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Improved method for selecting RNA-binding activities *in vivo*

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ABSTRACT

RNA challenge phages are modified versions of bacteriophage P22 that allow one to select directly for a specific RNA–protein interaction *in vivo*. The original construction method for generating a bacteriophage that encodes a specific RNA target requires two homologous recombination reactions between plasmids and phages in bacteria. An improved method is described that enables one to readily construct RNA challenge phages through a single homologous recombination reaction *in vivo*. We have applied the new method to construct a derivative of P22$_{R17}$, an RNA challenge phage that undergoes lysogenic development in bacterial cells that express the bacteriophage R17/MS2 coat protein.

We recently reported an RNA challenge phage system that enables one to genetically select proteins with RNA-binding activity in bacteria (1). RNA challenge phages are modified versions of bacteriophage P22 in which post-transcriptional regulatory events control the developmental fate of the phage (Fig. 1). The original method for constructing RNA challenge phages was developed to study the RNA-binding activities for which the RNA sequence and the RNA-binding activity are known (1). In many systems, however, the molecular identity of the RNA target remains unknown. Challenge phage libraries that encode a population of candidate RNA-binding sites cannot be constructed with current phage methods. In this system, an RNA target site is introduced into the challenge phage through homologous recombination in *Salmonella typhimurium*. Alleles of the *arc* gene are used to identify P22 derivatives that contain the desired RNA-binding site (1,2). However, the *arc*(Am) allele that was located on the recipient phage is replaced by an *arc*$^+$ allele for progeny phage acquiring the RNA-binding sequence. To restore the *arc*(Am) allele to the P22 challenge phage that encodes the RNA-binding site, a second homologous recombination reaction between two P22 bacteriophages in *S.typhimurium* is required (Fig. 2a). We now report the design and structure of new reagents that enable RNA challenge phages to be constructed in a single homologous recombination reaction *in vivo*. The revised recombination substrates swap *arc* alleles between the donor plasmid and the recipient P22 phage so that all recombinant progeny phages that encode the desired RNA-binding site will contain *arc*(Am) as shown in Figure 2b.

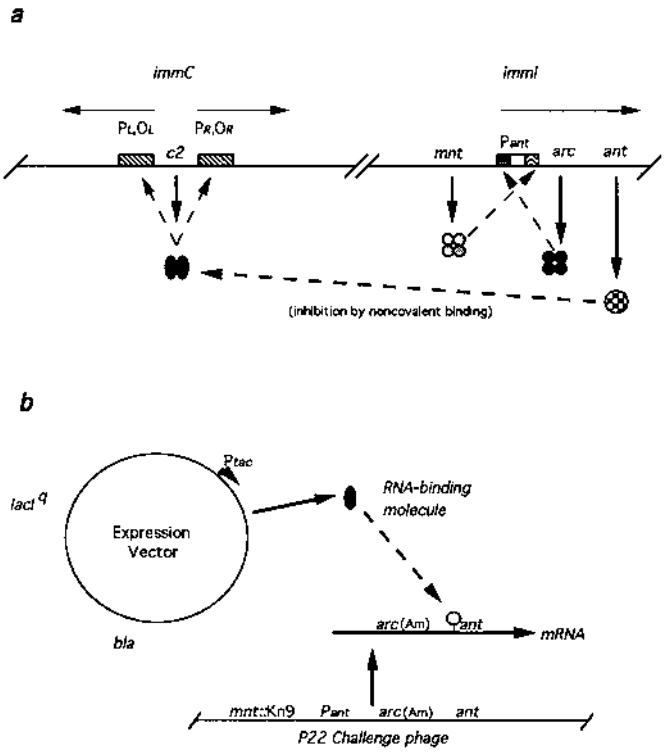

Figure 1. (**a**) P22 bacteriophage development. Solid lines refer to expression of regulatory gene products. Dashed lines refer to negative regulatory pathways. The c2 repressor protein, which maintains the lysogenic state of the phage, can be inactivated if bound by the Ant protein. The *ant* gene is transcriptionally repressed by the Mnt and Arc proteins. (**b**) RNA challenge phage development. Challenge phages carry a kanamycin resistance gene in the *mnt* gene (*mnt*::Kn9) and encode an amber mutation in the *arc* gene [*arc*(Am); (2)]. When these two repressor proteins are inactivated, the developmental fate of a challenge phage depends upon the relative abundance of the c2 protein and the Ant protein. Lysogenic development of the phage therefore relies upon expression of an RNA-binding activity in the susceptible host cell and the availability of an RNA-binding site encoded by the phage genome (1).

The recombination proficiencies of the new reagents were evaluated by performing homologous recombination reactions in MS1883. Following P22 *mnt*::Kn9 *arc*(Am) infection of MS1883 containing either pΦGen1, pPY190 (3) or pMMW20 (1), the progeny phages obtained were assessed for their ability to plaque on MS1582. P22 progeny phages derived from infection of cells

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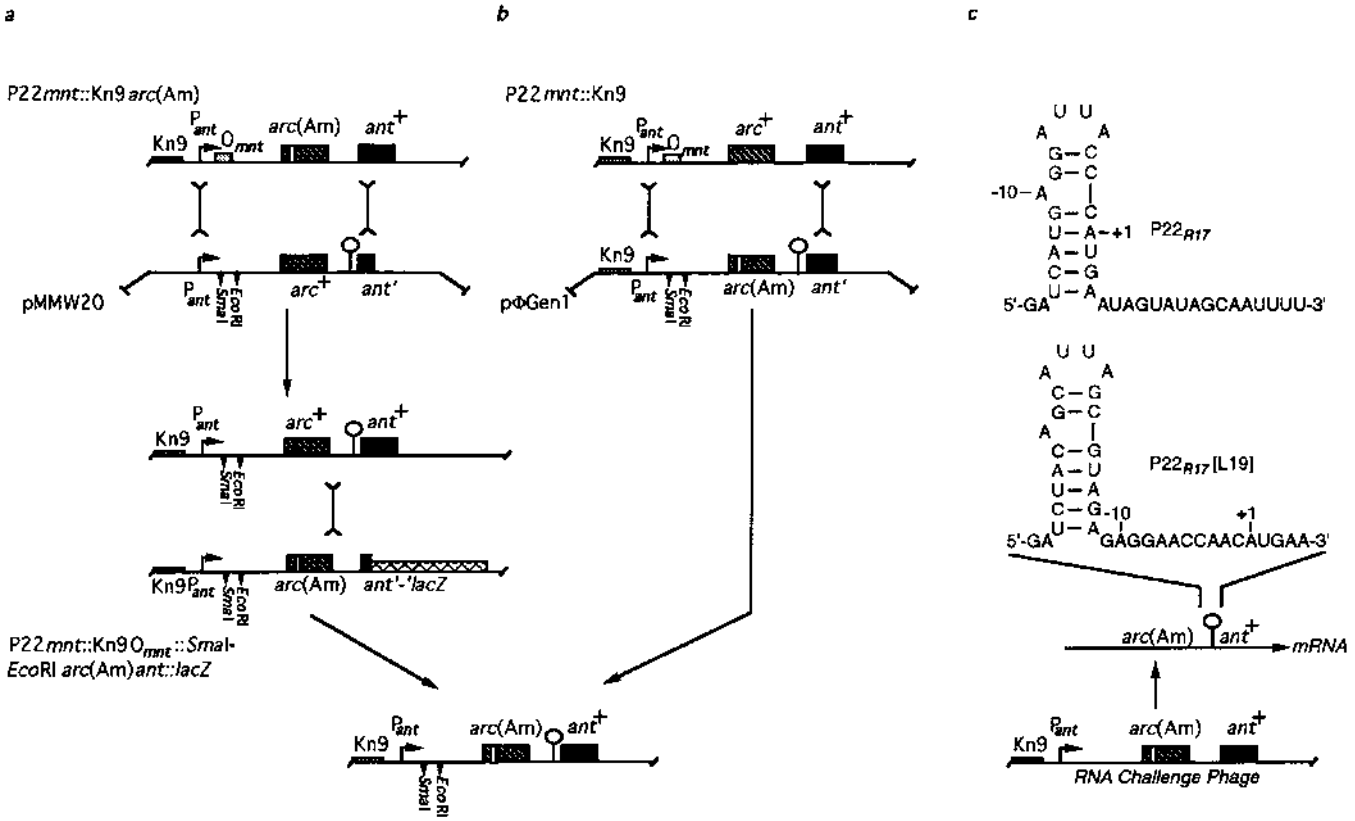

Figure 2. (**a**) Original method for RNA challenge phage construction. The bacteriophages that encode the RNA target sequence (designated by the lollipop) can be identified in the progeny phage pool by the segregation of bacteriophage genetic markers that are linked to the DNA encoding the target RNA sequence. These genetic markers report the functional status of Mnt and Arc, and the DNA operators, O_{mnt} and O_{arc}, to which these transcriptional repressors bind, respectively (reviewed in ref. 3). The plasmid recombination substrate [pMMW20; (1)] that encodes the RNA-binding site in*ant* contains *mnt*, *arc*$^+$, and a substitution for O_{mnt} (O_{mnt}::*Sma*I–*Eco*RI; referred to as O_{mnt}^- in the text). The parental phage [P22 *mnt*::Kn9 *arc*(Am)] used in the initial recombination reaction *in vivo* carries O_{mnt}^+ and *arc*(Am). The progeny phages that are obtained from *in vivo* recombination of pMMW20 and P22 *mnt*::Kn9 *arc*(Am) in *S.typhimurium* strain MS1883 [*leuA414* Fels$^-$ *hsdSB*(r$^-$m$^+$) *supE40*] are plated onto *S.typhimurium* strain MS1582 {*leuA414* (Am) Fels$^-$ *supE40 ataP*::[P22 *sieA44* 16(Am)*H1455* Tpfr49]}. Only progeny phages that carry an O_{mnt}^- will plaque on MS1582. An O_{mnt}^- phage that possesses either an *arc*$^+$ or *arc*(Am) allele will form a turbid plaque or a clear plaque on MS1582 respectively. The final *arc*(Am)-containing RNA challenge phages are generated following a second homologous recombination reaction *in vivo* between the *arc*$^+$ phage that carries the RNA target site and a replication-defective *arc*(Am) phage that lacks *ant* [P22 *mnt*::Kn9 O_{mnt}::*Sma*I–*Eco*RI *arc*(Am)*ant*::*lacZ* ; (1)]. (**b**) Revised method for RNA challenge phage construction. The desired RNA-binding site (designated by the lollipop) is introduced into pΦGen1 according to standard methods (1). The RNA challenge phage is obtained following homologous recombination between pΦGen1 and P22 *mnt*::Kn9. The P22 *mnt*::Kn9 *arc*(Am) progeny phages that contain the RNA-binding site are verified by PCR–RFLP and sequence analyses. For simplicity, only progeny phages acquiring the RNA target site are shown in the recombination reactions in (a) and (b). The new donor plasmid (pΦGen1) was constructed in the following fashion. A 1.95 kb *Eco*RI–*Eco*RV fragment that contains the entire *mnt*::Kn9 region, *arc*(Am), and the 5′ portion of *ant* from P22 *mnt*::Kn9 *arc*(Am) was inserted into pMMW20 to yield pMMW20-RR. Since pMMW20-RR contains O_{mnt}^+, the O_{mnt}^- site was incorporated into pMMW20-RR using the Kunkel method (4), resulting in pΦGen1. P22 *mnt*::Kn9 was obtained at a frequency of 0.2% following P22 *mnt*::Kn9 *arc*(Am) infection of *S.typhimurium* strain MS1883 containing pPY190 [O_{mnt}::*Sma*I–*Eco*RI *arc*(Am); (3)]. Recombinant P22 progeny phages that formed turbid plaques on MS1883 were found to contain *arc*$^+$, as assessed by PCR–RFLP analysis. The *arc*$^+$ P22 phages that contained an O_{mnt}^+ allele were identified by their inability to plaque on MS1582 and confirmed by PCR–RFLP and DNA sequence analyses of the O_{mnt} region of the P22 phage genome. (**c**) Structure and construction of $P22_{R17}$[L19] RNA challenge phage**.** The structure of the RNA region encompassing the 5′-end of the *ant* mRNA is illustrated; the corresponding region of $P22_{R17}$ is shown for comparison. The positions of the ribosome-binding site (GAGG) and the first nucleotide of the *ant* initiation codon are indicated by –10 and +1 respectively. The new P22 phage derivative was constructed according to the scheme outlined in (b). The DNA sequence that encodes the modified RNA target site was incorporated into pΦGen1 according to standard procedures (4) using a single-stranded phagemid DNA template of pΦGen1 that contained deoxyuridine residues and the following DNA oligonucleotide (Operon Technologies, Inc.): 5′-GGCTTCGGTTGTCAGATCTACAGCATTAGCGTAGAGAGGAACCAACATGAATAG-3′.

containing pΦGen1 were able to plaque on MS1582 at frequencies 10- and 20-fold greater than progeny phages obtained from infection of cells containing pPY190 and pMMW20 respectively (Table 1); a similar trend is observed when P22 *mnt*::Kn9 is used as the infecting phage (Table 1). These data indicate that the transfer of the O_{mnt}^- allele from the respective plasmid to the P22 infecting phage occurs more readily with pΦGen1 than with either pPY190 or pMMW20. The transfer frequency of the *arc* allele was determined by calculating the ratio of clear plaques to turbid plaques obtained for P22 progeny plated on MS1582. The P22 phages recombined with the *arc* allele from pΦGen1 at a greater frequency than observed from either pPY190 or pMMW20 (Table 1). The increased recombination frequencies of P22 phages with pΦGen1 may be attributed to the larger region of homologous P22 *mnt*::Kn9 phage DNA that is present in this plasmid—1.95 kb, in comparison with 0.235 and 0.495 kb regions of homology on pPY190 and pMMW20 respectively.

Table 1. Recombination and lysogen frequency data for P22 phages

Recipient cell plasmid	Parental P22 phages[a]		Progeny P22 challenge phages[b]	
	P22 *mnt*::Kn9 *arc*(Am)	P22 *mnt*::Kn9	P22$_{R17}$[L19]	P22$_{R17}$
O_{mnt}^- transfer frequency:[c]				
None	$2.2 \times 10^{-5} \pm 8 \times 10^{-6}$%	$3 \times 10^{-6} \pm 2 \times 10^{-6}$%	N.D.[f]	N.D.[f]
pPY190	$3.8 \times 10^{-3} \pm 2.4 \times 10^{-3}$%	$2.0 \times 10^{-3} \pm 7 \times 10^{-4}$%	N.D.[f]	N.D.[f]
pMMW20	$1.9 \times 10^{-3} \pm 8 \times 10^{-4}$%	$5.1 \times 10^{-3} \pm 2.8 \times 10^{-3}$%	N.D.[f]	N.D.[f]
pΦGen1	$4.6 \times 10^{-2} \pm 1.4 \times 10^{-2}$%	$3.0 \times 10^{-2} \pm 2.5 \times 10^{-2}$%	N.D.[f]	N.D.[f]
arc allele transfer frequency:[d]				
pPY190 (*arc*$^+$)	25.8 ± 4.7%	N.A.[g]	N.D.[f]	N.D.[f]
pMMW20 (*arc*$^+$)	37.7 ± 14.6%	N.A.[g]	N.D.[f]	N.D.[f]
pΦGen1 [*arc*(Am)]	N.A.[g]	66.5 ± 14.7%	N.D.[f]	N.D.[f]
Lysogen frequency:[e]				
None	N.D.[f]	N.D.[f]	$<1 \times 10^{-6}$%	$<1 \times 10^{-6}$%
pR17coat(+)	N.D.[f]	N.D.[f]	33.1 ± 11.0%	19.9 ± 4.6%

[a]MS1883 recipients that carry the indicated plasmids (5×10^7 cells) were inoculated with the indicated parental P22 phages at an M.O.I. = 10. Following growth at 37°C for 3 h, progeny phage lysates were prepared (ref. 3) and diluted for plating onto MS1582. Data reflect results obtained from three independent experiments.

[b]MS1868 recipients that carry the indicated plasmids (5×10^7 cells) were inoculated with the indicated P22 challenge phages at an M.O.I. = 10–20. Following phage adsorption at 20°C for 20 min, the infected cells were plated onto LB-agar plates containing selective media (ref. 1). Data reflect results obtained from three independent experiments.

[c]Recombination frequency (F_r) was determined by the following formula:

F_r = [(p.f.u/ml on MS1582)/(p.f.u/ml on MS1883)] × 100%

[d]Recombination frequency (F_r) was determined by the following formulae:

For *arc*$^+$ transfer, F_r = [(turbid p.f.u/ml on MS1582)/(total p.f.u/ml on MS1582)] × 100%

For *arc*(Am) transfer, F_r = [(clear p.f.u/ml on MS1582)/(total p.f.u/ml on MS1582)] × 100%

[e]Lysogen frequency (F_l) was determined by the following formula:

F_l = [(kanamycin resistant lysogens)/(viable number of MS1868 cells used in the infection)] × 100%

[f]Not determined.

[g]Not applicable. The donor plasmid and recipient phage carry isogenic *arc* alleles.

We evaluated the new reagents in RNA challenge phage construction by introducing a modified R17 RNA operator site into the P22 *mnt*::Kn9 phage as outlined in Figure 2. PCR/RFLP studies and direct DNA sequence analysis demonstrated that ~50% of the progeny phages that were obtained from homologous recombination of the P22 *mnt*::Kn9 phage with plasmid DNA in MS1883 and that formed clear plaques on the MS1582 host contain both the *arc*(Am) allele and the desired RNA operator site. As expected, all the P22 phage derivatives that encoded the desired RNA operator site also carried the *arc*(Am) allele.

The RNA operator site that is encoded by P22$_{R17}$[L19] contains an RNA structure that normally should be recognized by the R17/MS2 coat protein because the structural features necessary for efficient coat protein binding are present (1,5); however, the ribosome-binding site and the initiator codon of the *ant* gene are positioned entirely *outside* this coat protein RNA-binding site (Fig. 2c). These elements are normally located within the context of the RNA secondary structure recognized by the R17/MS2 coat protein (5). The bacteriophage P22$_{R17}$, which encodes the wild-type R17/MS2 RNA operator site in the 5′-end of the phage *ant* gene, forms lysogens in *S.typhimurium* strains that express the R17/MS2 coat protein (1). To determine whether the R17/MS2 coat protein could direct lysogen formation for P22$_{R17}$[L19], challenge phage assays were performed using this phage in *S.typhimurium* recipient strains that differ in the expression status of the coat gene. Lysogens were obtained at high frequencies following infection of only *S.typhimurium* strains that express the R17/MS2 coat protein (MS1868 [pR17coat(+)]; ref. 1) with P22$_{R17}$[L19] (Table 1). These frequencies of lysogen formation are comparable with those observed following infection of the corresponding bacterial strains with P22$_{R17}$ (Table 1; ref. 1). Thus, one should be able to select for a particular RNA–protein interaction *in vivo* using the RNA challenge phage system so long as the RNA–protein interaction of interest prevents translation of the *ant* mRNA.

In summary, we describe new reagents that enable RNA challenge phages to be constructed following a single recombination reaction *in vivo*. The improved efficiency of the described method makes it feasible to readily construct either RNA challenge phages that encode a well defined RNA-binding site or a phage library containing a population of randomly cloned RNA-binding sites from which the RNA component may be selected for a particular RNA-binding activity.

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