SUPPLEMENTAL MATERIAL

Expression of a peptidoglycan hydrolase from lytic bacteriophages Atu_ph02 and Atu_ph03 triggers lysis of *Agrobacterium tumefaciens*

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Running Head: Lytic bacteriophages and lysis proteins impact *A. tumefaciens* growth

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SUPPLEMENTAL METHODS

Clonal isolation of bacteriophage strains. Water samples were filtered by passing through a 0.45 μm membrane (Millipore Ultrafree – CL, Low-binding Durapore PVDP membrane) and 890 μl of filtrate was mixed with 100 μl 10X LB and 10 μl *A. tumefaciens* C58 at a starting OD_{600} of ~0.2. Cultures were incubated at 28°C in LB broth for 16 h while shaking. Cultures that appeared clear were screened for plaque formation. If the culture was turbid, supernatants were collected by centrifugation at 3,000 x g for 10 min and 100 μl filtrate were mixed with 100 μl bacteria (OD_{600} ~0.2) for another round of amplification. If cultures remained turbid after 5 rounds of amplification, the filtrate was considered to be negative for lytic activity. Filtrates that caused clearing of the bacterial culture within 5 rounds of amplification were examined for evidence of phage activity. Filtrates were screened for phage activity using a spot assay for detection of plaques. Whole plate plaque assays were performed using filtrates which produced plaques in the spot assay. Individual plaques were suspended in Dulbecco's phosphate-buffered saline (DPBS, Mediatech, Inc., Manassas, VA, USA) with gelatin added (1:20). Three rounds of purification comprised of selecting individual plaques after whole plate plaque assays were completed for each bacteriophage to ensure homogenous bacteriophage populations.

Concentration and partial purification of virions. Concentrated phage stocks were produced by polyethylene glycol (PEG) precipitation. For PEG precipitation, filtered lysates were scaled up to 1 L and centrifuged at 11,000 x g for 20 min at 4°C to remove bacterial cells. To the supernatants, 400 μl 10 mg/ml RNase A (Sigma) and 1 ml 3.45 mg/ml DNase I (Sigma) were added for removal of bacterial genomic DNA and RNAs.

After 1 h of stirring at room temperature, NaCl (final concentration of 500 mM) and 10% w/w PEG 8000 (Fisher) were added and the solution was stirred for 2 h until dissolved. Bacteriophages were precipitated for 16 h at 4°C. Precipitated bacteriophages were collected by centrifugation at 11,000 x g for 30 min at 4°C and resuspended in 30 ml DPBS. The bacteriophage solution was incubated with shaking overnight at 4°C. Insoluble material was removed and the supernatant was recovered. NaCl (final concentration 0.5M) and PEG 8000 (10% w/w) were added to the supernatant and the solution rotated for 2 h at 4°C. Bacteriophages were precipitated, collected by centrifuging for 20 min at 17,000 x g at 4°C, and resuspended 8 ml DPBS. The bacteriophage solution was rotated overnight at 4°C. This viscous solution was centrifuged at 17,000 x g for 2 min at 4°C and the supernatant was collected. The bacteriophage solution was mixed with 300 µl 10 mM phenol red and 22 ml DPBS and centrifuged at 17,000 x g for 30 min and the supernatant was collected. The supernatant was overlayed with a 2 ml sucrose (5% w/w) cushion and ultracentrifuged at 141,000 x g for 2 h at 4°C. The supernatant was removed and the pellet was washed in DPBS and dissolved in 1 ml DPBS. All phage stocks were stored at 4°C.

Preparation of virion DNA. Two 500-µl portions of partially purified virions in 1.5-ml microtubes were extracted twice with 500 µl neutralized phenol (water-saturated phenol shaken twice with 1/10 vol 1 M Tris.HCl pH 8, discarding the upper phase each time) and once with 500 µl chloroform (a 24:1 mixture of chloroform and isoamyl alcohol), each time discarding the organic (lower) phases. To the final aqueous phases were added 40 µl 3 M sodium acetate, pH adjusted to 6 with acetic acid, and 1 ml ethanol; precipitates

were pelleted by a 10-min centrifugation in a microfuge; supernatants were aspirated; pellets were gently washed by adding 1 ml freezer-cold 70% v/v ethanol and aspirating the liquid; pellets were air-dried, dissolved in 100 µl TE (10 mM Tris.HCl pH 7.5, 1 mM Na_2EDTA), and centrifuged 10 min in a microfuge to clear insoluble material; supernatants were pooled and stored at -20°C.

DNA restriction analysis. Phage genomic DNA was digested with restriction endonucleases from New England Biolabs using the standard protocol. All reactions contained 2.5 µg DNA and were incubated at 37°C for 2 h. Restriction patterns were analyzed on a 0.7% agarose gel, which ran at 100 V for 1 h and was stained with SYBR Safe DNA Gel Stain (Thermo Scientific).

SUPPLEMENTAL TABLES

Table S1. Comparison of gene products encoded in Atu_ph02 and Atu_ph03

Atu_ph03		Atu_ph02		
gene product	length in AA[a]	gene product	length in AA[a]	percent identity[b]
gp1	41	gp1	41	72.5
gp2	173	gp2	173	100
gp3	336	gp3	336	100
gp4	415	gp4	409	99.26
gp5	134	gp5	134	100
gp6	449	gp6	449	100
gp7	68	gp7	68	83.58
gp8	39	gp8	163	77.78
gp9	123	gp8	163	92.62
gp10	486	gp9	486	99.79
gp11	224	gp10	210	69.78
gp12	55			
gp13	331	gp11	337	93.75
gp14	180	gp12	181	88.27
gp15	786	gp13	787	98.09
gp16	291	gp14	291	100
gp17	77	gp15	77	100
gp18	38	gp16	38	100
gp19	317	gp17	317	98.73
gp20	61	gp18	61	100
gp21	77	gp19	77	96.05
gp22	128	gp20	128	100
gp23	816	gp21	816	99.88
gp24	57	gp22	57	100
gp25	66	gp23	66	100
gp26	155	gp24	155	100
gp27	88			
gp28	68	gp25	68	100
gp29	533	gp26	533	99.62
gp30	296	gp27	296	99.66

Atu_ph03		Atu_ph02		
gene product	length in AA[a]	gene product	length in AA[a]	percent identity[b]
gp31	327	gp28	327	100
gp32	212	gp29	212	99.53
gp33	823	gp30	823	99.64
gp34	169	gp31	169	99.4
gp35	1192	gp32	1192	99.83
gp36	1255	gp33	1255	99.68
gp37	507	gp34	507	98.62
gp38	181	gp35	181	98.33
gp39	59	gp36	59	100
gp40	107	gp37	107	100
gp41	612	gp38	623	100
gp42	122	gp39	122	100
gp43	91	gp40	91	100
gp44	149	gp41	149	99.32
gp45	64	gp42	64	100
gp46	289	gp43	289	98.96
gp47	47	gp44	47	94.59
gp48	222	gp45	222	98.19
gp49	124	gp46	124	100
gp50	61	gp47	60	93.22
		gp48	104	
gp51	65	gp49	65	85.94
gp52	88	gp50	88	49.33
gp53	50			
gp54	89	gp51	105	59.00
gp55	107	gp52	107	93.40
gp56	78	gp53	78	98.70
gp57	91	gp54	95	100
gp58	40	gp55	40	100

[a]Length of each gene product is given in amino acids (AA)
[b]Percent identites were determined by blastx analysis using each predicted ORF in Atu_ph03 as query against the protein database for Atu_ph02

Table S2. Similarity of putative Atu_ph03 proteins to proteins in select bacteriophages

		Similarity of putative proteins in select bacteriophages E-value (percent identity)[a]						
Atu_ph03		Rhe_phe2	Rhe_phe8	MedPE-SWcel-C56	T7	phiKMV	T5	Putative Function[b]
gene product	length in AA							
gp1	41	-	-	-	-	-	-	ORFan
gp2	173	gp014 $8e^{-10}$ (35%)	gp013 $2e^{-09}$ (34%)	-	-	-	-	Hypothetical protein
gp3	336	-	-	-	-	-	-	Hypothetical peptidoglycan binding protein (PPH)
gp4	415	-	-	-	-	-	-	DNA primase
gp5	134	gp020 $8e^{-24}$ (42%)	gp020 $9e^{-24}$ (42%)	gp17 $5e^{-08}$ (28%)	-	-	-	DNA primase
gp6	449	gp021 $2e^{-141}$ (49%)	gp021 $2e^{-141}$ (49%)	gp19 $1e^{-124}$ (43%)	-	gp15 $7e^{-59}$ (33%)	-	DNA helicase
gp7	68	-	-	-	-	-	-	ORFan
gp8	39	-	-	-	-	-	-	ORFan
gp9	123	-	-	-	-	-	-	ORFan
gp10	486	gp022 $3e^{-159}$ (49%)	gp022 $3e^{-159}$ (48%)	gp20 $6e^{-119}$ (43%)	-	-	gp004 $7e^{-103}$ (38%)	A1 protein
gp11	224	-	-	-	-	-	-	Hypothetical protein
gp12	55	-	-	-	-	-	-	ORFan
gp13	331	gp026 $2e^{46}$ (35%)	gp026 $2e^{-46}$ (34%)	gp23 $3e^{-21}$ (28%)	-	-	-	ATP-dependent DNA ligase
gp14	180	-	-	-	-	-	-	ORFan
gp15	786	gp028 0.0 (52%)	gp028 0.0 (52%)	gp26 0.0 (49%)	-	gp 19 $5e^{-114}$ (32%)	-	DNA-directed DNA polymerase
gp16	291	gp029 $9e^{-91}$ (52%)	gp029 $8e^{-92}$ (53%)	gp28 $3e^{-39}$ (35%)	-	gp21 $3e^{-27}$ (33%)	-	Hypothetical protein
gp17	77	-	-	-	-	-	-	ORFan
gp18	38	-	-	-	-	-	-	ORFan
gp19	317	gp031 $2e^{-125}$ (56%)	gp031 $2e^{-125}$ (56%)	gp29 $3e^{-76}$ (41%)	-	gp22 $6e^{-28}$ (32%)	-	5'-3' exonuclease
gp20	61	-	-	-	-	-	-	ORFan
gp21	77	-	-	-	-	-	-	ORFan
gp22	128	gp034 $2e^{-20}$ (42%)	gp034 $1e^{-26}$ (42%)	gp32 $1e^{-25}$ (37%)	-	gp23 $2e^{-16}$ (38%)	-	Recombination endonuclease VII
gp23	816	gp036 0.0 (49%)	gp037 0.0 (48%)	gp33 0.0 (42%)	gp1 $3e^{-123}$ (49%)	gp26 $1e^{-96}$ (29%)	-	T7-like RNA polymerase
gp24	57	gp037 $4e^{-15}$ (60%)	gp038 $3e^{-13}$ (60%)	-	-	-	-	Hypothetical protein

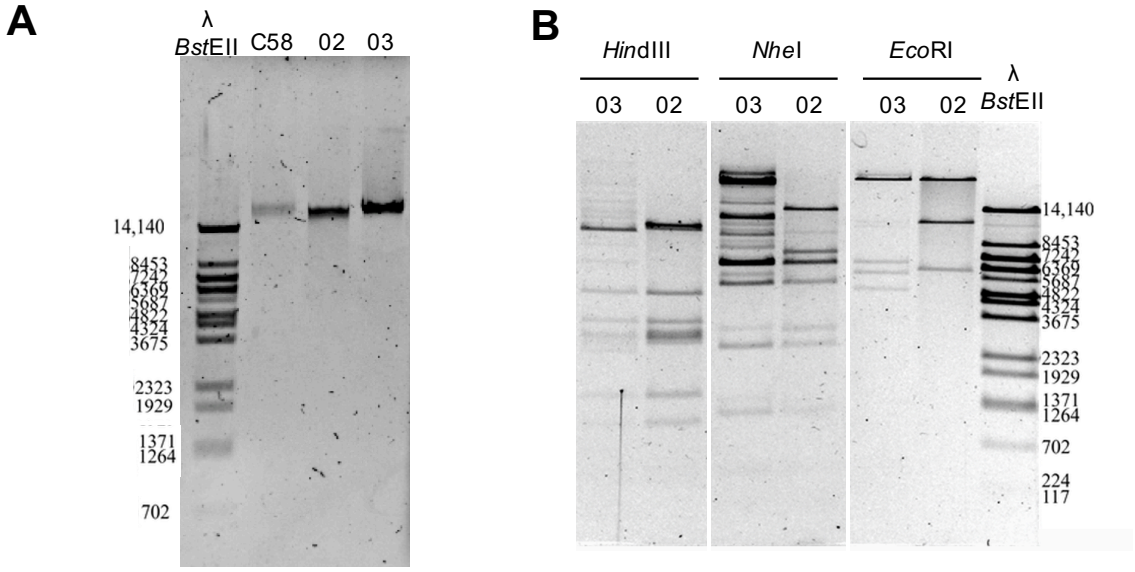
gp25	66	-	-	-	-	-	-	Hypothetical protein
gp26	155	-	-	-	-	-	-	N-acetyltransferase
gp27	88	-	-	-	-	-	-	ORFan
gp28	68	-	-	-	-	-	-	ORFan
gp29	533	gp043 $1e^{-173}$ (50%)	gp042 $9e^{-176}$ (48%)	gp37 $9e^{-104}$ (36%)	gp8 $8e^{-55}$ (29%)	gp30 $3e^{-37}$ (29%)	-	Tail-head connector protein
gp30	296	gp043 $1e^{-41}$ (38%)	gp044 $1e^{-40}$ (38%)	gp38 $8e^{-19}$ (31%)	-	gp31 0.001 (30%)	-	Capsid assembly protein
gp31	327	gp044 $7e^{-154}$ (66%)	gp045 $2e^{-153}$ (66%)	gp39 $8e^{-123}$ (53%)	-	gp32 $3e^{-33}$ (27%)	-	Major capsid protein
gp32	212	gp045 $5e^{-60}$ (43%)	gp046 $5e^{-60}$ (43%)	-	-	-	-	Tail tubular protein A
gp33	823	gp046 0.0 (44%)	gp047 0.0 (44%)	gp41 $9e^{-92}$ (29%)	gp12 $5e^{-62}$ (27%)	gp34 $1e^{-46}$ (28%)	-	Tail tubular protein B
gp34	169	gp047 $4e^{-18}$ (40%)	gp048 $4e^{-18}$ (40%)	-	-	-	-	Internal virion protein
gp35	1192	gp048 $1e^{-68}$ (43%)	gp049 $1e^{-68}$ (43%)	-	-	-	-	Cell wall hydrolase; M15 peptidase
gp36	1255	gp049 0.0 (33%)	gp050 0.0 (33%)	gp44 $5e^{-78}$ (27%)	-	gp37 $1e^{-19}$ (24%)	-	Internal virion protein
gp37	507	gp050 $3e^{-29}$ (46%)	gp051 $2e^{-28}$ (46%)	gp45 $1e^{-19}$ (41%)	-	-	-	Tail fiber protein
gp38	181	-	-	-	-	-	-	Hypothetical protein
gp39	59	gp052 $3e^{-13}$ (41%)	gp053 $3e^{-12}$ (36%)	-	-	-	-	Hypothetical protein
gp40	107	gp053 $2e^{-20}$ (41%)	-	gp47 $1e^{-09}$ (33%)	-	gp42 $5e^{-05}$ (33%)	-	Terminase, small subunit
gp41	612	gp054 0.0 (65%)	gp055 0.0 (64%)	gp48 0/0 (58%)	gp19 $4e^{-87}$ (35%)	gp43 $1e^{-132}$ (40%)	-	Terminase, large subunit
gp42	122	gp055 $1e^{-14}$ (37%)	gp056 $1e^{-14}$ (37%)	-	-	-	-	Hypothetical protein
gp43	91	-	-	-	-	-	-	ORFan
gp44	149	-	-	-	-	-	-	ORFan
gp45	64	-	-	-	-	-	-	ORFan
gp46	289	-	-	-	-	-	-	Hypothetical protein
gp47	47	-	-	-	-	-	-	ORFan
gp48	222	gp006 $1e^{-25}$ (34%)	gp004 $6e^{-26}$ (35%)	-	-	-	-	Hypothetical protein
gp49	124	-	-	-	-	-	-	ORFan
gp50	61	-	-	-	-	-	-	ORFan

gp51	65	-	-	-	-	-	-	ORFan
gp52	88	-	-	-	-	-	-	ORFan
gp53	50	-	-	-	-	-	-	ORFan
gp54	89	-	-	-	-	-	-	ORFan
gp55	107	-	-	-	-	-	-	ORFan
gp56	78	-	-	-	-	-	-	ORFan
gp57	91	-	-	-	-	-	-	ORFan
gp58	40	-	-	-	-	-	-	ORFan

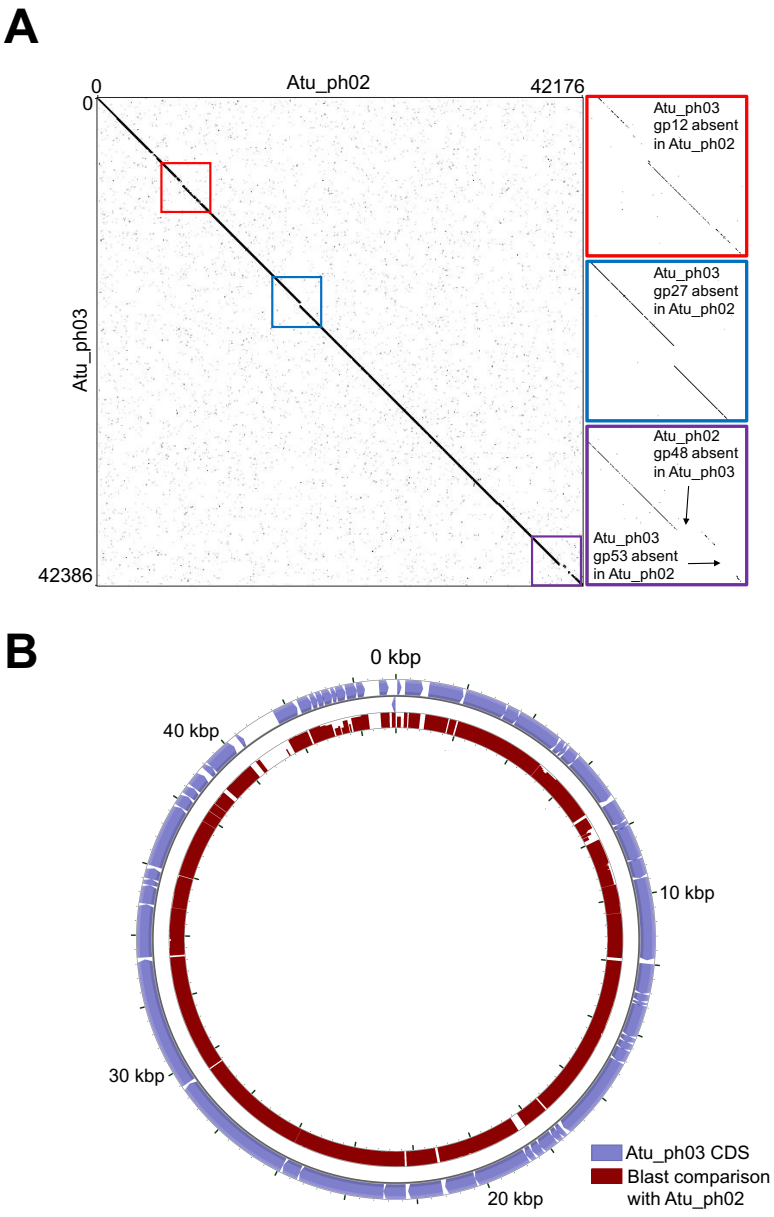
[a]E-values and percent identity were determined by blastp analysis using each predicted ORF in Atu_ph03 as query against the protein databases for Rhizobium phage RHEph02 (taxid:1220602), Rhizobium phage RHEph08 (taxid:1220715), Phage MedPE-SWcel-C56 (taxid: 1871314), Bacteriophage T7 (taxid: 10760), Bacteriophage phiKMV (taxid: 204270) and Bacteriophage T5 (taxid: 10726). – indicates that a significant hit was not detected in the pairwise comparison.

[b] ORFans are predicted proteins that do not have significant hits in the nr database (1). Hypothetical proteins share homology with proteins in the nr database.

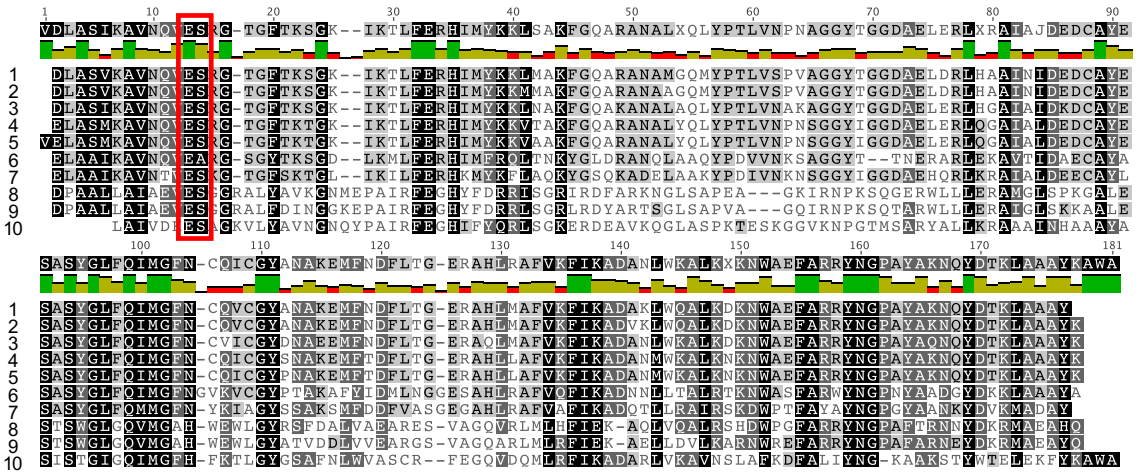
SUPPLEMENTAL FIGURES



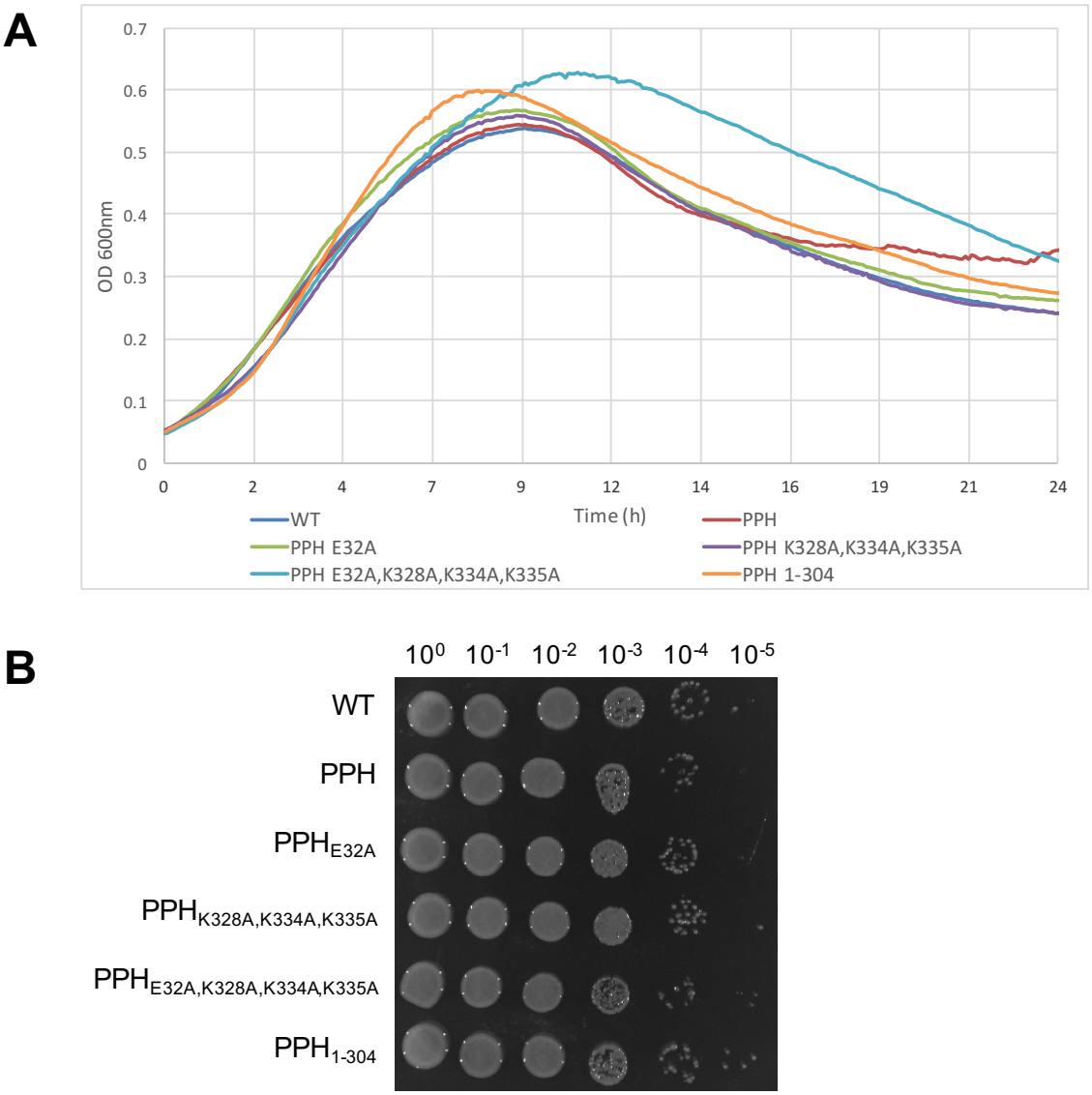
Supplemental Figure S1. Initial characterization of phage genomic DNA shows Atu_ph02 and Atu_ph03 are distinct. (A) Agarose gel containing undigested genomic DNA extracted from *A. tumefaciens* strain C58, phage Atu_ph02 (02), and phage Atu_ph03 (03). (B) Restriction fragment pattern analysis of Atu_ph02 (02) and Atu_ph03 (03) genomic DNA digested with *Eco*RI, *Nhe*I, and *Hin*dIII.



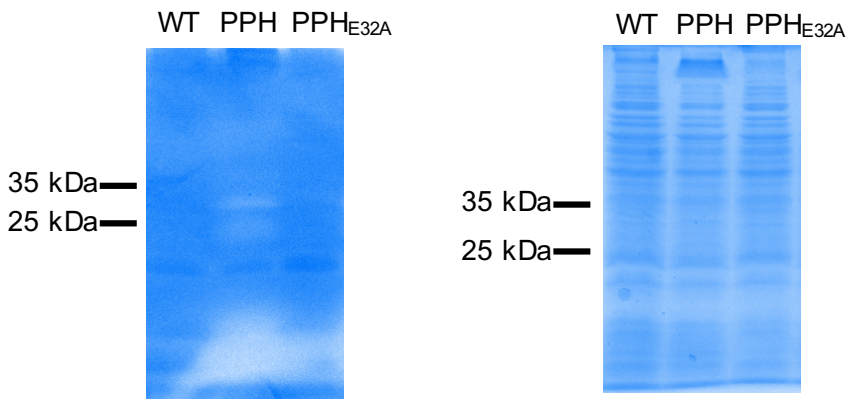
Supplemental Figure S2. Phage Atu_ph03 and Atu_ph02 are very similar. (A) Dot plot analysis comparing the nucleotide sequences of phages Atu_ph03 and Atu_ph02 genomes. Insets highlight areas of difference. (B) Blast analysis of the protein sequences encoded in Atu_ph02 and Atu_ph03. The CDS of Atu_ph03 (blue arrows) and the Blast comparison with Atu_ph02 (maroon) are shown.



Supplemental Figure S3. Clustal alignment of DUF3380 domains from various phage proteins with similarity to the DUF3380 from *Salmonella* phage 10 endolysin and bacterial PG-binding proteins with similarity to the DUF3380 found in PPH. Conserved ES residues are present in all sequences and shown in a red box. Consensus identity for the sequence is mapped along the top of the alignment. Green = 100% identical, gold = 30-100%, red = <30%, no color = 0%. (1) *Dickeya* virus Limestone putative endolysin YP_007237392.1 (aa 90-260), (2) *Shigella* phage Ag3 hypothetical protein YP_003358573.1 (aa 90-261), (3) *Klebsiella* phage 0507-KN2-1 phage-encoded PG binding protein YP_008531963.1 (aa 90-261), (4) *Salmonella* phage 10 endolysin gp110 ANK36008.1 (aa 90-261), (5) *Salmonella* phage Vil phage encoded PG-binding protein YP_004327457.1 (aa 90-261), (6) *Serratia* phage phiMAM1 PG-binding protein YP_007349105.1 (aa 90-261), (7) *Erwinia* phage phiEa2809 putative PG binding protein YP_009147574.1 (90-261), (8) *Brucella abortus* hypothetical protein WP_006091019.1 (20-190), (9) *Ochrobacterium anthropis* PG-binding protein WP_061347616.1 (aa 20-190), (10) *Agrobacterium* phage Atu_ph03 PPH (aa 26-196).



Supplemental Figure S4. Growth of *A. tumefaciens* with plasmids to express variants of *pph* under uninduced conditions. (A) Growth curve of *A. tumefaciens* growth when expressing plasmid pSRKKm with variants of *pph* under uninduced conditions. (B) Cell viability of *A. tumefaciens* containing plasmids to express variants of *pph* grown under uninduced conditions.



Supplemental Figure S5. Clearing of peptidoglycan is observed when PPH is expressed in *A. tumefaciens*. Zymogram (left) and SDS polyacrylamide gel (right) loaded with 30 μg whole cell lysates of *A. tumefaciens* lacking PPH, expressing PPH, and expressing PPH_{E32A}.

SUPPLEMENTAL MOVIE LEGENDS

Supplemental Movie S1. Time-lapse microscopy in DIC of *A. tumefaciens* strain C58 growing for 7 h, 10 frames per second (fps). Scale bar = 10 μm. White asterisk indicates the cell shown in Figure 2B, upper panel.

Supplemental Movie S2. Time-lapse microscopy in DIC of cells infected with Atu_ph03 at an MOI of 0.01 for 7 h, 10 fps. Scale bar = 10 μm. White asterisk indicates the cell shown in Figure 2B, lower panel. Fields 1 and 2 show separate representative fields.

Supplemental Movie S3. Time-lapse microscopy in DIC of cells infected with Atu_ph02 at an MOI of 0.01 for 7 h, 10 fps. Scale bar = 10 μm. White asterisk indicates the cell shown in Figure 2B, center panel.

Supplemental Movie S4. Time-lapse microscopy in DIC of cells expressing pSRKKm-Plac-sf*gfp* for 16 h, 10 fps. Scale bar = 10 μm. White asterisk indicates the cell shown in Figure 5E.

Supplemental Movie S5. Time-lapse microscopy in DIC of cells expressing pSRKKm-Plac-PPH growing for 16 h, 10 fps. Scale bar = 10 μm. White asterisk indicates the cell shown in Figure 5F.

Supplemental Movie S6. Time-lapse microscopy in DIC of cells expressing pSRKKm-Plac-PPH_{E32A} growing for 20 h, 10 fps. Scale bar = 10 μm. White asterisk indicates the cell shown in Figure 5G.

Supplemental Movie S7. Time-lapse microscopy in DIC of cells expressing pSRKKm-Plac-$PPH_{K328A,K334A,K335A}$ growing for 7 h, 10 fps. Scale bar = 10 μm. White asterisk indicates the cell shown in Figure 5H.

Supplemental Movie S8. Time-lapse microscopy in DIC of cells expressing pSRKKm-Plac-$PPH_{E32A,K328A,K334A,K335A}$ growing for 16 h, 10 fps. Scale bar = 10 μm. White asterisk indicates the cell shown in Figure 5I.

Supplemental Movie S9. Time-lapse microscopy in DIC of cells expressing pSRKKm-Plac-PPH_{1-304} growing for 16 h, 10 fps. Scale bar = 10 μm. White asterisk indicates the cell shown in Figure 5J.

SUPPLEMENTAL REFERENCES

1. **Yin Y, Fischer D.** 2008. Identification and investigation of ORFans in the viral world. BMC Genomics **9:**24.