A Rapid and Sensitive Microbiological Assay for Phaseolotoxin

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ABSTRACT

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A rapid and sensitive bioassay for phaseolotoxin, a phytotoxic compound produced by *Pseudomonas phaseolicola*, has been developed. Apparently phaseolotoxin inhibits the growth of *Escherichia coli* strain K-12 in minimal-glucose medium by inhibiting L-ornithine carbamyltransferase (OCT), thereby creating a phenotypic requirement for arginine. Inhibition is reversed by citrulline and arginine, but not by ornithine. A dose-response curve was established with purified phaseolotoxin preparations and as little as 10–12 pg of phaseolotoxin was detected. Growth inhibition also was observed with cell-free culture filtrates and around colonies of *P. phaseolicola*. Maximum zones of inhibition were observed when *P. phaseolicola* was grown at 18 C, the optimum temperature for toxin production and induction of chlorosis in planta. There was a 100% correlation between the ability of *P. phaseolicola* strains to induce chlorosis in bean and to inhibit *E. coli*. This assay technique greatly facilitates screening for nontoxigenic mutants of *P. phaseolicola*.

Additional key words: halo blight toxin.

Pseudomonas phaseolicola (Burkh.) Dowson produces a thermostable, low molecular weight exotoxin(s), here referred to as "halo-blight toxin(s)" (HBT), which is (are) the principal cause of chlorosis in infected plants (22, 31). HBT has been implicated directly or indirectly in several pathophysiological phenomena observed in infected plants: accumulation of ornithine in infected or toxin-treated tissues (16,18,21,29), ability of the bacterium to invade the plant systemically (25), abrogation of the hypersensitive-response of bean leaves that are genetically tolerant to certain (incompatible) strains of *P. phaseolicola* (6,24,28), and prevention or suppression of the formation of bean phytoalexins upon inoculation with incompatible pathogens (5,6,24).

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HBT is a potent and specific inhibitor of the enzyme L-ornithine-carbamyltransferase (EC 2·1·3·, abbreviated OCT), which catalyzes the conversion of ornithine to citrulline (4,16,23,25,26). This reaction is common to both arginine biosynthesis and the urea cycle. Inhibition of this reaction has been proposed as an explanation for the accumulation of ornithine in infected or toxin-treated plant tissues (18,29).

Recently the toxins from *P. phaseolicola* culture filtrates were purified and chemically characterized by two groups of researchers (13, 14, 27). Patil et al (27) reported the presence of four OCT-inhibiting compounds (A, B, C, D); the first component (A) was reported to be N-phosphoglutamic acid and was given the trivial

name phaseotoxin A. Patil et al (27) obtained inhibition of OCT from bean plants in vitro with chemically synthesized N-phosphoglutamate, suggesting that phaseotoxin A is indeed a "halo blight" toxin. These conclusions recently were questioned by Mitchell (13–18), who identified the toxin as (N^{δ}-phosphosulphamyl) ornithylalanylhomoarginine and gave it the trivial name phaseolotoxin. Mitchell (16) was unable to demonstrate inhibition of OCT by chemically synthesized N-phosphoglutamate, although he used an enzyme from a different source (*Streptococcus faecalis*). He claimed (15) that phaseolotoxin quantitatively accounts for all halo blight toxin activity detected in culture filtrates of *P. phaseolicola*.

When phaseolotoxin is assayed by the induction of chlorosis in bean leaves, the detection threshold is in the range of 10–20 ng, whereas in the in vitro OCT reaction assay 170 μg are required for 50% inhibition (16). In this paper, we describe a microbiological assay of picogram levels of phaseolotoxin.

MATERIALS AND METHODS

Bacterial strains. These are described in Table 1.

Media. Unless specified otherwise, cultures were grown and maintained on solid (2% agar) or liquid King's B Medium (KB) (11). For toxin bioassays, the organisms were grown in minimal salts medium (minimal A medium, [12]), with or without agar (2%), and with a carbon source (0.3%), amino acids (0.1–0.05%), and other supplements as specified in the Results.

Chemicals. Nalidixic acid, rifampicin, streptomycin, histidine, proline, ornithine, citrulline, and arginine were obtained from Sigma Chemical Co., St. Louis, MO 63178. N-methyl-N′-nitro-N-nitrosoguanidine (NG) was obtained from the Aldrich Chemical Co., Inc., Milwaukee, WI 53233. All chemicals were filter-sterilized (0.22-μm filters, Millipore Corp., Bedford, MA 01730).

Toxins. Purifed, lyophilized phaseolotoxin was kindly supplied by R. E. Mitchell. This preparation probably was a mixture of phaseolotoxin (>80%), its 2-serine analogue, and a minor fraction of breakdown products (Mitchell, *personal communication*). The toxin titer was determined by Mitchell with the bean leaf bioassay as previously described (18). Two sets of serial dilutions were prepared, one in sterile distilled water and the other in sterile phosphate buffer (0.01 M, pH 7.0) and stored frozen at −15 C.

Toxin assay. Cultures of *P. phaseolicola* grown on minimal agar at 18 C, the optimum temperature for toxin production (7,8,15), were overlaid with *E. coli* strain K-12 by mixing 2 ml of a log-phase culture (~5.0×10^8 cells/ml) of the indicator strain grown in minimal glucose medium, with an equal volume of molten water agar (2%) kept at 65 C, and incubating at 37 C for 2 hr.

Cell-free culture filtrates, prepared by filtering 1-ml samples through 0.22-μm Millipore filters, and serial dilutions of phaseolotoxin preparations were assayed by adding 20-μl volumes to individual Whatman No. 1 paper disks (0.5-cm diameter) or to wells cut into the agar plates with a No. 1 cork borer (0.5-cm diameter) after overlaying. For microassays, 1- or 2-μl volumes were delivered directly with a 1-μl syringe onto the overlaid plates. For reproducible, quantitative assays, the volume of minimal agar medium was standardized at 10 ml/plate. The size of the zones varied with the dryness of the plates and the temperature of incubation. Usually, when determining toxin concentrations from crude filtrates, an internal standard of known titer was assayed under identical conditions.

Isolation of antibiotic-resistant mutants, phaseolotoxin resistant mutants, and auxotrophs. Mutants resistant to nalidixic acid (Nal^R) rifampycin (Rif^R) and streptomycin (Str^R) were obtained by plating 0.1 ml of 10^9 cells per milliliter onto KB medium containing 100 μg/ml of the antibiotic. Spontaneous mutants appeared in 3–4 days and subsequently were purified on King's B Medium with and without the antibiotics. Spontaneous mutants of *E. coli* K-12 resistant to phaseolotoxin were isolated and purified from colonies growing within inhibition zones in bioassay plates. Auxotrophic mutants were obtained by NG mutagenesis and penicillin enrichment as described by Miller (12).

Isolation of nontoxigenic (Tox^-) mutants. These were isolated after mutagenesis with NG by the following procedures:

Suspensions of mutagenized populations of toxigenic strains of *P. phaseolicola* were diluted to give about 50 colonies per plate and then plated on minimal medium. Growth factors required by the parent strain were added when necessary. After incubation at 30 C for 2–3 days to allow for the colonies to grow, the petri dish cultures were incubated for 5–6 hr at 18 C, the optimal temperature for toxin production, and were overlaid with soft agar containing the indicator strain. Presumptive Tox^- mutants were retested for toxin production and for the parental phenotype by means of the bacteria and bean leaf (18) bioassay (see Tables 1, 2).

Plant inoculations. The corundum and vacuum infiltration tech-

TABLE 1. Bacterial strains used in the development of a microbiological assay for phaseolotoxin

Strains	Source and description
Pseudomonas phaseolicola:	
HB-2, HB-8, HB-9, HB-13, HB-18, HB-20, HB-26, HB-28, HB-30, HB-31, HB-33, HB-36, HB-43	M. N. Schroth[a]
NPPH1032	HB-36, Nal^R, Rif^R, this study
NPPH2017	A. K. Vidaver R_2QHB, Str^R
NPPH3000	S. S. Patil, G50
NPPH3001	S. S. Patil, G50 Tox^-, UV mutagenesis (24)
NPPH3007	NPPH3000 His^-, NG mutagenesis, this study
NPPH3051	NPPH3007 His^-, Str^R, Tox^-, this study
HB-526	UCPPB 526
Escherichia coli K-12	M. van Montague, N100[b]

[a]Sources of these isolates are reported in reference (30).
[b]The pedigree of this strain is given in reference 1. His, Tox, Nal, Rif, and Str stand for histidine, toxin, nalidixic acid, rifampicin, and streptomycin, respectively.

TABLE 2. Correlation between ability of *P. phaseolicola* strains and mutants to cause chlorosis in bean leaves and growth inhibition of *E. coli*

Strain or mutant	Ability of *P. phaseolicola* strains to cause: Chlorosis in bean leaves[a]	Inhibition of *E. coli*	Reversal of *E. coli* inhibition by either citrulline or arginine
HB-2	−	−	
HB-8	−	−	
HB-9	−	−	
HB-13	−	−	
HB-16	+	+	+
HB-18	−	−	
HB-20[b]	−	−	
HB-26	−	−	
HB-28	+	+	+
HB-30	−	−	
HB-31	+	+	+
HB-33[c]	+	+	+
HB-36[d]	+	+	+
NPPH1032	+	+	+
HB-43	+	+	+
HB-526	−	−	
NPPH2017	−	−	
NPPH3000[e]	+	+	+
NPPH3001[f]	−	−	+
NPPH3007[g]	+	+	
NPPH3051	−	−	

[a]Cultivar Red Kidney.
[b,c,d,e,f] Strains HB-20 and NPPH3001 do not produce toxins assayable by the in vitro L-ornithine carbamyltransferase inhibition test, but HB-36 and NPPH3000 do so (4,6,24).
[g]L-Histidine requirement (His^-) does not interfere with production of water-soaked lesions.

niques for inoculation of bean leaves, *Phaseolus vulgaris* 'Red Kidney', were used as described previously (19). Plants were kept in a growth chamber with a 12- hr day-night photoperiod and a 20–24 C temperature regime.

RESULTS

Toxin detection and specificity for OCT. Chlorosis-inducing toxigenic strains of *P. phaseolicola* produced diffusible substance(s) which inhibited the growth of *E. coli* in minimal agar medium. Cell-free filtrates from cultures grown in minimal glucose medium also were inhibitory to *E. coli* (Fig. 1). Inhibition zones were clear and were observed 1.5–2.0 hr following incubation at 37 C. Inhibition of growth apparently resulted from inhibition of OCT which resulted in a phenotypic requirement for arginine (Fig. 2). Inhibition was reversed by arginine or citrulline (500 μg/ml) added either to the overlay or to the samples being assayed (Fig. 1). The specificity of the assay for HBT was further supported by the 100% correlation between ability of different *P. phaseolicola* strains to cause typical chlorosis in the plant and their ability to inhibit the indicator strain under the conditions described (Table 2). Furthermore, the temperature dependence of the inhibitory activity observed in our assays was similar to that reported previously for HBT production and for the chlorosis-inducing effects of *P. phaseolicola* on bean leaves. These occurred optimally at 18 C and are progressively reduced as the temperature approaches 25–30 C (7,8,10,15).

Specificity for phaseolotoxin. Purified phaseolotoxin, obtained from R. E. Mitchell, inhibited *E. coli* in a manner suggesting specific inhibition of OCT (ie, reversal by citrulline or arginine, but not by ornithine). In addition, mutants selected for resistance to purified phaseolotoxin were not inhibited either by crude culture filtrates or by substances diffusing from colonies of *P. phaseolicola* grown as previously described. Thus, we conclude that zones of inhibition in our bioassay are specifically related to the presence of phaseolotoxin.

Dose-response curve for phaseolotoxin. Dose-response curves (Fig. 3) showed a linear relationship between the logarithm of phaseolotoxin concentration and the diameter of the inhibition zones. This bioassay detected 8–12 pg of phaseolotoxin spotted directly on the overlay. Thus, its sensitivity greatly surpasses that of the leaf bioassay and the in vitro OCT reaction (16,18).

Isolation of nontoxigenic mutants. We have used this bioassay successfully to isolate several nontoxigenic (Tox^-) mutants of *P. phaseolicola*. Mutants lacking inhibition zones were obtained and unambiguous identification of their origin was made by testing for

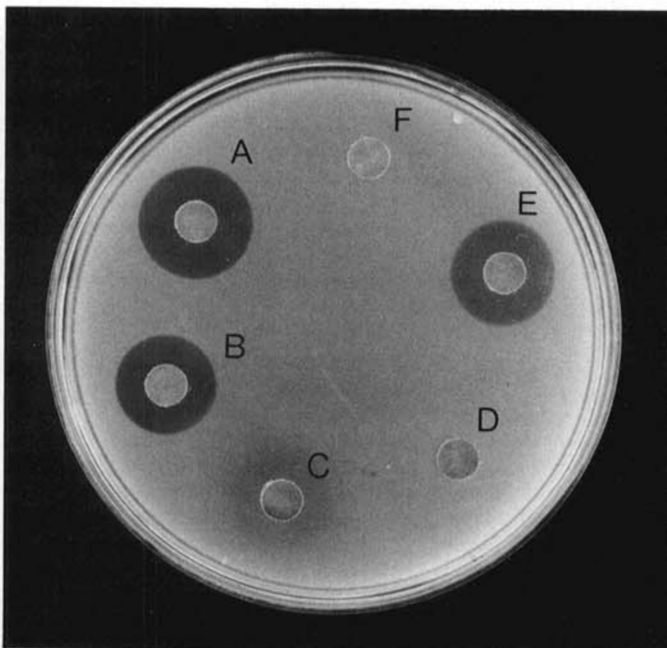

Fig. 1. Growth inhibition of *Escherichia coli* strain K12 by cell-free culture filtrates of *Pseudomonas phaseolicola* and its reversal by citrulline. **A**, HB-36 (20 μl); **B**, HB-36 10 μl + ornithine (10 μl of a 5% solution); **C**, HB-36 10 μl + citrulline (10 μl of a 5% solution); **D-F**, HB-20, G50, G50Tox$^-$, respectively (20 μl each).

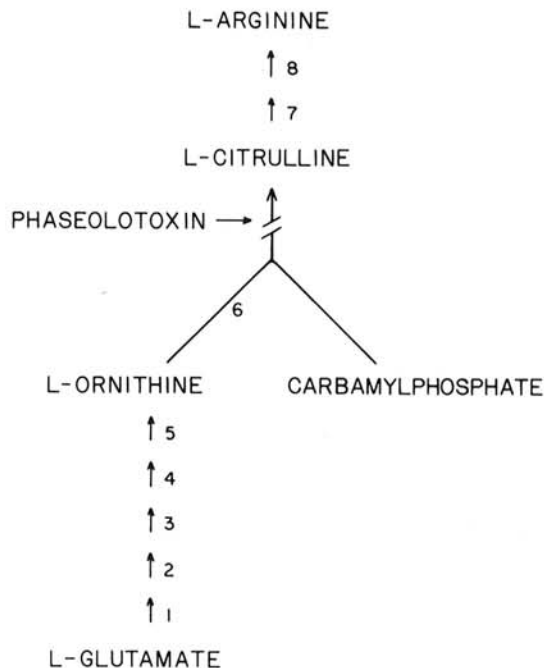

Fig. 2. The arginine biosynthetic pathway in *Escherichia coli* strain K12. Ornithine carbamyltransferase catalyzes reaction 6, the conversion of ornithine to citrulline. Phaseolotoxin inhibits this reaction.

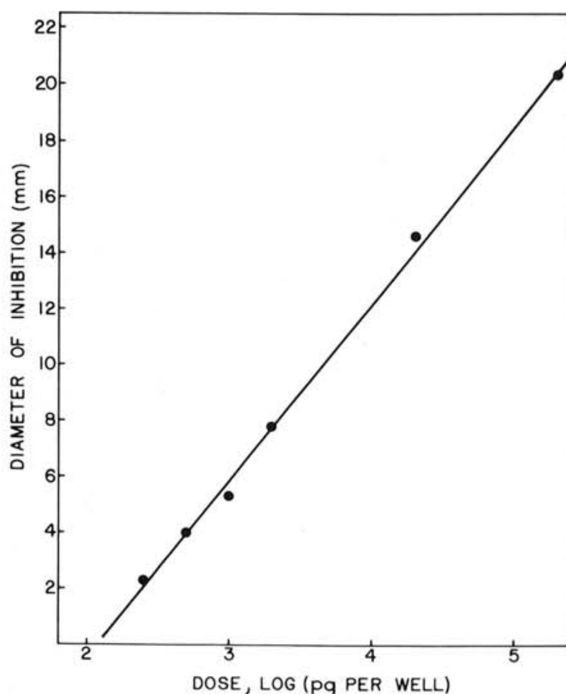

Fig. 3. Dose-response curve for inhibition of *Escherichia coli* strain N100 by phaseolotoxin. Twenty-μliter volumes of serial dilutions of phaseolotoxin made in phosphate buffer were delivered into 5-m-diameter wells cut into the minimal agar medium after it was overlaid with the indicator strain. The petri dishes contained 10 ml of agar medium plus 4 ml of overlay agar. Each point represents the average of three replicates; line of best fit is $Y = 6.35X - 13.2$ in which X is log [picograms] of phaseolotoxin and Y is millimeters inhibition.

the presence of the parental markers. Subsequently several Tox$^-$ mutants that were tested on plants were unable to induce chlorosis, although they grew normally and caused typical watersoaked lesions indistinguishable from those produced by the parent strains. Spontaneous Tox$^-$ mutants of G50 His$^-$ Str^R were not obtained from over 1,000 individual colonies screened with the bioassay.

DISCUSSION

The bioassay for phaseolotoxin was rapid and convenient, and its sensitivity greatly surpassed that of the bean leaf and in vitro OCT reaction assays (16,18). Results of experiments reported elsewhere (20) indicate that phaseolotoxin is transported into bacterial cells via the oligopeptide permease and that its peptide moiety is essential for transport. Mutants of *E. coli* and *S. typhimurium* that lacked this permease were not inhibited by phaseolotoxin or by crude culture filtrates of toxgenic *P. phaseolicola* strains. Furthermore, treatment of phaseolotoxin wth leucine amino peptidase for 1 hr at 40 C abolished its potency against *E. coli* by 98% (Staskawicz, *unpublished*). This enzyme yields (N^{δ}-phosphosulfamyl)ornithine (PSOrn) as one of the products of degradation (18). These observations add further evidence to the specificity for phaseolotoxin in this bioassay.

The choice of the *E. coli* indicator was based primarily on its rapid growth rate and its unrelatedness to *P. phaseolicola.* This reduces the chance of artifacts due to bacteriocins which generally inhibit organisms that are closely related to the producer strain. However, other HBT-sensitive organisms can be used as indicators. Strains of *S. typhimurium* were equally sensitive to phaseolotoxin under the conditions described (20).

Hoitink and Sinden (9) reported that HBT does not inhibit *E. coli*. The authors described a minimal-glucose-salts medium as well as complex medium containing yeast extract but did not specify which one was used in their bioassay. In our experience, HBT inhibition of *E. coli* was not observed on complex (KB) medium and was reversed when yeast extract was added to our bioassay medium.

Rudolph reported that HBT inhibited the growth of *Euglena gracilis* (28). In these experiments, citrulline reversed the inhibition, even though relatively high concentrations were needed for complete reversal. Orotic acid also gave partial reversal. In our experiments, orotate did not reverse HBT inhibition of *E. coli* on minimal medium. The halo blight toxin also has been reported to inhibit the growth of bean callus (2). Whether this is due to inhibition of OCT is not known since data on reversal by citrulline or arginine were not reported.

During the course of this investigation we became aware of an independent preliminary report on the inhibition of *E. coli* by toxigenic strains of *P. phaseolicola* (3). Our results confirm these observations and demonstrate that inhibition is specifically related to the presence of phaseolotoxin.

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