCCGGGGCTGACATGGCGCGCTTGC
 L E Y G L C E V I N L P G A D M A R L L

1981 2040
 TGGGTCTGTTCGCAGCGAATGACGCAACTGAAATCCGTGAGTAAGCCAGCTGATACGTT
 G L F A A N D A T E I R R V S Q L I R D

2041 2100
 ATTTGATTGCGGCATTCCCGGGCGAGTTGGTTCGATTGCTGCAAGCGCACAACCAGGGAG
 L I A A F P G E L V D S L Q A H N Q G G

Fig 3.2 (CONT'D)

2101 2160
 GCGTTCTGCGTTTTCTTCGGTCTTCCGACAAGGCGGTGTACCGCGTTGGGTTGCGATGCCA
 V L R F L R S S D K A C T A L G C D A S

2161 2220
 S6uni --
 GTTTTCTGGTGCCACTGATGAAATCCCTATGAACACTGAACTGACTGGACGGTCTGTCCC
 F L V P L M K S L * <----- HB37T7B

2221 2280
 ----->
 GGTGCCGAAGCAATCCGAGCGTAAAAGCGCCTTCGTTGATCGCTATGGTGCTGTCTTCGG

2281 2340
 TCGTCAACAGGGCCTGATGTTGAAGATGTGCGGGTTGTCCGCATCCGAAGCGCGTGGCGA
 M L K M S G L S A S E A R G D
 ORF3⇒

2341 2400
 CGGTGCGCTGGGTCGAAGATACTCAGGGTGGACGCTGGCTGGACTTCGGTTCGTTCCGGTGT
 G A W V E D T Q G G R W L D F G S F G V

2401 2460
 S6uniA ----->
 GCACCTACTCGGCCATAGTCATTCAGGCGTGGTATCTGCCCTGGTGGAAACAGATCCAGCG
 H L L G H S H S G V V S A L V E Q I Q R

2461 2520
 TTTTGGGCTTTCCACGAAAATCCTGTGCAACGAACCCATCGTACTGGCGGCCGAGCGGCT
 F G L S T K I L S N E P I V L A A E R L
 <----- HB37T7A

2521 2580
 ATTGGTCATGGCGGGACCTGAGAAGGATAAGGTGATTTTCGGCAATACCGGTTCCGAAGT
 L V M A G P E K D K V I F G N T G S E V

2581 2640
 CGTCGAAGCGGCATTGAACTTGCCAGGATCGTCACTGGACGGCGGAGGGTCATTGCCTT
 V E A A L K L A R I V T G R R R V I A F

2641 2700
 CGAGCAGGCTTACCACGGGCGCACCGCGGCTGCCTTGAGTGTGTCAGTCATGGCTACATGCG
 E Q A Y H G R T A A A L S V S H G Y M R

2701 2760
 GCATGCCGGTCTTCTGACTGAAGGTGACGTGGTTTTTTGTCCAATCGACGATCTCGATGC
 H A G L L T E G D V V F C P I D D L D A

Fig 3.2 (CONT'D)

2761 2820
S6uniB ----->
TGTCGCCAACGCGCTGGAGGCAGGCGACATCGCGGCGATCATTATTGAGCCCATACAGGG
V A N A L E A G D I A A I I I E P I Q G
<----- **HB37T7**

2821 2880
CGAGGGCGGGATACGCCCTGTGGAGAATGAATTTCTTCGAGGTCTTGGCGACCTGGCCCA
E G G I R P V E N E F L R G L G D L A Q

2881 2940
GGCTTTTGACTGCCGCTTGATATTTGACGAGATCCAAACGGGCCTGGGGCGTATCGGTAG
A F D C R L I F D E I Q T G L G R I G S

2941 3000
CCTGCGTGTGACGTTCCCTTGGACATCCTGCTGTTGGGCAAGGTTCTGGGCGGTGGTGT
L R V D V P C D I L L L G K V L G G G V

3001 3060
TTTCCCgATCTCGGCAGCCTTGTTTACGTGGAAGCGTTTTGATGCGGCTGCCcgGGATCC
F P I S A A L F T S K R F D A A A R D P

3061 3120
GGTAGTCCACGCTTCGTCTTTTCCCGGTAGTGCCCTGGCCGGTGCCGTCGTCAACGCGGT
V V H A S S F A G S A L A G A V V N A V

3121 3180
GCTGGATGTTGTGTCGCTCCCGATTTTACCCTCAGGGTGCAGCGCTTAGGCGATCGGGT
L D V V S A P D F T L R V Q R L G D R V

3181 3240
CTTGAGCTATCTCGGCCAGCGCCTTGCAGATAATCCCTTGATTACCGACATCCGTGGCCA
L S Y L G Q R L A D N P L I T D I R G Q

3241 3300
B4uni ----->
GGGCTTGATGATCGGCGTTGAGTTCAAGCGTCTCGATCATGTCGGGGAGATGATCATCGA
G L M I G V E F K R L D H V G E M I I E
<----- **HE45T7**

3301 3360
GGCCGCCAAACGGCACTTGTTGCTCGCTTTCTGTCTCACCGATCCGAAAGTTTTGCGTCT
A A K R H L L L A F C L T D P K V L R L

Fig 3.2 (CONT'D)

3361 3420
 CTACCCGCCAGCCGTCATCAGCGACGCAGATCTCGAAACAGGGCTGGAGCACTTTTGCCA
 Y P P A V I S D A D L E T G L E H F C Q

3421 3480
 GAGTGTGAAGCGATCACTCGAACAGAATTCGATA**AAGGAAAT**CCCGTTATGCCAGCAGTA
 S V E A I T R T E F D K E I P L C Q Q *
 M P A V
ORF4⇒

3481 3540
 AATACATCACTGAATCTTGGGTCTCTTGAGCTGGAGCGAGTCTGGTTCGACCCTTTGTGAG
 N T S L N L G S L E L E R V W S T L C E

3541 3600
 TTTCAGCGTTATCCGGAATTCATGAAGGACGTACTTGACGTCACTATCGAGCAACGTACC
 F Q R Y P E F M K D V L D V T I E Q R T

3601 3660
 GGAAACGAAATCATCAGCACCTGGCGTGTATTGCTAAACGGAAGTGAGTTGACCTGGACC
 G N E I I S T W R V L L N G S E L T W T

3661 3720
 GAGCGGATCTATTGCTGCCCGACCACCGTATCGTGTTCAGCAAATCGACGGGGACCTG
 E R D L L L P D H R I V F Q Q I D G D L

3721 3780
 GAAGTCTGGTCAGGGGAGTGGTCCATCCAGCAGGCGGGGACGACCTTAGTGTGCAACTG
 E V W S G E W S I Q Q A G D D L S V Q L
 <----- **HP46T7**

3781 3840
HE25T7 ----->
 GATGTCTTGTTTCGATCTTGGCATTCCGTCCCTGGCCGACGTTCTTCATCCGATCGGGGAG
 D V L F D L G I P S L A D V L H P I G E

3841 3900
 CGAGCGATCCGCGCCAATAGCCGGCAGATGCTGGAGGGTATTCGTGATCGAATCCTGCTA
 R A I R A N S R Q M L E G I R D R I L L

3901 3960
 TCGCAACTATCGACAGTTGCGGT**CGAGGC**ATAAACGATGTCTGCGCAGACCCCTTTGCAA
 S Q L S T V A V E A * M S A Q T P L Q
ORF5⇒

3961 4020
 TTGATACTGACTGGCGCCAACGGCACCCCTCGGCATTCCATTGGTTCGTTCTACTCCAG
 L I L T G A N G T L G I P L V R S L L Q

Fig 3.2 (CONT'D)

4681 4740
 AAGCGTATCGGCTACGAACACTTGGTTTCGCTGGTTCGAAAATCAACTGGGCGAGGCACGC
 K R I G Y E H L V R L V E N Q L G E A R

4741 4800
 GAAACGGCCGGCCTGGGAGCCCAGGATCCCGTGCCATTCTCAAAAGCCGCCAGTGGGAT
 E T A G L G A Q D P V P I L K S R Q W D

4801 4860
 TTCTTGAAGCGTTTCGCTATCGGCCTGGAGTCCTGAAGGCATCTCCAAAAATGAGTTTCGT
 F L K R S L S A W S P E G I S K N E F R

4861 4920
 TACTTGAACGGTTGTTACAAGTCTACGGGGTGTATGCCAGCAGCGACAGGGTTCCGGGCG
 Y F E R L L Q V Y G V Y A S S D R V R A

4921 4980
HB12T7 ----->
 CCGTTGAATGTTTCTCGCCATCACCCGGATCCCGTCGAGTTTCTGCCACAGGTCATCAAG
 P L N V S R P S P D P V E F L P Q V I K

4981 5040
 TTCTGGCTTCAACAACATCCACGGGCATTCCGCTCCCGCTCATTGACGTCTTCCGAGGTT
 F W L Q Q H P R A F R S R S L T S S E V

5041 5100
 CAAGGCGCTGCCACAGGATTGGTTCGAGGAGTAAGAGGGATGAGTCTGGCAACCGCGGTAG
 Q G A A T G L V E E * M S L A T A V V
 ORF6⇒

5101 5160
 TTTGCGAAAGAAAAGAACGATGTCGACATGAAAACTGCGTGTGATCCTCACGTAGTCC
 C E R K E R C R H E K L R V D P H V V H

5161 5220
 ACGACTTGATGAAGTTGCGTCCAGGTAATGTTTCATACAGATAGGCATGCAATGGTTAT
 D L M K L R P G K C F I Q I G M Q W L L

5221 5280
HB12T7A ----->
 TGATTGTGTTCCCGTTTTTTTTTCGCTAAGTGGGCCAGCAGTTGGCTGGCCTATAGTATTT
 I V F A V F F A K W A S S W L A Y S I S

5281 5340
 CCATTGTATGGATCGCTACACGTCAACATGCGTTATTGGTACTGATGCATGATGCTTCGC
 I V W I A T R Q H A L L V L M H D A S H

Fig 3.2 (CONT'D)

```

5341 . . . . . 5400
ATTATTTGATCAGTCGTCGGCGCTGGCTCAATGATACGGTGGGTAATCTGTTCCCTGGCTT
  Y L I S R R R W L N D T V G N L F L A F
                                     <----

5401 . . . . . 5460
TTCCATTGAGCGTTTGTGTATCCCGTTATCGCCGGCACCATTTGCTCCATCATCGCCATC
  P L S V C V S R Y R R H H L L H H R H L
----- HB12uniB

5461 . . . . . 5520
TCAATACCGAGCTTGATCCCGATATAAAAAGATTCACAACTGCCGAAGACACGTATCAGGT
  N T E L D P D I K D S Q L P K T R I R F

5521 . . . . . 5580
                                     HB12T7B -----
TTTATGGGCTGTTGCTGCGGATGCACTGGGTGTTTCGACACTGATGACACTTAGATCGG
  Y G L L L R D A L G V S T L M T L R S V
5581 . . . . . 5640
->
TCAACAACCTTTGGGCTGTTGGGGTTGTTCCGAGAGGCAGTCAcTGCTCGCGGCTTGATC
  N N F G L L G L F A R G S H C S R L D R
                                     <-

5641 . . . . . 5700
GGCGTCTGGCAATGGCTTTTATTATTGCGGTGGGCGGGGGGATAACTTGGGTAGGTGGCG
  R L A M A F I I A V G G G I T W V G G G
----- HB12uniA

5701 . . . . . 5760
GGTGGGACGTGTTATGGCTTTGGGTTGTTCCGGCGTTTACGATACTTCCGTTGATCCTGC
  W D V L W L W V V P A F T I L P L I L R

5761 . . . . . 5820
GTGTACGCAGTATTGCCGAACACGGGGGAAGGTTAGACCATCCGAATGCCAGTAATGCCC
  V R S I A E H G G R L D H P N A S N A R

5821 . . . . . 5880
GAAGTATCGATGTGGGTATTATCGAGCGGTTTCTCTGGGCGCCCTGTCAATCAATCGAC
  S I D V G I I E R F L W A P C H I N R H

5881 . . . . . 5940
ACTGGGAACATCATCTCTGTCCGAGTGCCCACGTATAACTTATCGCTGCTTACTGCACG
  W E H H L C P Q C P R I T Y R C L L H D
<----- HB12uni

5941 . . . . . 6000
ACTGGCTAGCTTTTTTCCCGAGTCGTCAGCGGCACAACGTACTCAAGGTTACTTTTTTAG
  W L A F F P S R Q R H N V L K V T F L V

```

Fig 3.2 (CONT'D)

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6001 . . . . . 6060
TGCTCGGTCTGGTGTCTGGAGTTGTACCCCAACACCACCTCCCCCATGGCTGGCAGATTG
  L G R W C R S C T P T P L P H G W Q I D

6061 . . . . . 6120
                                     HindIII
ATGTTTCGAGTAAGCCAGCGAATGGCAGGTGTTTTGAGGAGAAGCTTTCATGATGCTGACG
  V R V S Q R M A G V L R R S F H D A D G

6121 . . . . . 6180
GGTAGTGAAATTCAACGAATGCGTGTGAATTGAAACGCATTGTGATCGAACCATTTCGATC
  *

6181 . . . . . 6240
CCCCTTTGATAGAGCAGAATTCATATGCCTGCCATTTGGGGAGCGAGATTATCGAGTATG

6241 . . . . . 6300
                                     H2T7 ----->
ACGCCGAACAGATCGATCCTCATGAGGGCCTGGGAACATTCAGCACACTATCCCGAAGA

6301 . . . . . 6360
-----> <-----
GGGGCTGGTCTCTGCAGCCGAGGCGTTTTTACCTGGGCTCTACCGTCGAATGTATCGGGCGG
  ^^^^^      ^^^^^

6361 . . . . . 6420
GCTGGATTTTTCGAGTGAACCTCTACGCCAATCTATCGACAGCGCTATGTGGCATGTTCAT

6421 . . . . . 6480
TCAAACCAGCGCGCCACTGGGGCACACAGGGGCAACGATCTGCTGGACGCTGGAAATTGT

6481 . . . . . 6540
GGTCACTCAACCGCTGCGTATCTATGCAGGGACGAAGATTGCCAAAGTGTGTTTCTGGAA
<----- H2T7Arev

6541 . . . . . 6600
AAATTTTGGTGCCGTTTCGCGCCTATGAAGGGCGTTATGCAGGCTCGCAAAGTGTGTTCC
                                     ^^^^^^

6601 . . . . . 6660
                                     H2T7A ----->
TTCAAGAATTATAATGGACCAACCATGATATTGACGGGTAATGAATAACCAATCGCGTTA
  ^^^^^^

6661 . . . . . 6720
TTGCAGGGGATATCGTGATCTCTCCTTTCTCAAAGAATAGCATCAATCCAAATAGCTATA
      V I S P F S K N S I N P N S Y N

6721 . . . . . 6780
ATTTTCGCCTGCATAATCATATGAAAGTCTATGAGGACGGCATTATCGACGTGAAGTCGG
  F R L H N H M K V Y E D G I I D V K S E

```

Fig 3.2 (CONT'D)

6781 6840
 AGCAGCCAACCCGTGATATTTATATCGGGCTCTGAAGGGTTTGTCTCGAACCGATGCGGC
 Q P T R D I Y I G S E G F V L E P M R L

6841 6900
 TGTACCTTGCCAGTTCAATCGAGACCATGGGCAGTACTCGTTTTGTACCGACTTATGCGG
 Y L A S S I E T M G S T R F V P T Y A A

6901 6960
 CGCGTTCTTCCATTGCTCGACTCGGCATGTTTATCAACCTGTCGGCACCGCTTGGTGATA
 R S S I A R L G M F I N L S A P L G D I
 <----- H2T7rev1

6961 7020
 TAGGATTTATAGGCCGTTGGACCATTCAACTTTTGGCCGTCAATAGAATTCGGGTATAACC
 G F I G R W T I Q L L A V N R I R V Y L

7021 7080
 TGGCATGGATATTGGCAATGATGTTCTGGCATGTTCAAGGCGAGATCGAGCTGTATACGC
 A W I L A M M F W H V Q G E I E L Y T Q

7081
 AAGT
 -

FIG 3.2 (CONT'D)

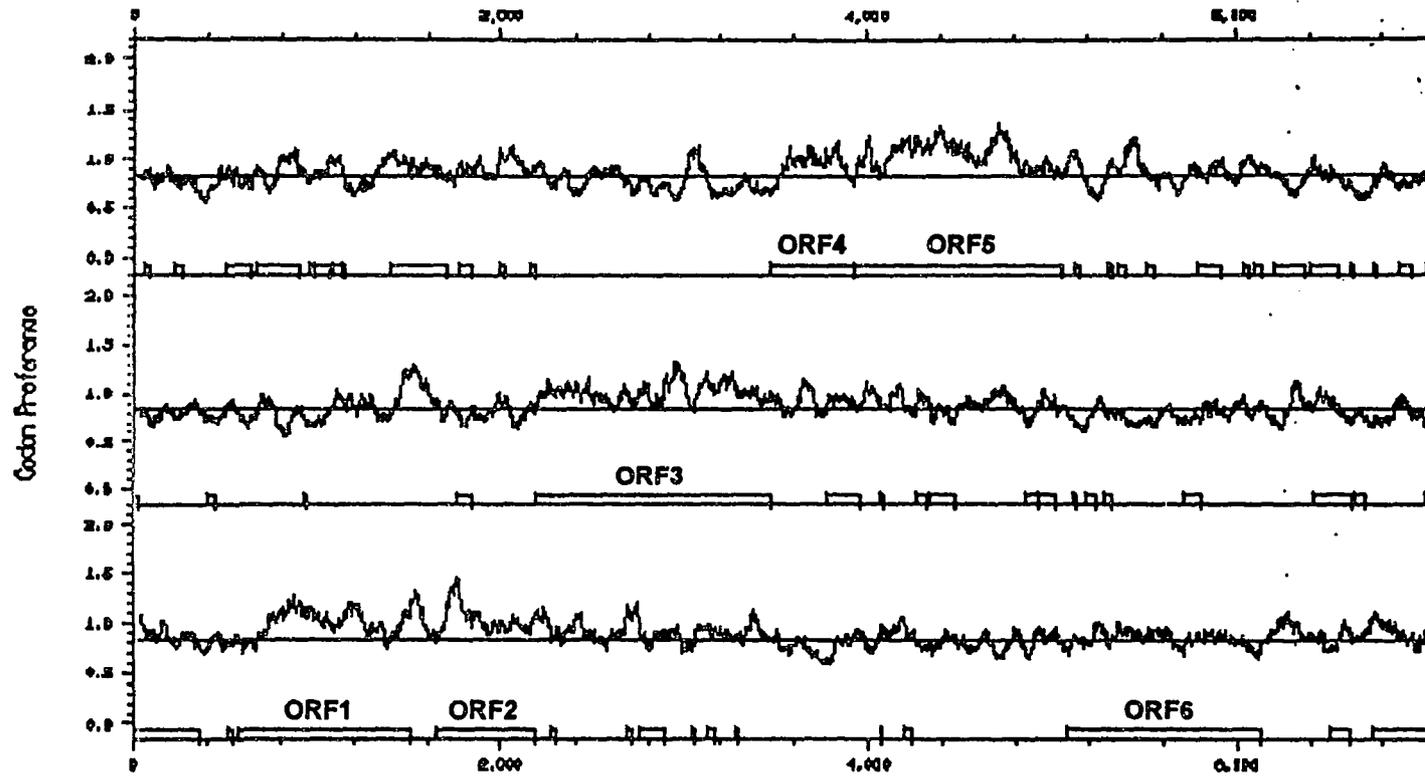
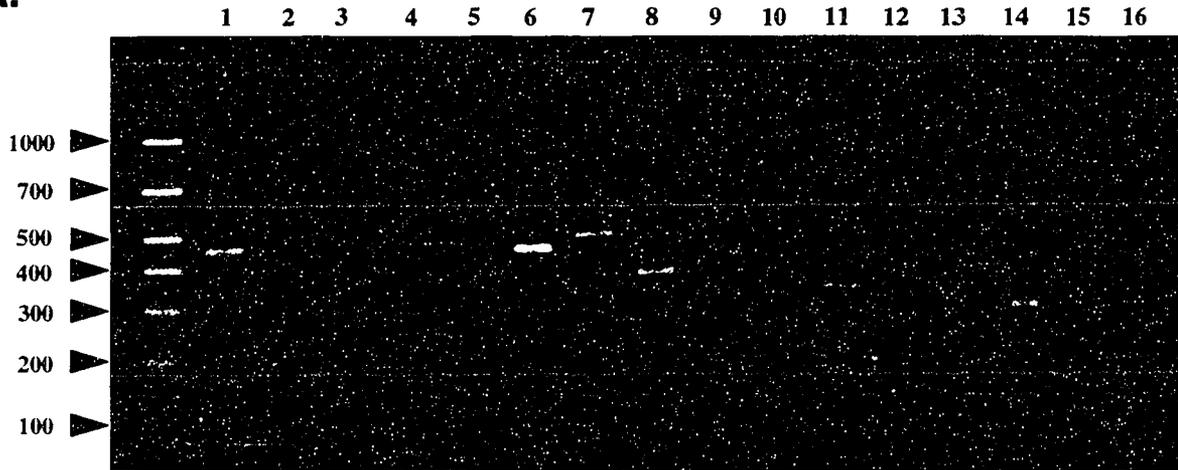


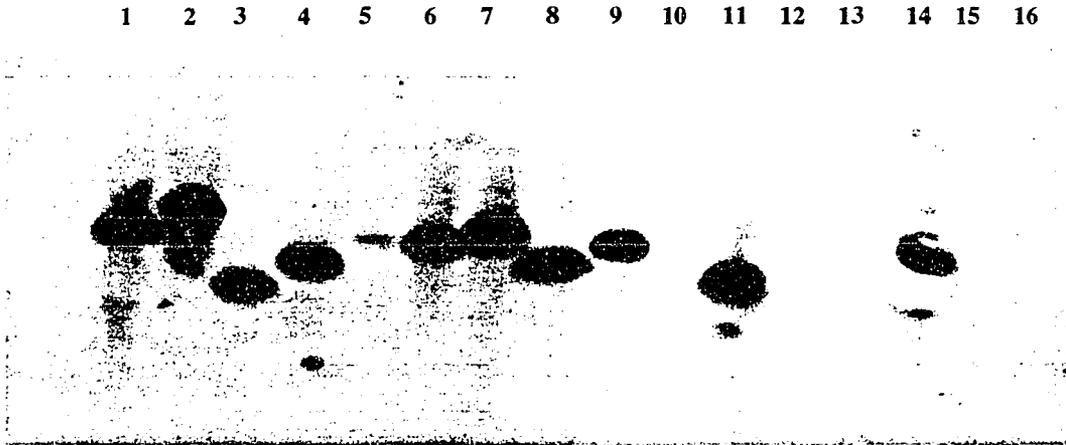
Fig. 3.3. Codon preference of the ORFs in the *phtE* locus as compared to a codon usage table of published *Pseudomonas* genes.

Fig. 3.4. Results of the RT-PCR experiment. The RNA was isolated from transconjugants 4612-1(pHK120). 2 µg of total RNA was treated with 40 units of RNase-free DNase I and used as the template for RT-PCR amplification in a final volume of 100 µl. **A.** 20 µl of RT-PCR products were separated on a 2% agarose gel. Lane 1: ET72rev, HS27T7; lane 2: ET72rev1, HB37T7B; lane 3: S6uni, HB37T7A; lane 4: S6uniA, HB37T7; lane 5: S6uniB, HE45T7; lane 6: B4uni, HP46T7; lane 7: HE25T7, B4T7A; lane 8: HP22T7, B4T7; lane 9: HB12T7, HB12uniB; lane 10: HB12T7A, HB12uniA; lane 11: HB12T7B, HB12uni; lane 12: H2T7, H2T7rev; lane 13: H2T7, H2T7Arev1; lane 14: H2T7A, H2T7Arev1; lane 15: internal control; lane 16: S6uni, HB37T7A (no RT). Shown at the left of the gel is the DNA marker (in bp). **B.** Southern blot analysis of the fragments as shown in A. Lane 1 to 11 were hybridized with ³²P-labeled H1 fragment (Fig. 2.1A). Lane 12 to 16 were hybridized with ³²P-labeled H2 fragment (Fig. 2.1A). **C.** The regions in the *phtE* locus covered by fragments generated in the RT-PCR experiment. The numbers on the top of each line corresponds to the lanes in A and B.

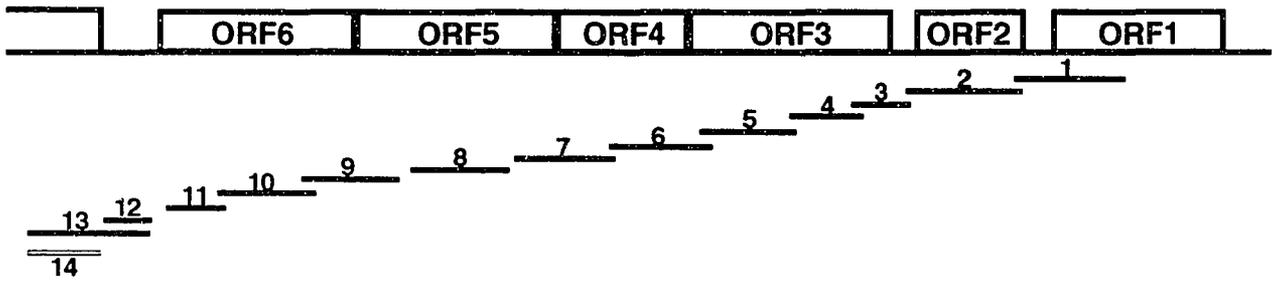
A.



B.



C.



(Fig. 3.4)

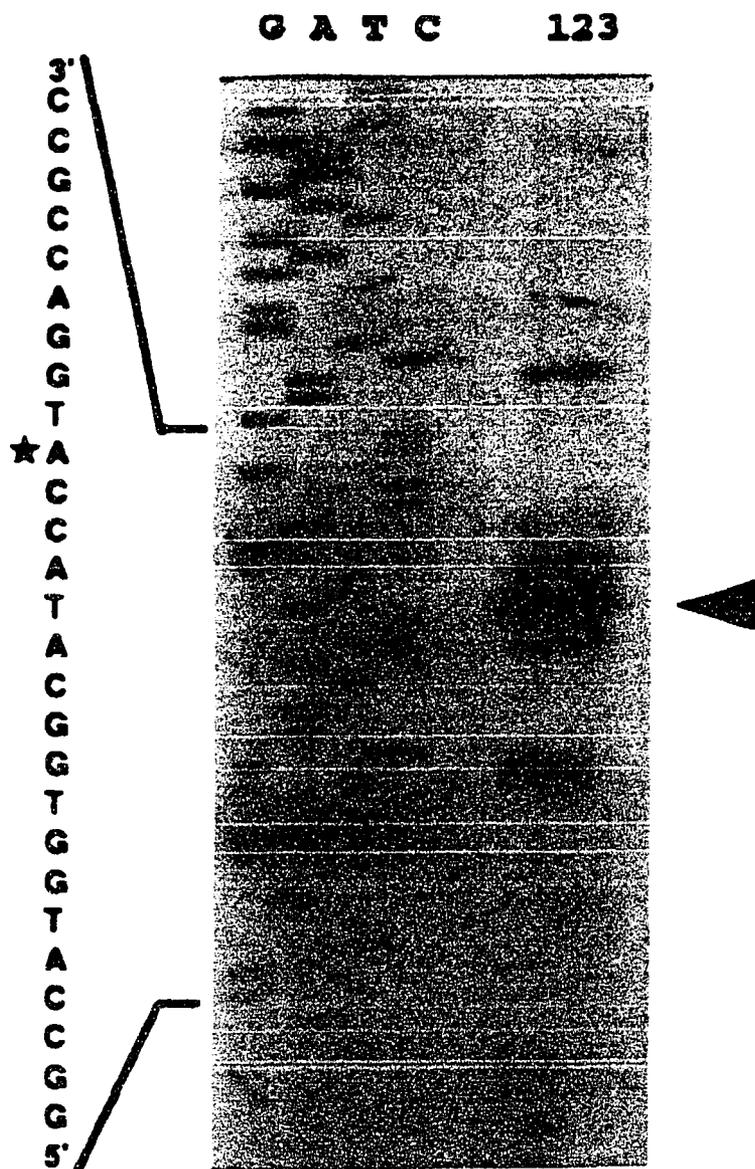


Fig. 3.5. Transcriptional start site of the *phtE* locus. Total RNA isolated from G50-1 grown at 18°C (lane 1), G50-1 grown at 28°C (lane 2) and 4612-1 (pHK120) grown at 18°C (lane 3) was used as template in the reverse transcription reaction. The primer used was St1rev (5'CGGTGCTGACAGGCTC). The arrowhead indicates the possible transcriptional start site of the message. A DNA sequence (complementary to the mRNA sequence) ladder generated from pHK120 DNA using the same primer as that in the primer extension experiment is shown at left. The nucleotide A (transcriptional start) is marked by a star.

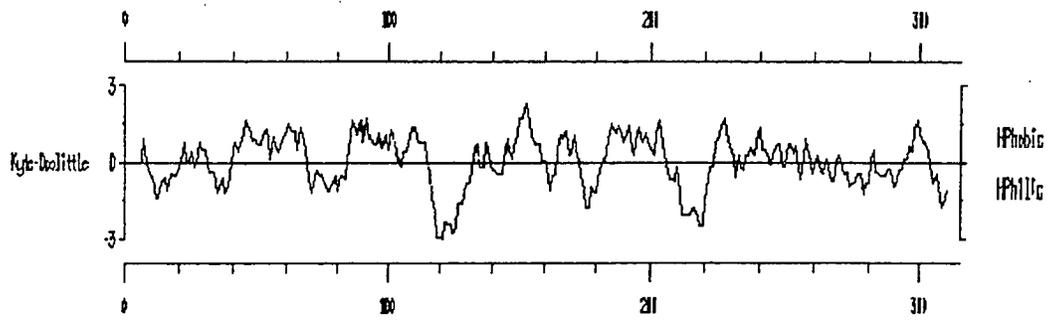


Fig. 3.6. Hydropathy profile of ORF1.

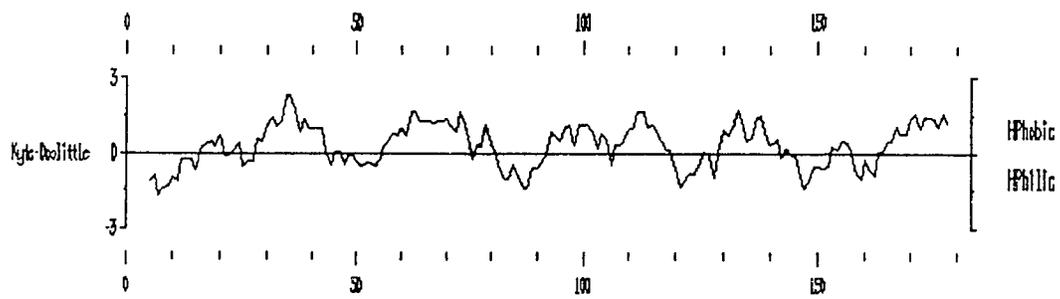


Fig. 3.7. Hydropathy profile of ORF2.

	161								240
Styhem1D	MVRMVNSGTE	ATMSAIRLAR	GF.....	TGRDKIIFEG	GCYHGHADCL	LVKAGSGA..	.LTLGQPNSP	
BachemaxcE	IVRMVSSGTE	ATMSALRLAR	GY.....	TGRNKILKFEG	CYHGHGDSL	LIKAGSGV..	.ATLGLPDSF	
Ntgsamr2E	MVRFVNSGTE	ACMGVLRRLAR	AF.....	TGRPKIIFEG	CYHGHADPF	LVKAGSGV..	.ATLGLPDSF	
Ratota	FNY.....N	KVLPMTGVE	AGETACKLAR	RWGYTVKGIQ	KYKAKIVFAVG	NFWGRTLSA	VSSSTDPTS	DGFGPFMPG.	
Hskoat	FNY.....H	KVLPMTGVE	AGETACKLAR	KWGYTVKGIQ	KYKAKIVFAAG	NFWGRTLSA	ISSSTDPTS	DGFGPFMPG.	
Yscarg8	KQFGGQHDS	RVFLCNSGTE	ANEAAALKFAK	KHGIMKN..	PSKQGIVAFEN	SFHGRMTGA	LSVTWNSK.Y	RTPFGDLVPH	
Ecoargd	F.....AE	RVVFMNSGTE	ANETAFKLAR	HYACVRHS..	PFKTKIIAFHN	AFHGRSLFT	VSVGGQPK.Y	SDGFGPKPAD	
Ecogabt	...PGDFAK	KTLVTTGSE	AVENAVKIA.RAA..	TKRSGTIAFSG	AYHGRTHYT	LALTGKVPY	SAGMGLMPGH	
ORF3	..VMAGPEKD	KVIFGNTGSE	VVEAALKLA.RIV..	TGRRRVIAFEQ	AYHGRTAAA	LSVSHGYMRH	AGLLTE..GD	
Consensus	FNY--G--A-	KVLF-N-GTE	AGETALKLAR	-WGY-RKGIQ	TGRAKIIAFE	G--HGRTLSA	LS-SG-P-SY	D--LGLMPGP	
	241								320
Styhem1	GVPADFAKHT	LTCTYNDLTS	VRAAFEQY..	.PQEIASIIIV	EPVAGNMNCV	PPLPEFLPGL	RALCDEFGAL	LIIDEVMTGF	
Bachemaxc	GVPEGIKNT	ITVPYNDLES	VKLAFQQF..	.GEDIAGVIV	EPVAGNMGVV	PPQEGFLQGL	RDITEQYGS	LIFDEVMTGF	
Ntgsamr2	GVPKAATTD	LTAPYNDISA	VESLFKEH..	.KGEVAAIL	EPVVGNAFVI	QPNPDLAAI	RKITKENDAL	LIFDEVMTGF	
RatotaF	ETIPYNDL..	...PALERAL	QDPNVAAFMV	EPIQGEAGVI	VPDPGYLTGV	RELCTRQV	FIADEIQTGL	
HskoatF	DIIPYNDL..	...PALERAL	QDPNVAAFMV	EPIQGEAGVV	VPDPGYLMGV	RELCTRQV	FIADEIQTGL	
Yscarg8	VSF.....	..LNLNDEMT	KLQSY..IET	KKDEIAGLIV	EPIQGEAGVF	PVEVEKLTGL	KKICQDNDVI	VIHDEIQCG	
Ecoargd	IIH.....	..VPFND...	.LHAV..KAV	MDDHCAVVV	EPIQGEAGVT	AATPEFLQGL	RELCDQHQA	LVFDEVQCGM	
Ecogabt	VYRALYPCPL	HGISEDDAIA	SIHRIFKND	APEDIAAIVI	EPVQGEAGFY	ASSPAFMQRL	RALCDEHGIM	LIADEVQSGA	
ORF3	VVFC.....PID	DLDAV..ANAL	EAGDIAAIII	EPIQGEAGGIR	PVNEFLRGL	GDLAQAFDCR	LIFDEIQTGL	
Consensus	VVPA--AK-F	ET-PYNDLI-	VLHAFLE-AL	QD--IAAIIIV	EPIQGE-GVV	PPDPEFL-GL	RELCD--VL	LI-DEVQTGL	
	321								400
Styhem1	RVA...LAGA	QDYGVVVDL	TCLGKIIGGG	M.PVGAFGGR	RDVMDALAPT	GPVYQAGTLS	GNPIAMAAGF	ACLNEVAQPG	
Bachemaxc	RVD...YNCA	QGYFGVTPDL	TCLGKVIGGG	L.PVGAYGGK	AEIMEQIAPS	GPIYQAGTLS	GNPLAMTAGL	ETLKQLT.PE	
Ntgsamr2	RLA...YGGA	QEYFGITPDL	TTLGKIIGGG	L.PVGAYGGR	RDIMEMVAPA	GPMYQAGTLS	GNPLAMTAGI	HTLKRLQGGP	
Ratota	ARTGRWLAVD	H..ENVRPDI	VLLGKALSGG	LYPVSAVLC	DDIMLTIK..	.PGEHGSTYG	GNPLGCRVAI	AALVLEEEH	
Hskoat	ARTGRWLAVD	Y..ENVRPDI	VLLGKALSGG	LYPVSAVLC	DDIMLTIK..	.PGEHGSTYG	GNPLGCRVAI	AALVLEEEH	
Yscarg8	GRSGKLWAHA	YLPSEAHPI	FTSAKALGNG	.FPIAATIVN	EKVNNALR..	.VGDHGTTYG	GNPLACSVSN	YVLDTIADDA	
Ecoargd	GRTGDLF..A	YMHYALAPDI	LTSKALGGG	.FPISAMLT	AEIASAFH..	.PGSHGSTYG	GNPLACAVAG	AAFDIINTPE	
Ecogabt	GRTGTLFAME	QM..GVAPDL	TTFAKSIAGG	.FPLAGVTGR	AEVMDAVA..	.PGGLGGTYA	GNPIACVAAL	EVLKVFEQEN	
ORF3	GRIGSLRV..DVPCDI	LLLGVVGGG	VFPISAAALFT	SKRFDAAAR.	DPVVHASSFA	GSALAGAVVN	AVLDVVSAPD	
Consensus	GRTGRLLAVA	QMY-GV-PD-	-TLGKALGGG	LFPVSAVLG-	DDIMDAIAP-	GPG-HG-TYG	GNPLACAVA-	A-LDVLEERPE	

Fig 3.8 (CONT'D)

	401							480
Styheml	IHETLDELTT	RLA EGLCEAA	QEAGIPLVNV	HVGGMFGIFF	TDAESVTCYQ	DVMACDVERF	KRFFHLMLEE	GVYLAPSAFE
Bachemaxc	SYKNFIKKGD	RLEEGISKTA	GAHGIPHTFN	RAGSMIGFFF	TN.EPVINYE	TAKSSDLKLF	ASYKGMANE	GVFLPPSQFE
Ntgsamr2	TYDYLDKITG	ELTQGILDAG	KKTGHAMCGG	YIRGMFGFFF	VE.GPVNNS	DAKSDTEKF	GRFYRGMLEE	GVYFAPSQFE
Ratota	LAENADKMGA	ILRKELMKLP	...SDVVTAV	RGKGLL....	.NAIVIRETK	DCD.....A	WKVCLRLRDN	GLL..AKPTH
Hskoat	LAENADKLG I	ILRNELMKLP	...SDVVTAV	RGKGLL....	.NAIVIKETK	DWD.....A	WKVCLRLRDN	GLL..AKPTH
Yscarg8	FLKQVSKKSD	ILQKRLREIQ	AKYPNQIKTI	RGKGLM....	.LGAE...F	VEP.....P	TEVIKKAREL	GLLIITAGK.
Ecoargd	VLEGIQAKRQ	RFVDHLQKID	QQY.DVFSDI	RGMGLL....	.IGAELKPQY	KGR.....A	RDFLYAGAEA	GVMVLNAGP.
Ecogabt	LLQKANDLGQ	KLKDGLLAIA	EKHPE.IGDV	RGLGAM....	.IAIELFEDG	DHNKPKAKLT	AEIVARARDK	GLILLSGCPY
ORF3	FTLRVQRLGD	RVLSYLGQRL	ADNP.LITDI	RGQGLM....	.IGVEFKR..LDHV	GEMIIEAAKR	HLLLAFLCLTD
Consensus	L-ENADKLG D	RLR-GL-K--	-K--DV-TDV	RGKGLLGFFF	TNAIE-KEYK	D-DKSDLELA	WEVCLRMREE	GLLLAP--TE
	481				556			
Styheml	AGFMSVAHSM	DDINNTIDAA	RRVFAKL*..
Bachemaxc	GLFLSTAHTD	EDIEN TIQAA	EKVFAEISRR	*.....
Ntgsamr2	AGFTSLAHTS	EDIQRTVAAA	EKVLKQI*..
Ratota	GDIIRLAPPL	VIKEDEIRES	VEIINKTILS	F*.....
Hskoat	GDIIRFAPPL	VIKEDELRES	IEIINKTILS	F.....
Yscarg8	.STVRFVPAL	TIEDELIEEG	MDAFEKAIEA	VYA*.....
Ecoargd	.DVMRFAPSL	VVEDADIDEG	MQRFAHAVAK	VVGA*.....
Ecogabt	YNVLRILVPL	TIEDAQIRQG	LEIISQCFDE	AKQ*.....
ORF3	PKVLRLYPPA	VISDADLETG	LEHFCQSVEA	ITRTEFDKEI	PLCQQ*
Consensus	GD-LRFAPPL	VI-D-DI-EG	-E-FAK-IE-	V-----	-----	-----	-----	-----

Fig 3.8 (CONT'D)

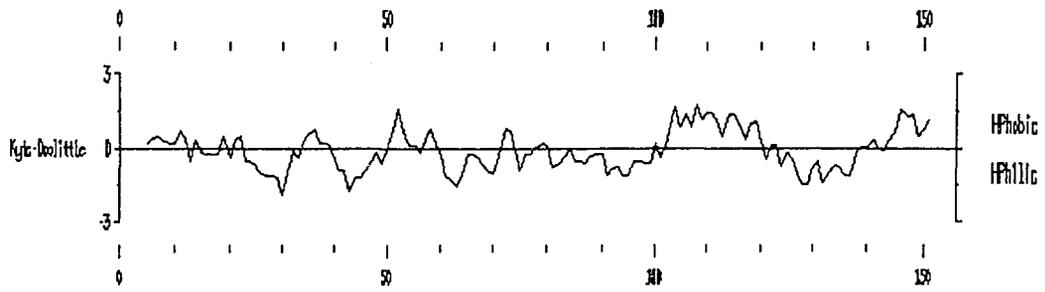


Fig. 3.9. Hydropathy profile of ORF4.

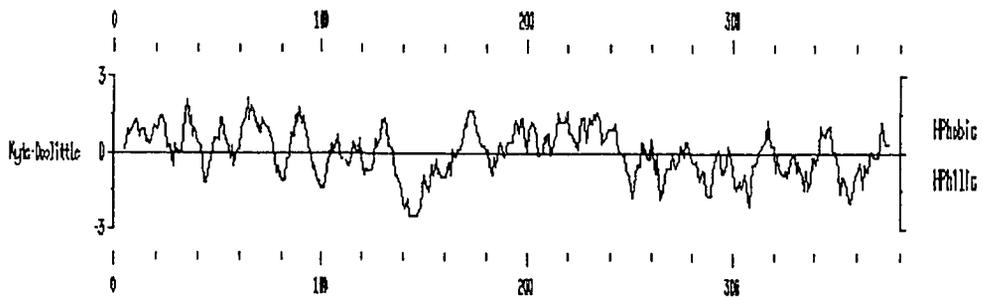


Fig. 3.10. Hydropathy profile of ORF5.

```

1                                                                                               80
Csdesa  MTATIPPLTP TVTPSNPDR. PIADLKLQDI IKTLPKCEFE KKASKAWASV LITLGAIAVG YLGIIYLPWY CLPITWIWTG
ORF6    MSLATAVVCE RKERCRHEKL RVDPHVVHDL MKLRPGKCFI QIGMQ...WL LIVFAV... FFAKWASSWL AYSISIVWIA
Consensus M----- ----- -D- -K--P--CF- ----- LI----- ------W- ---I---W--

81                                                                                               160
Csdesa  TALTGAFVVG HDCGHRSEFAK KRWNNDLVGH IAFA...PLI YPFHSWRLH DHHHLHTNKI EVDNAWDPWS VEAFAQASPAI
ORF6    TRQHALLVLM HDASHYLISR RRWLNDTVGN LFLAFPLSVC VSRYRRHLL HHRHLNTE.. . . . . LDP.D IKDSQLPKTR
Consensus T-----V-- HD--H----- -RW-ND-VG- ---A----- -----L- -H-HL-T--- -----DP-- -----Q-----

161                                                                                               240
Csdesa  VRLFYRAIRG PFWWTGSIFH WSLMHFKLSN FAQRDRNKVK LSIADVFLFA AIAFPALIIT TGVWGFVKFW LMP.WLVYHF
ORF6    IRFYGLLLRD ALGVSTLMTL RSVNMFGLLG LFARGSHCSR LDRRLAMAFI IAVGGGITWV GGGWDVLWLW VVPAFTILPL
Consensus -R-----R- ----- -S---F-L-- ---R----- L-----F- ----- -G-W-----W --P-----

241                                                                                               300
Csdesa  WMSTFTIVHH TIPEIRFRPA ADWSAAEAQL NGTVHCDYPR WVEVLCHDIN VHIPPHLSVA IPSYNLRLAH GSLKENWGPF
ORF6    ILRVRZIAEH GGRLDHPNAS NARSIDVGII E.....R FLWAPCH.IN RHWEHHLCPQ CP...RITY RCLLHDWLAF
Consensus -----I--H ----- ---S----- ------R ------CH-IN -H--HHL--- -P----R--- --L---W--F
                                     ^ ^ ^ ^ ^ ^ ^ ^
301                                                                                               357
Csdesa  LYER..... TFNWQLMQQI SGQCHLYDPE HGY..... .RTFGSLKKV .....
ORF6    FPSRQRHNVL KVTFLVLGRW CRSCTPTPLP HGWQIDVRVS QRMAGVLRRS FHDADG*
Consensus ---R----- ----- ---C----- HG----- -R--G-L--- -----

```

Fig. 3.11. Alignment of the ORF6 amino acid sequence with that of the *desA* gene of *Synechocystis* Pcc6803. A consensus sequence is shown in bold at the bottom. The aa residues in ORF6 that are the same as in the consensus sequence are also shown in bold. Amino acid residues marked by the symbol ^ are the copper B binding signature similar to that of cytochrome c oxidase subunit I.

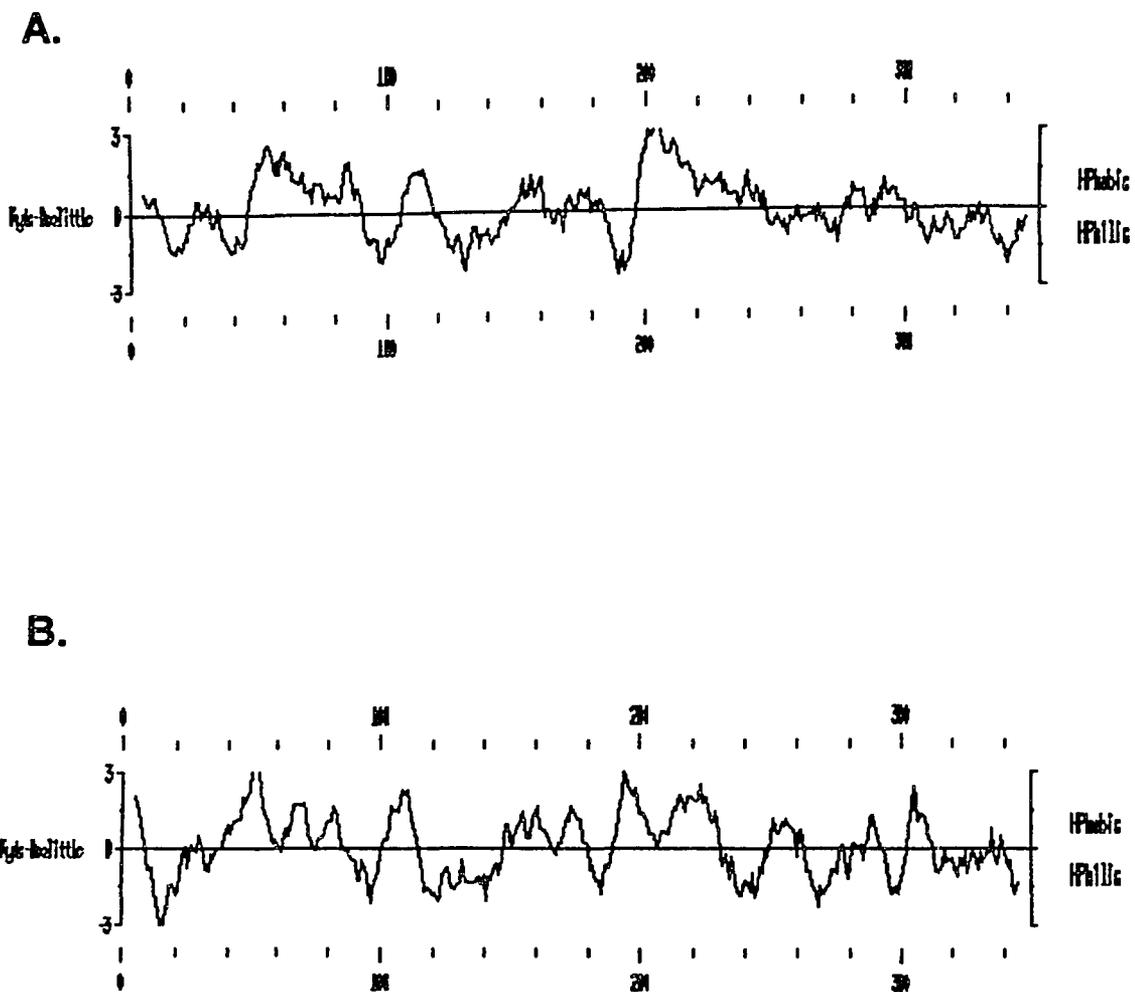


Fig. 3.12. Hydropathy profiles of *desA* of *Synechocystis* Pcc6803 (A) and ORF6 (B).

E. DISCUSSION

The sequence data on phaseolotoxin genes reported here for the first time, show that the *phtE* locus contains 6 ORFs and that they constitute an operon. I deduced the presence of an operon from the sequence for the following reasons: a, two terminator-like sequences are located upstream of ORF1 and downstream of ORF6, respectively; b, a promoter-like sequence is located downstream of ORF6; c, ORFs from ORF3 through ORF6 overlap in that the ribosomal binding site for ORF4 is within the C-terminal end of ORF3; the ribosomal binding site of ORF5 is within the C-terminal end of ORF4, and the ribosomal binding site of ORF6 is within the C-terminal end of ORF5. Furthermore, the results of the RT-PCR experiments indicated that the message covers all six ORFs and is continuous. I considered the possibility that I might have failed to detect the message transcribed by the DNA sequence in *phtE* because large messages are more likely to be degraded. However, the Northern hybridization analysis (data not shown) and the fact that clone pYZH102 failed to complement HOC25 (Fig. 2.1, Chapter II) collectively support the conclusion that a large transcript is transcribed from the DNA sequence in *phtE*.

Often the proteins encoded by the genes in one operon are functionally related, *viz.* the genes in the *virB* operon of *Agrobacterium tumefaciens* (33), or they participate in the same pathway, such as genes in the *lacZ* operon from *E. coli* (6). Another advantage of the operon organization is that the message is expressed as a polycistronic message and therefore the transcription of the genes in an operon is coordinately regulated. (5). Although the functions of the products transcribed by the *phtE* locus are not completely elucidated, the fact that all of them except ORF3 contain one to several hydrophobic regions suggests that they may be functionally related. Phaseolotoxin production in *P. syringae* pv. *phaseolicola* is temperature-regulated and some of the toxin genes are not

expressed at 28°C because of a temperature regulated repressor which negatively controls these genes (36). However, the genes in the *phtE* locus do not appear to be among those regulated by temperature, because the same primer extension product was obtained using RNA templates prepared from cells grown at both 18°C as well as 28°C. It is also noteworthy that ORF4 and ORF3 overlap, and the ribosomal binding sites for ORF5 and ORF6 are located within the preceding ORFs. This indicates that the genes corresponding to ORF3 through ORF6 are translationally coupled, to assure that their gene products are produced in equimolar quantities (32).

The deduced amino acid sequence of ORF3 shows significant homology to acetylornithine aminotransferase, an enzyme which catalyzes the formation of N-acetylornithine and α -ketoglutarate from N-acetylglutamate semialdehyde and glutamate (1). The pyridoxal phosphate and α -carboxyl substrate binding sites are conserved in ORF3 which strongly suggests that the gene product of ORF3 is an acetylornithine aminotransferase homologue, and it may be involved in the production of ornithine. Its homology not only to acetylornithine aminotransferase, but also to ornithine aminotransferase, and glutamine-1-semialdehyde aminotransferase, indicates that it is a member of this large aminotransferase family. It is not surprising that the ORF3 product is homologous to ACOAT, because the end product of the reaction ACOAT catalyzes is ornithine which is a constituent of phaseolotoxin. What is surprising is that the homogenotes containing Tn5 insertions upstream of ORF3 (HOC28 through HOA21, Fig. 2.1C, Chapter II) are not auxotrophic for arginine, indicating that the ORF3 product is not involved in the biosynthesis of ornithine used in arginine synthesis and the house keeping gene required for ornithine synthesis for this purpose is located at a site different from the ACOAT homologue present in ORF3. It is known that the ornithine pool in bacteria is tightly regulated because ornithine is not a common amino acid found in most proteins. To

produce phaseolotoxin, additional ornithine may be needed and this may be provided by the gene product of ORF3. This hypothesis can be tested by examining whether acetylornithine accumulates in homogenotes containing a Tn5 in the *phlE* locus, and determining whether supplementing the media with ornithine complements these mutants.

ACOAT has been reported to display substrate ambiguity in both *E. coli* (7) and *Pseudomonas aeruginosa* (44). This enzyme utilizes acetylornithine as well as ornithine as substrates thus playing dual role in arginine biosynthesis and arginine catabolism (20). Its real function in arginine catabolism is to transaminate succinylornithine (42), an intermediate in a newly discovered catabolic pathway responsible for the breakdown of arginine in a number of bacteria (43). In light of this, I propose that the product of ORF3 may generate ornithine needed for phaseolotoxin production using the arginine pool. Two lines of evidence that support this hypothesis are that arginine enhances toxin production (A. Aslamkhan, 1992, MS thesis, University of Hawaii) and overrides thermoregulation of *argK* (18) in *P. syringae* pv. *phaseolicola*.

ORF6 is homologous to *desA*, a gene encoding the fatty acid desaturase in the cyanobacterium, *Synechocystis* Pcc6803. Although the amino acid identity between these two genes is not very high, the striking resemblance in their hydropathy profiles strongly suggests that the ORF6 product has a fatty acid desaturase-like function. Fatty acid desaturase (or steroyl-CoA desaturase) generates unsaturated fatty acids in the synthesis of phospholipids located in the cytoplasmic membrane (47). Phospholipids consist of saturated and/or unsaturated fatty acids and the content of the unsaturated fatty acid affects the fluidity of the membrane. The higher the unsaturated fatty acid content in the phospholipids, the more fluid the membrane, possibly due to the formation of double bonds in the unsaturated fatty acids, which prevent close packing of the acyl chains in the phospholipids (10). Higher fluidity of the cell membrane facilitates transport of solutes

across the membrane (10). Interestingly, the desaturase activity in many organisms, including *E. coli* (11), Cyanobacterium (46), *Bacillus* (13), and Plant (8) are all temperature-dependent, i. e. the activity is low at higher temperature (37-45°C) but higher at lower temperature (18-25°C). It has been proposed that the changes in the desaturase activity in *Bacillus magaterium* (16) and *Synechocystis* Pcc6803 (47) involves transient synthesis of the enzyme, while in *Pseudomonas* E-3 (45) and *Tetrahymena pyriformis* (40) these changes are due to the activation of pre-existing enzymes. Based on the structure similarity between the ORF6 product and fatty acid desaturase, it is tempting to postulate that the ORF6 gene product may be involved in the modulation of membrane composition so that secretion of phaseolotoxin is facilitated at the lower temperature (18°C), the permissive temperature for toxin production. Recently, Quigley et al. (31) isolated and sequenced a gene from *P. syringae* pv. *syringae* which encodes an ATP-binding membrane protein, which is probably involved in the secretion of syringomycin. They proposed that most, if not all, phytotoxins are secreted by a similar membrane protein in other phytotoxin-producing bacteria. It is possible that a similar ATP-binding membrane protein is directly involved in the secretion of phaseolotoxin by forming a pore, and the ORF6 product modifies the membrane to facilitate the transport of toxin through the pore. Recently, our laboratory (Rowley et al, unpublished data) has isolated a mutant that contains a *TnphoA* insertion in ORF6. This result confirms that the ORF6 product is a membrane associated protein.

No specific function has been proposed for the products of the remaining ORFs because no significant homologous gene sequences were found in the data bases. Although ORF2, ORF4 and ORF5 showed some homology with certain genes, the similarities were limited to only small regions to be significant. However, with a better understanding of the structure-function relationships, these localized homologies will be helpful in the

elucidation of the function of these ORFs, or to deduce at least the functional domains of the gene. ORF1, ORF2, ORF4 and ORF5 all have more than one membrane-spanning region, indicating that all the gene products of these ORFs are membrane associated, and may be involved in the secretion of the toxin.

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CHAPTER IV. CONCLUSIONS

The first objective of this dissertation was to determine the number and organization of genes involved in the production of phaseolotoxin in the insert of the cosmid clone, pHK120 (2). The second objective was to shed light on the functions of the genes in the largest locus, *phtE*, by doing sequence analysis of the DNA in this locus. Both objectives were accomplished in this study and the results are presented in Chapters II and III, respectively.

Eight transcriptional units or loci that are involved in toxin production in the insert of pHK120 have been identified by Tn5 mutagenesis, marker exchange and pair complementation. The eight transcriptional units (or complementation groups), designated *phtA* through *phtH*, were mapped and they are located close to each other in a cluster. This organization is similar to many antibiotic biosynthetic genes (4) and genes involved in phytotoxin production (9) which are also clustered. The boundaries of the eight units were defined on the basis of pair complementation data. The sizes of these units are varied, and range from 1 kb to 6.5 kb. Although pHK120 overlaps with pRCP17 (6), a cosmid clone that also complements *Tox⁻* mutants, it differs from pHK120 in two aspects: pHK120 does not contain the ROCT gene; instead, it contains 4 kb of DNA that is absent in pRCP17. On the other hand, pRCP17 contains 5 kb of DNA not found in pHK120. I also concluded that pHK120 does not contain all the genes that are necessary for toxin production because mobilization of this plasmid into a phaseolotoxin-non producing strain, *P. syringae* pv. *syringae*, did not result in the phaseolotoxin production. However, pHK120 abolishes thermoregulation and increases toxin production about 3 fold in the wild-type strain. I have proposed three hypotheses to explain this phenomenon: a) pHK120 contains a gene encoding a positive regulator of phaseolotoxin genes; b) pHK120 encodes a rate-limiting

factor in the biosynthetic pathway of the toxin; c) pHK120 contains multiple binding sites for a repressor protein (7) which suppresses toxin production in the wild-type strain at the non-permissive temperature.

In the process of defining the complementation groups in pHK120, I have generated 29 Tox^- mutants, each containing a Tn5 insertion at a known position in the chromosome. These mutants will be useful in the elucidation of the steps involved in the pathway of the biosynthesis of phaseolotoxin.

Chapter III describes the complete sequence of the *phtE* locus, the largest locus in the pHK120 insert. Small peptides are synthesized through a unique non-ribosomal pathway by large, multifunctional synthetases (3). These synthetases have characteristic domains involved in activation of individual amino acids. The size of the *phtE* locus falls in the range of many synthetase genes, raising the possibility that this locus may harbor a peptide synthetase-like gene. However, the sequence of the *phtE* locus revealed six ORFs and none of them showed homology to any known synthetases. The RT-PCR experiments showed that these six ORFs are transcribed into one large transcript, indicating that the *phtE* locus is an operon. Computer analysis indicated that ORF3 has homology with the bacterial acetylornithine aminotransferase gene and ornithine aminotransferase genes from several other organisms. These data suggest that the ORF3 product may be involved in ornithine synthesis; ornithine is a constituent of phaseolotoxin. ORF6 is homologous to the fatty acid desaturase gene from a cyanobacterium, *Synechocystis* Pcc6803 (8). On the basis of this finding, I have proposed that the ORF6 product may be involved in modulation of the cytoplasmic membrane and in facilitating the secretion of phaseolotoxin. The functions of other ORFs are still unknown because no statistically homologous gene sequences were found in the data base. However, hydropathy searches showed that these ORFs have one to

several hydrophobic regions, implying that they may also be involved in the secretion of phaseolotoxin.

The transcriptional start site of the operon in the *phtE* locus was determined by doing a primer extension experiment. The transcript starts at a T located 31 bp upstream of ORF1. This experiment also indicated that the transcription of this operon is not subject to repression by temperature. A computer search failed to find a sequence homologous to the putative repressor binding motif (Rowley, unpublished data) in the promoter region of the *phtE* locus. Even though the transcription of the *phtE* locus is temperature-independent, the activity of one of its products, the ORF6 product, may be temperature-dependent because it is homologous to the fatty acid desaturase, which shows the highest activity around 18°C and the lowest around 30°C (1).

The RT-PCR technique was used to detect the message transcribed by the *phtE* locus. The principle of this method is that RT utilizes RNA only as a template to synthesize the first strand DNA (cDNA) which is used as a template for PCR amplification. The drawback of the RT-PCR method is that contamination of the RNA template with even a single molecule of genomic DNA can give misleading results. When appropriate controls were used this method in my hands turned out to be more reliable than the standard Northern blot technique.

This work is the first detailed study on the organization of genes involved in phaseolotoxin production by *P. syringae* pv. *phaseolicola* and is the first step toward elucidating the biosynthetic pathway of phaseolotoxin.

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