

## A fully decompressed synthetic bacteriophage øX174 genome assembled and archived in yeast

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### ABSTRACT

The 5386 nucleotide bacteriophage øX174 genome has a complicated architecture that encodes 11 gene products via overlapping protein coding sequences spanning multiple reading frames. We designed a 6302 nucleotide synthetic surrogate, øX174.1, that fully separates all primary phage protein coding sequences along with cognate translation control elements. To specify øX174.1f, a decompressed genome the same length as wild type, we truncated the gene F coding sequence. We synthesized DNA encoding fragments of øX174.1f and used a combination of *in vitro*- and yeast-based assembly to produce yeast vectors encoding natural or designer bacteriophage genomes. We isolated clonal preparations of yeast plasmid DNA and transfected *E. coli* C strains. We recovered viable øX174 particles containing the øX174.1f genome from *E. coli* C strains that independently express full-length gene F. We expect that yeast can serve as a genomic 'drydock' within which to maintain and manipulate clonal lineages of other obligate lytic phage.

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### Introduction

Small icosahedral virus particles containing single stranded DNA genomes encoding various *Microviridae* have been found in many environments, from seawater to the human gut (Roux et al., 2012). Given its intrinsic natural importance plus accumulated relevance as an experimentally tractable model system, bacteriophage øX174 is now the best-studied member of the *Microviridae* (Hayashi et al., 1988; Fane et al., 2005). øX174 is also widely used to explore topics ranging from adaptive evolution, to molecular self-assembly, molecular systems biology, and virus particle transmission (Wichman and Brown, 2010; Gordon et al., 2012; Markely and Yin, 2007; Julian et al., 2010).

One notable feature of the øX174 genome is the extent to which the coding sequences for seemingly independent gene products overlap (Sanger et al., 1977). For example, gene E, whose encoded product is responsible for host cell lysis, is encoded entirely within an alternate reading frame internal to gene D, which encodes an external scaffold protein (Barrell et al., 1976; Fane et al., 2005). Overall, 16.8% of the øX174 genome encodes for more than one gene, and there are two genomic regions in which three genes overlap (Fig. 1A, top).

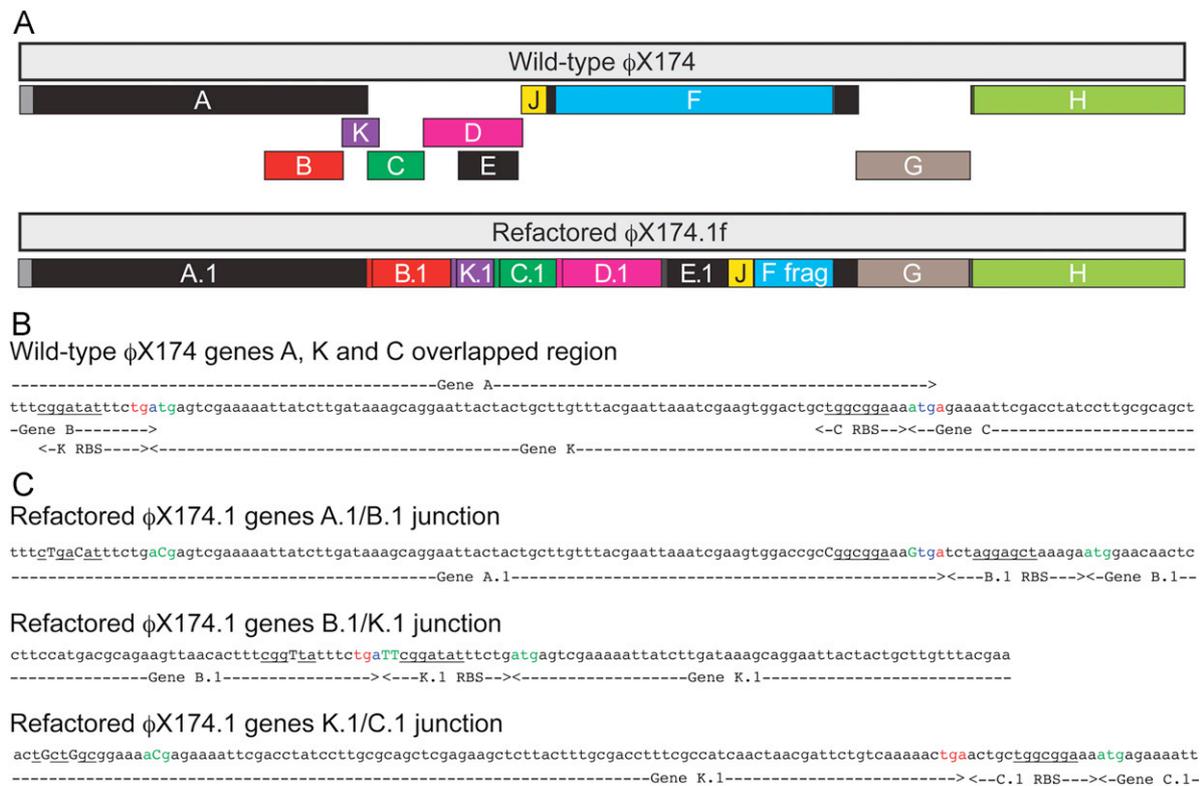
For broader context, ~30% of prokaryotic genes overlap with the coding sequences of neighboring genes (Kingsford et al., 2007), and several models have been proposed to explain the existence of

overlapping genetic elements. For example, Yokoo and Oshima (1979) hypothesized that the øX174 genome encodes information from an advanced alien civilization, with overlapping genes as the most likely candidate regions in which to find such messages. Researchers have also considered co-regulation of gene expression, genetic coding efficiency as driven by genome replication or packaging constraints, and coupling of evolutionary selection(s) as terrestrial factors leading to overlapping elements (Chirico et al., 2010). However, direct experimental tests demonstrating the spontaneous emergence of overlapping genes and other genetic elements have been difficult to implement given the starting complexity of already evolved natural systems.

Previously, using the bacteriophage T7 genome as a test case we introduced and explored the idea that natural genetic systems might be 'refactored' in order to produce engineered surrogates that are easier to study and apply (Chan et al., 2005). Our refactoring process reflected six engineering goals: (1) well-defined boundaries for all genetic elements, (2) no overlapping elements, (3) only one encoded function per element, (4) enable precise and independent manipulation of elements, (5) a genome that is possible to construct, (6) a genome that encodes a viable system. We constructed 12,179 base pairs of the T7.1 genome and recovered viable chimeric phage, albeit with significantly reduced fitness. Subsequent phage evolution experiments by others reported that ~35% of the T7.1 design changes were lost while selecting for mutant phage that recovered to a near wild-type fitness level (Springman et al., 2012). Taken together, these two studies suggest that genome refactoring to remove overlapping elements followed by experiments that attempt to select for "repacking" might be fruitful.

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**Fig. 1.** Design of a fully decompressed bacteriophage  $\phi$ X174 genome. (A) Reading frame comparison of the wild type and decompressed  $\phi$ X174.1f. Wild-type genome reading frames +1 to +3 are given from top to bottom. (B) Expanded view of the wild-type  $\phi$ X174 genes A, K, C junction. (C) Decompression of genes A, K, and C. To separate gene K from gene A (top) the start codon of K was changed from ATG to ACG and its RBS weakened. To decompress gene C from gene A (top) the start codon was changed from ATG to GTG and its RBS weakened. To decompress gene K from gene B (middle) the start codon was changed from ATG to ATT and its RBS weakened. To decompress gene C from gene K (bottom) the start codon was changed from ATG to ACG and its RBS weakened. Start and stop codons are green and red, respectively, while overlapping start/stop codons are blue. RBSs are underlined. Changes made during gene decompression are shown as capital letters.

However, given our prior experiences with T7, we first sought to develop a new system and methods that would be better suited for developing and applying ideas related to refactoring to obligate lytic phage. For example, many DNA fragments encoding phage genetic elements cannot be readily propagated using typical *E. coli* cloning strains and vectors. As a second example, we were limited in working on T7.1 by concerns that any individual genetic design change might produce a non-viable genome that could not be physically recovered, propagated, or amplified; since refactoring projects often attempt to change hundreds of nucleotides simultaneously (Temme et al., 2012), non-viable designs that cannot be independently cloned or propagated are difficult if not impossible to work with.

There have been notable advances in the synthesis, assembly, and handling of nucleic acids over the past decade (Carr and Church, 2009). For example, the genomes of several viruses and one microbe have been assembled from chemically synthesized oligonucleotides, with viable systems being recovered in each case (Cello et al., 2002; Smith et al., 2003; Gibson et al., 2010; Yang et al., 2011). Also of note, a bacterial genome was assembled via transformation-associated recombination in yeast, wherein the yeast-based bacterial genome might be maintained and further manipulated without immediate concern for the viability of the microbe resulting from the so-encoded bacterial genome (Gibson et al., 2008).

Thus, we sought to establish methods enabling the synthesis, assembly, and recovery of a bacteriophage genome via yeast. We first choose to work with bacteriophage  $\phi$ X174 given its small circular genome. We were then further motivated to explore if all genetic element overlaps within the  $\phi$ X174 genome might be eliminated and shown to be non-essential, generating the first fully decompressed phage genome.

## Results and discussion

### Designing a decompressed bacteriophage $\phi$ X174

We designed a completely decompressed version of  $\phi$ X174 in order to test the limits of refactoring with a highly overlapping natural genetic system. Our first design,  $\phi$ X174.1, eliminated all gene overlaps while preserving the use of native ribosome binding sites (RBSs) and promoters as much as possible (Fig. 1A, bottom). Our genetic decompression algorithm was similar to that used with T7: (i) overlapping coding sequences were fully separated, with sequence duplication as needed, into physically distinct open reading frames and (ii) vestiges of regulatory elements such as start codons and legacy RBSs were weakened or erased via silent point mutations so as to make translation initiation of legacy reading frames less likely (Fig. 1B and C). We generated synonymous mutations to remove remnant RBSs by reducing the frequency of A and G nucleotides. We used a published RBS strength calculator to confirm that the predicted strengths of so-changed RBSs decreased from wild-type strengths (Salis et al., 2009). Again following Chan et al. (2005), in making synonymous changes we sought to maintain codon usage bias but, when required, choose higher frequency codons.

Next, we used these design rules to separate genes B, K, C from each other and gene A, gene D from gene C, and genes E and J from gene D (Fig. 1, Supplementary Files). We also implemented 22 simultaneous point mutations from the wild-type sequence in order to disrupt legacy start codons and RBSs (Table 1). We referred to the so-changed coding and RBS sequences via a “letter.1” notation; for example, gene A.1 replaces gene A. In total, the process of genome decompression added 909 nucleotides to the wild-type genome.

**Table 1**  
Single nucleotide changes in the decompressed øX174.1f design to remove legacy gene sequences.

Position <sup>a</sup>	Refactored gene	Refactored sequence	Position <sup>b</sup>	Original gene <sup>c</sup>	Original sequence	Effect
1149	A.1	T	5066	B	A	Gene B RBS remnant weakened
1152	A.1	C	5069	B	T	Gene B RBS remnant weakened
1158	A.1	G	5075	B	A	Gene B remnant start codon changed ATG- > GTG
1509	A.1	T	40	K	G	Gene K RBS remnant weakened
1512	A.1	C	43	K	T	Gene K RBS remnant weakened
1521	A.1	C	52	K	T	Gene K remnant start codon changed ATG- > ACG
1590	A.1	C	121	C	T	Gene C RBS remnant weakened
1593	A.1	C	124	C	T	Gene C RBS remnant weakened
1602	A.1	G	133	C	A	Gene C remnant start codon changed ATG- > GTG
1974	B.1	T	42	K	A	Gene K RBS remnant weakened
1985	K.1 RBS	T	53	K	G	Gene K remnant start codon changed ATG- > ATT
2069	K.1	C	122	C	G	Gene C RBS remnant weakened
2072	K.1	C	125	C	G	Gene C RBS remnant weakened
2081	K.1	C	134	C	T	Gene C remnant start codon changed ATG- > ACG
2428	C.1	C	378	D	A	Gene D RBS remnant weakened
2431	C.1	C	381	D	T	Gene D RBS remnant weakened
2440	C.1	T	390	D	A	Gene D remnant start codon changed ATG- > TTG
2626	D.1	A	557	E	G	Gene E RBS remnant weakened
2638	D.1	C	569	E	T	Gene E remnant start codon changed ATG- > ACG
2905	D.1	G	836	J	A	Gene J RBS remnant weakened
2908	D.1	T	839	J	A	Gene J RBS remnant weakened
2919	E.1 RBS	T	850	J	G	Gene J remnant start codon changed ATG- > ATT
3378–3385	After J/F-intergenic	GCGATCGC	–	–	–	AsiSI site added J/F-intergenic

<sup>a</sup> Coordinates of refactored øX174.1f (see [Supplemental Data](#) for sequence file), beginning at the start of the H/A-intergenic region.

<sup>b</sup> Coordinates according to original øX174 sequence numbering scheme.

<sup>c</sup> Sequence belonging to original gene RBS or start codon although it is embedded in another gene in the wild type sequence.

We next addressed practical constraints arising from the length of DNA that can be physically packaged within a øX174 capsid without impacts to reproductive fitness. Previous work has shown that the length of a øX174 genome, when packaged *in vitro*, must be kept within a few percent of the 5386 nucleotide wild-type length in order to avoid any significant fitness decrease (Aoyama and Hayashi, 1985). Similar results were shown *in vivo* (Russell and Muller, 1984). To reduce the decompressed genome length we removed the first 916 nucleotides of gene F, encoding the coat protein (Air et al., 1978). We chose gene F because a plasmid containing a restriction fragment encoding wild-type gene F was able to complement two conditional gene F mutations (Avoort et al., 1983). Additionally, the gene F coding sequence is greater than the total of the combined increases needed to implement the øX174.1 genome design. The truncated gene F version of the decompressed genome was named øX174.1f. To complement øX174.1f when transformed into host cells we designed a medium copy vector expressing gene F under control of a rhamnose-inducible promoter (Fig. S1).

#### Building bacteriophage genomes in yeast

Our workflow to construct bacteriophage genomes in yeast consisted of several steps (Fig. 2). After designing a genome on a computer we obtained all genomic elements to be assembled via DNA synthesis or PCR cloning. We then used PCR amplification to add homologous overhangs to adjacent elements and any short functional sequences, such as restriction enzyme recognition sites, required for later steps. We mixed all DNA fragments together with a yeast/*E. coli* shuttle vector backbone and, if needed, performed *in vitro* isothermal assembly (Gibson et al., 2009) followed by transformation into yeast spheroplasts (Kouprina and Larionov, 2008; Orr-Weaver et al., 1983). We screened yeast colonies using multiplex PCR followed by plasmid purification from positive clones. These plasmids were subsequently digested to release the phage genome and ligated to reform a circular phage genome. Re-circularized genomes were

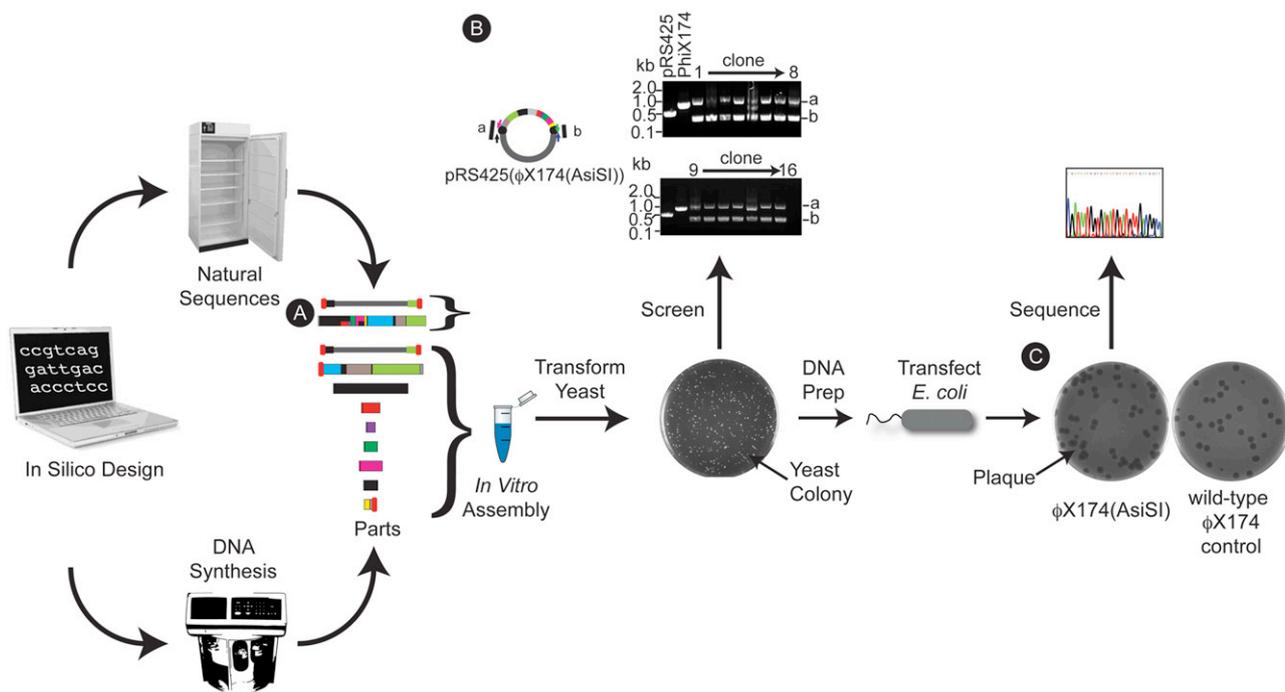
then transfected into suitable host cells. Finally, when our design and assembly process resulted in viable phage, we picked plaques and sequenced genomic DNA from clonal lineages.

#### Transplantation of wild-type øX174 to and from yeast

To test the validity of our assembly workflow and to determine whether bacteriophage genomes propagated in yeast remain viable, we first attempted to capture and then recover a wild-type øX174 genome from yeast. We used PCR to linearize the yeast shuttle vector pRS425 while also adding overhangs homologous to the ends of a linearized wild-type øX174 genome plus AsiSI restriction sites (Fig. 2A). Both DNA fragments were co-transformed into yeast spheroplasts. Plasmids from positive screened clones (Fig. 2B) were processed via restriction digestion and ligation and transformed into *E. coli* C to establish that a recovered øX174 genome would still initiate infection. We recovered equivalent size plaques following overnight plating for both a wild-type positive control and also with øX174(AsiSI) (Fig. 2C, Table 2). We did not recover plaques from transformations for which the yeast vector backbone was not removed via AsiSI digestion. Overall, of the 16 yeast clones we screened, 11 were positive by PCR for both the phage-vector junctions and 7 of these clones produced phage, giving an overall yeast assembly followed by redeployment process efficiency of ~44%.

#### Assembly and recovery of a decompressed bacteriophage øX174

We next sought to construct and test a øX174.1f genome. We assembled the øX174.1f genome from nine DNA fragments encoding independent phage elements plus the shuttle vector backbone. Because assembly efficiencies decline with increasing numbers of fragments (Liang et al., 2012), we used longer 30 base pair homologous fragment overhangs. Yeast-based assembly alone yielded no functional phage. Therefore, to increase the proportion of complete assemblies, we used an *in vitro* isothermal assembly



**Fig. 2.** Building bacteriophage with yeast. Complete workflow for assembling and recovering a bacteriophage genome from a combination of natural and synthesized DNA parts. From left to right, the DNA sequence of a new bacteriophage genome is designed using software tools. The required parts are sourced from either natural sequences or DNA synthesis. Parts are amplified by PCR. Assemblies involving more than two parts are assembled *in vitro* followed by transformation into yeast spheroplasts. An auxotrophic marker encoded on the yeast vector is selected for. Transformants are screened using colony multiplex PCR with primer sets spanning key assembly junctions. Cultures of positively screened colonies are grown and plasmid DNA is purified directly from cell pellets. Phage genomic DNA is then released from the yeast vector, religated, and transformed into bacterial host cells. Resultant plaques are sequenced to verify correctness of the assembly. (A) A linearized wild-type øX174 genome assembled with yeast vector pRS425. Flanking AsiSI restriction sites were added to the assembly via PCR primers used to linearize the vector, so that the phage genome can be released from the vector and re-circularized prior to bacterial transfection. (B) Multiplex PCR screening of pRS425(øX174(AsiSI)) assemblies. The left schematic shows amplicon locations for junction testing. Letters (right) note expected amplicon sizes for correct assemblies. (C) Infection of *E. coli* C with øX174(AsiSI) genomes recovered from yeast.

**Table 2**

Plaque sizes of wild type and refactored øX174.1f strains grown with varying amounts of gene F induction.

Host strain	Phage	Rhamnose ( $\mu\text{M}$ )	Plaque size <sup>a</sup>
<i>E. coli</i> C	wt øX174	–	6(1)
<i>E. coli</i> C	ø174X(AsiSI)	–	6(1)
<i>E. coli</i> C	Refactored øX174.1f	–	n/a
<i>E. coli</i> C(p804(Gene F))	wt øX174	0	4.9(0.7)
<i>E. coli</i> C(p804(Gene F))	wt øX174	1	4.6(0.6)
<i>E. coli</i> C(p804(Gene F))	wt øX174	10	4(1)
<i>E. coli</i> C(p804(Gene F))	wt øX174	100	4(1)
<i>E. coli</i> C(p804(Gene F))	wt øX174	1000	4.4(0.8)
<i>E. coli</i> C(p804(Gene F))	Refactored øX174.1f	0	n/a
<i>E. coli</i> C(p804(Gene F))	Refactored øX174.1f	1	0.6(0.1)
<i>E. coli</i> C(p804(Gene F))	Refactored øX174.1f	10	0.9(0.2)
<i>E. coli</i> C(p804(Gene F))	Refactored øX174.1f	100	2.2(0.7)
<i>E. coli</i> C(p804(Gene F))	Refactored øX174.1f	1000	4.1(0.6)

<sup>a</sup> Plaque sizes in mm  $\pm$  standard deviation in brackets.

step (Gibson et al., 2009) prior to yeast transformation. We screened resulting yeast clones using two multiplex primer pairs targeting both phage-vector junctions (Fig. 3A). Positive clones were processed as before and transformed into an *E. coli* C strain expressing gene F under the control of rhamnose induction. Full genome sequencing of phage DNA recovered from a clonal lineage cultured from a plaque confirmed that all designed changes to the wild-type genome were present in øX174.1f. Additionally, in gene G, where our design recapitulated the wild-type sequence (Sanger et al., 1977) we identified two unexpected nucleotide changes: a GAT (Asn) to AAT (Asp) substitution in the 113th codon, and an AAC to AAT silent mutation in the 139th codon. Both of these differences

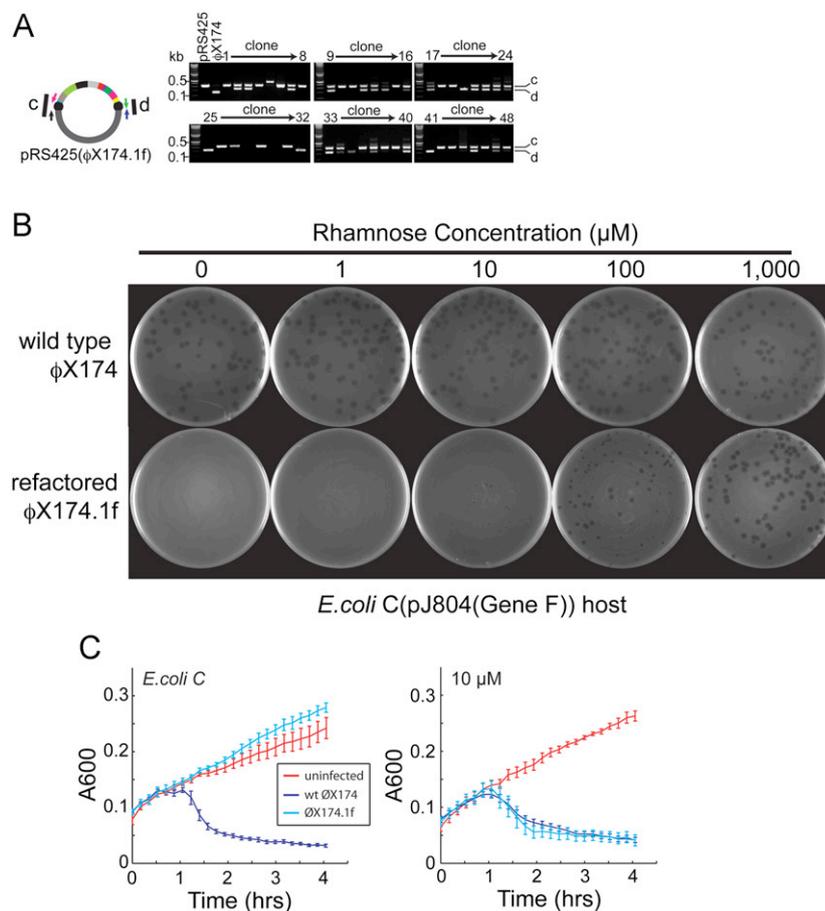
from our design likely arose from sourcing of the DNA fragment encoding gene G via PCR from our laboratory phage stock (as opposed to *de novo* DNA synthesis). Both polymorphisms are consistent with observed variation in the øX174 genome, as Smith et al. (2003) also reported these exact two changes from sequencing of laboratory stocks of øX174.

#### Initial phenotypic characterization of øX174.1f

We checked for first-order phenotypic effects, if any, resulting from the øX174.1f genome architecture. For reference, separation of bacteriophage T7 early genes resulted in a 50% reduction in plaque size and lysis times (Chan et al., 2005). First, we compared wild-type and decompressed phage plaque formation on an *E. coli* C host expressing various amounts of gene F under the control of a rhamnose inducible promoter (Fig. 3B). Wild-type plaque sizes did not change significantly with varying gene F induction (Table 2). No plaques were observed for øX174.1f when 0  $\mu\text{M}$  rhamnose was added. øX174.1f plaque sizes increased from  $\sim 7.5$ -fold smaller than wild-type at 1  $\mu\text{M}$  rhamnose to virtually identical to wild-type at 1000  $\mu\text{M}$  (Table 2). These results suggest that gene F expression levels are closely coupled to plaque size.

We also assayed the growth properties of øX174.1f in liquid culture. As expected, the decompressed øX174.1f phage was unable to lyse *E. coli* C lacking the gene F plasmid (Fig. 3C, left panel). We observed that both wild-type øX174 and øX174.1f phage lysed *E. coli* C (pJ804(Gene F)) with similar efficiency (Fig. 3C, right panel).

Taken together, when sufficient gene F is supplied *in trans* the first-order phenotype of øX174.1f is indistinguishable from the wild type. In an artificial fashion, our decompression of the øX174 genome has allowed us to travel ‘backwards in time’ regarding *Microviridae* evolution. For example, previous bioinformatic analysis has suggested



**Fig. 3.** Assembly and characterization of a the fully decompressed  $\phi$ X174.1f genome. (A) Multiplex PCR screening of pRS425( $\phi$ X174.1f) assemblies. Schematic on left shows locations of PCR amplicons. Letters on right denote expected amplicon sizes for correct assembly of the first and last phage parts with the yeast vector. (B) Characterization of wild-type  $\phi$ X174 and decompressed  $\phi$ X174.1f plaque size in *E. coli C* strain expressing gene F under the control of a rhamnose inducible promoter. (C) Characterization of wild-type  $\phi$ X174 and decompressed  $\phi$ X174.1f liquid lysis dynamics. Left panel depicts infection of *E. coli C*; right panel depict infection of *E. coli C*(pJ804(Gene F)) in media supplemented with 10  $\mu$ M rhamnose. Cells were grown in TK broth with 5 mM CaCl<sub>2</sub> and 10 mM MgCl<sub>2</sub> added just prior to infection. Assays were carried out at 37C with an initial average MOI of  $\sim$ 5. Uninfected control (red), decompressed  $\phi$ X174.1f (light blue), and wild-type  $\phi$ X174 phage (dark blue). Error bars give standard deviations from four replicates.

that  $\phi$ X174 genes evolved in a step-wise fashion, whereby gene A first expanded its 3' end to overlap gene B, followed by overprinting of genes K and E (Pavesi, 2006). Such findings are supported by the non-essential nature of genes A\*, K and E (Colasanti and Denhardt, 1987; Gillam et al., 1985; Pollock et al., 1978). Our aggregate finding that no essential information is lost by full  $\phi$ X174 gene decompression may further support the hypothesis that genome compression results from evolutionary pressure to package more genetic information within a limited capsid size (Chirico et al., 2010). Now that the  $\phi$ X174 genetic sequences have been separated from one another further tests of such evolutionary relationships can be pursued via direct experiments.

## Materials and methods

### Bacterial, yeast and bacteriophage strains and propagation

Wild-type  $\phi$ X174 (ATCC # 13706-B1) and *E. coli C* host (ATCC # 13706) were used throughout. All competent cells were made by the TSS method (Chung et al., 1989). Bacterial cells were propagated with LB and TK broth (Fane and Hayashi, 1991). *E. coli C* (pJ804(Gene F)) was routinely grown with carbenicillin at 50  $\mu$ g/mL concentration. Yeast strain W303 was used.  $\phi$ X174 was propagated following published methods (Fane and Hayashi, 1991).

### DNA design and manipulation and sequencing

$\phi$ X174 wild-type sequence NC\_001422.1 was used throughout as the reference  $\phi$ X174 sequence. All wild-type sequences are numbered as in NC\_001422.1 (from the single PstI cleavage site) as originally designated (Sanger et al., 1977). All refactored sequence coordinates are referenced from the first nucleotide of the H/A-intergenic region. A plasmid Editor (ApE, <<http://biologylabs.utah.edu/jorgensen/wayned/ape/>>) was used to visualize and manipulate all sequences. The Primer3Plus web interface (<<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>>) (Rozen and Skaletsky, 2000), NCBI Primer-BLAST (Ye et al., 2012), and NetPrimer (Premier Biosoft) were used for designing and analyzing oligonucleotides. Synthetic DNA for genes A.1, B.1, K.1, C.1, D.1, E.1 and the pJ804(Gene F) plasmid were synthesized by DNA2.0, Inc. The decompressed  $\phi$ X174.1f genome was sequenced via Sanger methods by Elim Biopharmaceuticals Inc., and we assembled sequencing reads into a contig using CodonCode Aligner. Average sequencing depth was greater than 3x at all positions.

### Building bacteriophage genomes in yeast

To capture the  $\phi$ X174 genome inside yeast we first linearized the yeast/*E. coli* shuttle vector pRS425 (Christianson et al., 1992) by using PCR amplification with opposite facing primers at

coordinates 2957 and 2971 Genbank Accession #U03452.1, leaving out a small part of the plasmid between the primers. The forward primer 1 (Table S1) encodes (5'–3') 20 nt of  $\phi$ X174 homologous sequence, AsiSI site, and pRS425 homologous sequence. The reverse primer 2 was the same layout as the forward primer, both binding within *lacZ*. *DpnI* digestion followed amplification.

Since the native  $\phi$ X174 genome is circular and single-stranded, we purified a dsDNA intermediate form of the genome (called RFI form) from infected cells (Burch et al., 1999). This purified circular dsDNA was linearized by PCR using opposite facing PCR primers (as for vector linearization above) that bind to the Gene H/A-intergenic region boundary. The forward primer 3 binds at the beginning of the H/A-intergenic region while the reverse primer 4 binds at the 3'-end of Gene H. This site of linearization of the  $\phi$ X174 genome was chosen because the region of the genome: (1) does not encode any known genes, (2) is upstream of the  $P_A$ -promoter and origin of viral strand replication located in Gene A (Hayashi et al., 1988), and (3) the junction is not known to encode any other function.

Linear  $\phi$ X174 genomes and pRS425 were co-transformed into yeast spheroplasts (strain W303) (Kouprina and Larionov, 2008; Orr-Weaver et al., 1983). Multiplex screening of the assemblies were performed on resuspended yeast colonies directly picked from 3 to 5 days re-grown spheroplast transformations. Yeast colonies were resuspended in 50  $\mu$ L Zymolyase 20 T (MP Biochemicals) and 0.05  $\mu$ L beta-mercaptoethanol, then incubated for 1 h at 37°C. Primer sets for multiplex PCR were designed using muPlex (Rachlin et al., 2005). See Supplementary materials for primer sequences 5–8. Qiagen Multiplex PCR Plus kit was used for all screens. For  $\phi$ X174(AsiSI) assembly screening the following PCR conditions were used: 95C/5:00, 35 cycles of 95C/0:30; 65C/1:30; 72C/1:00, 68C/10:00, 8C/hold. 1–2  $\mu$ L of yeast resuspensions were used for PCR template, while 1 ng of purified  $\phi$ X174 RFI (New England Biolabs) and pRS425 control templates used per 10  $\mu$ L reaction. We cultured positive clones and purified plasmid DNA using a modified miniprep procedure (Singh and Weil, 2002).

The decompressed  $\phi$ X174.1f genome was built identically to  $\phi$ X174(AsiSI), but with the following exceptions. Yeast vector pRS425 was linearized by *SmaI* digestion, followed by gel purification. The middle of gene F to the end of the H/A-intergenic region (coordinates 1917–3980) and gene J (coordinates 832–1000) parts of the assembly were amplified from wild-type  $\phi$ X174 RFI DNA generated from ATCC # 13706-B1. All other parts were amplified from plasmids synthesized by DNA2.0. All parts were assembled *in vitro* using the Gibson isothermal assembly method (Gibson et al., 2009), with 10 fmol of each part for 15 min at 50C, prior to yeast spheroplast transformation. Yeast clones were screened with multiplex primers 9–12. We initially screened yeast clones with denser multiplex PCR amplicons covering all part-part assembly junctions but dropped this expanded testing since we found the results to be difficult to interpret due to the highly repetitive nature of the  $\phi$ X174.1f genome. Out of the 48 yeast clones we screened, 18 were positive by PCR for both phage-vector junctions and only 1 of the prepared and processed vectors resulted in plaque formation, giving an overall success rate of ~2%. We picked plaques, produced liquid culture phage lysates, and sequenced single-stranded genomic DNA from purified  $\phi$ X174.1f phage particles.

#### $\phi$ X174 plaque assays

For plaque assays, we added 100  $\mu$ L phage in BE or 100  $\mu$ L  $\phi$ X174 transformed *E. coli* C cells, plus 100  $\mu$ L untransformed host *E. coli* C grown in LB broth, to 5 mL 0.7% TK agar at 46C. This mixture of cells and agar was poured over 15 mL 1.2% TK agar

plate and incubated at 37C for 12 h. For *E. coli* C(pJ804(Gene F)), antibiotics and rhamnose were added to the bottom agar in amounts needed for the total volume of bottom plus top agar.

#### Lysis curves

To obtain lysis curve data, 96-well plate experiments were set up in a Victor3 plate reader at 37C. A600 measurements were taken every 5 min, and MATLAB was used to process the data. The volume of each 200  $\mu$ L well was comprised of phage, *E. coli* C(p804(Gene F)), 5 mM CaCl<sub>2</sub>, 10 mM MgCl<sub>2</sub>, variable amounts of rhamnose, and TK broth for the remaining volume.

#### Author contributions

PRJ and DE conceived the study. PRJ, JR, and AS designed  $\phi$ X174.1. PRJ and EKL performed all experiments. PRJ, EKL, and DE wrote the paper.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2012.09.020>.

#### References

- Air, G.M., Coulson, A.R., Fiddes, J.C., Friedmann, T., Hutchison III, C.A., Sanger, F., Slocombe, P.M., Smith, A.J.H., 1978. Nucleotide sequence of the F protein coding region of bacteriophage  $\phi$ X174 and the amino acid sequence of its product. *J. Mol. Biol.* 125 (2), 247–248.
- Aoyama, A., Hayashi, M., 1985. Effects of genome size on bacteriophage  $\phi$ X174 DNA packaging *in vitro*. *J. Biol. Chem.* 260 (20), 11033.
- Avoort, H.G., Van der Teertstra, R., Versteeg, R., Weisbeek, P.J., 1983. Genes and regulatory sequences of bacteriophage  $\phi$ X174. *Biochim. Biophys. Acta* 741, 94–102.
- Barrell, B., Air, G., Hutchison III, C., 1976. Overlapping genes in bacteriophage  $\phi$ X174. *Nature* 264, 34–41.
- Burch, A.D., Ta, J., Fane, B.A., 1999. Cross-functional analysis of the microviridae internal scaffolding protein. *J. Mol. Biol.* 286 (1), 95–104.
- Carr, P.A., Church, G.M., 2009. Genome engineering. *Nat. Biotechnol.* 27, 1151–1162.
- Cello, J., Paul, A.V., Wimmer, E., 2002. Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. *Science* 297, 1016–1018.
- Chan, L.Y., Kosuri, S., Endy, D., 2005. Refactoring bacteriophage T7. *Mol. Syst. Biol.* 1, E1–E10.
- Chirico, N., Vianelli, A., Belshaw, R., 2010. Why genes overlap in viruses. *Proc. R. Soc. London B Biol. Sci.* 277, 3809–3817.
- Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., Hieter, P., 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene* 110 (1), 119–122.
- Chung, C.T., Niemela, S.L., Miller, R.H., 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Sci. USA* 86, 2172–2175.
- Colasanti, J., Denhardt, D.T., 1987. Mechanism of replication of bacteriophage  $\phi$ X174 XXII. Site-specific mutagenesis of the A\* gene reveals that A\* protein is not essential for  $\phi$ X174 DNA replication. *J. Mol. Biol.* 197 (1), 47–54.
- Fane, B.A., Hayashi, M., 1991. Second-site suppressors of a cold-sensitive prohead accessory protein bacteriophage  $\phi$ X174. *Genetics* 128, 663–671.
- Fane, B.A., Brentlinger, K.L., Burch, A.D., Chen, M.I.N., Hafenstein, S., Moore, E., Novak, C.R., Uchiyama, A., 2005.  $\phi$ X174 et al., the *Microviridae*. In: Abedon, S., Calendar, R.L. (Eds.), *The Bacteriophages*, second ed. Oxford University Press, USA, pp. 129–145.
- Gibson, D.G., Benders, G.A., Andrews-Pfannkoch, C., Denisova, E.A., Baden-Tillson, H., Zaveri, J., Stockwell, T.B., Brownley, A., Thomas, D.W., Algire, M.A., Merryman, C.,

- Young, T., Noskov, V.N., Glass, J.I., Venter, J.C., Hutchison, C.A., Smith, H.O., 2008. Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science* 319, 1215–1220.
- Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6, 343–345.
- Gibson, D.G., Glass, J.I., Lartigue, C., Noskov, V.N., Chuang, R.-Y., Algire, M.A., Benders, G.A., Montague, M.G., Ma, L., Moodie, M.M., Merryman, C., Vashee, S., Krishnakumar, R., Assad-Garcia, N., Andrews-Pfannkoch, C., Denisova, E.A., Young, L., Qi, Z.-Q., Segall-Shapiro, T.H., Calvey, C.H., Parmar, P.P., Hutchison, C.A., Smith, H.O., Venter, J.C., 2010. Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 329, 52–56.
- Gillam, S., Atkinson, T., Markham, A., Smith, M., 1985. Gene *K* of bacteriophage  $\phi$ X174 codes for a protein which affects the burst size of phage production. *J. Virol.* 53, 708–709.
- Gordon, E.B., Knuff, C.J., Fane, B.A., 2012. Conformational switch-defective  $\phi$ X174 internal scaffolding proteins kinetically trap assembly intermediates before procapsid formation. *J. Virol.* 86, 9911–9918.
- Hayashi, M., Aoyama, A., Richardson, D.L., Hayashi, M.N., 1988. Biology of the bacteriophage  $\phi$ X174. (Ed.) In: Calendar, R. (Ed.), *The Bacteriophages*, 2. Plenum Press, New York, NY, pp. 1–71.
- Julian, T.R., Leckie, J.O., Boehm, A.B., 2010. Virus transfer between fingerpads and fomites. *J. Appl. Microbiol.* 109 (6), 1868–1874.
- Kingsford, C., Delcher, A.L., Salzberg, S.L., 2007. A unified model explaining the offsets of overlapping and near-overlapping prokaryotic genes. *Mol. Biol. Evol.* 24, 2091–2098.
- Kouprina, N., Larionov, V., 2008. Selective isolation of genomic loci from complex genomes by transformation-associated recombination cloning in the yeast *Saccharomyces cerevisiae*. *Nat. Protocols* 3, 371–377.
- Liang, X., Peng, L., Tsvetanova, B., Li, K., 2012. Recombination-based DNA assembly and mutagenesis methods for metabolic engineering. In: Cheng, Q. (Ed.), *Methods in Molecular Biology*. Springer New York, New York, NY.
- Markely, L.R.A., Yin, J., 2007. Intracellular dynamics of bacteriophage  $\phi$ X174 gene expression. *Journal of Young Investigators* 16, 5.
- Orr-Weaver, T.L., Szostak, J.W., Rothstein, R.J., 1983. Genetic applications of yeast transformation with linear and gapped plasmids. *Meth. Enzymol.* 101, 228–245.
- Pavesi, A., 2006. Origin and evolution of overlapping genes in the family *Microviridae*. *J. Gen. Virol.* 87, 1013–1017.
- Pollock, T.J., Tessman, E.S., Tessman, I., 1978. Identification of lysis protein E of bacteriophage  $\phi$ X174. *J. Virol.* 28, 408–410.
- Rachlin, J., Ding, C.M., Cantor, C., Kasif, S., 2005. muPlex: A multi-objective approach to multiplex PCR assay design. *Nucleic Acids Res.* 33, W544–W547. (Web Server Issue).
- Roux, S., Krupovic, M., Poulet, A., Debroas, D., Enault, F., 2012. Evolution and diversity of the *Microviridae* viral family through a collection of 81 new complete genomes assembled from virome reads. *PLoS ONE* 7 (7), e40418. <http://dx.doi.org/10.1371/journal.pone.0040418>.
- Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S., Misener, S (Eds.), *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp. 365–386.
- Russell, P.W., Muller, U.W.E.R., 1984. Construction of bacteriophage  $\phi$ X174 mutants with maximum genome sizes. *J. Virol.* 52, 822–827.
- Salis, H.M., Mirsky, E.A., Voigt, C.A., 2009. Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol.* 27, 946–950.
- Sanger, F., Barrell, B.G., Brown, N.L., Coulson, A.R., Fiddes, J.C., Hutchison III, C.A., Slocombe, P.M., Smith, M., 1977. The nucleotide sequence of bacteriophage  $\phi$ X174. *Nature* 265, 687–695.
- Singh, M.V., Weil, P.A., 2002. A method for plasmid purification directly from yeast. *Anal. Biochem.* 307, 13–17.
- Smith III, H., Pfannkoch, C.H., Venter, J.C., 2003. Generating a synthetic genome by whole genome assembly:  $\phi$ X174 bacteriophage from synthetic oligonucleotides. *Proc. Natl. Acad. Sci. USA*, 1–6.
- Springman, R., Kapadia-Desai, D.S., Molineux, I.J., Bull, J.J., 2012. Evolutionary recovery of a recombinant viral genome. *G3: Genes|Genomes|Genetics* 2, 825–830.
- Temme, K., Zhao, D., Voigt, C.A., 2012. Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca*. *Proc. Natl. Acad. Sci. USA* 109, 2–7.
- Wichman, H.A., Brown, C.J., 2010. Experimental evolution of viruses: Microviridae as a model system. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 365 (1552), 2495–2501.
- Yang, R., Han, Y., Ye, Y., Liu, Y., Jiang, Z., Gui, Y., Cai, Z., 2011. Chemical synthesis of bacteriophage G4. *PloS ONE* 6, e27062.
- Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T., 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13, 134.
- Yokoo, H., Oshima, T., 1979. Is bacteriophage  $\phi$ X174 DNA a message from an extraterrestrial intelligence? *Icarus* 38, 148–153.