MICROBIOLOGY

TIR signaling activates caspase-like immunity in bacteria

François Rousset[1]†‡, Ilya Osterman[1]†, Tali Scherf[2], Alla H. Falkovich[2], Azita Leavitt[1], Gil Amitai[1], Sapir Shir[1], Sergey Malitsky[3], Maxim Itkin[3], Alon Savidor[4], Rotem Sorek[1]*

Caspase family proteases and Toll/interleukin-1 receptor (TIR)-domain proteins have central roles in innate immunity and regulated cell death in humans. We describe a bacterial immune system comprising both a caspase-like protease and a TIR-domain protein. We found that the TIR protein, once it recognizes phage invasion, produces the previously unknown immune signaling molecule adenosine 5′-diphosphate-cyclo[N7:1″]-ribose (N7-cADPR). This molecule specifically activates the bacterial caspase-like protease, which then indiscriminately degrades cellular proteins to halt phage replication. The TIR-caspase defense system, which we denote as type IV Thoeris, is abundant in bacteria and efficiently protects against phage propagation. Our study highlights the diversity of TIR-produced immune signaling molecules and demonstrates that cell death regulated by proteases of the caspase family is an ancient mechanism of innate immunity.

Caspases are proteases with central roles in innate immunity and regulated cell death in humans (*1*). The human genome harbors 12 caspase-encoding genes, with some promoting inflammation and others functioning in the execution of cell death (*1*). Inflammatory caspases, including caspases 1, 4, and 5, are recruited to activated inflammasomes following pathogen recognition, and are responsible for the proteolytic activation of cytokines and gasdermin D, promoting cell death through pyroptosis (*2*). Executioner caspases (e.g., caspases 3, 6, and 7), once activated by initiator caspases (8, 9, and 10), cleave hundreds of protein substrates to promote apoptotic cell death (*1*, *3*). Proteases of the caspase family (Pfam PF00656) also exist in prokaryotes (*4*) and were proposed to cleave bacterial gasdermins to activate immunity in response to phage infection (*5*). Bacterial proteins of the caspase family were also shown to promote cell death when activated by type III CRISPR-Cas systems (*6*–*8*). However, the molecular functions of most caspase-like proteins in bacteria remain poorly understood (*4*).

Toll/interleukin-1 receptor (TIR) domain proteins also play key roles in innate immunity. Initially described as protein-protein interaction modules in human Toll-like and interleukin-1 receptors (*9*), TIR domains in bacteria and plants were later shown to be enzymes that produce immune signaling molecules using nicotinamide adenine dinucleotide (NAD^+) as a substrate (*10*). In the bacterial defense system type I Thoeris, the TIR-domain protein, once it senses phage infection, produces the signaling molecule 1″-3′ glycocyclic adenosine 5′-diphosphate (ADP)-ribose (gcADPR) (*11*, *12*). This molecule activates an effector protein that depletes the cell of NAD^+ and aborts phage replication (*13*, *14*). The TIR-domain protein in type II Thoeris, by contrast, produces the signaling molecule histidine-ADP-ribose (His-ADPR) (*15*), which activates an effector protein that disrupts the cell membrane to abort phage propagation. A third type of Thoeris was recently proposed but its mechanism of action and molecular signaling remains unknown (*16*). In plants, TIR-derived signaling molecules are diverse and include phosphoribosyl-AMP/ADP (*17*), 1″-2′ gcADPR (*11*, *12*, *18*), di-ADPR (*19*), ATP-ADPR (*19*), and 2′,3′-cAMP/cGMP (*20*). Current evidence suggests that the repertoire of immune signaling molecules produced by TIR domains has not been fully unveiled (*10*).

In this study, we describe type IV Thoeris, a bacterial defense system encoding both a TIR-domain protein and a caspase-like protease. We show that upon phage infection, the TIR-domain protein produces the signaling molecule N7-cADPR that binds the caspase-like protease and triggers promiscuous arginine-specific protease activity. Protease activation leads to indiscriminate cleavage of multiple proteins, including elongation factor Tu (EF-Tu), thereby aborting phage infection. Our study establishes a direct functional connection between TIR signaling and caspase activation in bacterial defense against phages.

Results

A TIR-caspase operon provides anti-phage immunity.

While examining the genomic environment of caspase-like proteins in bacteria, we noticed an abundant two-gene operon encoding a short TIR-domain protein and a caspase-like protease (Fig. 1A). Operons with this gene organization were frequently encoded in the vicinity of bacterial defense systems, suggesting a defensive function (fig. S1). We synthesized and cloned two such operons, one from *Escherichia coli* 328 and the other from *Pseudomonas* sp. 1-7 and expressed these in *E. coli* K-12 MG1655 under the control of an arabinose-inducible promoter. Following a challenge by a panel of phages, we observed that the infectivity of phage T6 was substantially reduced when plated on cells expressing either of the operons, showing that this TIR- and caspase-encoding operon is a defense system (Fig. 1B). Since the *E. coli* 328 operon exhibited slight toxicity upon expression induction, we selected the *Pseudomonas* sp. 1-7 (*Ps*) homolog for further study. A sensitivity screen against phages of the BASEL collection (*21*) revealed that phages Bas18, Bas25, and Bas43 are also blocked by the defense system from *Pseudomonas* sp. 1-7 (fig. S2).

Single amino acid substitutions in the predicted catalytic sites of *Ps*TIR (E79Q) and *Ps*Caspase (C129A) proteins abolished defense (Fig. 1B), suggesting that the enzymatic activity of both proteins is required for immunity. Bacterial cells expressing the system were able to survive when infected with T6 in liquid culture at a low multiplicity of infection (MOI) but died when infected at a high MOI (Fig. 1C). In addition, infected cells expressing the system did not release phage progeny (Fig. 1D). These results suggest that this defense system functions through regulated cell death or dormancy (*22*) and provides population-level protection. Homology-based searches in a database of ~38,000 prokaryotic genomes revealed that this defense system is found in hundreds of bacteria and archaea belonging to diverse phyla (Fig. 1E and table S1).

Caspase cleaves EF-Tu during phage infection

TIR domains were described as NAD^+-degrading effectors in diverse families of defense systems, including Pycsar, CBASS, and prokaryotic argonautes, where their function is to deplete cells of NAD^+ once triggered by phage infection (*23*–*26*). Bacterial caspase-like proteases, on the other hand, were suggested to cleave bacterial gasdermins into active pore-forming effectors to induce cell death following infection (*5*). For this reason, we initially hypothesized that *Ps*Caspase would cleave *Ps*TIR into an active NAD^+-depleting form during infection. However, we did not detect any change in the molecular weight of *Ps*TIR during T6 infection (fig. S3A). In addition, we observed only mild reduction in NAD^+ levels during infection of cells expressing the system, which contrasts with the >95% reduction typically observed in NAD^+-depleting defense systems (*13*, *25*–*29*) (fig. S3B). These results suggest that *Ps*TIR is

[1]Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel. [2]Department of Chemical Research Support, Weizmann Institute of Science, Rehovot, Israel. [3]Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot, Israel. [4]The Nancy and Stephen Grand Israel National Center for Personalized Medicine, Weizmann Institute of Science, Rehovot, Israel.
*Corresponding author. Email: rotem.sorek@weizmann.ac.il
†These authors contributed equally to this work.
‡Present address: CIRI, Centre International de Recherche en Infectiologie, INSERM U1111, CNRS UMR5308, Université Claude Bernard Lyon 1, Ecole Normale Supérieure de Lyon, Lyon, France.

unlikely to be an NAD^+-depleting effector activated by *Ps*Caspase.

In Thoeris defense systems, bacterial TIR domains have an alternative role that does not involve NAD^+ depletion. In these systems, TIR domains generate signaling molecules that activate downstream effectors (*10*). We therefore hypothesized that *Ps*TIR may produce a signaling molecule during infection, and that this signaling molecule would bind and activate the effector *Ps*Caspase. Under this hypothesis, *Ps*Caspase is expected to cleave cellular or phage target proteins and halt phage infection.

To explore possible target proteins of *Ps*Caspase, we chemically labeled NH_2 groups in proteins present in lysates of T6-infected cells and subjected the labeled proteins to mass spectrometry (MS). This technique enables the identification of new protein N-termini specifically found in cells expressing the defense system as compared with control cells. A preliminary MS analysis following free amine tagging identified multiple candidate *Ps*Caspase targets, including the elongation factor Tu (EF-Tu), a highly abundant cellular protein essential for protein translation, which was previously shown to be the target of the Lit protease (*30*) (fig. S4). To verify that EF-Tu is a target of *Ps*Caspase, we co-expressed the TIR-caspase system with a C-terminally tagged copy of EF-Tu in an *E. coli* K-12 strain in which *lit* was deleted, and monitored EF-Tu cleavage during phage infection by Western blot (Fig. 2A). Two EF-Tu cleavage products were visible in cells expressing the defense system following T6 infection (Fig. 2B). These cleavage products were absent when the catalytic cysteine in *Ps*Caspase or the catalytic glutamate in *Ps*TIR were mutated to alanine, showing that EF-Tu cleavage requires both caspase and TIR activities (Fig. 2B). The molecular weight of cleavage products and the exploratory MS data suggested that EF-Tu is cleaved after two specific arginine residues (R45 and R59) (Fig. 2, C and D), which was confirmed by the loss of EF-Tu cleavage upon mutation of these two residues into glutamines (Fig. 2B). Taken together, our results suggest that *Ps*Caspase is specifically activated during phage infection and cleaves EF-Tu downstream of arginine residues at positions 45 and 59.

Caspase activity is triggered by a TIR-derived signaling molecule

We then investigated whether, as in the Thoeris family of defense systems, *Ps*TIR produces a signaling molecule that activates *Ps*Caspase during infection. To do so, we collected lysates from cells expressing *Ps*TIR only and filtered these lysates with a 3-kDa cutoff to retain small metabolites. We then tested the ability of these metabolite extracts to activate EF-Tu cleavage by *Ps*Caspase (Fig. 3A). EF-Tu cleavage was visible when lysates from cells expressing both

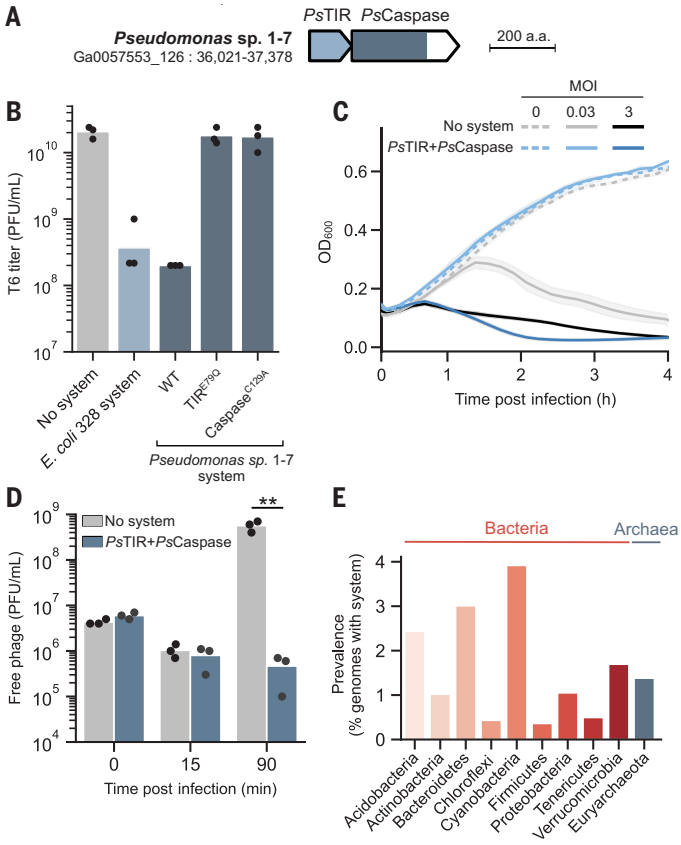


Fig. 1. A genetic system encoding a TIR-domain protein and a caspase-like protease provides anti-phage defense. (**A**) Genetic architecture of a system from *Pseudomonas* sp. 1-7. IMG (*44*) genome ID and gene coordinates are displayed below strain name. TIR and caspase-like domains are shown as blue shades. (**B**) Quantification of phage infection efficiency through plaque assays. Tenfold serial dilutions of phage T6 were spotted on a lawn of *E. coli* K-12 MG1655 cells expressing an empty vector (no system), the system from *E. coli* 328, or the system from *Pseudomonas* sp. 1-7, either wild type or mutated in the predicted active sites of the TIR (E79Q) or caspase-like protease (C129A). Bars show the mean of three replicates with individual data points overlaid. (**C**) Growth curves of *E. coli* K-12 MG1655 cells expressing an empty vector or the system from *Pseudomonas* sp. 1-7, infected by phage T6 at an MOI of 0.03 or 3 (or 0 for uninfected cells). Curves show the mean of three replicates with the standard deviation indicated by the shaded area. (**D**) Plaque-forming units of phage T6 sampled from the supernatant of *E. coli* K-12 MG1655 cells expressing an empty vector or the system from *Pseudomonas* sp. 1-7. Cells were infected at an MOI of 0.1. Bars represent the mean of three replicates with individual data points overlaid. Stars show significance of a two-sided *t*-test (**$P < 0.01$). (**E**) Detection of the defense system in prokaryotic genomes, shown for phyla with at least 50 genomes and where at least one system was detected.

*Ps*Caspase and tagged EF-Tu were incubated with metabolite extracts derived from infected *Ps*TIR-expressing cells (Fig. 3B). By contrast, EF-Tu cleavage was absent when the metabolites were extracted from infected cells expressing red fluorescent protein (RFP) or $PsTIR^{E79Q}$ instead of *Ps*TIR, or from uninfected *Ps*TIR-expressing cells (Fig. 3B). These results suggest that *Ps*TIR produces a *Ps*Caspase-activating signaling molecule during infection.

To develop a more streamlined assay for *Ps*Caspase activation, we used a synthetic five amino acid peptide comprising the residues preceding the target cleavage site in EF-Tu (positions 55 to 59, EEKAR) fused to 7-amino-4-methylcoumarin (AMC). In this assay, which is similar to assays previously used to study human caspases (*31*) and caspase-like proteins in bacteria (*8*), peptide cleavage downstream of the arginine is expected to release free AMC and emit a fluorescence signal (Fig. 3C). *Ps*Caspase was able to cleave the synthetic EEKAR-AMC peptide when incubated with metabolites derived from infected cells expressing *Ps*TIR, but not when incubated with metabolites extracted from infected RFP-expressing control cells (Fig. 3D). *Ps*Caspase activity was even more pronounced upon incubation with metabolites derived from infected cells expressing the TIR protein from the *E. coli* 328 TIR-caspase system (*Ec*TIR) (Fig. 3D), suggesting that in the experimental conditions used here, *Ec*TIR produces higher amounts of signaling molecule than *Ps*TIR. Together, these results demonstrate that the caspase-like protein is activated by a small molecule specifically produced by the TIR-domain protein during phage infection. We therefore name this defense system type IV Thoeris.

*Ps*Caspase is a promiscuous arginine-specific protease

We then investigated the substrate specificity of the caspase-like protein using synthetic peptides with mutations in the wild-type (WT) EF-Tu sequence (EEKAR). Activated *Ps*Caspase was able to cleave EEAAR-AMC and EEKLR-AMC

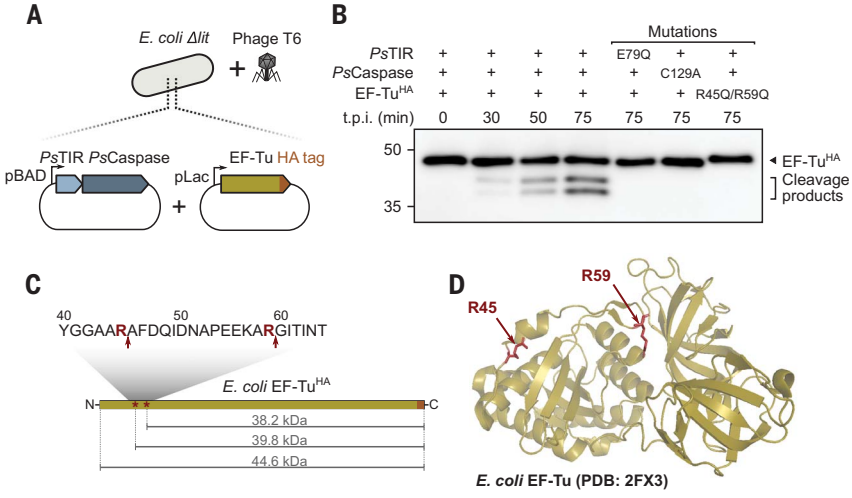


Fig. 2. EF-Tu is a target of *Ps*Caspase during phage T6 infection. (**A**) *E. coli* K-12 MG1655Δ*lit* cells co-expressing the *Pseudomonas* sp. 1-7 defense system and an HA-tagged copy of EF-Tu were infected with phage T6. (**B**) Western blot analysis of infected cells co-expressing WT or mutated *Ps*TIR and *Ps*Caspase, and WT or mutated HA-tagged EF-Tu. t.p.i., time post infection. (**C**) and (**D**) Locations of cleavage sites in the EF-Tu protein sequence (C) and structure (D) are indicated with red arrows.

but not EEKAA-AMC peptides, indicating that the arginine residue at the P1 position is strictly required for cleavage, but other residues are not (fig. S5). These results suggest that *Ps*Caspase does not specifically recognize EF-Tu but might rather be a promiscuous protease.

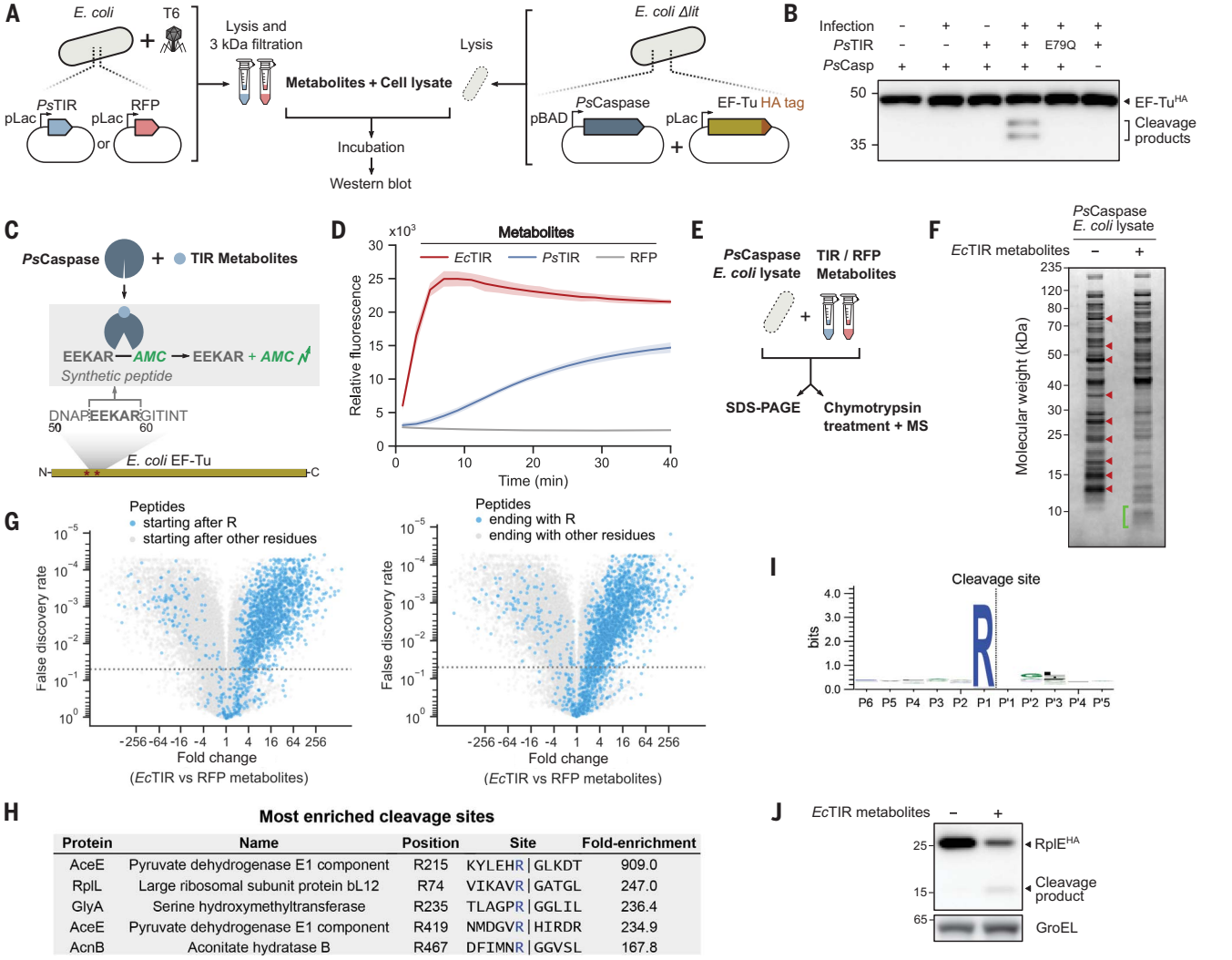
To systematically search for additional cellular targets of *Ps*Caspase beyond EF-Tu, we incubated lysates of *Ps*Caspase-expressing *E. coli* cells with metabolites extracted from infected *Ec*TIR-expressing cells (Fig. 3E). SDS-PAGE analysis revealed the loss of multiple abundant proteins in the presence of *Ec*TIR-derived metabolites, combined with the presence of a smear of low-molecular weight protein products, suggesting massive *Ps*Caspase-dependent protein degradation (Fig. 3F). We subjected these lysates to chymotrypsin digestion and mass spectrometry analysis to identify cleavage sites in a systematic manner. This analysis revealed that peptides starting directly downstream of arginine, or ending with arginine, were strongly enriched upon incubation with *Ec*TIR-derived metabolites (Fig. 3G), indicating that cleavage occurs downstream of arginine residues. Cleavage events were observed in multiple essential proteins of *E. coli* (Fig. 3H), with a strict requirement for an arginine in the P1 position and a slight preference for small nonpolar residues surrounding the cleavage site (Fig. 3I). We confirmed the cleavage of one of these target proteins, ribosomal protein RplE, by Western blot using cell lysates incubated with *Ec*TIR-derived metabolites (Fig. 3J). Altogether, our results show that *Ps*Caspase is a promiscuous arginine-specific protease that cleaves multiple target proteins once activated by the *Ps*TIR-derived signaling molecule.

Type IV Thoeris produces signaling molecule N7-cADPR

We next examined the properties of the *Ps*Caspase-activating signaling molecule produced by type IV Thoeris. For this, we sought to take advantage of previously described phage proteins that sequester specific Thoeris-derived signaling molecules as a means to inhibit bacterial defense. Thoeris anti-defense 1 (Tad1) from phage SBSphiJ7 and Tad2 from phage SPO1 are known to sequester the 1”-3′ gcADPR signaling molecule produced by type I Thoeris (*12*, *32*), and Tad2 from *Myroides odoratus* (ModTad2) sequesters His-ADPR molecules and inhibits type II Thoeris (*15*). However, co-expression of Tad1, Tad2, or ModTad2 with type IV Thoeris did not inhibit the defensive activity of the system (Fig. 4A), suggesting that type IV Thoeris does not utilize the same molecule as types I or II Thoeris. Furthermore, incubation of metabolites derived from *Ec*TIR-expressing infected cells with purified Tad proteins did not affect the ability of these metabolites to activate *Ps*Caspase in our reporter assay, suggesting that Tad proteins are unable to sequester the signaling molecule of type IV Thoeris (fig. S6A). Finally, purified 1”-3′ gcADPR, 1”-2’ gcADPR, ADPR, and cyclic-ADPR molecules were unable to activate *Ps*Caspase (Fig. 4B), and conversely, *Ec*TIR-derived metabolites were unable to activate the ThsA NADase effector of type I Thoeris (*13*) in vitro (fig. S6B). Together, these results indicate that the signaling molecule of type IV Thoeris is distinct from that of type I and type II Thoeris.

To further examine the nature of the signaling molecule produced by type IV Thoeris, we used HPLC fractionation to purify the molecule from lysates of phage-infected *Ec*TIR-expressing cells, using the peptide-AMC cleavage assay as a reporter system to assess the activity of HPLC fractions (fig. S7). Initial purification in phosphate buffer (pH 8.0) showed that the molecule rapidly lost activity under these conditions, and we therefore purified it in water (pH 7.0). Liquid chromatography coupled with mass spectrometry (LC-MS) showed that the molecule has the same mass as the canonical cyclic ADP-ribose (cADPR) (Fig. 4C) and MS-MS analysis showed that the fragmentation pattern of the molecule was indistinguishable from that of cADPR (fig. S7E). However, the molecule had a different LC retention time and absorbed light at a different wavelength than cADPR, suggesting that the type IV Thoeris molecule is distinct from cADPR (Fig. 4C). Indeed, synthetic cADPR was unable to activate *Ps*Caspase (Fig. 4B).

We subjected the purified molecule to nuclear magnetic resonance (NMR) analysis. Data from 1D ^{1}H, 2D homonuclear COSY and TOCSY, and 2D heteronuclear ^{1}H-^{13}C HSQC and HMBC NMR experiments, showed that the molecule comprises two ribose moieties and an adenine base, supporting the notion that the molecule is a variant of cADPR (fig. S8). However, ribose carbon 1”, which in the canonical cADPR is naturally found attached to N1 in the adenine ring, showed a 3-bond HMBC correlation to H8 in the adenine ring (Fig. 4D, inferred from the H8-C1” and H1”-C8 correlations in the HMBC spectrum). Moreover, similar intensities were observed for peaks correlating with position 8 on the adenine base and 1’ and 1” in the two ribose moieties (H1’-C8 and H1”-C8, and H8-C1’ and H8-C1”), indicating a symmetrical orientation of the two ribose moieties relative to carbon atom C8 of the adenine base (Fig. 4D). Given that ribose carbon 1’ was verified as attached to N9 in the adenine base (H1’-C4 correlation), our results indicate that ribose carbon 1” is attached to nitrogen atom N7 in the adenine base (Fig. 4D). Altogether, our MS and NMR experiments indicate that the molecule that activates *Ps*Caspase is ADP-cyclo[N7:1″]-ribose (N7-cADPR), a molecule that, to our knowledge, has not been described before in natural systems (Fig. 4E). It was previously shown that N7-methylation of adenosine shifts the wavelength of the absorption maximum from 257 to 272 nm (*33*), which is in line with the shift in absorption that we observe in N7-cADPR as compared with cADPR (Fig. 4C).



Protein	Name	Position	Site	Fold-enrichment
AceE	Pyruvate dehydrogenase E1 component	R215	KYLEHR | GLKDT	909.0
RplL	Large ribosomal subunit protein bL12	R74	VIKAVR | GATGL	247.0
GlyA	Serine hydroxymethyltransferase	R235	TLAGPR | GGLIL	236.4
AceE	Pyruvate dehydrogenase E1 component	R419	NMDGVR | HIRDR	234.9
AcnB	Aconitate hydratase B	R467	DFIMNR | GGVSL	167.8

Fig. 3. A TIR-derived signaling molecule triggers *Ps*Caspase arginine-specific proteolytic activity. (**A**) Experimental setup to test for *Ps*Caspase activation by small molecules. Lysates of T6-infected cells expressing *Ps*TIR or RFP were passed through 3-kDa filters to collect small molecules. Separately, *E. coli* K-12 MG1655Δ*lit* cells co-expressing *Ps*Caspase and a C-terminally HA-tagged copy of EF-Tu were lysed and incubated with the small molecules collected from infected *Ps*TIR- or RFP-expressing cells. Reactions were resolved by SDS-PAGE and western blot. (**B**) Western blot analysis shows EF-Tu cleavage only in the presence of metabolites derived from infected *Ps*TIR-expressing cells. (**C**) Schematic representation of the *Ps*Caspase reporter assay. A peptide comprising the residues preceding one of the cleavage sites in EF-Tu (EEKAR, positions 55 to 59) fused to 7-amino-4-methylcoumarin (AMC) was synthesized. The synthetic peptide was incubated with lysates from *Ps*Caspase-expressing *E. coli* K-12 MG1655Δ*lit* cells and small molecules collected from infected TIR- or RFP-expressing cells. *Ps*Caspase activation leads to peptide cleavage downstream of the arginine residue, thereby releasing the free fluorophore. (**D**) *Ps*Caspase reporter assay with small molecules collected from infected *Ec*TIR-, *Ps*TIR- or RFP-expressing cells. Curves show the mean of three replicates with the standard deviation shown as a shaded area. (**E**) Schematic of the in vitro cleavage assays, using lysates from *E. coli* K-12 MG1655Δ*lit* cells that express *Ps*Caspase, incubated with small molecules collected from T6-infected cells that express either *Ec*TIR or RFP. (**F**) SDS-PAGE of in vitro reactions followed by Coomassie staining. Red arrows indicate protein bands that are depleted in the presence of *Ec*TIR metabolites. Green bracket indicates a smear of low molecular weight fragments that are enriched in the presence of *Ec*TIR metabolites. (**G**) Volcano plots showing differential abundance of peptides in *Ps*Caspase cell lysates following incubation with *Ec*TIR-derived small molecules or with small molecules from control RFP-expressing cells. Peptides starting directly after an arginine residue (left) or ending with an arginine residue (right) are marked in blue. (**H**) Peptides enriched >fivefold in the *Ec*TIR sample, and depleted >twofold when incubated with RFP versus *Ec*TIR metabolites were selected for motif analysis (*n* = 83 peptides from 70 proteins). Shown are the five strongest hits. Fold enrichment relates to peptides starting at the position after arginine. (**I**) Sequence logo of the 83 cleavage sites. (**J**) Cleavage of C-terminally HA-tagged RplE from cell lysates in the presence of *Ec*TIR metabolites as shown by western blot. GroEL is shown as a loading control.

Two types of Thoeris systems were previously studied in detail, and a third type (type III Thoeris) was suggested recently based on an operon architecture that contains TIR domains (*16*). In type I Thoeris, the effector protein ThsA harbors a SLOG domain that binds the signaling molecule gcADPR (*14*), and in type II Thoeris, the signaling molecule His-ADPR is perceived by a Macro domain found in the effector protein (*15*). In type IV Thoeris, the caspase-like protease effector encodes a C-terminal domain of unknown function (fig. S9A). Structural modeling (*34*) suggests that *Ps*Caspase forms a dimer in which the C-terminal domains are brought in close proximity to form

a putative ligand-binding pocket (fig. S9B). When co-folding the *Ps*Caspase dimer with N7-cADPR, AlphaFold3 (*34*) confidently placed N7-cADPR within this pocket, predicting multiple residues in *Ps*Caspase to interact with N7-cADPR through hydrogen bonds (E255, Q284, R288, H299) or π-π stacking with the adenine base (W301) (fig. S9C). Mutations in any of these residues abolished defense against phage T6 (fig. S9D), together suggesting that the pocket formed by the C-termini of the *Ps*Caspase dimer is responsible for binding N7-cADPR to trigger the proteolytic activity of the caspase-like domain.

Discussion

Together, our data provide a model for the mechanism of type IV Thoeris (Fig. 4F). Once infection is sensed in the cell, the TIR-domain protein utilizes NAD^+ as a substrate and generates a cyclized ADPR molecule where the carbon previously connected to the nicotinamide

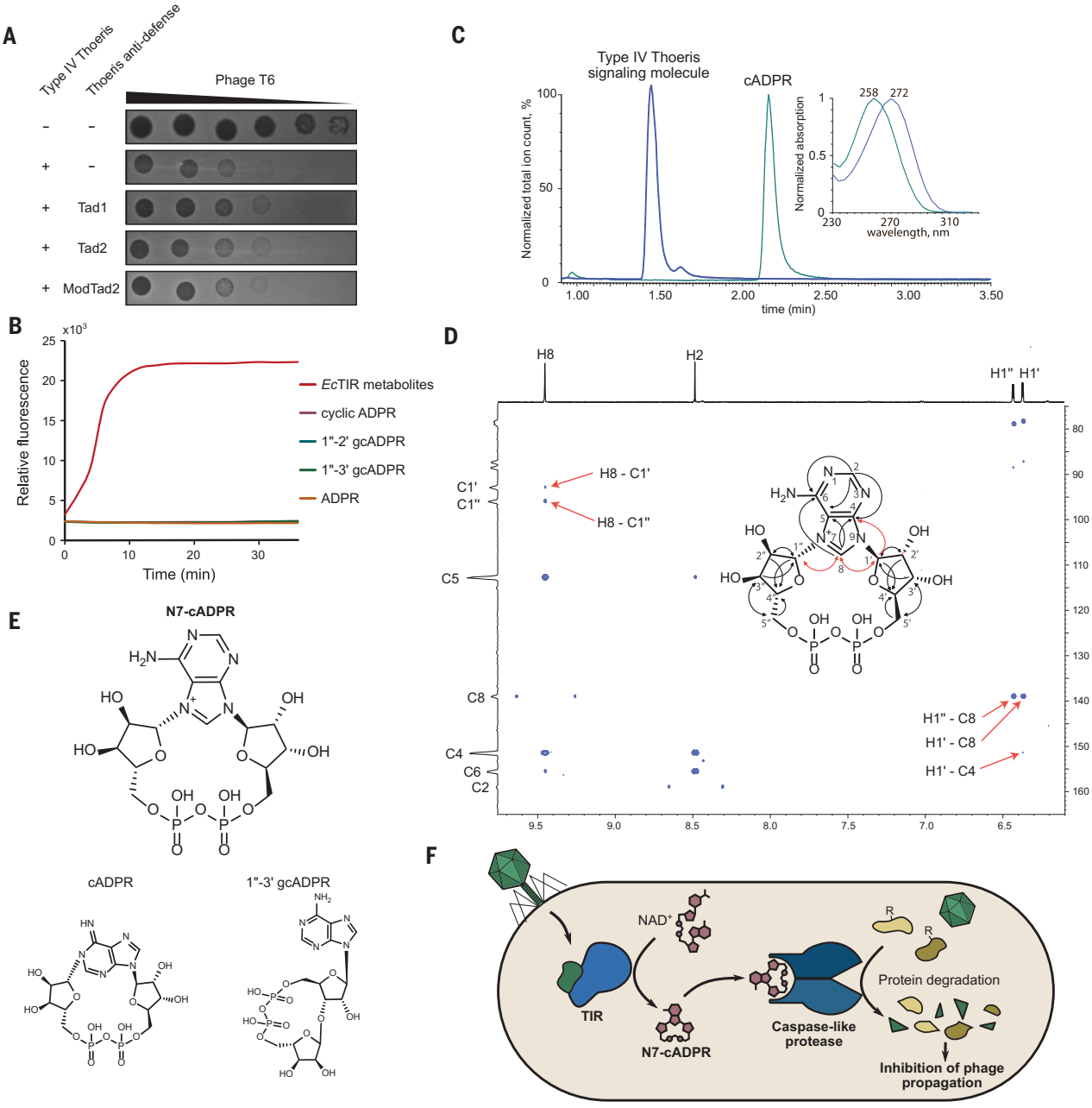


Fig. 4. Type IV Thoeris produces the immune signaling molecule N7-cADPR. (**A**) Plaque assay of phage T6 on cells co-expressing the type IV Thoeris system from *Pseudomonas* sp. 1-7 with RFP control, Tad1 from phage SBSphiJ7 (*12*), Tad2 from phage SPO1 (*32*), or Tad2 from *M. odoratus* (ModTad2) (*15*). None of the anti-defense proteins inhibit type IV Thoeris defense. (**B**) *Ps*Caspase reporter assays using metabolites derived from *Ec*TIR-expressing cells following phage infection, or using 100 μM of pure cyclic ADPR, 1"-2' gcADPR, 1"-3' gcADPR or ADPR. Only *Ec*TIR-derived metabolites activate *Ps*Caspase. (**C**) Chromatograms and UV-spectra (inset) of the type IV Thoeris signaling molecule (blue) and canonical cADPR (N1-cADPR, green). Both molecules have m/z = 540.0543 in negative ionization mode. (**D**) Correlations observed for N7-cADPR in the 2D ^{1}H-^{13}C HMBC NMR spectrum. Correlations between the adenine and the two ribose moieties are marked by red arrows both in the spectrum and on the molecule; other HBMC-derived correlations are shown by black arrows on the molecule. (**E**) Structure of N7-cADPR, compared with structures of canonical cADPR and 1"-3' gcADPR. (**F**) A model for the type IV Thoeris mechanism of immunity.

ring becomes covalently attached to the N7 nitrogen atom in the adenine base. This N7-cADPR molecule specifically activates the caspase-like effector of the defense system which then indiscriminately cleaves cellular and potentially phage proteins after arginine residues, causing massive protein degradation in the cell and incapacitating the phage reproductive cycle (Fig. 4F). The phage-derived component that is sensed by type IV Thoeris as a signature for infection is currently unknown and awaits future study.

Our data show that the molecule N7-cADPR functions as an immune signaling molecule in bacteria. Past studies showed that immune signaling molecules tend to be shared between domains of life. For example, 2′3′ cyclic GMP-AMP functions as an immune signaling molecule in the human cGAS-STING pathway (*35*), as well as in defense systems of the CBASS family (*36*). Similarly, 3′3′ cyclic UMP-AMP, originally discovered as a signaling molecule in bacterial CBASS systems (*37*), was later shown to be produced by animal immune proteins in response to dsRNA stimulation (*38*). TIR-derived immune signaling molecules also have parallels between bacteria, animals, and plants. For example, 1″-3′ gcADPR, produced by type I Thoeris, was shown to also be produced by the plant immune protein BdTIR (*18*) and the human TIR-domain protein SARM1 (*39*), although the biological roles of this molecule in plants and humans are currently unclear. It is therefore anticipated that the molecule discovered here, N7-cADPR, could also be found to participate in immune signaling in multicellular eukaryotes in future studies.

Human caspases can function both as initiators of cell death, where they activate specific death-promoting proteins by proteolytic cleavage (*1*), or as executioners of cell death, where they cleave hundreds of proteins to promote apoptosis (*3*). Our findings strengthen the understanding that caspase-like proteins have similar roles in bacterial immunity. Bacterial caspase-like proteases were previously shown to cleave bacterial gasdermins into pore-forming effectors that execute a form of pyroptotic-like cell death (*5*), paralleling inflammatory caspases in humans (*1*, *2*). In type IV Thoeris immunity, the role of the caspase-like protease is analogous to executioner caspases, as it directly executes cell death by cleaving many cellular target proteins, likely shutting off essential processes in the cell and preventing viral replication. Recent work has shown that some type III CRISPR-Cas systems also co-opted caspase-like proteases as a part of their immune mechanism (*6*–*8*). In particular, a type III-B CRISPR-Cas system employs a cascade of proteolytic events, in which CRISPR-Cas signaling activates a SAVED-CHAT protease to specifically process *PCaspase*, a caspase-like promiscuous protease that cleaves multiple proteins possibly downstream of arginine residues (*8*). Our results point to a common molecular function of *PCaspase* and the type IV Thoeris caspase as arginine-specific promiscuous proteases responsible for executing cell death or dormancy. Caspase-like proteases were predicted to be associated with other families of defense systems such as CBASS (*40*) and Avs (*41*), but their roles in these systems are currently unknown. It is possible that these proteases mediate bacterial defense through a mechanism similar to that of *Ps*Caspase. Our results show that proteases of the caspase family are ancient modules in the toolbox of immune-related cell death machineries shared by bacteria, fungi (*42*), plants (*43*), and humans (*1*).

REFERENCES AND NOTES

1. N. Van Opdenbosch, M. Lamkanfi, *Immunity* **50**, 1352–1364 (2019).
2. J. Shi *et al*., *Nature* **526**, 660–665 (2015).
3. S. Nagata, *Annu. Rev. Immunol.* **36**, 489–517 (2018).
4. J. Asplund-Samuelsson, B. Bergman, J. Larsson, *PLOS ONE* **7**, e49888 (2012).
5. A. G. Johnson *et al*., *Science* **375**, 221–225 (2022).
6. C. Hu *et al*., *Science* **377**, 1278–1285 (2022).
7. J. Strecker *et al*., *Science* **378**, 874–881 (2022).
8. J. A. Steens *et al*., *Science* **383**, 512–519 (2024).
9. K. Takeda, S. Akira, *Int. Immunol.* **17**, 1–14 (2005).
10. K. Essuman, J. Milbrandt, J. L. Dangl, M. T. Nishimura, *Science* **377**, eabo0001 (2022).
11. M. K. Manik *et al*., *Science* **377**, eadc8969 (2022).
12. A. Leavitt *et al*., *Nature* **611**, 326–331 (2022).
13. G. Ofir *et al*., *Nature* **600**, 116–120 (2021).
14. G. Tamulaitiene *et al*., *Nature* **627**, 431–436 (2024).
15. D. Sabonis *et al*., TIR domains produce histidine-ADPR conjugates as immune signaling molecules in bacteria. bioRxiv 2024.01.03.573942 (2024); doi:10.1101/2024.01.03.573942.
16. D. F. van den Berg *et al*., *Cell Host Microbe* **32**, 1427–1443.e8 (2024).
17. S. Huang *et al*., *Science* **377**, eabq3297 (2022).
18. A. M. Bayless *et al*., *Sci. Adv.* **9**, eade8487 (2023).
19. A. Jia *et al*., *Science* **377**, eabq8180 (2022).
20. D. Yu *et al*., *Cell* **185**, 2370–2386.e18 (2022).
21. E. Maffei *et al*., *PLOS Biol.* **19**, e3001424 (2021).
22. F. Rousset, R. Sorek, *Curr. Opin. Microbiol.* **74**, 102312 (2023).
23. G. Hogrel *et al*., *Nature* **608**, 808–812 (2022).
24. B. R. Morehouse *et al*., *Nature* **608**, 803–807 (2022).
25. N. Tal *et al*., *Cell* **184**, 5728–5739.e16 (2021).
26. B. Koopal *et al*., *Cell* **185**, 1471–1486.e19 (2022).
27. J. Garb *et al*., *Nat. Microbiol.* **7**, 1849–1856 (2022).
28. A. Millman *et al*., *Cell Host Microbe* **30**, 1556–1569.e5 (2022).
29. M. Zaremba *et al*., *Nat. Microbiol.* **7**, 1857–1869 (2022).
30. Y. T. N. Yu, L. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 802–806 (1994).
31. H. R. Stennicke, G. S. Salvesen, *Methods Enzymol.* **322**, 91–100 (2000).
32. E. Yirmiya *et al*., *Nature* **625**, 352–359 (2024).
33. S. P. Assenza, P. R. Brown, *J. Chromatogr. A* **289**, 355–365 (1984).
34. Willmore, A. J. *et al*., *Nature* **630**, 493–500 (2024).
35. A. Ablasser *et al*., *Nature* **498**, 380–384 (2013).
36. U. Tak, A. T. Whiteley, Bacterial cGAS-like enzymes produce 2′,3′-cGAMP to activate an ion channel that restricts phage replication. bioRxiv [preprint] 2023.07.24.550367 (2023);
37. A. T. Whiteley *et al*., *Nature* **567**, 194–199 (2019).
38. Y. Li *et al*., *Cell* **186**, 3261–3276.e20 (2023).
39. J. Garb *et al*., *PLOS ONE* **19**, e0302251 (2024).
40. A. Millman, S. Melamed, G. Amitai, R. Sorek, *Nat. Microbiol.* **5**, 1608–1615 (2020).
41. L. A. Gao *et al*., *Science* **377**, eabm4096 (2022).
42. A. Daskalov, *iScience* **26**, 106793 (2023).
43. Q. Xu, L. Zhang, *Plant Signal. Behav.* **4**, 902–904 (2009).
44. I. A. Chen *et al*., *Nucleic Acids Res.* **47**, D666–D677 (2019).

ACKNOWLEDGMENTS

We acknowledge C. Katina for preparing protein samples for mass spectrometry, N. Sumbatyan for useful discussions, and all members of the Sorek lab for constructive criticism on the manuscript. **Funding:** F.R. was supported by the Clore Foundation postdoctoral fellowship and by the Dean of Faculty fellowship from the Weizmann Institute of Science. I.O. was supported by the Ministry of Absorption New Immigrant program. T. Scherf is the incumbent of the Monroy-Marks Research Fellow Chair. R.S. was supported, in part, by the European Research Council (grant ERC-AdG GA 101018520), the Israel Science Foundation (MAPATS grant 2720/22), the Deutsche Forschungsgemeinschaft (SPP 2330, grant 464312965), the Minerva Foundation with funding from the Federal German Ministry for Education and Research, the Ernest and Bonnie Beutler Research Program of Excellence in Genomic Medicine, a research grant from the Estate of Marjorie Plesset, the Institute for Environmental Sustainability (IES), the Center for Immunotherapy at the Weizmann Institute of Science, and the Knell Family Center for Microbiology. M.I. and S.M. are supported by the Vera and John Schwartz Family Center for Metabolic Biology. **Author contributions:** Conceptualization: F.R., I.O., and R.S. Phage infection experiments: F.R. Cloning: F.R. and A.L. Production and purification of the signaling molecule and analytical chemistry data analysis: I.O. LC-MS experiments: A.H.F., M.I., and S.M. NMR analysis: T.S. EF-Tu cleavage experiments and cleavage sites identification: F.R. Protein mass spectrometry analysis: A.S. Design of the peptide-based reporter assay: G.A. Testing of Thoeris anti-defense proteins: S.S. Bioinformatic detection of Type IV Thoeris: F.R. Writing: F.R., I.O., and R.S. **Competing interests:** R.S. is a scientific cofounder and advisor of BiomX and Ecophage. Other authors declare that they have no competing interests. **Data and materials availability:** All data from the manuscript are available in the manuscript or in supplementary materials. Other materials are available upon request.

SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.adu2262
Materials and Methods
Figs. S1 to S9
Tables S1 to S4
References (*45*–*58*)
MDAR Reproducibility Checklist

Submitted 29 October 2024; accepted 30 December 2024
10.1126/science.adu2262