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Phaseolotoxin Transport in *Escherichia coli* and *Salmonella typhimurium* via the Oligopeptide Permease

BRIAN J. STASKAWICZ AND NICKOLAS J. PANOPOULOS*

Department of Plant Pathology, University of California, Berkeley, California 94720

Phaseolotoxin [(N^{δ}-phosphosulfamyl)ornithylalanylhomoarginine], a phytotoxic tripeptide produced by *Pseudomonas syringae* pv. *phaseolicola* that inhibits ornithine carbamoyltransferase, is transported into *Escherichia coli* and *Salmonella typhimurium* via the oligopeptide transport system (Opp). Mutants defective in oligopeptide permease (Opp^-) were resistant to phaseolotoxin. Spontaneous phaseolotoxin-resistant mutants (Tox^r) lacked the Opp function as evidenced by their cross-resistance to triornithine and failure to utilize glycylhistidylglycine as a source of histidine. Growth inhibition by phaseolotoxin was prevented by peptides known to be transported via the Opp system and by treatment of the toxin with L-aminopeptidase. In both *E. coli* and *S. typhimurium*, Tox^r mutations were cotransducible with *trp*, suggesting that the *opp* locus occupies similar positions in genetic maps of these bacteria.

Phaseolotoxin [(N^{δ}-phosphosulfamyl)ornithylalanylhomoarginine] (17) is a phytotoxic tripeptide produced by *Pseudomonas syringae* pv. *phaseolicola* (34), the causal agent of the bean halo blight disease. The toxin inhibits the enzyme ornithine carbamoyltransferase (OCTase) from a variety of organisms in vitro (18, 31; A. R. Ferguson and J. S. Johnson, Physiol. Plant Pathol., in press; this laboratory, unpublished data), causes chlorosis and ornithine accumulation in plant leaves (19), and inhibits the growth of plant cells in tissue culture (this laboratory, unpublished data; and Z. R. Sung, unpublished data) and of bacteria (31) in arginine-deficient media. The in vivo effects are OCTase specific, as shown by the ability of citrulline or arginine to reverse or prevent them (31; this laboratory, unpublished data; Sung, unpublished data).

In a previous study (31), we surveyed several bacterial strains to determine those which are most sensitive to inhibition by phaseolotoxin for use as bioassay indicators. Although all *Escherichia coli* K-12 and most *Salmonella typhimurium* LT2 strains were sensitive, several stocks of the latter were resistant. Furthermore, resistant mutants of all sensitive strains were frequently observed within the inhibition zones in the bioassay plates. We have studied these mutants further in hopes that the elucidation of the biochemical and genetic defects might lead to a better understanding of phaseolotoxin-plant cell interactions. In this report, we present genetic and physiological evidence that phaseolotoxin is transported into bacterial cells via the oligopeptide permease (Opp) and that the phaseolotoxin-resistant phenotype is a result of mutations affecting this system. A preliminary report of these studies has been presented elsewhere (23).

MATERIALS AND METHODS

Strains. Bacterial and phage strains are listed in Table 1.

Phaseolotoxin and peptides. A sample of phaseolotoxin was supplied by R. E. Mitchell and contained about 80% phaseolotoxin, 5 to 10% 2-serine-phaseolotoxin (20), and minor amounts of other impurities (R. E. Mitchell, personal communication). Other phaseolotoxin samples were purified in this laboratory by the procedure described previously (17) and modified by us (Staskawicz and Panopoulos, manuscript in preparation) in that the toxin was assayed by the quantitative *E. coli* inhibition test (31). The purity of phaseolotoxin-containing fractions from quaternary aminoethyl-Sephadex and LH-20 columns was examined by thin-layer chromatography-electrophoresis (17); only the fractions containing phaseolotoxin as the major ninhydrin-reactive spot and 2-serine-phaseolotoxin as a minor spot and no traces of other ninhydrin-positive substances were used. (N^{δ}-phosphosulfamyl)ornithine (PSorn) was prepared from phaseolotoxin by L-aminopeptidase (EC 3.4.11.1) as described below and was purified through an LH-20 column by the method of Mitchell (17). Triornithine was purchased from Vega Biochemicals; all other peptides, L-amino acids, adenosine, and vitamins were from Sigma Chemical Co., St. Louis, Mo. Ornithylalanylhomoarginine was a gift from R. E. Mitchell.

Media. Nutrient agar or Luria agar (16) and the minimal salts-glucose (MMG) medium described previously (31) were used as complex agar and defined medium, respectively. Casamino Acids agar contained 2% agar and 1% casein hydrolysate. Shake cultures were grown in nutrient broth, Luria broth, or liquid MMG medium (31). The Casamino Acids and minimal

TABLE 1. *Bacterial strains*[a] *and phage*[b]

Strain	Markers	Source/description
E. coli K-12		
J53-1	*pro met nalA*	J. E. Berringer
JC8630	*trp metB his thi lac rpsL*	A. J. Clark
N100	(Mu) *gal recA rpsL* Mu^r	M. van Montague
SK1592	*gal thi endA hsdM$^+$ hsdR*	S. Kushner
1556-026	(P1 *clm clr*100) *purE*::Tn*5*	D. Berg (16)
S. typhimurium LT2		
GW45	*hisG46*	P. Hartman via G. F. Ames (1, 33)
TA2872	*hisG46 opp-7*	G. F. Ames (1)
TA2874	*hisG46 opp-10*	G. F. Ames (1)
PM786	*trpE747 araB9 gal-205*[c]	P. Margolin through G. F. Ames
TYP10	*trpA109*	M. Demerec through G. F. Ames
Phage		
P1 *clm clr*100		Prepared by heat induction from strain 1556-026 (16)
P22HT105/1 *int*201		J. Roth through G. F. Ames (2, 6)

[a] TA2872 and TA2874 had been isolated from GW45 by selecting for resistance to tri-lysine and norleucylglycylglycine, respectively (1), and were resistant to phaseolotoxin (Tox^r). All other strains were phaseolotoxin sensitive (Tox^s) and, based on the results of this study, are considered to be *opp*$^+$. TYP numbers were assigned by us. Allele designations for *trp* genes in *Salmonella* are as previously described (29).

[b] The properties of P1 *clm clr*100 are described in reference 16, and those of P22HT105/1 *int*201 are described in references 2, 6, and 30 (see also reference 32). *clm*, Chloramphenicol resistance; *clr*, clear plaques (at 42°C); HT, increased frequency of generalized transduction; *int*, integration deficient (inability to form stable lysogens).

[c] The *gal-205* mutation has not been assigned to a specific *gal* cistron. PM786 is insensitive to galactose and sensitive to P22HT105/1 *int*201 in the absence of exogenous galactose.

media were supplemented with 0.5% glucose, autoclaved separately, and, when required, supplemented with 20 μg of an amino acid or adenosine or 1 μg of thiamine per ml (filter sterilized).

Transductional methods. Phage P22 containing the mutation HT105/1 (30), which causes an increased frequency of generalized transduction, and the mutation *int*201 (2), which prevents stable lysogen formation, was used for transductions in *S. typhimurium* (6). Transducing lysates were obtained by infection of logarithmically growing cultures with phage at a multiplicity of 0.05 plaque-forming unit per cell in nutrient broth. After 160 min of incubation at 37°C with shaking, the culture was centrifuged at 8,000 × *g* for 10 min, and the supernatant fluid was collected and stored over chloroform at 5°C. Transductions in *E. coli* were performed with phage P1 *cml clr*100 (16). Lysogens, prepared by selecting for resistance to chloramphenicol at 30°C, were tested for temperature sensitivity at 40°C (16). P1 *cml clr*100 lysates were obtained by heat induction of such lysogens and titrated as described previously (16). For transduction, phage was added to 1 ml of mid-log-phase cultures at a multiplicity of 0.1 plaque-forming unit per cell in the presence of 100 mM Mg^{2+} and 5 mM Ca^{2+} (16). Transductants were selected for tryptophan independence (Trp$^+$) by plating on Casamino Acids medium supplemented with thiamine, when required, at 30°C. Between 90 and 170 transductants from each experiment were purified on Casamino Acids medium and tested for growth on MMG agar containing phaseolotoxin (50 μg/ml) and any required growth factors.

Isolation of mutants and peptide utilization tests. Mutants resistant to phaseolotoxin (Tox^r) and triornithine (Tor^r) were obtained by streaking a loopful of cells on MMG agar, supplemented with required growth factors, and the respective compound was supplied on a filter paper disk. Resistant colonies were purified and retested for resistance to the inhibitor used in their selection and for cross-resistance as above. Utilization of peptides was tested by standard methods (16).

Aminopeptidase treatment. Phaseolotoxin (1 mg in 1 ml of 0.25 M NH_4HCO_3 buffer, pH 8) was treated with L-aminopeptidase (chromatographically purified type V from porcine kidney, Sigma Chemical Co.) at 40°C as described by Mitchell (17). Samples (3 μl) were removed periodically and analyzed by thin-layer chromatography on MN300 cellulose plates (Brinkmann Instruments Inc., Westbury, N.Y.). Inhibitory titers against *E. coli* N100 also were determined by the standard bioassay (20-μl samples) as described previously (31). Inhibition zones were measured after incubation for 2 h at 37°C.

Peptide competition studies. Mixtures of phaseolotoxin and various peptides were prepared and added, in 20-μl volumes, to 5-mm-diameter agar wells in bioassay plates overlaid with strain N100 as described previously (31).

RESULTS

Phaseolotoxin transport system: general considerations. Two possible mechanisms for resistance to phaseolotoxin were considered: (i) mutational inactivation of a transport system and (ii) structural alteration in the OCTase enzyme leading to a reduced binding affinity for the phaseolotoxin molecule. A transport system was considered necessary for toxicity against bacteria in vivo becasue of the highly charged nature, which would prevent free passage across the cell membrane (9), and the high potency of the toxin against *E. coli* (detection threshold, 0.02 pmol) (31), compared with the concentrations required for a 50% inhibition of OCTase in vitro (0.3 μM) (20; Ferguson and Johnson, in press). The tripeptide structure of the toxin further suggested that an oligopeptide permease was a likely transport system. The structure of phaseolotoxin (Fig. 1) fulfills the specific require-

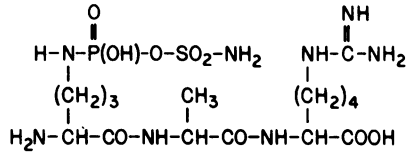

FIG. 1. *Structure of phaseolotoxin (17).*

ments of oligopeptide transport systems, namely, a protonated α-amino group and size (566 daltons) not exceeding that of pentalysine (25) or the molecular exclusion limit of the outer membrane of enteric bacteria (7).

Insensitivity of Opp$^-$ *S. typhimurium* mutants to phaseolotoxin. *S. typhimurium* has an oligopeptide permease capable of transporting several tripeptides, among them glycylhistidylglycine (Gly-His-Gly) (1). Initially we tested strain GW45 and its two *opp* derivatives, TA2872 and TA2874, for sensitivity to phaseolotoxin and for ability to grow in the presence of Gly-His-Gly as a source of histidine. Only GW45 was sensitive to the toxin and responded positively to the tripeptide although all three responded normally to histidine (Table 2). The inability of TA2872 and TA2874 to utilize Gly-His-Gly as a substitute source for histidine is an indication of their Opp$^-$ phenotype (1). The minimal inhibitory concentration of phaseolotoxin for strain GW45 is 10 to 20 pg when applied directly on the agar overlay, which is similar to that for our standard indicator *E. coli* N100 (31). The level of resistance conferred by the *opp* mutations in TA2872 and TA2874 is at least 50 μg of toxin per well. The above results suggested that Gly-His-Gly and phaseolotoxin share a common transport system in *S. typhimurium*. This system is also capable of transporting triornithine as evidenced by the ability of this peptide to inhibit GW45 but not TA2872 or TA2874 (Table 2).

Isolation and characterization of phaseolotoxin- and triornithine-resistant mutants. Several phaseolotoxin-resistant (Tox^r) and triornithine-resistant (Tor^r) mutants of GW45 were isolated and tested for their cross-resistance to these compounds and for their ability to grow on Gly-His-Gly as a source of histidine. In all cases these mutants were cross-resistant to triornithine and phaseolotoxin and failed to respond to Gly-His-Gly, although they responded normally to histidine (Table 2). Therefore, these results support the above conclusion that the Opp system of *S. typhimurium* mediates the uptake of phaseolotoxin.

Tox^r and Tor^r mutants were also isolated in several *E. coli* strains. As with *Salmonella*, *E. coli* strains sensitive to triornithine were also sensitive to phaseolotoxin, and all Tox^r mutants isolated were also Tor^r and vice versa (Table 2). Since triornithine is transported in *E. coli*, as in *S. typhimurium*, via the Opp system (4, 5, 22, 25, 26), our findings suggest that this system mediates the transport of phaseolotoxin in *E. coli*.

E. coli has, in addition to a general oligopeptide permease (Opp) analogous to that of *S. typhimurium*, a second more specialized system (Opr) for the uptake of methionine-containing peptides, trileucine, trivaline, and trithreonine (5, 22). This system does not transport triornithine (4, 5, 22, 25, 26). However, its involvement in phaseolotoxin transport is not clear at present. The Opp and Opr systems are physiologically and genetically distinct (5, 28). Nevertheless, common regulatory gene mutations affecting both systems in our mutants cannot be excluded. Tox^r and Tor^r mutants of methionine-requiring strains J53-1 and JC8630 were cross-resistant to triornithine and phaseolotoxin, respectively, and responded normally to methionylalanylserine. In JC8630, such mutants failed to utilize Gly-His-Gly as a source of histidine, as expected of Opp$^-$ mutants (1; see above). However, their ability to utilize methionylalanylserine as a source of methionine cannot be safely taken as an indicator that these mutants had a functional Opr system because some methionine-contain-

TABLE 2. *Sensitivity to phaseolotoxin and triornithine and utilization of peptides by S. typhimurium and E. coli strains*[a]

Strain	No. of mutants studied	Sensitivity to:		Utilization of:	
		Phaseolotoxin	Triornithine	Gly-His-Gly	Met-Ala-Ser[b]
TA2872		r	r	−	
TA2874		r	r	−	
GW45		s	s	+	
GW45 Tor^r	10	r	r	−	
GW45 Tor^r	5	r	r	−	
PM786		s	s		
PM786 Tox^r	5	r	r		
JC8630		s	s	+	+
JC8630 Tox^r	7	r	r	−	+
J53-1		s	s		+
J53-1 Tox^r	12	r	r		+
J53-1 Tor^r	4	r	r		+
N100		s	s		
N100 Tox^r	10	r	r		
N100 Tor^r	12	r	r		
SK1592		s	s		
SK1592 Tox^r	8	r	r		
SK1592 Tor^r	13	r	r		
1556-026		s	s		
1556-026 Tox^r	6	r	r		

[a] Tox^r and Tor^r indicate resistance to phaseolotoxin and triornithine, respectively. Sensitivity or resistance to these compounds and utilization of the peptides were determined as described in the text. r, s, +, and − indicate resistance, sensitivity, growth, and no growth response, respectively.

[b] Met-Ala-Ser, Methionylalanylserine.

ing peptides are reported to compete for the uptake of, and presumably share a common uptake system with, methionine (13, 14). Thus, the above results do not permit a decision as to whether the Opr system of *E. coli* is capable of mediating the transport of phaseolotoxin.

Peptide competition experiments and L-aminopeptidase treatment. The involvement of a peptide transport system in the uptake of phaseolotoxin was further examined by competition experiments with several tripeptides and by treatment of the toxin with L-aminopeptidase. Triglycine, triserine, alanylglycylglycine, methionylalanylserine, and ornithylalanylhomoarginine all prevented inhibition of *E. coli* N100 by phaseolotoxin. Quantitative experiments with the first three peptides (Table 3) showed that high peptide/toxin molar ratios were required for a complete prevention of toxicity. At molar ratios of 250 or lower, an apparent potentiation of inhibition by phaseolotoxin resulted. The reason for the high molar ratio requirement is not known but may be related to the physiological and kinetic parameters determining toxin-OCTase interaction. The apparent potentiation of toxicity at low molar ratios is presumed to be a result of either increased rates of toxin diffusion in the presence of high concentrations of solutes in the agar wells and matrix or hyperinduction of the Opp system in the presence of the added peptides.

Treatment of phaseolotoxin with aminopeptidase resulted in a drastic reduction of its inhibitory titer which paralleled the disappearance of the corresponding spot on the thin-layer chromatogram in the initial period (spot was not visible after 30 min) and the appearance of three new compounds with R_f values characteristic of PSorn, alanine, and homoarginine. The residual inhibitory activity in the treated samples was not observed when these were bioassayed with Opp^- indicator strains, suggesting that it was due to undegraded phaseolotoxin. This conclusion is further supported by the lack of inhibitory activity associated with the PSorn, alanine, or homoarginine spots from the thin-layer plates against either Opp^+ or Opp^- indicator strains, whereas such activity was easily detected in the phaseolotoxin spot.

Transductional mapping of Tox^r mutations in *E. coli* and *S. typhimurium*. We mapped the mutations leading to phaseolotoxin resistance in *E. coli*, using P1-mediated transduction, and in *S. typhimurium*, using P22-mediated transduction. The *opp* locus had been mapped at 34 min on the *E. coli* linkage map between *trp* and *cysB* (3, 4). To our knowledge, neither the genes encoding for the Opr permease of *E. coli* nor those encoding for the Opp system of *S. typhimurium* have been genetically mapped (5, 22, 29). The chromosomal segment between 25.5 and 35.5 map units on the *S. typhimurium* linkage map, which carries the *trp-cysB* region, is inverted with respect to its homologous region on the *E. coli* chromosome, but the *trp* and *cysB* genes lie within the inversion (3, 29). This suggested that the *opp* locus and Tox^r mutations in *S. typhimurium* should also map near *trp*. The results of Table 4 bear out this prediction. The cotransduction frequency between *trp* and *opp-7* in *S. typhimurium* was 32%, whereas that for two independent Tox^r mutations was 28 and 34%. The linkage value for two Tox^r mutations and *trp* in *E. coli* (85 to 93% [Table 4]) is similar to that reported previously for *opp* and *trp* (4). Thus, the mapping data support the earlier conclusion regarding the role of the Opp permease in phaseolotoxin transport.

DISCUSSION

The data presented show that the general

TABLE 3. *Effect of tripeptides on the inhibitory activity of phaseotoxin against E. coli N100*[a]

Peptide[b]	Phaseolotoxin (μg/well)	Peptide/phaseolotoxin molar ratio	Diam of inhibition zone (mm)[c]	Decrease (%) in inhibitory titer[d]
None	10		18.5	
Gly-Gly-Gly	10	1.4×10^3	5.5	99
	10	2.8×10^3	3.5	99.5
	10	1.4×10^4	0	100
Ala-Gly-Gly	5	2.6×10^3	0	100
Ser-Ser-Ser	5	4.0×10^3	0	100

[a] Ten microliters of a phaseolotoxin solution (17 μM) was mixed with equal volumes of peptide solutions, and the mixtures were placed in 5-mm-diameter wells in bioassay agar plates (31; see the text).

[b] Gly-Gly-Gly, Triglycine, Ala-Gly-Gly, alanylglycylglycine; Ser-Ser-Ser, triserine.

[c] Total diameter of zone minus diameter of the agar well (5 mm).

[d] Estimated from a standard (log linear phaseolotoxin dose-response plot (31).

TABLE 4. *Transductional mapping of Tox^r mutation in S. typhimurium and E. coli*

Phage grown on:	Recipient	Trp^+ transductants expressing donor Tox phenotype[a] (%)
GW45 Tox-1	PM786[b]	28
GW45	PM786 Tox-1[b]	34
TA2872	TYP10	32
1556-026	JC8630 Tox-1	93
1556-026 Tox-1	JC8630	85

[a] Trp^+ transductants were selected on Casamino Acids agar plates, purified on Luria agar, and streaked on MMG agar plates containing 50 μg of phaseolotoxin per ml. Amino acids or thiamine required by the recipient parent was added as indicated in the text.

[b] See Table 1, footnote *c*.

oligopeptide permease system (Opp) of *E. coli* and its analogous system in *S. typhimurium* mediate the uptake of phaseolotoxin. Since all Tox^r mutants studied were phenotypically Opp$^-$, no tests were performed regarding the in vitro sensitivity of their OCTases. Mutations resulting in a toxin-insensitive OCTase, if possible, may be more rare than those causing the Opp$^-$ phenotype, which are known to occur with high frequency in *E. coli* (4). Our results further show that the *opp* locus in *S. typhimurium*, which has not been previously mapped (29), also maps near *trp*, as in *E. coli*.

The transport of phaseolotoxin via the bacterial Opp system is another example of "illicit transport" (1, 9) of a natural antimetabolite via a peptide permease. Three other instances involving oligopeptide antibiotics were studied recently by Diddens et al. (8) and by Kening and Abraham (15). In the present case, several lines of evidence suggest that the chief, if not the sole, function of the peptide backbone of phaseolotoxin is to facilitate transmembrane passage. PSorn, which is inactive against *E. coli* in vivo, is equally as or more active against OCTase in vitro as phaseolotoxin (our unpublished data). Its highly charged nature would be a barrier to its transport across membranes in the absence of an uptake system (9). At present we have no direct evidence that an uptake system capable of transporting PSorn is absent in *E. coli*. Since the compound is structurally similar to ornithine, we considered the possibility that it may be transported via the ornithine-specific or the general permease for basic amino acids (28). We found (unpublished data) that an ornithine-responding Arg$^-$ strain (C-3, from R. Calendar) was sensitive to phaseolotoxin but insensitive to PSorn (10 μg/well), suggesting that Psorn and ornithine do not share the transport system(s) present in this strain. It is possible that the acidic nature of PSorn precludes its passage through basic amino acid permeases.

In a recent study, Penninckx and Gigot (27) reported that *N*-δ-(phosphonoacetyl)-L-ornithine (PALO), a chemically synthesized OCTase inhibitor (12, 21), was nontoxic to *E. coli* cells but that its tripeptide form, PALO-Gly-Gly, was highly toxic. As with phaseolotoxin, the toxicity of PALO-Gly-Gly was *opp* dependent. These results further support our conclusions concerning the role of the peptide backbone of phaseolotoxin in transmembrane passage.

The antibacterial properties of phaseolotoxin against *E. coli* and *S. typhimurium* are probably incidental rather than essential to the biology of *P. syringae* pv. *phaseolicola* since the organisms do not share common habitats in nature. Although the toxin inhibits other microorganisms, it is inactive against many *Pseudomonas* spp. and yellow-pigmented bacteria found in naturally infected bean seed (31; our unpublished data). Furthermore, *P. syringae* pv. *phaseolicola* is not well adapted for survival in soil where microbial competition would be strong and arginine and citrulline (which reverse the effect of phaseolotoxin) or high concentrations of peptides (which prevent phaseolotoxin uptake) would be unavailable. More likely, the toxin-derived advantages are mainly associated with the biology of *P. syringae* pv. *phaseolicola* as a plant pathogen. Toxigenicity is a secondary, although not a primary, determinant of pathogenicity in the halo blight disease affecting the ability of this pathogen to cause leaf chlorosis and to invade the plant systemically (24) and, thereby, to infect the seed internally. Furthermore, peptide uptake systems in plants have been reported (10, 11) and are presumed to play a useful role in plant development. Preliminary experiments indicate that phaseolotoxin is transported into plant cells via a peptide permease (this laboratory, unpublished data; Sung, unpublished data). We speculate, therefore, that the peptide structure of this toxin has evolved as a means of securing or maximizing the biological potency of PSorn in planta.

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