Genetic Organization of a Cluster of Genes Involved in the Production of Phaseolotoxin, a Toxin Produced by *Pseudomonas syringae* pv. phaseolicola

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Phaseolotoxin \(N^k(N’\text{-sulfo-diaminophosphinyl})-\text{ornithyl-alanyl-homoarginine}\) 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 *Tn5* 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 (R. C. Peet, P. B. Lindgren, D. K. Wills, and N. J. Panopoulos, J. Bacteriol. 166:1096–1105, 1986) 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.

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 \(N^k(N’\text{-sulfo-diaminophosphinyl})-\text{ornithyl-alanyl-homoarginine}\) (23) and its nonpeptide degradation product ochtadin \(N^k(N’\text{-sulfo-diaminophosphinyl})-\text{ornithine}\) (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 nonoverlapping classes of genomic cosmid clones from a library of *P. syringae* pv. phaseolicola that complements 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. (30) 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 we describe the genetic characterization of the pHK120 insert, which was performed by *Tn5* mutagenesis of pHK120, marker exchange 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.

Preliminary reports of this study were presented at the 80th Annual Meeting of the American Phytopathological Society held in St. Louis, Mo., in 1991 and at the 5th Biennial Meeting

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of the International Society of Molecular Plant-Microbe Inter-
actions held in Seattle, Wash., in July 1992.)

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The
bacterial strains and plasmids used in this study are listed in
Table 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
or 28°C. When necessary, the following antibiotics were used:
rifampicin (50 μg/ml), tetracycline hydrochloride (20 μg/ml),
kanamycin (50 μg/ml), ampicillin (50 μg/ml), spectinomycin
(50 μg/ml), and chloramphenicol (15 μg/ml).

Tn5 mutagenesis. Tn5 mutagenesis was carried out accord-
ing the method of de Bruijn and Lupski (4). E. coli Q1
harboring pHK120 was mixed with λA67 phage at a multiplicity
of infection of 1:10 and incubated for 2 h at 30°C. Aliquots (0.2
ml) were spread on LB agar plates containing kanamycin and
tetracycline and incubated for 48 h at 30°C. Colonies were
pooled with 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. The location of Tn5 in the plasmids was
determined by single and double restriction endonuclease
digestion (3) with HindIII, BamHI, or Smal.

Triparental mating, electroporation, and marker exchange.
Bacterial conjugation was carried out according to the method
of Kamdar et al. (13) with E. coli (pKK2073) as an intermediate
donor in triparental matings. Electroporation was performed
as described by Rowley et al. (32), except that the field strength
was raised to 7.1 kV. To verify constructs, plasmid DNA was
extracted from transconjugants and transformed into E. coli
DH5α in order to segregate the plasmid of interest from
indigenous plasmids in the transconjugant. Marker exchange in
transconjugants was forced by withdrawing tetracycline from
the media and by repeated subculturing of the transconjugants
in the presence of kanamycin (8). The marker exchange
mutants were examined for tetracycline sensitivity and
screened by the E. coli toxin assay (16). Southern hybridiz-
ation analysis of HindIII- or BamHI-digested genomic DNA
from each marker exchange mutant was done by using the
32P-labeled internal fragment from Tn5 as a probe to verify the
position of Tn5 in the homogenate. Appropriate pHK120::Tn5
plasmid DNA was used as the control.

DNA isolation and manipulation. Plasmid DNA was ex-
tracted 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-point agarose after electrophoresis and purified
with the USBioltec kit (U.S. Biochemicals, Cleveland, Ohio).
Restriction enzyme digestion and ligation of DNA were car-
ried out by standard protocols (2).

RESULTS

Restriction map, Tn5 mutagenesis of pHK120, and marker
exchange in the wild-type strain. The restriction map of
pHK120 was constructed, and the HindIII, BamHI, and Smal
sites and relevant Srl and PslI sites are shown in Fig. 1A. The
plasmid was mutagenized with Tn5, and of the 350
pHK120::Tn5 hybrid plasmids examined by digestion with
HindIII, 200 contained a Tn5 within the insert. The position of
the Tn5 insertion in each clone was mapped, and those which
mapped to a unique site within the insert of pHK120 are shown
in Fig. 1B (represented by an arrowhead or an open circle; the
symbol # represents the chromosomal Tn5 mutants, as de-
scribed below). A total of 44 plasmids containing Tn5 inserts
at unique sites were mobilized into P. syringae pv.
phaseolicola 4612-1 (Tox+) by triparental mating. The Tn5 was
marker exchanged into the chromosome of the transconjugant
by double homologous recombination, as described in Materi-
als and Methods. Sixteen randomly chosen Tox Km+ colonies
from a single mating for each marker exchange mutant (des-
ignated by the prefix HO and a number representing the
position of the Tn5, as shown in Fig. 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. 1B, shaded arrowheads) failed to produce toxin,
indicating that the Tn5 insertion in these mutants had inter-
rupted genes necessary for toxin production. Eleven marker
exchange mutants (Fig. 1B, open arrowheads) retained their
ability to produce toxin, suggesting that the Tn5 insertions in
these mutants were outside regions essential for toxin produc-
tion. 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. 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 plas-
mid in E. coli was approximately 10%. It is possible that
the frequency of secondary transposition of Tn5 in P. syringae
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 four
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 with that of pHK120 revealed that the
insertions were located within the pHK120 region at the
positions denoted by the symbol # (Fig. 1B).

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 and indicated in Fig. 1B
by the symbol ⋆) and one random Tn5 mutant (TNM7) (Table
2 and Fig. 1B) were chosen for complementation by
pHK120::Tn5 hybrid plasmids. Since these experiments in-
volved 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.
TABLE 1. Bacterial strains and plasmids used in this study

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<th>Strains</th>
<th>Genotype, phenotype, or description</th>
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To ensure that recombination had not occurred in these transconjugants, the plasmids were retrieved from the transconjugants, transformed into *E. coli* DH5α, 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. 1B)).

(i) The *phtA* locus. Since hybrid plasmids containing Tn5 insertions at positions AF4, A46, A41, and A13 (Fig. 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.

(ii) The *phtB*, *phtC*, and *phtD* loci. The second Tox⁻ region, encompassing a 6.7-kb region between C37 and D19 (Fig. 1B), was divided into three transcriptional units on the basis of pair complementation experiments (Table 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*. HOA44 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 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 BamHI site. Similarly, the left boundary of the *phtD* locus is located right of the fourth SstI site, whereas its right boundary is delimited by a Tox⁻ mutant, HOD19.

(iii) The *phtE*, *phtF*, and *phtG* loci. Another Tox⁻ region, encompassing an 8.0-kb region between C7 and G4 (Fig. 1B), consists of three transcriptional units designated *phtE*, *phtF*, and *phtG*, respectively (Table 2). Nine marker exchange mutants, HOA14, HOE17, HOG1, HOA21, HOB23, HOD13, HOC25, HOD18, and HOC28, and the 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.

(iv) 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. 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). Hybrid plasmids with Tn5 insertions to the
left of A47, i.e., in the \( \text{phtF} \) locus, complemented the mutants with mutations in the \( \text{phtH} \) locus. These results suggest that the \( \text{phtG} \) locus is required for the \( \text{phtH} \) locus to be functional.

**Complementation of selected mutants by subclones of \( \text{pHK120} \).** Selected Hind III, \( \text{BamHI} \), \( \text{PstI} \), and \( \text{SalI} \) fragments from \( \text{pHK120} \) were cloned into the broad-host-range vectors \( \text{pLAFR3} \), \( \text{pRK404} \), and \( \text{pLAFR376} \) and mobilized into one or more of the marker exchange mutants or the random chromosomal \( \text{Tn5} \) mutants by electroporation or conjugation (Table 2 and Fig. 1C). The plasmid \( \text{pYZB2102} \), containing the 4.9-kb \( \text{BamHI} \) (B21) fragment (Fig. 1A and C), restored toxin production to the marker exchange mutants \( \text{HOA41} \) and \( \text{HOA46} \), which contain mutations in the \( \text{phtA} \) locus. The \( \text{phtB} \) locus, represented by the mutant \( \text{TNN7} \), was not complemented by either the plasmid \( \text{pYZB2102} \) or the plasmid \( \text{pYZH302} \), containing the 4.9-kb \( \text{BamHI} \) (B21) or the 4.3-kb \( \text{HindIII} \) (H3) fragment, respectively (Fig. 1A and C). Therefore, the \( \text{phtB} \) locus must extend beyond the third \( \text{HindIII} \) site in \( \text{pHK120} \) (Fig. 1A). The plasmid \( \text{pYZB302} \), containing a 2.2-kb \( \text{BamHI} \) (B3) fragment (Fig. 1A and C), partially complemented \( \text{HOA25} \) (Table 2), a mutant with a mutation in the \( \text{phtC} \) locus. Mutant \( \text{HOA44} \), containing a mutation in the \( \text{phtD} \) locus, was partially complemented by the plasmid that contains the 4.6-kb \( \text{HindIII} \) (H2) fragment from \( \text{pHK120} \), \( \text{pYZH201} \), and this plasmid also complemented the \( \text{phtE} \) locus.

**Table 2. Results of pair complementation and complementation with \( \text{pHK120} \):**

<table>
<thead>
<tr>
<th>Hybrid plasmid or subclone</th>
<th>HOA46</th>
<th>HOA41</th>
<th>TNN7</th>
<th>HOA25</th>
<th>HOA44</th>
<th>HOA47</th>
<th>HOA51</th>
<th>HOA54</th>
<th>HOA58</th>
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<tr>
<td>( \text{pHK120}::\text{Tn5-B25} )</td>
<td>+</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( \text{pHK120}::\text{Tn5-AF4} )</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( \text{pHK120}::\text{Tn5-A13} )</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
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<tr>
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<td>ND</td>
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</tr>
<tr>
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<td>+</td>
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<tr>
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<tr>
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<td>ND</td>
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<td>( \text{pHK120}::\text{Tn5-A25} )</td>
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<td>+</td>
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<td>ND</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
</tbody>
</table>

* +, toxin was detected in the transconjugant; --, toxin was not detected in the transconjugant; +/-, a limited amount of toxin was detected in the transconjugant; ND, test was not done.
(Fig. 1A and C), 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 (StI) fragment from pHK120 (Fig. 1A and C). Surprisingly, the plasmid pYZB2202, which contains the 4.9-kb BamHI (B22) fragment (Fig. 1A and C), failed to complement HOA4. The Tox− mutants with mutations in the phtF 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 SstI (StI) fragment (Fig. 1A and C), which covers the putative phtF locus. However, mutants HOA14 and HOAC5 were not complemented by pYZST102. Three other plasmids, pYZH102, containing the 7.0-kb HindIII (H1) fragment; pYZB102, containing the 7.8-kb BamHI (B1) fragment (Fig. 1A and C); and pYZP102, containing the 8.2-kb Psfl (P1) fragment (Fig. 1A and C), also failed to complement HOAC5. 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 HindIII site. On the basis of the pair complementation analysis, the phtG and phtH loci are located within the B1 or P1 fragment of the pHK120 insert (Fig. 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. 1A and C).

**A comparison of the genomic fragments from pHK120 and pYZB2202.** Previously, Peet et al. (30) reported that pRCPI7, 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 with pRCPI7 as the probe revealed that these two clones have overlapping inserts (Fig. 1D). pHK120 contains about 4.8 kb of DNA (Fig. 1D, H4 and part of H3) at the left end of its insert which is not present in pRCPI7, whereas pRCPI7 harbors about 5 kb of DNA which is not present in the insert of pHK120.

Because pRCPI7 contains the ROCT gene (31), transformants of *E. coli* HB101 containing pRCPI7 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, we conclude that the ROCT gene must be located within the 5-kb DNA fragment in pRCPI7 that is not present in pHK120.

**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 or higher, 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). The level of toxin produced by the transconjugants 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 at a level roughly three times 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.

**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 (pRCPI7) in this study indicated that the two clones differ from each other in only two respects: unlike pRCPI7, pHK120 does not harbor the ROCT gene, and pHK120 contains a region of DNA (Fig. 1D, fragment H4 and part of H3) that is not shared by pRCPI7. 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 HOA44 and HOA46 (with mutations in the phtA locus) containing pYZB2102 and in transconjugants of mutant HOB36 (with a mutation in the phtF locus) containing plasmid pYZB102 as well as plasmid 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 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.

Transcriptional complementation was also observed in mutants 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. 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. 1C complemented these mutants. We 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 HOAC5). The
fact that such complementation did not occur raises the possibility that pytZST102 does not contain the entire phfE locus. We have recently sequenced 6.7 kb of DNA (41) downstream of the phfE locus, and this analysis reveals that the fifth SstI site (Fig. 1A) interrupts a putative open reading frame (ORF). If this ORF is part of the phfE locus, then this locus must be larger than the predicted one (Fig. 1B), and this may explain why pytZST102 does not complement the phfE mutants (41). A similar discrepancy between complementation analysis and sequencing data was reported by Jacobs et al. (12) for nodulation genes in Rhizobium meliloti. We 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 SstI site is part of the phfE locus. If this is the case, the reason for the failure of the first SstI 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 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 downregulate toxin synthesis by a feedback mechanism.

The last two loci, phfG and phfH, are located in the B1 or the P1 fragment of pHK120 according to pair complementation analysis. However, neither pytZB102 nor pytZP102 complemented the mutants with mutations located 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, we have no explanation for this unexpected result. Pair complementation analysis also indicated that the pHK120: Tn5 hybrid plasmids containing Tn5 insertions within the phfG locus failed to complement the phfH mutants, but pHK120: Tn5 hybrid plasmids containing Tn5 insertions within the phfH locus complemented the phfG mutants, indicating that the phfG locus is required for the function of the phfH 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 phfG and phfH loci constitute one locus and that the two Tox" mutants with mutations between the phfG and phfH loci are nonpolar mutants.

Although pHK120 contains most of the genes involved in the production of phaseolotoxin, we conclude that it does not contain all of them. This conclusion is based on the fact that the transconjugants of E. coli containing pHK120 grew on minimal media (data not shown). Since pHK120 does not contain the ROC7 gene and E. coli is sensitive to phaseolotoxin, production of the toxin should have resulted in growth inhibition of the transconjugants of E. coli on minimal media. Another observation which supports our conclusion that 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 biosassay (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 coelicolor, 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- to 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, overproduction 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 the 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 gene 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 an 490-bp fragment from the argK promoter region also specifically binds to a protein(s) produced by the wild-type strain at 28°C. We are currently examining the DNA sequence of the phfE 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 determine their gene products, and to determine whether these genes are thermoregulated. These studies are currently under way. 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.

ACKNOWLEDGMENTS

This work was supported in part by USDA-CRGO grant no. 85-CRRCR-1-1897. Y.Z. was partially supported by the University of Hawaii Graduate Specialization in Cell, Molecular, and Neurosciences.

JoAnn Dileanis helped with the construction of the preliminary restriction map of the pHK120 insert. We thank Nikolos Panopoulos for providing cosmids pRPC17 and pLAFR376 and Dulal Borthakur for useful discussions and suggestions about the manuscript.

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