

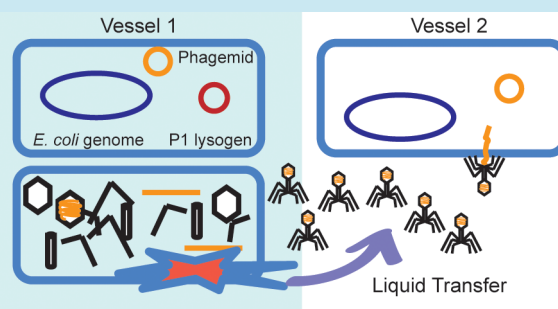
Scalable Plasmid Transfer using Engineered P1-based Phagemids

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ABSTRACT: Dramatic improvements to computational, robotic, and biological tools have enabled genetic engineers to conduct increasingly sophisticated experiments. Further development of biological tools offers a route to bypass complex or expensive mechanical operations, thereby reducing the time and cost of highly parallelized experiments. Here, we engineer a system based on bacteriophage P1 to transfer DNA from one *E. coli* cell to another, bypassing the need for intermediate DNA isolation (e.g., minipreps). To initiate plasmid transfer, we refactored a native phage element into a DNA module capable of heterologously inducing phage lysis. After incorporating known *cis*-acting elements, we identified a novel *cis*-acting element that further improves transduction efficiency,

exemplifying the ability of synthetic systems to offer insight into native ones. The system transfers DNAs up to 25 kilobases, the maximum assayed size, and operates well at microliter volumes, enabling manipulation of most routinely used DNAs. The system's large DNA capacity and physical coupling of phage particles to phagemid DNA suggest applicability to biosynthetic pathway evolution, functional proteomics, and ultimately, diverse molecular biology operations including DNA fabrication.

KEYWORDS: phagemid, bacteriophage P1, high-throughput, DNA transfer



Biological discovery and design relies on a wide array of software, hardware, and bioware tools. The last describes biological entities or their derivatives, such as DNA manipulation enzymes, recombinant cell lines, and general transducing phages. Importantly, bioware such as yeast two-hybrid systems (Y2H)¹ reduce otherwise complex and expensive operations into simple, highly parallelized procedures. Absent bioware such as Y2H, identifying interactions between a target protein and ~3000 other proteins requires time- and resource-consuming expression and purification of each protein, followed by an appropriate *in vitro* binding assay. By contrast, traditional Y2H requires only plasmid transformation, population selection, and interaction pair sequencing. With the advent of next generation sequencing, Y2H has been extended to provide data on an entire interactome from a single experiment.² Similarly, the mating-assisted genetically integrated cloning (MAGIC) strategy takes advantage of bacterial conjugation, recombination, and selection to simplify the cloning process.³ Incorporation of bioware into MAGIC eliminated the costs and complications associated with lysing cells, purifying DNA from the lysate, and prepping cells for DNA transformation, thereby achieving greatly improved throughput. These examples highlight how transformative jumps in throughput can be accomplished by performing otherwise slow, laborious, or expensive unit operations with appropriately designed bioware. Development of bioware tools compatible with high-throughput robotics for routine oper-

ations could dramatically reduce the costs and improve the throughput of diverse experimental approaches.

In particular, routine methods of DNA manipulation rely principally on *E. coli* for plasmid amplification and propagation, taking advantage of its fast growth, high competency, and high DNA yields. While generally effective, such methods are still subject to a number of constraints: isolation of high quality DNA from *E. coli* usually requires either centrifugation or vacuum application, analysis of the DNA frequently relies on gel electrophoresis, growth cycles of *E. coli* in liquid and solid media require on the order of 12 h, and clonal selection methods require isolation of a single colony from a plate. These difficult hardware operations limit automatability, with expensive, specialized robotics required to achieve even moderate throughput using a 96-well format (e.g., the Qiagen BioRobot 8000). If the operations were instead implemented as bioware designed to function using purely liquid handling manipulations, they could instead be conducted at the nanoliter scale with throughput in the hundreds of thousands using technologies such as acoustic liquid handlers and microfluidics.⁴ To overcome the inherent limitations of relying on bacteria alone, here we investigate using phage-based DNA transfer to address the essential problem of moving DNAs efficiently between cells.

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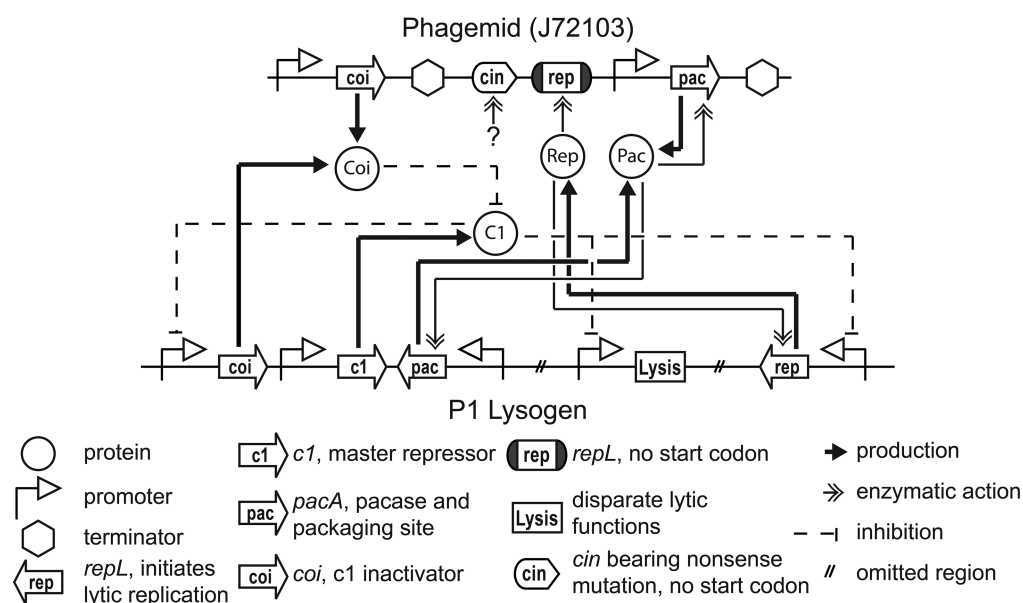


Figure 1. Genetic interaction of phage lysogen and phagemid. The interaction between a complete phagemid, J72103, and a simplified representation of the P1 genome is shown. For simplicity, only interactions relevant to the system behavior or mentioned in the main text are presented here. C1 is the master repressor of lysis and can be inhibited by *Coi*. It also downregulates expression of the native *coi*, but not the refactored version on the phagemid. The phage lysogen provides both *repL* and *pacA*, which act on sites within their respective coding regions. The phagemid also expresses *pacA* but has a truncated version of *repL* encoding only the *cis* element.

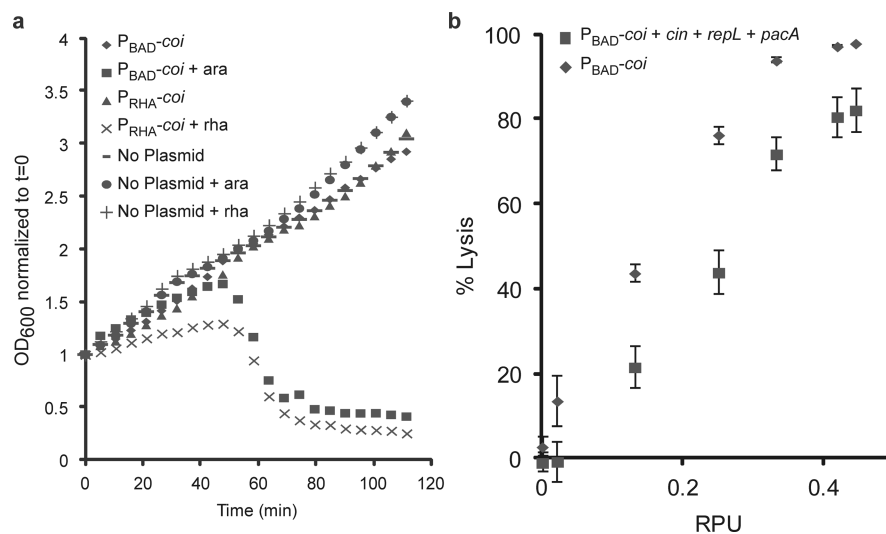


Figure 2. Expression of *coi* induces lysis. (a) MC1061 P1 lysogens harboring a plasmid with an arabinose-inducible *coi* (P_{BAD}-*coi*, J72110-J72094), a plasmid with a rhamnose-inducible *coi* (P_{RHA}-*coi*, J72110-J72097), or no plasmid were incubated in the presence or absence of inducer, and the OD₆₀₀ was monitored. Each construct was run in triplicate; error bars have been omitted for clarity, with all standard deviations falling below 16% of the mean, except the P_{BAD}-*coi* construct induced with arabinose, which had standard deviations as high as 67% of the mean. (b) P1 lysogenized MC1061 containing either a plasmid with only an arabinose-inducible *coi* (P_{BAD}-*coi*, J72110-J72094) or a complete phagemid (P_{BAD}-*coi* + *cin* + *repL* + *pacA*, J72110-J72103) were induced to lyse by addition of arabinose. The corresponding arabinose promoter activity, reported in relative promoter units (RPU), is plotted against the resulting percent lysis, calculated as 1 - (OD₆₀₀ sample/OD₆₀₀ uninduced control). Error bars indicate the standard deviation of quadruplicate samples.

Many phage can infect, reproduce, and lyse their hosts in less than an hour,⁵ and they have evolved to transmit genetic information from one host to another through aqueous intermediates. We selected phage P1 to use as a starting point to take advantage of its extensive characterization⁶ and ability to package large DNAs from a well-defined packaging site. Because P1 employs a headful packing mechanism, any sized DNA up to 90 kb can be packaged into a P1 particle, since smaller DNAs can either be concatenated before uptake or

packaged serially.⁷ Further, another group has already demonstrated that a P1 phagemid, a plasmid containing *cis* phage elements, can be successfully packaged and delivered into new cells.⁸ In that study, a temperature-inducible P1 lysogen was used to generate particles containing a plasmid with a packaging site and a P1 lytic origin of replication. In this work, we employ a transcriptionally activated mechanism for inducing lysis to generate a small molecule responsive phagemid system. In an effort to improve efficiency, we also isolate an additional

cis element that enhances phagemid transduction. Finally, we characterize behaviors of the system relevant to its potential applications. The resulting DNA transfer system operates at low volumes under isothermal conditions and should find application in improving continuous selection schemes, identifying protein–protein interactions, and streamlining diverse molecular biology operations including DNA fabrication.

Transcription of *coi* Triggers the P1 Lytic Cycle. We sought a *trans*-acting protein capable of inducing the P1 lytic cycle, reasoning that transcription of such a protein would provide a mechanism for controlling phage induction. The P1 lysis/lysogeny decision is regulated by the activity a master repressor, C1. Although a complex genetic circuit controls C1 synthesis and activity during P1 infection,⁶ heterologous expression of a C1 inhibitory protein, *Coi*, blocks C1 activity, thereby inducing the lytic cycle.⁹ Here, we similarly employ overexpression of *coi* from a phagemid to induce the P1 lytic cycle, and the interactions between a P1 lysogen and a *coi*-based phagemid are summarized in Figure 1. To validate this approach, we first refactored *coi* into a synthetic module activated by transcription, removing all sequence 5' of the ribosome binding site, taken to be the 17 base pairs immediately 5' of the start codon,¹⁰ and adding a terminator 3' of the stop codon. We then constructed plasmids with the refactored *coi* under the transcriptional control of one of two small-molecule inducible promoters, P_{BAD} or P_{RHA} , which are induced by arabinose or rhamnose, respectively. Addition of the appropriate inducer to P1 lysogens transformed with these plasmids resulted in cell lysis, while uninduced controls and controls lacking a phagemid failed to lyse (Figure 2a). Consistent with previous results,⁹ this confirms that induced transcription of *coi* from phagemids initiates lysis.

Although we focus here on the use of small-molecule inducible promoters to control lysis, other applications may require the use of a different triggering mechanism. To aid in the development of alternative *coi*-based lysis circuits, we quantified lysis induction efficiency as a function of P_{BAD} transcriptional activity. As illustrated in Figure 2b, lysis of both an arabinose-driven *coi*-only phagemid and a phagemid bearing additional *cis* elements (*coi* + *cin* + *repL* + *pacA*, discussed below) exhibited dose dependence, appearing to saturate at the maximal observed promoter strength of 0.4 relative promoter units (RPU).¹¹ We expect that any alternative phagemid design that provides the same level of *coi* transcription would also function to induce lysis.

***cin* Is a *cis* Element That Improves Phagemid Transduction.** Motivated by the observation that plasmids not harboring phage *cis* elements are also transduced, albeit infrequently, we sought to improve the relative efficiency with which desired plasmid DNA is transduced. Incorporation of a packaging site (which resides within *pacA*) and a lytic origin of replication (which resides within *repL*) both enhance packaging.⁸ Although no other packaging or delivery enhancers have been reported, we reasoned that there might be additional regions of the P1 genome that improve DNA packaging or delivery. We therefore inserted a library of P1 genomic fragments into a phagemid harboring complete *pacA* and *repL* ORFs. The library was enriched for elements that improve transduction by generating phagemid lysate from pooled library members, and then infecting naïve lysogens with the lysate. After several rounds of such enrichment, individual clones were isolated and sequenced to identify the inserted genomic

fragment. Sequences of interest were PCR-amplified out of the P1 genome and cloned into phagemids, which were subsequently tested individually for improved function. One region, which contained the *cin* ORF, consistently showed improvement (data not shown). Although there is no published *cis* role for *cin* in the packaging or delivery of P1 phage DNA, it has been shown to encode a site-specific recombinase responsible for switching the host specificity of the phage.¹² To determine if the improvement was an unexpected function of the coding sequence or an overlying *cis* element, a noncoding version of the ORF lacking a start codon and bearing a nonsense mutation was created. Inclusion of the noncoding *cin* DNA improved the relative transduction efficiency of phagemids harboring *coi* and any combination of *repL* and *pacA* by 3–11 fold (Figure 3), suggesting that *cin* operates as a

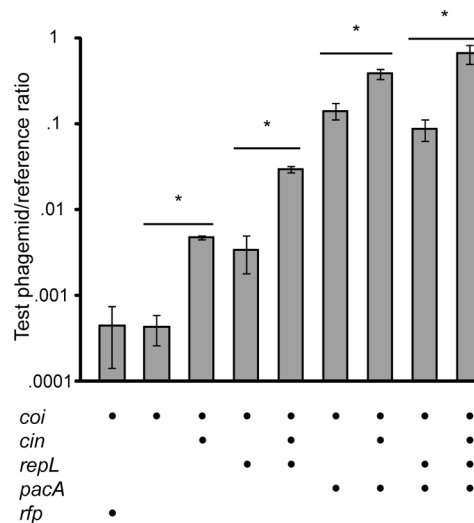


Figure 3. Impact of *cis* elements on packaging. Test phagemids harboring various combinations of putative *cis* packaging elements were competed against a reference phagemid containing all of the elements (J72109–J72105: *cin* + *repL* + *pacA*). The resulting ratio of test phagemid to reference phagemid indicates the relative efficiency of test phagemid packaging. Error bars indicate the standard deviation of triplicate biological samples. Starred pairs show a statistically significant difference ($p < 0.05$ in an unpaired two-tailed *t* test). The test phagemids are J72113–J72106: *coi* + *rfp*, J72113–J72094: *coi*, J72113–J72102: *coi* + *cin*, J72113–J72095: *coi* + *repL*, J72113–J72115: *coi* + *cin* + *repL*, J72113–J72101: *coi* + *pacA*, J72113–J72116: *coi* + *cin* + *pacA*, J72113–J72096: *coi* + *pacA* + *repL*, and J72113–J72103: *coi* + *cin* + *repL* + *pacA*.

cis element. We also examined the possibility that increased plasmid size alone might lead to improved transduction, but inclusion of a gene coding for red fluorescent protein had no effect on transduction efficiency. Importantly, a phagemid harboring all three *cis* elements showed an approximately 1600 fold improved yield over a plasmid lacking any *cis* elements. This suggests that the system very specifically transduces desired phagemid DNA.

Phagemids Exhibit Robust and Faithful Transduction.

Because we envision utilization of the phagemid in high-throughput liquid handling operations with diverse genetic material, it is important for phagemid transduction to be robust to both reaction volume and phagemid size. To establish the effect of reaction volume on lysis, we generated lysate from P1 lysogenized cells bearing phagemids in different vessels and used the lysate to transduce naïve cells to titer viable phagemid-

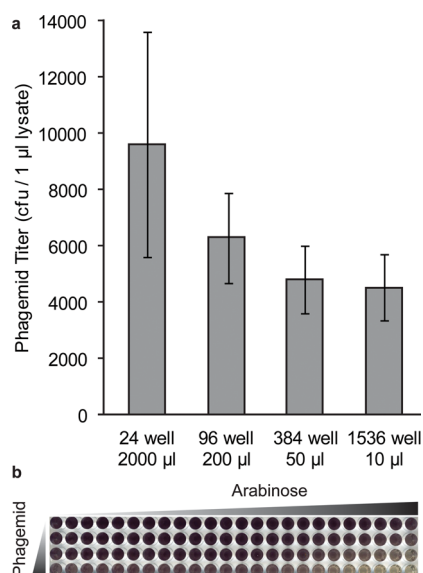


Figure 4. Impact of volume on phagemid efficiency. (a) MC1061 P1 lysogens containing a complete phagemid (J72110-J72103) were induced to lyse at 2000, 200, 50, and 10 μL scale in 24-well blocks, 96-well plates, 384-well plates, and 1536-well plates, respectively, and the lysate used to transduce naïve JTK029 cells for titer determination. Error bars indicate the standard deviation of biological triplicates. Differences between conditions are not statistically significant ($p > 0.05$ in an unpaired two-tailed t test). (b) P1 lysogenized JTK160C harboring plasmid J72111-J72098 was transduced with 2-fold serial dilutions of J72110-J72103 lysate from (a) in the presence of 2-fold serial dilutions of arabinose (highest concentration, 13 mM) in a 384-well plate. Tetrazolium violet was added to visualize unlysed cells.

carrying phage particles (Figure 4a). Although the average titer dropped approximately 2-fold between a 2000 μL lysis volume and a 200 μL lysis volume, this difference is not statistically significant ($p > 0.05$), and there is little impact on the average titer from further scale reduction. To ascertain if transduction also operates at small volumes, we tested the ability of phagemid lysate to transduce naïve lysogens and initiate lysis in a 384-well plate (50 μL scale). Figure 4b illustrates that a high titer of phagemid, in the presence of sufficient inducer, leads to complete lysis of the recipient cells. This suggests that transduction functions at small volumes, as expected.

To probe the size constraints of the system, we tested the transduction efficiency of a modest (10 kb), a medium (14 kb), and a large (25 kb) phagemid; larger constructs are not readily supported by the p15a origin of replication.¹³ We observed that although the smallest phagemid transduced with 2.5-fold higher efficiency than the larger phagemids, all of the phagemids generated hundreds to thousands of transductants (Figure 5). Therefore, this system is capable of operating on large DNAs, such as those encoding multigene genetic circuits.

Some applications of the phagemid system may involve manipulation of libraries of genetic constructs. Under conditions such as those in Figure 4a, 1 μL of phagemid lysate is sufficient to generate up to 10,000 transductants. Scaled up to higher volumes, this implies that 100 mL of lysate could generate up to 10^9 clones. However, library manipulations should also preserve the relative abundance of each clone. As a simple probe of this functionality, we generated a library of phagemids expressing different levels of green fluorescent protein (GFP) in P1-lysogenized cells, illustrated in Figure 6a. We pooled the clones, induced lysis, and transduced naïve P1-

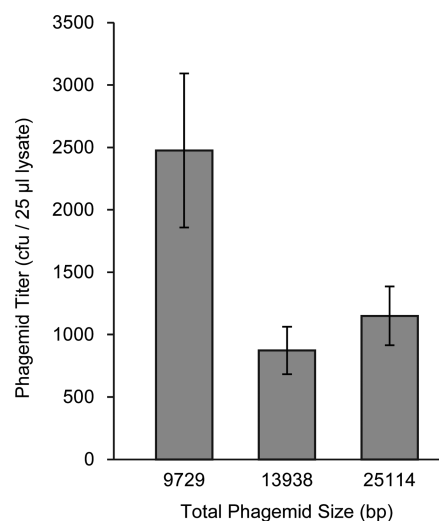


Figure 5. Impact of phagemid size on efficiency. MC1061 P1 lysogens containing one of three phagemids (J72114-J72100, 9729 bp; J72114-J72090, 13938 bp; or J72114-J72104, 25114 bp) were induced to lyse with arabinose, and the lysate was used to transduce naïve MC1061 cells for titer determination. Error bars indicate the standard deviation of biological triplicates. The 9729 bp phagemid is significantly different from the other two phagemids ($p < 0.05$ in an unpaired two-tailed t test), which are not significantly different from each other ($p > 0.05$ in an unpaired two-tailed t test).

lysogenized cells with the lysate. The resulting clones (Figure 6b) show a similar diversity of GFP expression levels. To better quantify the fidelity of library transfer, we used a mixture of GFP-expressing and non-GFP-expressing phagemids to generate a library with 10% GFP-expressing clones. Lysate generated from pooled library members was used to transduce naïve P1-lysogenized cells and resulted in an average of 9% GFP-expressing clones, which is not significantly different from the original library ($p > 0.05$, Figure 6c). The ability to produce large numbers of clones without significantly perturbing the ratio of variants confirms the utility of phagemids for transduction of genetic libraries.

Summary and Conclusions. We have engineered and characterized a P1-based phagemid that requires only liquid handling for the isothermal, high efficiency transfer of plasmids from one cell to another. As part of this process, we isolated an additional *cis* element that lies inside of the *cin* gene and enhances transduction of the phagemid. Because there is no known role for *cin* in the packaging or delivery of P1 phage DNA, the biological mechanism of improved transduction remains to be elucidated, illustrating how engineering novel synthetic systems can probe our understanding of native systems.

By refactoring the natural transcriptional regulation of *coi*, we gain control of the lysogeny-to-lysis switch. Here, we demonstrated simple control by a heterologous input, arabinose or rhamnose, using an inducible promoter to drive *coi* expression. In theory, however, any genetic circuit could be used. For example, recent work by Esvelt and colleagues¹⁴ demonstrated the power of connecting phage viability to an encoded gene function with the development of phage-assisted continuous evolution (PACE). An analogous approach could be used to evolve an entire biosynthetic pathway encoded on a P1 phagemid by using a biosensor¹⁵ for the product of the pathway to trigger expression of *coi*.

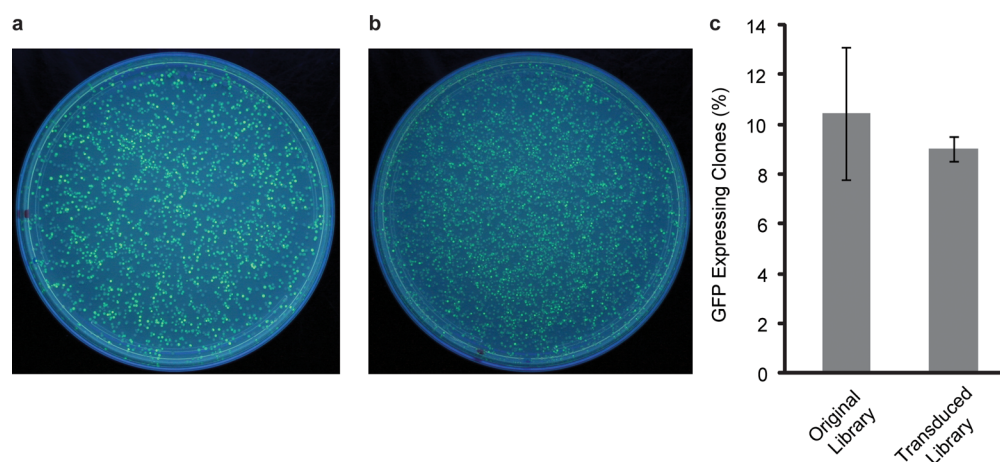


Figure 6. Library transfer using the phagemid. MC1061 P1 lysogens containing a library of GFP expressing phagemids (J72110–J72152 library) (a) were pooled and induced to lyse with arabinose, and the lysate used to transduce naïve MC1061 P1 lysogens (b). (c) MC1061 P1 lysogens were transformed with a mixture of GFP expressing (J72113–J72152) and non-GFP expressing (J72113–J72103) phagemids, and the percent of GFP expressing clones was counted (the original library). The clones were pooled and induced to lyse, and the lysate was used to transduce naïve MC1061 P1 lysogens. The resulting clones (the transduced library) were counted, and the percent expressing GFP was calculated. Error bars indicate the standard deviation from three replicates. The difference between the original library and the transduced library is not statistically significant ($p > 0.05$ in an unpaired two-tailed t test).

Addition of *cin* to the phagemid improved transduction efficiency, enabling a phagemid bearing all relevant *cis* elements to be transduced 1600-fold more efficiently than a plasmid lacking any such elements. Although further work is required to elucidate the relative packaging efficiency of phagemids and wild-type P1 phage genomes, which may be important to continuous selection schemes such as PACE, the current system is clearly sufficient to transfer both individual clones and libraries of genetic elements. The ability to specifically couple at least 25 kb of DNA to a phage particle takes a step toward enabling P1 phage display for functional proteomics. Based on work with other well characterized phages,^{10,16–18} we speculate that phagemid-encoded protein complexes could be attached to the particle surface, e.g., via a short tag,¹⁹ allowing functional complexes to be enriched by targeted binding to individual complex components.

Transcriptional control of lysis provides a final advantage: isothermal phage induction, which confers compatibility with simple liquid handling robotics including microfluidic technologies. The robustness of the system at microliter volumes suggests the potential for operation on nanoliter scale platforms. DNA transfer, in combination with as yet undeveloped bioware operations such as restriction and ligation, may ultimately provide a mechanism for fast, cheap DNA fabrication. Although development of such bioware is challenging to engineer today due to the general difficulties of creating complex genetic systems,²⁰ improvements in our ability to design²¹ and fabricate²² genetic systems will continue to make bioware development easier. An initial investment in the development of additional liquid handling-only bioware tools for common procedures would reduce the cost of genetic engineering to the purchase of a standard liquid handling platform, accelerating development of useful biotechnologies.

METHODS

Plasmids, Strains, Phage, and Growth Media. Plasmids used in this study are diagrammed in Supplementary Figure S1 and were constructed using BglBrick standard assembly.²³ *E. coli* strain MC1061²⁴ or derivatives were used for all studies.

Derivatives are diagrammed in Supplementary Figure S1 and were generated by the procedure of Datsenko and Wanner.¹⁷ Full descriptions of plasmids and strain modifications are available in the Supporting Information, and 56 DNA sequences have been deposited in the Registry of Standard Biological Parts (<http://partsregistry.org>).² For brevity, the BBa_ prefix of sequence names has been omitted throughout the manuscript. For example, to access the sequence for vector J72113, search for BBa_J72113 on <http://partsregistry.org> and select the “get selected sequence” option. P1kc²⁵ (obtained in strain KL739 from the Yale Coli Genetic Stock Center) was used for experiments involving phage lysogens and will be referred to simply as P1 for convenience. P1vir²⁶ (gift from Carlos Bustamante) DNA was used for library preparation. Bacterial cells were grown in Luria–Bertani medium (LB) or phage lysis medium (PLM; LB containing 100 mM MgCl₂ and 5 mM CaCl₂).

Generation of Phage Lysates. Stationary phase cultures of a P1 lysogenized strain harboring a phagemid were diluted 100-fold into PLM and incubated for 1 h at 37 °C. Lysis was then induced by addition of either 13 mM arabinose or 10 mM rhamnose. After 1–4 h of further incubation at 37 °C, chloroform (2.5% v/v) was added, and the culture was vortexed for 30 s. The culture was then clarified by centrifugation (12,100g for 90 s), and the supernatant was recovered. For characterization of lysis efficiency in different volumes, the chloroform and clarification steps were omitted, and the crude lysate was instead used directly for subsequent transduction.

Transduction of Phage Lysates. A stationary phase culture of the host strain was isolated by centrifugation and resuspended to an OD₆₀₀ of 2 in PLM. An equal volume of phage lysate was mixed with resuspended cells and allowed to adsorb at 37 °C without agitation for 30 min. The reaction was quenched with 5 volumes of 2YT medium containing 200 mM sodium citrate, pH 5.5, and the infected cells were incubated for an additional 1 h at 37 °C before titering transductants on plates with appropriate antibiotics.

Timecourse of Phage Lysis. Stationary phase cultures of MC1061 P1 lysogens were diluted 100-fold into PLM. After

growth for 1 h at 37 °C with agitation, 200 μ L of cells was transferred to a Corning flat-bottom 96-well microplate and induced by addition of either 13 mM arabinose or 10 mM rhamnose. Culture OD₆₀₀ was then monitored every 5 min in a Tecan Sapphire instrument with continued incubation at 37 °C with agitation. OD₆₀₀ measurements were then normalized to the first OD₆₀₀ reading (OD₆₀₀ at time t divided by OD₆₀₀ at time 0).

Measurement of Relative Promoter Units (RPU). The RPU of the P_{BAD} promoter with different levels of arabinose induction (0–13 mM) was measured essentially as previously described.¹¹ Briefly, samples of stationary phase MC1061 P1 lysogen cells harboring a p15a plasmid with either a constitutive reference promoter (P_{REF}, J72110-J72107) or P_{BAD} (J72110-J72108) driving expression of green fluorescent protein were diluted 100-fold into fresh inducing media and grown until mid exponential phase. At that point, the OD₆₀₀ (OD) and fluorescence (F) were measured. RPU was then calculated using eq 10 from ref 11, with the approximation that all relevant growth rates are equal: $[F(P_{BAD})/OD(P_{BAD})]/[F(P_{REF})/OD(P_{REF})]$.

Visualization of Serial Lysis. Stationary phase culture of the host strain was diluted 100-fold into PLM and incubated for 75 min at 37 °C. Subsequently, 25 μ L aliquots of cells were arrayed into a Genetix flat-bottom 384-well microplate and incubated with 25 μ L of phagemid lysate for 2 h at 37 °C in the presence of arabinose. Cells were then stained by addition of 0.04% w/v tetrazolium violet, and the plate was incubated for an additional 2 h at 37 °C before image acquisition.

Phagemid Library Construction and Enrichment. To isolate P1 *vir* DNA, 250 μ L of P1 *vir* lysate was treated with 5 μ L of Qiagen buffer L1 (300 mM NaCl; 100 mM Tris-Cl, pH 7.5; 10 mM EDTA; 0.2 mg/mL BSA; 20 mg/mL RNase A; 6 mg/mL DNase I) and incubated for 30 min at 37 °C. After incubation, 400 μ L of 4% SDS was added, and the mixture was further incubated for 15 min at 55 °C. Then 350 μ L of Qiagen buffer N3 was added, and the mixture applied to a Qiagen Qiaprep Spin Column. DNA isolation then proceeded according to the manufacturer's instructions for plasmid DNA purification.

Purified P1 *vir* DNA was then amplified using Phi29 polymerase. A typical reaction consisted of 2 μ L 10x Phi29 buffer, 5 U Phi29 polymerase, 400 ng thiophosphate protected random hexamers,²⁷ 500 μ M each dNTP, approximately 25 ng template DNA, and water to 20 μ L. The reaction mixture was incubated at 30 °C for 16 h and then purified using Zymo-Spin I columns according to the manufacturer's instructions.

Amplified P1 *vir* DNA was then partially digested with Sau3AI, and fragments of 1–4 kilobases ligated into *Bam*HI digested and CIP treated J72112-J72099 plasmid DNA. Plasmids were inserted into a JTK030 P1kc lysogen by transformation, generating a library of 3×10^6 members. Library members were pooled and then used to generate phagemid lysate. This lysate was used to transduce phagemids into naïve JTK030 P1kc lysogen cells, and the cycle of pooling, lysis, and transduction was repeated.

Competition between Phagemids. MC1061 P1 lysogens were cotransformed with two phagemids, a noninducible "reference" phagemid (J72109-J72105) composed of *cin*, *pacA*, and *repL* and a "test" phagemid (in vector J72113) composed of arabinose-inducible *coi* and a subset of the elements *pacA*, *repL*, and *cin*. Phage lysate was generated as

described above, and then used to transduce naïve MC1061 cells for titering of each phagemid.

Construction of Library of GFP Expressing Phagemids. Splicing by overlap extension SOEing PCR²⁸ with degenerate oligos (Outer forward: 5'-GTATCACGAGGCA-GAATTTTCAG-3', Outer reverse: 5'-ATTACCGCCTTT-GAGTGAGC-3', Inner forward: 5'-GCACGGATCTTAGC-TACTAGAGAAAGANNNGAAANNNNNNATGCG-TAAAGGCGAAGAGC-3', Inner reverse: 5'-TCTTTCTCTAGTAGCTAAGATCCGTGC-3') on template plasmid J72111-J72155 was used to generate RBS variants (NNNGAAANNNNNNATG) of the GFP expression cassette. The SOEing PCR product was digested with *Eco*RI and *Bam*HI and cloned into the *Eco*RI and *Bam*HI sites of vector J72154. The resulting library of clones was pooled before DNA extraction and subsequent assembly with plasmid J72153-J72103 using the 2ab strategy²⁹ to generate a library of variants of J72110-J72152.

■ ASSOCIATED CONTENT

● Supporting Information

Details of the constructs used in this study, This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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