Phage detection by a bacterial NLR-related protein is mediated by DnaJ

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Bacteria encode a wide range of antiphage systems and a subset of these proteins are homologous to components of the human innate immune system. Mammalian nucleotide-binding and leucine-rich repeat containing proteins (NLRs) and bacterial NLR-related proteins use a central NACHT domain to link infection detection with initiation of an antimicrobial response. Bacterial NACHT proteins provide defense against both DNA and RNA phages. Here we determine the mechanism of RNA phage detection by the bacterial NLR-related protein bNACHT25 in *E. coli*. bNACHT25 was specifically activated by *Emesvirus* ssRNA phages and analysis of MS2 phage suppressor mutants that evaded detection revealed Coat Protein (CP) was sufficient for activation. bNACHT25 and CP did not physically interact. Instead, we found bNACHT25 requires the host chaperone DnaJ to detect CP. Our data suggest that bNACHT25 detects a wide range of phages by guarding a host cell process rather than binding a specific phage-derived molecule.

Keywords: innate immunity, bacteria, bacteriophage, phage, NLR, antiphage, DnaJ, MS2, capsid, coat protein, NACHT, bNACHT, chaperone

Introduction

The coevolution of bacteria and their viruses, bacteriophages (phages), has led bacteria to evolve diverse antiphage systems that halt infection. These systems can be a single gene or an operon of genes whose products cooperate to sense phage, amplify that signal, and activate an effector response that is antiviral[1,2]. Antiphage systems are distributed in the pangenome for each bacterial species and any one bacterial strain has a subset of these systems, which tend to colocalize and move between bacteria on mobile genetic elements[3–5]. The collection of antiphage systems within a given bacterium is referred to as the bacterial immune system[1,2].

Bacterial antiphage systems, like all immune pathways, can be categorized as adaptive or innate. Adaptive immune systems, such as CRISPR-Cas, are targeted to specific pathogens and specialize as a result of previous exposure or "immunization". Innate immune systems, on the other hand, target a wide range of pathogens in their native form by detecting conserved features. In this way, innate immune pathways act as the first line of defense.

One component of the innate immune systems of humans, plants, and bacteria are nucleotide-binding domain and leucine-rich repeat containing proteins (NLRs)[6–9]. These proteins are grouped based on their gross similarity to each other, i.e. inclusion of a nucleotide binding domain and leucine-rich repeats, however, their evolutionary histories and relatedness are complicated. The nucleotide binding domains of all NLRs belong to the STAND NTPase family, which can be further subdivided into related but distinct sister clades[10]. Human NLRs (such as NLRC4, NLRP3, and other components of the inflammasome) use NACHT modules[11]. Plant NLRs (such as ZAR1 and other R-proteins that form the resistosome) use AP-ATPase modules[10]. Previously, we found that bacteria encode NACHT modules in open reading frames with leucine-rich repeats, bona fide bacterial NLRs[9]. In addition, bacteria encode many NACHT modules in proteins with other domains in place of leucine-rich repeats. These proteins are "NLR-related" and we used NACHT domains to reconstruct the evolutionary history of how NACHT domains originated in bacteria and were horizontally acquired by eukaryotes[9]. More broadly, bacteria also encode proteins with AP-ATPase modules and other STAND NTPases. Multiple clades of antiviral ATPases/NTPases of the STAND superfamily (AVAST) systems have been described[10,12,13]. In humans, there are also antiviral STAND NTPases that are not NLRs, such as SAMD9[14,15].

Bacterial NACHT module-containing (bNACHT) proteins, as with all STAND NTPases, have a tripartite domain architecture[9,10]. The C-terminus is a sensing domain, the central NACHT domain enables oligomerization and signal transduction, and the N-terminus is an effector domain. While the effector domain function can often be predicted bioinformatically (e.g., identification of a nuclease domain suggests the antiphage system targets phage/host DNA), the stimuli that activates bNACHT proteins is challenging to discover[16].

We investigated the mechanism of phage detection by bNACHT proteins. Specifically, we focused on sensing of RNA phage because bNACHT proteins are the only innate immune pathway currently identified that are capable of protecting against RNA phage. We found that bNACHT25 is indirectly activated by the Coat Protein (CP) of the model ssRNA phage MS2, which suggests that host cell processes or components are required for sensing. Our analysis led us to discover that activation of bNACHT25 by phage or CP requires the host chaperone DnaJ.

Results

Breadth of RNA phage defense by bNACHT proteins

We previously reported that four bacterial NLR-related proteins provided robust phage defense against both DNA and RNA phages[9]: bNACHT02, 12, 25, and 32. Each encode a central NACHT module. bNACHT02 is 39% identical to bNACHT12 and bNACHT25 is 81% identical to bNACHT32. bNACHT02 and bNACHT12 fall within clade 14 of bacterial NLR-related proteins, which all contain short C-terminal NACHT-associated (SNaCT) domains but lack readily discernible effector domains[9]

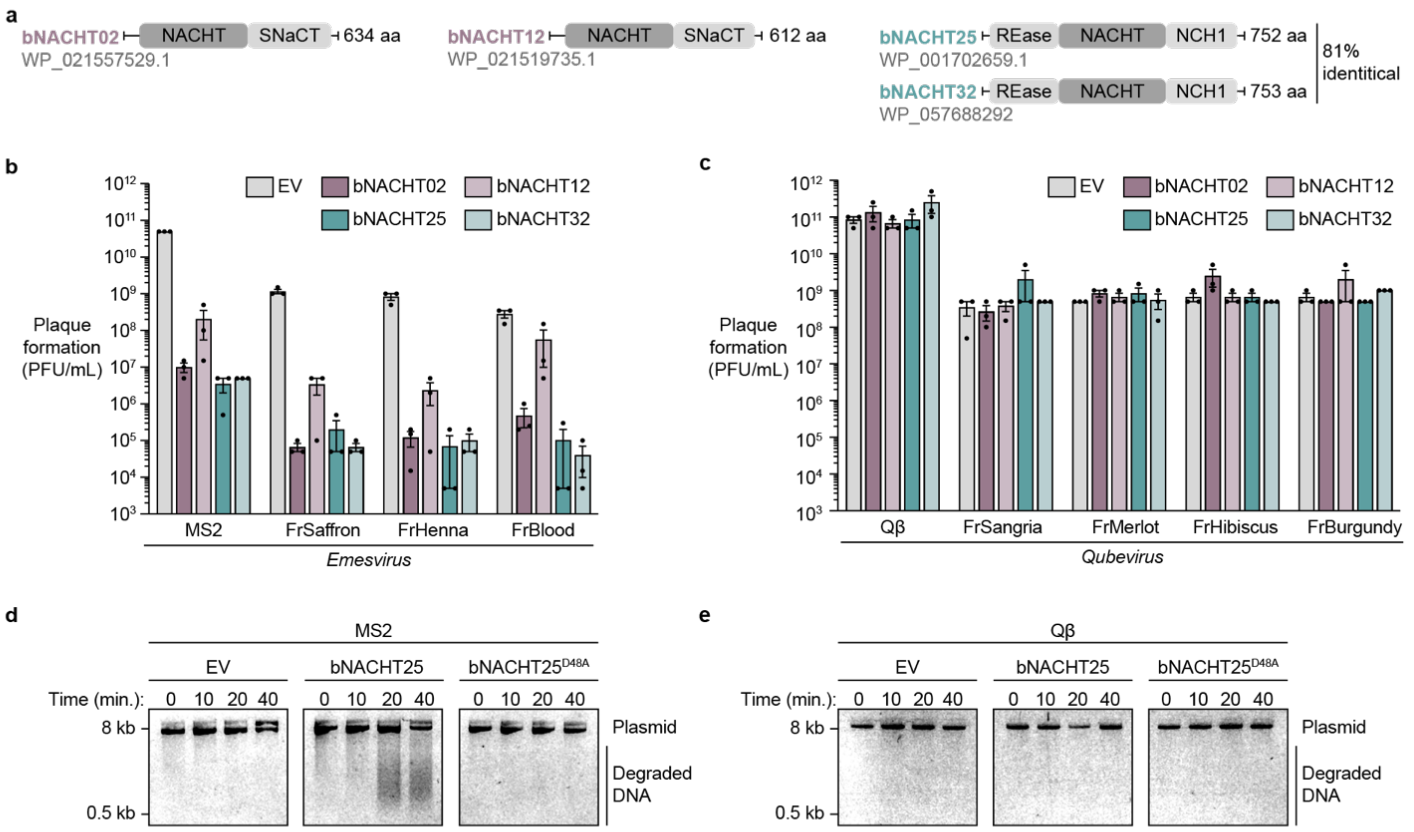

Figure 1: bNACHT proteins protect against ssRNA phages of the *Emesvirus* genus.
(a) Domain architectures of the bNACHT proteins that protect against MS2. **(b-c)** Efficiency of plating in plaque forming units per milliliter (PFU/mL) of the indicated *Emesvirus* or *Qubevirus* ssRNA phages on *E. coli* expressing the indicated defense system. Data are the mean ± standard error of the mean (SEM) of n=3 biological replicates. **(d-e)** Visualization of plasmid integrity in *E. coli* expressing empty vector (EV), bNACHT25, or bNACHT25^{D48A} infected with MS2 or Qβ at a multiplicity of infection (MOI) of 2. Plasmid DNA was harvested at the indicated timepoints post-infection and analyzed on an agarose gel. Data are representative images of n=3 biological replicates.

(**Fig. 1a**). bNACHT25 and bNACHT32 are from clade 6, contain NACHT C-terminal helical domain 1 (NCH1) domains, and N-terminal PD-(D/E)XK family DNase effector domains[9,17] (**Fig. 1a**).

To investigate the breadth of RNA phages restricted by NLR-related proteins, we challenged *E. coli* MG1655 expressing empty vector (EV) or bacterial NLR-related proteins with diverse ssRNA phages from the *Fiersviridae* family (**Fig. 1b-c**). All four systems defended against MS2 and MS2-like phages (the *Emesvirus* genus), however, none of these systems were capable of defending against Qβ or Qβ-like phages (the *Qubevirus* genus). While MS2 and Qβ have similar genome organizations and infectious cycles[18], they possess little sequence identity (38% identical). Within the *Emesvirus* and *Qubevirus* genera, the phages tested are highly related (≥92% identical)[19].

We confirmed our findings in liquid culture by infecting MG1655 expressing EV or bNACHT25 with either MS2 or Qβ. At a multiplicity of infection (MOI) of 0.2, bacteria expressing EV were lysed by MS2 and Qβ, while cultures expressing bNACHT25 continued to grow after infection with MS2 (**Extended Data Fig. 1a-d**). At a MOI of 2, the OD_{600} of bNACHT25-expressing bacteria stopped increasing upon MS2 infection (**Extended Data Fig. 1c**).

We hypothesized that OD_{600} plateaued when bNACHT25-expressing bacteria were infected due to activation of the endonuclease effector domain and tested this by measuring intracellular DNA degradation following infection with MS2. Plasmid DNA was harvested to represent total host DNA. Infection of bNACHT25-expressing bacteria with MS2 caused DNA degradation indicative of bNACHT25 effector activation (**Fig. 1d**). Nuclease activity was ablated by mutating a catalytic residue of the endonuclease domain (D48A). Nuclease activity was not observed when cultures were infected with Qβ (**Fig. 1e**); of note, we cannot distinguish whether Qβ evades detection by bNACHT25 or instead encodes a bNACHT25 inhibitor.

Mutations in CP enable MS2 to evade bNACHT proteins

ssRNA phages have some of the smallest known viral genomes, with MS2 only possessing four genes in its 3.6 kb genome: mat encodes maturation protein (MP), which enables attachment to the F pilus and RNA entry into the host; cp encodes coat protein (CP), which forms the viral capsid; lys encodes lysis protein (L), which allows for host lysis; and rep encodes replicase (Rep), which is an RNA-dependent RNA polymerase that replicates the ssRNA viral genome[20,21] (**Fig. 2a**). We sought to determine how MS2 activated bNACHT proteins and generated spontaneous MS2 suppressor mutants that evaded these systems. We isolated

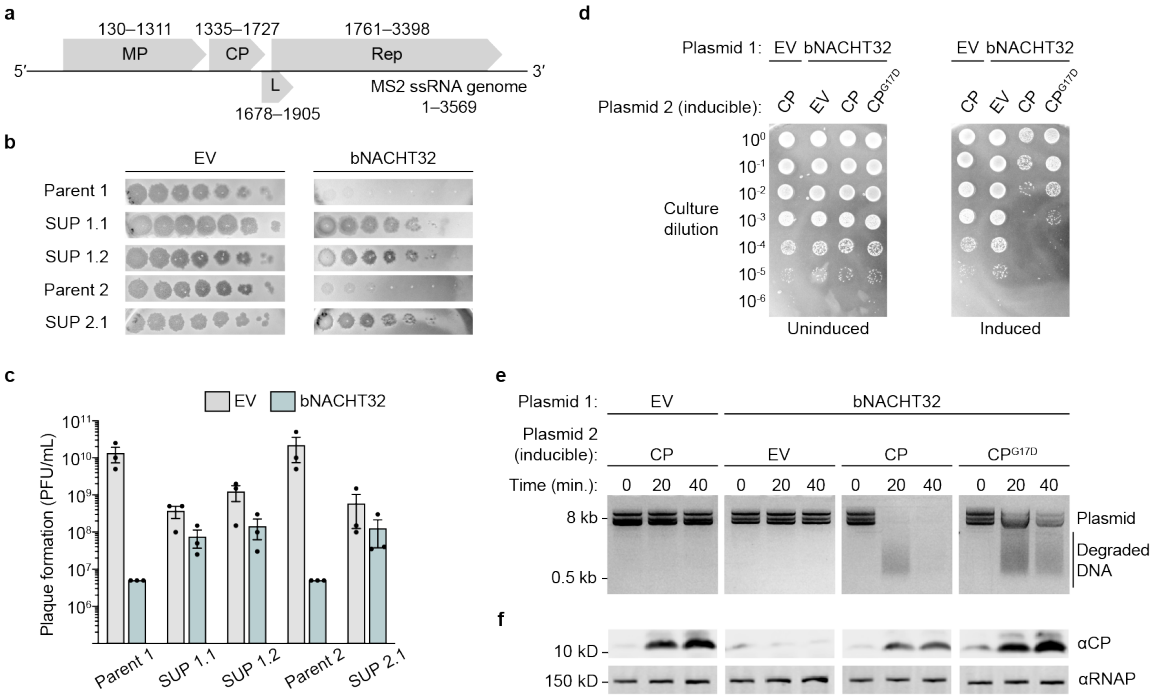

Figure 2: MS2 phage suppressor mutants reveal a role for CP in bNACHT activation
(a) ssRNA genome of phage MS2. Each gene is labeled with the protein it encodes and the nucleotide positions of the coding sequences. Maturation protein (MP), coat protein (CP), lysis protein (L), replicase (Rep). **(b-c)** Efficiency of plating of MS2 parent phages and corresponding suppressor mutant phages (Suppressor, SUP X.Y, where X indicates the parent phage number and Y indicates the suppressor phage number) on *E. coli* expressing EV or bNACHT32 from a plasmid. Images in (b) are representative of n=3 biological replicates that were quantified in (c) as PFU/mL with data plotted as in **Fig. 1b**. See **Table 1 and Supplementary Table 1** for bNACHT32 suppressor mutations. **(d)** Colony formation of *E. coli* expressing the indicated plasmids with or without IPTG induction of plasmid 2. Data are representative images of n=3 biological replicates. **(e)** Visualization of plasmid integrity in *E. coli* expressing the indicated proteins. Plasmid DNA was harvested at indicated timepoints post-induction of CP or EV with IPTG. Data are representative images of n=3 biological replicates. **(f)** Western blot analysis of *E. coli* lysates from the genotypes, timepoints, and conditions indicated in (e).

28 MS2 suppressor mutants that successfully formed plaques in the presence of either bNACHT02, bNACHT12, or bNACHT32 from three separate, clonal parent lineages. We were unable to isolate MS2 mutants that grew on bNACHT25. Mutant phage genomes were sequenced and variations from parent genomes were determined (**Table 1**, **Supplementary Table 1**).

Interestingly, MS2 mutants generated on bNACHT32 from two different parent lineages had acquired the same missense mutation in the cp gene, resulting in G17D (**Table 1**). Suppressor mutant phages encoding CP^{G17D} evaded bNACHT32-mediated defense compared to their parent strains (**Fig. 2b-c**). CP is the major structural protein that forms the viral capsid of MS2 and has been long-studied for its role in RNA binding[22]. The G17D mutation is located on the AB loop of CP, a surface-exposed loop that is not predicted to interact with the RNA genome or participate in the CP dimerization interface[23,24]. Additional mutations in cp were also found in MS2 suppressor mutants generated on bNACHT02 and bNACHT12 (**Supplementary Table 1**).

bNACHT32 and the highly similar bNACHT25 were selected for further investigation due to their readily assayable DNase effector domains, which provide unambiguous evidence of activation during phage infection. We hypothesized that CP activated bNACHT32, leading to effector activation and cell death, and tested this by inducing expression of CP in the presence of bNACHT32 and measuring colony formation. Significant growth inhibition was observed in the bNACHT32 and CP coexpression condition that was not observed when either was expressed individually (**Fig. 2d**). As suggested by our suppressor analysis, CP^{G17D} did not reduce colony formation to the same degree as wild-type CP when coexpressed with bNACHT32 (**Fig. 2d**).

DNA was purified from cultures expressing CP, bNACHT32, or both proteins combined to visualize effector domain activation. DNA degradation was only observed in the bNACHT32 and CP coexpression condition (**Fig. 2e**), indicating that CP is sufficient to activate the endonuclease domain of bNACHT32. The CP^{G17D} mutant activated bNACHT32 to a lesser extent than wild-type CP (**Fig. 2e**). The decrease in activation was not due to decreased protein expression as CP^{G17D} was produced in higher amounts than wild-type (**Fig. 2f**). Together, these data suggest that CP plays a role in bNACHT32 activation and the G17D mutations in CP allow MS2 to evade detection.

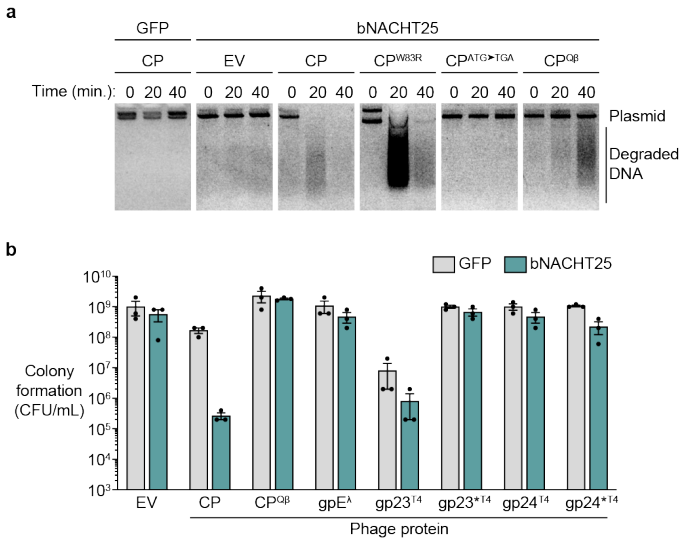

Figure 3: CP is sufficient to activate bNACHT25.
(a) Visualization of plasmid integrity in *E. coli* expressing GFP or VSV-G-bNACHT25 from the chromosome and the indicated CP alleles from an inducible plasmid. Plasmid DNA was harvested at indicated timepoints post-induction with IPTG. Data are representative images of n=3 biological replicates. **(b)** Colony formation of *E. coli* expressing GFP or VSV-G-bNACHT25 on the chromosome and indicated phage protein from an inducible plasmid. gp23* and gp24* indicate the truncated versions of gp23 and gp24, respectively, which are produced upon proteolytic cleavage during T4 infection (Fokine et al., 2005). Data are the mean ± SEM of n=3 biological replicates.

CP synthesis is sufficient to activate bNACHT25

We next interrogated the qualities of CP that are required for bNACHT activation using a genetic assay. bNACHT25 was used in place of bNACHT32 for these and future assays because bNACHT25 provided more robust effector activation in response to phage (**Fig. 1d and data not shown**). Expression of wild-type CP in the presence of bNACHT25 resulted in DNA degradation and inhibition of colony formation (**Fig. 3a-b**). Expression of CP^{W83R}, which is unable to oligomerize beyond dimerization and limits capsid assembly[25], similarly induced DNA degradation (**Fig. 3a**). However, mutation of the cp start codon completely abrogated DNA degradation phenotypes, demonstrating that translation of CP is required to activate bNACHT25 (**Fig. 3a**). Expression levels of CP were confirmed for each of these mutants by western blot (**Extended Data Fig. 2a**). Despite the similar structure to CP from MS2[26], $CP^{Q\beta}$ only modestly induced DNA degradation and did not impact colony formation (**Fig. 3a-b**). These findings suggest a mechanism for why bNACHT25 provides resistance against MS2 but not Qβ or Qβ-like phages.

We next tested if bNACHT25 could be activated by capsid proteins from dsDNA phages that bNACHT25 provides resistance to. The major and minor capsid proteins of phage T4, gp23* and gp24*, and the major capsid protein of phage λ, gpE, did not inhibit colony formation in the presence of bNACHT25 (**Fig. 3b**)[27,28]. These findings are, perhaps, unsurprising as CP from MS2 adopts a fold unique to the ssRNA phages that is not related to the HK97 fold capsid proteins from contractile tailed dsDNA phages[29–31].

The other three proteins encoded by MS2 were also interrogated for activation of bNACHT25. Interestingly, significant DNA degradation was observed upon induction of the lysis protein L (**Extended Data Fig. 2b**). A slight amount of activation was also observed upon induction of genes encoding MP and Rep that resembled expression of $CP^{Q\beta}$ (**Fig. 3a, Extended Data Fig. 2b**). The relevance of these data is unclear as expression of L is extremely toxic to the bacteria. Of note, the inducible vector in the genetic assay used expressed CP slightly higher than MS2 infection (**Extended Data Fig. 2c**). Our findings suggest that synthesis of CP, but not formation of higher-order CP oligomers, is required for activation of bNACHT25.

CP does not directly interact with bNACHT25

We next sought to understand the mechanism of CP activation of bNACHT25. AVAST systems encode central STAND NTPase modules that are related to NACHT modules and are activated by binding of phage proteins[12,13,32]. We tested whether CP similarly activates bNACHT25 by direct binding using immunoprecipitation (IP) assays. CP failed to IP from bacteria expressing epitope-tagged, catalytically inactive bNACHT25 (FLAG-$bNACHT25^{D48A}$) (**Fig. 4a**). $bNACHT25^{D48A}$ lacking a FLAG-tag was used as a negative control and the nuclease domain was catalytically inactivated to enrich for active complexes in the bacterial cytoplasm and prevent death of bacteria.

Our genetic data unambiguously showed bNACHT25 activation upon CP synthesis (**Fig. 3**) yet the two proteins did not form a complex (**Fig. 4a**). We therefore hypothesized that bNACHT25 may instead be activated by a host protein that is specifically changed during phage infection. To address this hypothesis, we performed Mass Spectrometry (MS) on tryptic digests of

Phage name	NTD (nt 1-130)	mat (nt 130-1311)	cp (nt 1335-1727)	lys (nt 1678-1905)	rep (nt 1761-3398)	CTD (nt 3398-end)
SUP 1.1		*g162a* Q233H L278P	G17D			
SUP 1.2		*a69u*	G17D	L44P*	*u48c**	
SUP 2.1		*a564c*	G17D			

Table 1: MS2 suppressor mutants capable of evading bNACHT32. Polymorphisms detected in MS2 mutant phages that evaded bNACHT32 (Suppressor, SUP X.Y, where X indicates the parent phage number and Y indicates the suppressor phage number). Suppressor mutants were derived from one of three WT parent lineages and selected on bacteria expressing bNACHT32. RNA genome mutations that did not result coding changes are lower-case, italics, and the number represents the nucleotide within the gene/locus. Coding mutations are indicated as capital letters, not italics, and the number represents the amino acid position within the protein. *Indicates SNP is located in overlapping region between lys and rep genes.

bNACHT25 IP samples to identify interacting proteins. A large cohort of proteins increased in abundance over the negative control (**Fig. 4b and Supplementary Table 2**). This analysis also confirmed bNACHT25 was successfully immunoprecipitated, while CP was not enriched (**Fig. 4b**).

To identify the specific host protein required for bNACHT25-mediated sensing of phage, we introduced plasmids expressing bNACHT25 or the bNACHT25^{D48A} catalytically inactive control into *E. coli* BW25113 carrying marked deletions of genes encoding each of 15 most enriched, non-essential IP-MS hits[33]. These strains were then challenged with phage T4. Only a mutation in *dnaJ* impacted bNACHT25-dependent phage resistance (**Fig. 4c**). These results suggest that DnaJ is required for bNACHT25 to sense phage.

bNACHT25 activation is modulated by the host chaperone DnaJ

DnaJ is an Hsp40-family chaperone whose J domain is conserved from humans to bacteria[34]. DnaJ combats misfolded proteins in the cell in two ways. First, DnaJ can bind misfolded proteins and prevent their aggregation[35]. Second, DnaJ can deliver misfolded proteins to DnaK, an Hsp70-family chaperone, and stimulate the ATPase activity of DnaK through the J domain of DnaJ[36]. DnaJ has many substrates and is famously required for replication of phage λ and certain plasmids[37,38]. To understand the role of DnaJ in bNACHT25-mediated phage resistance, we constructed a marked *dnaJ* deletion (*dnaJ::cat*) in bacteria expressing VSV-G-bNACHT25 or a GFP negative control from the chromosome. Western blots confirmed *dnaJ* deletion and that the level of bNACHT25 was unperturbed (**Fig. 5a**). bNACHT25 lost the ability to protect against the phages T2 and T4 in the absence of *dnaJ* (**Fig. 5b**). We were not able to measure changes to MS2 protection because MS2 failed to form plaques in *dnaJ::cat* bacteria (data not shown). Similar results were obtained with VSV-G-bNACHT32 expressed from the chromosome, however bNACHT32 expression was slightly decreased in the *dnaJ::cat* background (**Extended Data Fig. 3a and b**). These data suggest that bNACHT25, and potentially bNACHT32, require host DnaJ to sense phage.

We hypothesized that if DnaJ was required for phage detection, it would also be required for bNACHT25 activation by CP in our genetic assay. Accordingly, coexpression of CP with bNACHT25 resulted in bacterial growth inhibition in wild-type bacteria, but this phenotype was completely abrogated in a *dnaJ::cat* background (**Fig. 5c**). DNA degradation caused by bNACHT25 and CP coexpression was also dependent on the presence of *dnaJ* (**Fig. 5d**), with expression levels of CP and bNACHT25 being comparable in wild-type and *dnaJ::cat* strains (**Fig. 5e**).

Our analysis of MS2 proteins that activated bNACHT25 unexpectantly revealed that L, in addition to CP, activated nuclease activity (**Extended Data Fig. 2b**). L is the single-gene lysis protein of MS2 and was previously shown to bind to DnaJ, and *dnaJ* mutations limited host lysis by MS2[39]. We hypothesized that L interactions with DnaJ were responsible for L-mediated bNACHT25 activation. Expression of L in a wild-type background induced strong activation of bNACHT25, and this activation was completely ablated in a *dnaJ::cat* background

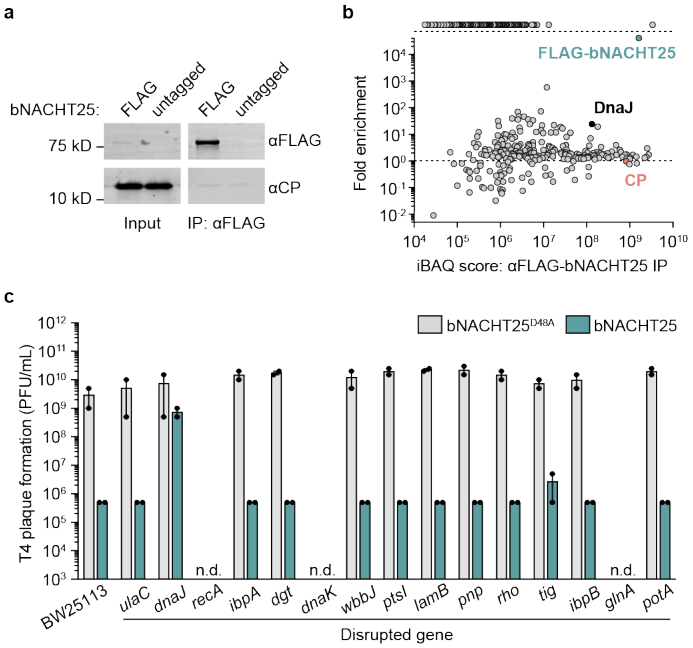

Figure 4: IP-MS of bNACHT25 reveals potential host interactors. **(a)** Western Blot analysis of αFLAG immunoprecipitation (IP) from *E. coli* expressing bNACHT25^{D48A} either with an N-terminal FLAG tag or an untagged allele. Input samples represent bacterial lysates prior to IP. Data are representative images of n=3 biological replicates. **(b)** Mass spectrometry (MS) of IP'd FLAG-bNACHT25. Data are intensity-based absolute quantification (iBAQ) score and fold enrichment comparing αFLAG IP from bacteria expressing bNACHT25^{D48A} either with an N-terminal FLAG tag or an untagged allele. Proteins only detected in FLAG-bNACHT25 IP are represented as data points positioned above the top of the y-axis (dashed line). Data is a representative plot of n=2 biological replicates. See **Supplementary Table 2** for full MS results. **(c)** Efficiency of plating of phage T4 on BW25113 mutants containing plasmids expressing bNACHT25 or bNACHT25^{D48A}. Disrupted genes are in order of abundance with the gene encoding the proteins with the highest iBAQ score listed first. Not determined (n.d.) indicates the strain was unable to grow with one or both plasmids. Data plotted as in **Fig. 1b**.

(**Extended Data Fig. 3c**), indicating that DnaJ is necessary for activation of bNACHT25 by both CP and L.

Finally, we investigated the specificity of DnaJ-mediated impacts on bNACHT25 by knocking out other *E. coli* chaperones, chaperone-related proteases, cold shock proteins, and phage shock proteins. bNACHT25 was expressed in bacteria deleted for *clpA/X*, *lon*, *hslO*, *spy*, *htpG*, *cspA/B/C*, *hscA/B*, and *pspB/C* and these strains were challenged with phage T4. bNACHT25 provided the same level of phage resistance for each of these as was observed in a wild-type background (**Extended Data Fig. 3d**). Taken together, these data support a model in which DnaJ is required for transmitting an activation signal from phage proteins to bNACHT25 and that bNACHT25 guards host processes or proteins that specifically involve DnaJ (**Fig. 5f**).

Discussion

In this study we investigated the mechanism by which bacterial NLR-related proteins are activated by DNA and RNA phages. bNACHT25 protects against multiple ssRNA phages belonging to the *Emesvirus* genus and the model ssRNA phage MS2 was used to identify phage proteins that activate bNACHT25. We found that CP, the capsid protein of MS2, was sufficient to

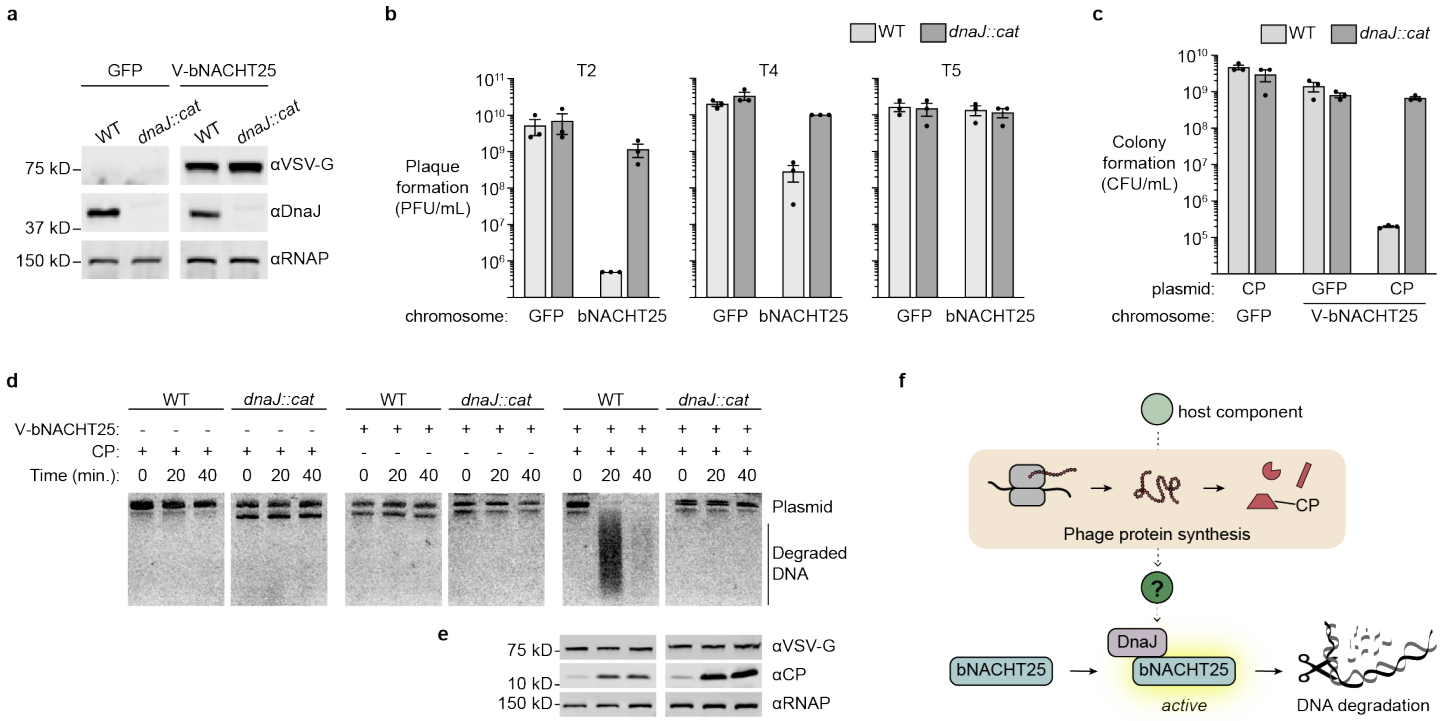

Figure 5: bNACHT25 activation is mediated by host DnaJ.
(a) Efficiency of plating of the indicated phage on wild-type (WT) or *dnaJ::cat E. coli* MG1655 expressing either GFP or VSV-G-bNACHT25 from the chromosome. Data plotted as in **Fig. 1b**. *cat*: chloramphenicol acetyltransferase. **(b)** Western blot analysis of cell lysates generated from *E. coli* with the indicated genotypes. VSV-G-bNACHT25 (V-bNACHT25). **(c)** Colony formation of *E. coli* with the indicated genotype following IPTG induction of GFP or CP. Data plotted as in **Fig. 3b**. **(d)** Visualization of plasmid integrity in *E. coli* with the indicated genotype at the indicated timepoints post-induction of CP with IPTG. bNACHT25 is on the chromosome and CP is on a plasmid as in (c). (+) represents indicated gene; (-) represents GFP negative control. **(e)** Western blot analysis of *E. coli* lysates from the genotypes, timepoints, and conditions indicated in (d). **(f)** Proposed model. Our data suggests that bNACHT25 monitors changes in the host that occur upon synthesis of phage proteins, such as CP, in a process that requires the chaperone DnaJ.

activate bNACHT25, however, this activation was not mediated by CP binding bNACHT25. Instead, CP induced changes to the cell that indirectly activated bNACHT25 in a process that depended on the host cell chaperone DnaJ. In addition, *dnaJ* was required for defense against the dsDNA phages T2 and T4. These data illustrate an effective strategy used by bNACHT25 to detect a wide range of phages that are not predicted to share homology between any of their proteins.

Two dominant mechanisms innate immune pathways use to sense invading pathogens are to detect either (1) pathogen-derived molecules or (2) pathogen-specific activities[2,40,41]. In the first mechanism, pathogen-associated molecular patterns (PAMPs) are directly sensed by pattern recognition receptors (PRRs). The PAMP-PRR model relies on PAMPs being invariant and crucial to the pathogen to limit immune evasion[42]. In the second mechanism, often termed effector-triggered immunity or the guard model, the innate immune system monitors for the biochemical activity of a pathogen-encoded effector[43]. The guard model relies on the function of pathogen effectors being essential to causing disease to limit immune evasion. NLRs in eukaryotes exemplify both of these mechanisms. The human NAIP/NLRC4 inflammasome is a PRR that detects structural components of bacterial type 3 secretion systems as a PAMP[44]. The *Arabidopsis* RPM1 R-protein is a guard for RIN4 and is activated by the pathogen effectors AvrRpm1 and AvrB[45]. bNACHT25 adopts a mechanism of sensing most similar to the guard model and senses perturbations to the host cell proteome. CP is not a conventional pathogen effector; however, it still impacts a core host process that bNACHT25 monitors as a proxy for infection. Across bacteria, bNACHT25 joins retrons[46,47] and ToxIN[48] in a growing list antiphage systems that indirectly survey the cell for signs of infection[1,2].

We propose that bNACHT25 is activated by binding DnaJ in complex with substrate proteins that become available during phage infection. Those substrates may be from the host cell as a result of CP expression or L protein itself interacting with DnaJ. In this way, bNACHT25 can guard both DnaJ and upstream host chaperones (e.g., DnaK or GroEL/ES) whose occupancy by CP may result in increased unfolded proteins. It is not clear what CP expression may specifically be doing to the cell, however, there are many lines of evidence linking viral infection and host chaperones. Several phages, including λ and T4, require the GroEL chaperonin to fold their capsid proteins[49,50]. Additionally, λ requires a functional DnaJ/DnaK chaperone system to complete its replication cycle[37], and MS2 experiences delayed lysis in a DnaJ mutant background, likely due to interactions between L and DnaJ[39].The dependence of some phage proteins, such as capsids, on chaperones to properly fold and assemble is curious. It suggests that perhaps there is a trade-off that a virus must make: in order to adopt the ideal capsid structure, they must use a host chaperone to assist in protein folding. Consistent with this, conservative point mutations in the major capsid protein of phage T4 can bypass the GroEL chaperonin, but these are not equivalently well-expressed to wild-type in GroEL proficient

strains[51]. Intriguingly, T4 bypass mutants that no longer require GroEL are reminiscent of the CP suppressor mutations identified here.

The requirement of phages for host chaperones provides the cell with a unique opportunity to detect a wide range of viruses through DnaJ without an easy path for the phage to escape immune detection. Guarding host chaperones may therefore be an effective strategy used by other uncharacterized antiphage systems. Given the highly conserved nature of DnaJ/Hsp40-family proteins throughout life, it may further be true that eukaryotic innate immune pathways also monitor their DnaJ homologs.

References

1. Georjon, H. & Bernheim, A. The highly diverse antiphage defence systems of bacteria. *Nat. Rev. Microbiol.* **21**, 686–700 (2023).
2. Ledvina, H. E. & Whiteley, A. T. Conservation and similarity of bacterial and eukaryotic innate immunity. *Nat. Rev. Microbiol.* 1–15 (2024) doi:10.1038/s41579-024-01017-1.
3. Bernheim, A. & Sorek, R. The pan-immune system of bacteria: antiviral defence as a community resource. *Nat. Rev. Microbiol.* **18**, 113–119 (2020).
4. Makarova, K. S., Wolf, Y. I., Snir, S. & Koonin, E. V. Defense islands in bacterial and archaeal genomes and prediction of novel defense systems. *J. Bacteriol.* **193**, 6039–6056 (2011).
5. Doron, S. *et al.* Systematic discovery of antiphage defense systems in the microbial pangenome. *Science* **359**, eaar4120 (2018).
6. Ting, J. P.-Y. *et al.* The NLR gene family: An official nomenclature. *Immunity* **28**, 285–287 (2008).
7. Chou, W.-C., Jha, S., Linhoff, M. W. & Ting, J. P.-Y. The NLR gene family: from discovery to present day. *Nat. Rev. Immunol.* **23**, 635–654 (2023).
8. Broz, P. & Dixit, V. M. Inflammasomes: mechanism of assembly, regulation and signalling. *Nat. Rev. Immunol.* **16**, 407–420 (2016).
9. Kibby, E. M. *et al.* Bacterial NLR-related proteins protect against phage. *Cell* **186**, 2410-2424.e18 (2023).
10. Leipe, D. D., Koonin, E. V. & Aravind, L. STAND, a Class of P-Loop NTPases Including Animal and Plant Regulators of Programmed Cell Death: Multiple, Complex Domain Architectures, Unusual Phyletic Patterns, and Evolution by Horizontal Gene Transfer. *J. Mol. Biol.* **343**, 1–28 (2004).
11. Koonin, E. V. & Aravind, L. The NACHT family – a new group of predicted NTPases implicated in apoptosis and MHC transcription activation. *Trends Biochem. Sci.* **25**, 223–224 (2000).
12. Gao, L. *et al.* Diverse enzymatic activities mediate antiviral immunity in prokaryotes. *Science* **369**, 1077–1084 (2020).
13. Gao, L. A. *et al.* Prokaryotic innate immunity through pattern recognition of conserved viral proteins. *Science* **377**, eabm4096 (2022).
14. Peng, S. *et al.* Structure and function of an effector domain in antiviral factors and tumor suppressors SAMD9 and SAMD9L. *Proc. Natl. Acad. Sci.* **119**, e2116550119 (2022).
15. Mekhedov, S. L., Makarova, K. S. & Koonin, E. V. The complex domain architecture of SAMD9 family proteins, predicted STAND-like NTPases, suggests new links to inflammation and apoptosis. *Biol. Direct* **12**, 13 (2017).
16. Aravind, L., Iyer, L. M. & Burroughs, A. M. Discovering Biological Conflict Systems Through Genome Analysis: Evolutionary Principles and Biochemical Novelty. *Annu. Rev. Biomed. Data Sci.* **5**, 367–391 (2022).
17. Knizewski, L., Kinch, L. N., Grishin, N. V., Rychlewski, L. & Ginalski, K. Realm of PD-(D/E)XK nuclease superfamily revisited: detection of novel families with modified transitive meta profile searches. *BMC Struct. Biol.* **7**, 40 (2007).
18. Tars, K. ssRNA Phages: Life Cycle, Structure and Applications. *Biocommunication Phages* 261–292 (2020) doi:10.1007/978-3-030-45885-0_13.
19. Quinones-Olvera, N. *et al.* Diverse and abundant phages exploit conjugative plasmids. *Nat. Commun.* **15**, 3197 (2024).
20. Fiers, W. *et al.* Complete nucleotide sequence of bacteriophage MS2 RNA: primary and secondary structure of the replicase gene. *Nature* **260**, 500–507 (1976).
21. Beremand, M. N. & Blumenthal, T. Overlapping genes in rna phage: a new protein implicated in lysis. *Cell* **18**, 257–266 (1979).
22. Valegård, K. *et al.* The three-dimensional structures of two complexes between recombinant MS2 capsids and RNA operator fragments reveal sequence-specific protein-RNA interactions. *J. Mol. Biol.* **270**, 724–738 (1997).
23. Peabody, D. S. *et al.* Immunogenic Display of Diverse Peptides on Virus-like Particles of RNA Phage MS2. *J. Mol. Biol.* **380**, 252–263 (2008).
24. Hobson, D. & Uhlenbeck, O. C. Alanine Scanning of MS2 Coat Protein Reveals Protein–Phosphate Contacts Involved in Thermodynamic Hot Spots. *J. Mol. Biol.* **356**, 613–624 (2006).
25. Stockley, P. G. *et al.* A Simple, RNA-Mediated Allosteric Switch Controls the Pathway to Formation of a T = 3 Viral Capsid. *J. Mol. Biol.* **369**, 541–52 (2007).
26. Golmohammadi, R., Fridborg, K., Bundule, M., Valegård, K. & Liljas, L. The crystal structure of bacteriophage Qβ at 3.5 å resolution. *Structure* **4**, 543–554 (1996).
27. Rao, V. B. & Black, L. W. Structure and assembly of bacteriophage T4 head. *Virol. J.* **7**, 356 (2010).
28. Murialdo, H. & Becker, A. Assembly of biologically active proheads of bacteriophage lambda in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **74**, 906–910 (1977).
29. Fokine, A. *et al.* Structural and functional similarities between the capsid proteins of bacteriophages T4 and HK97 point to a common ancestry. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 7163–7168 (2005).
30. Rūmnieks, J. *et al.* Three-dimensional structure of 22 uncultured ssRNA bacteriophages: Flexibility of the coat protein fold and variations in particle shapes. *Sci. Adv.* **6**, eabc0023 (2020).
31. Nasir, A. & Caetano-Anollés, G. Identification of Capsid/Coat Related Protein Folds and Their Utility for Virus Classification. *Front. Microbiol.* **8**, (2017).
32. Béchon, N. *et al.* Diversification of molecular pattern recognition in bacterial NLR-like proteins. 2024.01.31.578182 Preprint at https://doi.org/10.1101/2024.01.31.578182 (2024).
33. Baba, T. *et al.* Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008 (2006).
34. Kampinga, H. H. & Craig, E. A. The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nat. Rev. Mol. Cell Biol.* **11**, 579–592 (2010).
35. Rüdiger, S., Schneider-Mergener, J. & Bukau, B. Its substrate specificity characterizes the DnaJ co-chaperone as a scanning factor for the DnaK chaperone. *EMBO J.* **20**, 1042–1050 (2001).
36. Liberek, K., Wall, D. & Georgopoulos, C. The DnaJ chaperone catalytically activates the DnaK chaperone to preferentially bind the sigma 32 heat shock transcriptional regulator. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 6224–6228 (1995).
37. Sell, S. M., Eisen, C., Ang, D., Zylicz, M. & Georgopoulos, C. Isolation and characterization of dnaJ null mutants of Escherichia coli. *J. Bacteriol.* **172**, 4827–4835 (1990).
38. Sozhamannan, S. & Chattoraj, D. K. Heat shock proteins DnaJ, DnaK, and GrpE stimulate P1 plasmid replication by promoting initiator binding to the origin. *J. Bacteriol.* **175**, 3546–3555 (1993).
39. Chamakura, K. R., Tran, J. S. & Young, R. MS2 Lysis of Escherichia coli Depends on Host Chaperone DnaJ. *J. Bacteriol.* **199**, e00058-17 (2017).
40. Vance, R. E., Isberg, R. R. & Portnoy, D. A. Patterns of Pathogenesis: Discrimination of Pathogenic and Nonpathogenic Microbes by the Innate Immune System. *Cell Host Microbe* **6**, 10–21 (2009).
41. Huiting, E. & Bondy-Denomy, J. Defining the expanding mechanisms of phage-mediated activation of bacterial immunity. *Curr. Opin. Microbiol.* **74**, 102325 (2023).
42. Janeway, C. A. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb. Symp. Quant. Biol.* **54 Pt 1**, 1–13 (1989).
43. Remick, B. C., Gaidt, M. M. & Vance, R. E. Effector-Triggered Immunity. *Annu. Rev. Immunol.* **41**, 453–481 (2023).
44. Reyes Ruiz, V. M. *et al.* Broad detection of bacterial type III secretion system and flagellin proteins by the human NAIP/NLRC4 inflammasome. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 13242–13247 (2017).
45. Mackey, D., Holt, B. F., Wiig, A. & Dangl, J. L. RIN4 Interacts with *Pseudomonas syringae* Type III Effector Molecules and Is Required for RPM1-Mediated Resistance in *Arabidopsis*. *Cell* **108**, 743–754 (2002).
46. Millman, A. *et al.* Bacterial Retrons Function In Anti-Phage Defense. *Cell* **183**, 1551-1561.e12 (2020).
47. Bobonis, J. *et al.* Bacterial retrons encode phage-defending tripartite toxin–antitoxin systems. *Nature* **609**, 144–150 (2022).

48. Guegler, C. K. & Laub, M. T. Shutoff of host transcription triggers a toxin-antitoxin system to cleave phage RNA and abort infection. *Mol. Cell* **81**, 2361-2373.e9 (2021).
49. van der Vies, S. M., Gatenby, A. A. & Georgopoulos, C. Bacteriophage T4 encodes a co-chaperonin that can substitute for Escherichia coli GroES in protein folding. *Nature* **368**, 654–656 (1994).
50. Georgopoulos, C. P., Hendrix, R. W., Casjens, S. R. & Kaiser, A. D. Host participation in bacteriophage lambda head assembly. *J. Mol. Biol.* **76**, 45–60 (1973).
51. Andreadis, J. D. & Black, L. W. Substrate Mutations That Bypass a Specific Cpn10 Chaperonin Requirement for Protein Folding. *J. Biol. Chem.* **273**, 34075–34086 (1998).
52. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci.* **97**, 6640–6645 (2000).
53. Gibson, D. G. *et al.* Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **6**, 343–345 (2009).
54. Valentine, R. C. & Strand, M. Complexes of F-Pili and RNA Bacteriophage. *Science* **148**, 511–513 (1965).
55. Loeb, T. & Zinder, N. D. A bacteriophage containing rna. *Proc. Natl. Acad. Sci.* **47**, 282–289 (1961).
56. Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E. & Johnson, R. P. Enumeration of Bacteriophages by Double Agar Overlay Plaque Assay. in *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions* (eds. Clokie, M. R. J. & Kropinski, A. M.) 69–76 (Humana Press, Totowa, NJ, 2009). doi:10.1007/978-1-60327-164-6_7.
57. Golec, P. *et al.* A reliable method for storage of tailed phages. *J. Microbiol. Methods* **84**, 486–489 (2011).
58. Harel, N., Meir, M., Gophna, U. & Stern, A. Direct sequencing of RNA with MinION Nanopore: detecting mutations based on associations. *Nucleic Acids Res.* **47**, e148 (2019).
59. Baym, M. *et al.* Inexpensive Multiplexed Library Preparation for Megabase-Sized Genomes. *PLOS ONE* **10**, e0128036 (2015).
60. Ledvina, H. E. *et al.* An E1–E2 fusion protein primes antiviral immune signalling in bacteria. *Nature* **616**, 319–325 (2023).

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Author contributions

Conceptualization, A.N.C and A.T.W.; methodology, A.N.C., E.M.K. and A.T.W.; investigation, A.N.C., S.M.R, M.E.R., and A.T.W; writing – original draft, A.N.C. and A.T.W.; writing – review and editing, all authors; visualization, A.N.C.; resources: T.A.N. and E.M.K; supervision, A.T.W.; funding acquisition, A.T.W.

Declaration of interests

The authors declare no competing interests.

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Materials availability: Strains, plasmids, and phages used in this study are available upon reasonable request.

Materials and Methods

Bacterial strains and culture conditions

E. coli strains used in this study are listed in **Supplementary Table 3a**. Unless otherwise indicated, all cultures were grown in 1–4 mL of media in 14 mL culture tubes shaking at 220 rpm at 37 °C. "Overnight" cultures were started from either a single colony or glycerol stock and grown for 16–20 hours following inoculation. Culture media was supplemented with carbenicillin (100 µg/mL), chloramphenicol (20 µg/mL), kanamycin (50 µg/mL), and/or tetracycline (15 µg/mL) when applicable for plasmid maintenance or strain verification. Experiments were performed with *E. coli* MG1655 (CGSC6300) or *E. coli* BW25113 (CGSC7636), and *E. coli* OmniPir was used for construction and storage of plasmids. Where indicated, bNACHT sequences were inserted into the *lacZ* locus of *E. coli* MG1655 using Lambda red methodology as previously described[9].

Bacterial cultures used for cloning, strain construction, indicated colony formation assays, and immunoprecipitation assays were grown in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Strains were frozen for long-term storage in LB supplemented with 30% glycerol at 70 °C. Bacteria used to perform phage propagation, phage infection assays, DNA degradation assays, and indicated colony formation assays were cultivated in "MMCG" minimal medium (47.8 mM Na_2HPO_4, 22 mM KH_2PO_4, 18.7 mM NH_4Cl, 8.6 mM NaCl, 22.2 mM glucose, 2 mM $MgSO_4$, 100 mM $CaCl_2$, 3 mM thiamine, Trace Metals at 0.01 % v/v (Trace Metals mixture T1001, Teknova, final concentration: 8.3 µM $FeCl_3$, 2.7 µM $CaCl_2$, 1.4 µM $MnCl_2$, 1.8 µM $ZnSO_4$, 370 nM $CoCl_2$, 250 nM $CuCl_2$, 350 nM $NiCl_2$, 240 nM Na_2MoO_4, 200 nM Na_2SeO_4, 200 nM H_3BO_3)). When a strain with two plasmids was cultivated in MMCG medium, bacteria were grown in carbenicillin (50 µg/mL) and chloramphenicol (10 µg/mL). MMCG and LB agar plates contain 1.6% agar and media components described above.

Conjugation of F′ plasmid into *E. coli* MG1655

The plasmid F′ was introduced into various strains of *E. coli* MG1655 via conjugation. Briefly, 400 µL of overnight cultures of donor (*E. coli* MG1655 + F′ donor grown in LB + tetracycline) and recipient (various *E. coli* strains grown with proper antibiotics) were pelleted at 10,000 ×g for 1 minute. Pellets were washed in 20 µL of LB, pelleted again, and resuspended in 20 µL of LB. Resuspensions of donor alone, recipient alone, and mixture of donor and recipient were spotted on an LB plate, which was incubated upright for 1 hour at 37 °C. Spots were then struck to single colonies on LB + tetracycline + appropriate antibiotics to select for recipients that had received F′.

Plasmid construction

Plasmids used in this study are listed in **Supplementary Table 3a**. MS2 and Qβ phage genes were amplified from synthesized cDNA (see below). T4 and λ genes were amplified from 1 µL of boiled, diluted phage lysate. bNACHT genes were cloned from plasmids previously generated[9]. See **Supplementary Table 3c** for protein accession numbers.

bNACHT genes along upstream native promoter regions/downstream terminator regions were cloned into the SbfI/NotI sites of the pLOCO2 vector as previously described[53]. All phage genes were cloned into the NotI and BamHI or BmtI sites of pTACxc (for chloramphenicol selection) or pTACx (for carbenicillin selection) vector for inducible expression with IPTG. Restriction enzymes were purchased from New England Biolabs. DNA sequences were amplified and amended with ≥18 bp homology to their destination vectors using Q5 Hot Start High Fidelity Master Mix (NEB, M0494L). Vectors were constructed using Gibson Assembly with HiFi DNA Assembly Master Mix (NEB, E2621L) as described[54,55] and transformed into OmniPir via heat shock or electroporation. To add epitope tags, tag sequences were included in 5′ sequences of primers that annealed to the gene of interest. Plasmid sequences were verified with Sanger sequencing (Quintara Biosciences or Azenta).

Phage amplification and storage

Phages used in this study are listed in **Supplementary Table 3b**. F-dependent RNA phages were amplified using the *E. coli* MG1655 + F′ host[56], and dsDNA phages were amplified using *E. coli* MG1655. A modified double agar overlay plate amplification was used to generate phage lysates[57]. Briefly, 400 µL of mid-logarithmic phase (OD_{600} = 0.2–0.8) bacterial cultures were combined with 5,000–15,000 plaque forming units (PFU) of phage and incubated for 1 minute at room temperature to allow for adsorption. 3.5 mL of "top agar" (0.35% agar, 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 100 µM $MnCl_2$, 0.01% v/v Trace Metals) was then added to the phage-bacteria mixture which was then plated directly onto an MMCG agar plate. Plates were incubated overnight at 37 °C. To recover the amplified phage from the top agar, 5 mL of SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% gelatin) was applied to the top of the plate and the plate was incubated for 1–6 hours at room temperature. Phages were then harvested by transferring SM buffer from the plate to a tube. To increase phage titers, top agar overlay was sometimes scraped and harvested along with the SM buffer. In this case, the SM buffer was first centrifuged at 4,000 ×g for 10 minutes and supernatant was transferred to a new tube. 2–3 drops of chloroform

were added to all phage stocks followed by vortexing to sterilize the lysate. Phages were stored at 4 °C.

MS2-like (FrSaffron, FrHenna, and FrBlood) and Qβ-like (FrSangria, FrMerlot, FrHibiscus, FrBurgundy) phages were kindly provided by Michael Baym and colleagues. To plaque purify, serial dilutions of these phage stocks were plated as described in the above paragraph, and single plaques were picked using a glass Pasteur pipet and soaked out in 500 µL SM buffer with 1-2 drops of chloroform.

We observed that MS2 and Qβ phage stocks were prone to loss of infectiousness over time at 4 °C, at times inexplicably losing over 10^4 PFU within 6 months. To maintain phage stocks, MS2 and Qβ were frozen for long term storage according to published methods[33]. Briefly, phage was added to mid-logarithmic phase *E. coli* MG1655 + F′ in MMCG medium at an MOI of 0.5. The phage and bacteria were incubated for 15 minutes at room temperature without shaking to allow for adsorption. The mixture was then combined with glycerol (final concentration 15%), flash frozen in liquid nitrogen, and stored at −70 °C. To recover and amplify phages from frozen glycerol stocks, a small amount of stock was scraped and swirled into 100 µL of SM buffer. This mixture was then used in a plate amplification with *E. coli* MG1655 + F′ as described above.

Phage infection assays estimated efficiency of plaque formation

To quantify efficiency of plaque formation or titer for phage lysates, including determining phage defense, 400 µL of mid-logarithmic phase MG1655 + F′ expressing the defense system from the indicated vector or from the chromosome was combined with 3.5 mL of top agar and plated onto an MMCG agar plate. Once solidified, 2 µL of 10-fold serial dilutions of phage in SM buffer were spotted onto the top agar overlay. Once dried, plates were incubated overnight at 37 °C. The next day, plaques were quantified. When single plaques were not distinguishable/countable, the most dilute spot with visible phage (i.e. a hazy zone of clearance) was recorded as 10 PFU. When no plaques or zone of clearance were visible at the most concentrated spot, plaque formation fell below the limit of detection and 0.9 PFU were recorded for that spot. Phage protection data was reported as PFU/mL ± standard error of the mean (S.E.M) of n=3 biological replicates.

Phage infection time course in liquid culture

Bacteria containing the indicated plasmid were grown to mid-logarithmic phase in 25-mL MMCG cultures. 150 µL of cultures OD_{600}-normalized to 0.15 were added to wells of a 96-well plate in triplicates. Phage was added to each bacterial culture at the indicated MOI. Final volume of phage added to each well was 5 µL. Beginning immediately after infection, OD_{600} readings were recorded every 2 minutes over the course of the experiment using the SPARK Plate Reader (TECAN). Bacterial growth curves are representative of n=3 biological replicates. The mean of 3 technical replicates was plotted for each timepoint.

Knockout strains used for testing bNACHT25-mediated phage protection

Gene deletion mutants described in **Fig. 4c** and **Extended Data Fig. 3d** were constructed by replacing the indicated gene with the kanamycin resistance cassette (KanR), as previously described (Keio collection)[9] and obtained as glycerol stock duplicates. Strains were streaked to single colonies on LB + kanamycin, cultivated overnight, and transformed with plasmids expressing bNACHT25 or bNACHT25^{D48A} via electroporation.

MS2 suppressor mutant generation

Suppressor mutants were generated from 3 independent, wild-type parent MS2 phage stocks that were separately plate amplified on *E. coli* lacking a defense system. To generate suppressor phages, ~5 × 10^6 PFU of each parent phage was used to infect *E. coli* carrying plasmids expressing either bNACHT02, bNACHT12, bNACHT25, or bNACHT32 using the modified double agar overlay assay described above. Six single plaques were picked from each amplification ("primary suppressors"), soaked out in 500 µL of SM buffer, and re-amplified on bacterial lawns expressing the corresponding defense system to repurify and confirm suppressor mutants ("secondary suppressors"). bNACHT02 and bNACHT12 primary suppressors were diluted 1:1000 to generate single secondary suppressor plaques. bNACHT32 primary suppressors required higher concentrations of phage lysates to obtain secondary suppressor plaques (undiluted or 1:100 dilutions), and no bNACHT25 primary suppressors generated plaques upon repurification. Single secondary suppressor plaques were picked and soaked out in SM buffer for storage. The resulting 28 suppressor phage lysates were plate amplified and suppressors of interest were spot plated onto bacteria expressing the corresponding bNACHT system to confirm suppressor phenotype.

Phage genome sequencing and suppressor analysis

RNA genomes of MS2 and Qβ were purified as previously described[9]. Briefly, phage lysates were treated with DNase I, and RNA was extracted using the PureLink RNA Minikit (Invitrogen) according to the manufacturer's instructions. On-column DNase treatment was omitted, and RNA was eluted in 30 µL of nuclease-free water. RNA phage cDNA was synthesized using the Invitrogen SuperScript III First-Strand Synthesis System as previously described[58]. MS2 cDNA was then PCR amplified in 3 overlapping fragments using OneTaq polymerase using previously described primers[59]. Amplified MS2 genome was prepared for Illumina sequencing using a modification of the Nextera kit protocol as previously described[52]. Samples were sequenced using the Illumina MiSeq V2 Micro 300-cycle kit (CU Anschutz Genomics and Microarray Core). Using the Map to Reference feature in Geneious software, reads were mapped to the MS2 reference genome (NCBI Genome accession NC_001417). Under the Trim Primers option, the Nextera Trimming Oligo (AGATGTGTATAAGAGACAG) was trimmed from reads; otherwise, default settings were used.

The Geneious feature "Find Variations/SNPs" was used to identify variants in suppressor genomes. Variants were identified as suppressor mutations if they were present in ≥70% of reads and were not present in parent genomes.

bNACHT25 (DNA degradation) activation assay

Bacteria containing the indicated plasmids were grown to mid-logarithmic phase in 30 mL MMCG containing appropriate antibiotics. If applicable, phage was added at an MOI of 2, or 500 µM IPTG was added to induce gene expression. Following infection/induction, 2 × 10^9 CFU were harvested from each strain by pelleting at 4,000 ×g for 10 minutes at 4 °C. Plasmid DNA was extracted using standard miniprep protocol (Qiagen) and eluted in 60 µL DNA elution buffer (10 mM Tris-HCl pH 8.0). 20 µL of eluted DNA was combined with 5 µL of 6× Loading Dye (final concentration 4 mM Tris-HCl, 3% Ficoll-400, 12 mM EDTA, 0.04% Orange G, pH 8) and run for 30 minutes at 130 V on a 1% agarose gel (1% agarose, 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, 0.02% v/v SYBR Safe DNA stain). Gels were imaged using an Azure Biosystems Azure 200 Bioanalytical Imaging System.

Colony formation assays for measuring growth inhibition

To test for bacterial growth inhibition, 5 µL of 10-fold serial dilutions of indicated cultures were spotted onto the appropriate agar plates. Data in **Fig. 2d** was collected by inducing mid-logarithmic phase MMCG cultures with 500 µM IPTG or no inducer, growing bacteria for 80 minutes, and plating 5 µL of 10-fold serial dilutions of each culture on MMCG plates. In all other cases, overnight cultures in LB were spotted onto LB plates containing appropriate antibiotic and either 0.2% glucose for uninduced conditions or 500 µM IPTG for induced conditions. Once dry, plates were incubated overnight at 37 °C. The next day, colonies were quantified. In instances where single colonies were not distinguishable at the most dilute spot with visible bacteria (i.e., hazy spot of bacteria), 10 colony forming units (CFU) were recorded. When no colonies were visible at the most concentrated spot, 0.9 CFU were recorded. Colony formation data was reported as CFU/mL ± S.E.M of n=3 biological replicates.

Construction of *dnaJ* knockout strains

The *E. coli* MG1655 *dnaJ* gene was replaced using Lambda red recombination with *cat*, a gene encoding chloramphenicol acetyltransferase, as described previously[60]. Briefly, *cat* and its promoter were amplified from pKD3 with overhangs containing homology to 50 bp immediately upstream and downstream of the *dnaJ* gene. *E. coli* with chromosomally inserted KanR-VSVG-bNACHT25, KanR-VSVG-bNACHT32, or KanR-GFPmut3 carrying the pKD46 plasmid were grown to mid-logarithmic phase in LB + 0.2% arabinose to induce the Lambda red system. These bacteria were then made electrocompetent, transformed with gel-purified PCR products containing the *cat* insert via electroporation, and recovered for 2 hours at 30 °C. Cultures were then plated on LB + chloramphenicol to select for acquisition of *cat*. PCR and western blot were used to confirm the replacement of *dnaJ* with *cat* (**Fig. 5a, Extended Data Fig. 3b,** and data not shown).

Western blots

To measure protein expression via western blot, bacterial strains expressing indicated gene(s) were grown to mid-logarithmic phase. When applicable, 500 µM IPTG or phage at an MOI of 2 was added, and cultures were grown for an additional 20 minutes or indicated timepoints before extracting whole cell lysates. To harvest, 5 × 10^8 CFU were pelleted and when testing for CP expression after infection with MS2, pellets were washed with 1 mL of sterile water and re-pelleted. Pellets were all resuspended in 50 µL of LDS buffer (106 mM Tris-HCl pH 7.4, 141 mM Tris Base, 2% w/v lithium dodecyl sulfate, 10% v/v glycerol, 0.51 mM EDTA, 0.05% Orange G). Samples were then incubated at 95 °C for 10

minutes and centrifuged at 20,000 ×g for 3–5 minutes to remove debris. Samples in LDS were loaded at equal volumes on a 4–20% SDS-PAGE gel, run for ~1–2 hours at 100–160 V, and transferred to PVDF membranes charged in methanol. Membranes were blocked in Licor Intercept Buffer for 1 hour at room temperature and incubated with primary antibodies diluted in Intercept buffer overnight at 4 °C with rocking. αVSV-G (Rockland, RRID:AB_217930) was used at 1:10,000 to detect VSV-G-bNACHT25 and VSV-G-bNACHT32, αCP (Millipore, RRID:AB_2827507) was used at 1:10,000 to detect CP, αFLAG (Sigma, RRID:AB_259529) was used at 1:10,000 to detect 3×FLAG-bNACHT25^{D48A}, and αDnaJ (Enzo, RRID:AB_2039063) was used at 1:2,500 to detect DnaJ. αRNAP (Biolegend) was used at 1:5,000 as a loading control for testing protein levels.

Membranes were washed 3 times for 10 minutes each in TBS-T (Tris-buffered saline, 0.1% Tween-20) then incubated in Licor infrared (800CW/680RD) αRabbit/Mouse secondary antibodies diluted 1:40,000 in TBS-T for 1 hour at room temperature. Blots were visualized with the Licor Odyssey CLx.

Immunoprecipitation (IP) Assays

Immunoprecipitation assays were performed as described previously[60]. Briefly, *E. coli* MG1655 expressing 3×FLAG-bNACHT25^{D48A} or bNACHT25^{D48A} from one plasmid and inducible CP from another plasmid were grown in 25 mL of LB with 500 µM IPTG until they reached mid-logarithmic phase. OD_{600}-normalized cultures (~1 × 10^{10} CFU) were then centrifuged to 4,000 ×g for 10 minutes at 4 °C. The resulting pellet was resuspended in 4.5 mL lysis buffer (400 mM NaCl, 20 mM Tris-HCl pH 7.5, 2% glycerol, 1% triton, and 1 mM β-mercaptoethanol). Cells were lysed by sonication and then centrifuged at 20,000 ×g and 4 °C to remove cellular debris. 50 µL of each soluble lysate was saved as "Input." Samples were then mixed with 30 µL magnetic beads covalently linked to the αFLAG M2 antibody (Sigma) overnight at 4 °C with end-over-end rotation. After 3 washes in lysis buffer, IP beads were resuspended in 50 µL LDS. SDS-PAGE and western blots were performed as described above.

Mass Spectrometry (MS) analysis

An IP was performed as described above with the following alterations. To increase protein yields, 100 mL of induced, logarithmic-phase culture was processed for each sample. Following incubation of bacterial lysates with αFLAG beads, beads were washed 3 times with 20 mM ammonium bicarbonate. Dry beads were then subjected to on-bead trypsin digest followed by analysis on a Thermo Obitrap Q-Exactive HF-X using nanoLC-MS MS, as previously described[60]. Peptides were mapped to the proteome of *E. coli* MG1655 (Uniprot ID: UP000000625) in addition to MS2 CP and 3×FLAG-bNACHT25^{D48A} sequences. Two biological replicates of the IP were performed and analyzed by MS. Data from replicate 1 can be found in **Supplementary Table 2a** and replicate 2 can be found in **Supplementary Table 2b**.

Accession numbers

See **Supplementary Table 3c** for complete list of protein accession numbers. Briefly, bNACHT02: WP_021557529.1, bNACHT12: WP_021519735.1, bNACHT25: WP_001702659.1, bNACHT32: WP_057688292, CP (MS2): YP_009640125.1, CP (Qβ): BAP18764.1, maturation protein (MS2): YP_009640124.1, lysis protein (MS2): YP_009640126.1, replicase (MS2): YP_009640127.1, gp23 (T4): AAD42428.1, gp24 (T4): AAD42429.1, gpE (λ): AAA96540.1, DnaJ (*E. coli* MG1655): NP_414556.1.

Data Availability

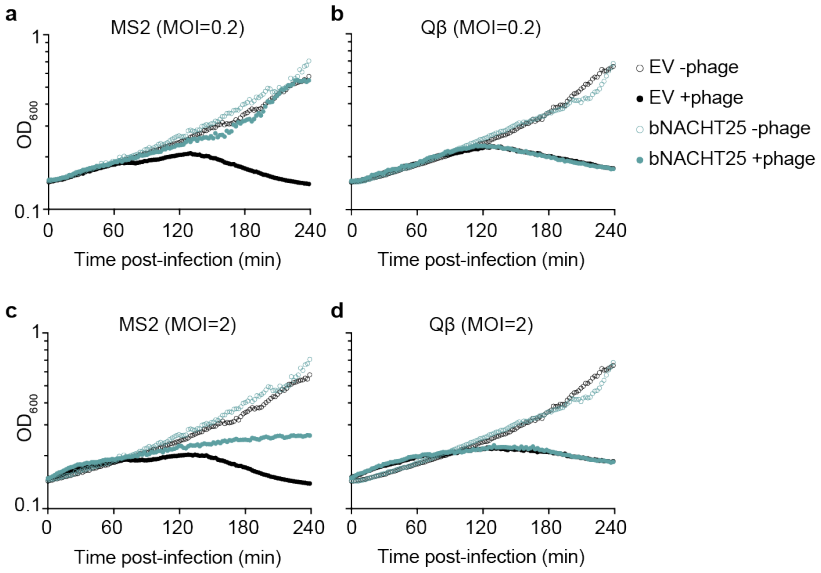
Data supporting the findings in this study are available within the article and its supplementary files.

Supplementary Figures and Tables

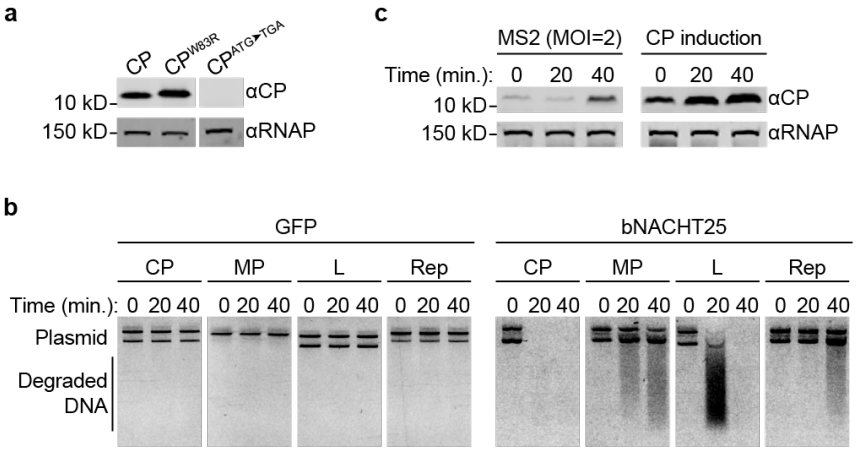
Supplementary Table 1. All MS2 suppressor mutations identified.

Supplementary Table 2. Mass spectrometry results for immunoprecipitated bNACHT25.

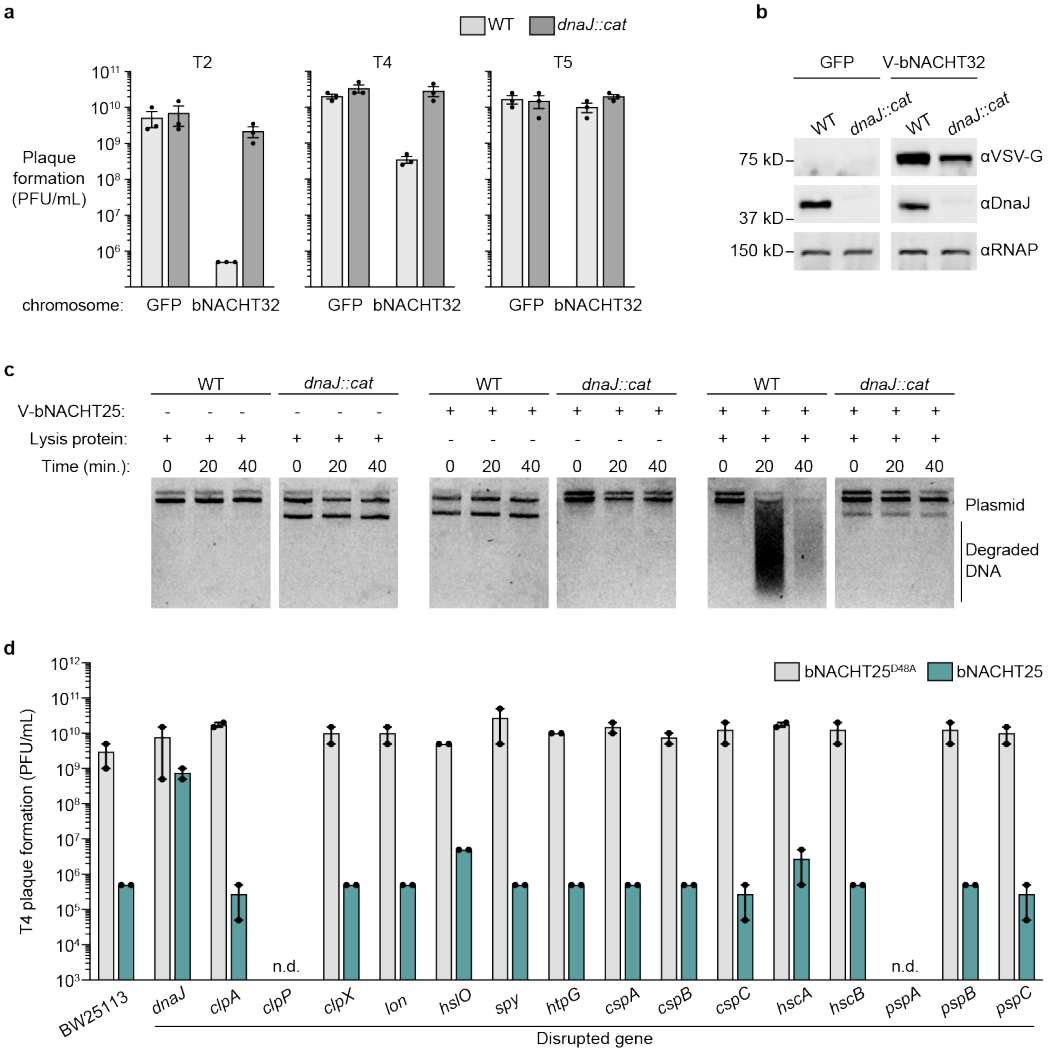
Supplementary Table 3. Strains, plasmids, phages, and accession numbers used in this study.



Extended Data Figure 1: bNACHT25 protects against infection by MS2 but not Qβ in liquid culture. (a-d) Growth curves of *E. coli* expressing either EV or bNACHT25. OD_{600} measurements began immediately following infection with the indicated phages at the indicated MOI. Data are representative of n=3 biological replicates. The mean of n=3 technical replicates is shown.



Extended Data Figure 2: Analysis of MS2 genes. (a) Western blot analysis of bacterial lysates generated from *E. coli* expressing the indicated CP alleles and GFP from the chromosome. Bacteria were harvested 20-minutes post-induction with IPTG. **(b)** Visualization of plasmid integrity in *E. coli* expressing either GFP or bNACHT25 on the chromosome coexpressed with the MS2 protein indicated via an inducible plasmid. Plasmid DNA was harvested at indicated timepoints post-induction with IPTG. **(c)** Western blot analysis of *E. coli* lysates generated from strains expressing bNACHT25 harvested at the indicated timepoints following infection with MS2 at MOI of 2 or induction of CP with IPTG. For (a-c), data are representative images of n=3 biological replicates.



Extended Data Figure 3: DnaJ is required for bNACHT25 and bNACHT32 activation by phage. (a) Efficiency of plating of the indicated phage on WT or *dnaJ::cat* *E. coli* MG1655 expressing either GFP or VSV-G-bNACHT32 from the chromosome. Data plotted as in **Fig. 1b**. chloramphenicol acetyltransferase (*cat*). **(b)** Western blot analysis of cell lysates generated from *E. coli* with the indicated genotypes. VSV-G-bNACHT32 (V-bNACHT32). **(c)** Visualization of plasmid integrity in *E. coli* with the indicated genotype at the indicated timepoints post-induction of L with IPTG. bNACHT25 is on the chromosome and L is on a plasmid. (+) represents indicated gene; (-) represents GFP negative control. **(d)** Efficiency of plating of phage T4 on *E. coli* BW25113 mutants containing plasmids expressing bNACHT25 or bNACHT25^{D48A}. Not determined (n.d.) indicates the strain was unable to grow with one or both plasmids. Data plotted as in **Fig. 1b**.