Supporting Information

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SI Methods.

Cloning and Plasmid Design. The construction of pEx6 was described in ref. 1. To create pEx6$^{TI\text{-}6}$, nucleotides 5068 to 5542 were amplified by PCR from the HK97 genome. This region encodes the 3′ end of gene *5* (the 3′ end of *5* is out of frame in the final plasmid and therefore is not translated), the intergenic region between genes *5* and *6*, and gene *6*. The resulting product was subcloned into pAD100 (2) such that transcription was IPTG inducible from the pTrc promoter, but the plasmid encoded ribosome binding site was far from the *6* translation start site and translation initiated from this site was terminated before the start of gene *6.* Amino acid substitutions to pEx6 and pEx6$^{TI\text{-}6}$ were made using a standard site-directed mutagenesis protocol, and mutations were confirmed by DNA sequencing.

Gene *7* was subcloned into pAD100 to create pEx7. To test the effect of the gene *6* translation rate on gene *7*, gene *7* was subcloned into pEx6$^{TI\text{-}6}$ to make pEx7$^{TI\text{-}6}$. pEx7$^{TI\text{-}7}$ was created by PCR amplification of nucleotides 5395 to 5880 from the HK97 genome as above. This region encodes the 3′ end of gene *6* (the 3′ end of *6* is out of frame in the final plasmid and therefore is not translated) and the entire gene *7*. This product was subcloned into pAD100 with the same strategy used for pEx6$^{TI\text{-}6}$.

Construction of a 7am Mutation in HK97. Genes *5–16* of HK97 were cloned into pAD100. Site-directed mutagenesis was performed to insert an amber codon (TAG) at the gene position encoding conserved Leu48 of gp7. A 594 (HK97 *6am*) lysogen containing this mutated plasmid was grown and induced with Mitomycin C. The phage-containing cell lysate was plated on *Escherichia coli* (*su6*), which inserts Leu at amber codons. Phages carrying the *6am* mutation will not grow on this strain; thus plaques were only formed by phages that had recombined with the plasmid to remove the *6am* mutation. These plaques were screened for isolates that had incorporated the *7am* mutation.

Electron Microscopy. The prophage of 594 (HK97 *6am*) was induced by treatment with Mitomycin C in the presence of plasmids overexpressing WT or mutant gp6 in the presence of IPTG. The resulting lysates were visualized by transmission electron microscopy (TEM) as described (1).

1. Cardarelli L, et al. (2010) The crystal structure of bacteriophage HK97 gp6: Defining a large family of head-tail connector proteins. *J Mol Biol* 395:754–768.
2. Davidson AR, Sauer RT (1994) Folded proteins occur frequently in libraries of random amino acid sequences. *Proc Natl Acad Sci USA* 91:2146–2150.

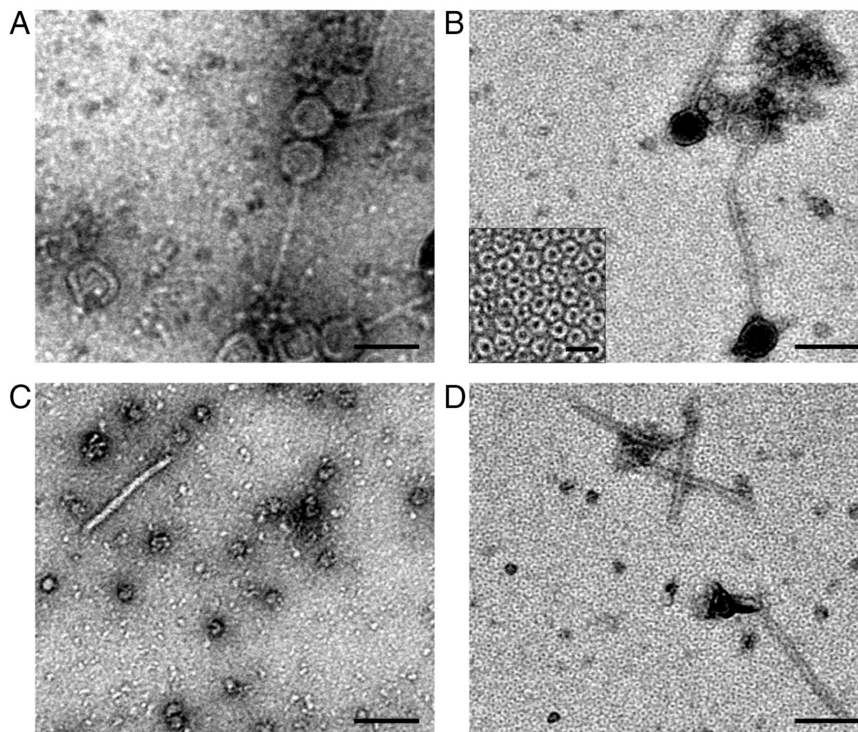

Fig. S1. Representative images of lysates of WT HK97 phage produced in the presence of gp6-overexpressing plasmids visualized by negatively stained transmission electron microscopy. Lysogens of WT HK97 transformed with plasmids expressing (*A*) no protein (i.e., empty vector), (*B*) WT gp6, (*C*) L28A, or (*D*) E89R were induced for phage production (with Mitomycin C) and protein expression (with IPTG) simultaneously. Lysates from these cells were visualized by negatively stained TEM. Lysates from cells that were transformed with empty vector contain the normal assembly intermediates heads, tails, and complete phage. Lysates of cells expressing WT or E89R gp6 contained an abundance of protein rings that are identical to rings previously identified as gp6 13-membered rings (1). (*B*, *Inset*) A higher magnification image of the ring complexes. Bars in large panels represent 90 nm, and the bar in the small inset panel is 20 nm.