Direct and General Selection for Lysogens of *Escherichia coli* by Phage λ Recombinant Clones

MICHAEL F. HENRY¹ AND JOHN E. CRONAN, JR.^{1,2*}

*Departments of Microbiology¹ and Biochemistry,² University of Illinois, 131 Burrill Hall,
407 South Goodwin Street, Urbana, Illinois 61801*

Received 13 November 1990/Accepted 30 March 1991

We report a simple *in vivo* technique for introducing an antibiotic resistance marker into phage λ . This technique could be used for direct selection of lysogens harboring recombinant phages from the Kohara λ bank (a collection of ordered λ clones carrying *Escherichia coli* DNA segments). The two-step method uses homologous recombination and λ DNA packaging to replace the nonessential λ DNA lying between the lysis genes and the right cohesive (*cos*) end with the neomycin phosphotransferase (*npt*) gene from Tn903. This occurs during lytic growth of the phage on a plasmid-containing host strain. Neomycin-resistant (*npt*⁺) recombinant phages are then selected from the lysates containing the progeny phage by transduction of a *polA1* λ lysogenic host strain to neomycin resistance. We have tested this method with two different Kohara λ phage clones; in both cases, neomycin resistance cotransduced with the auxotrophic marker carried by the λ clone, indicating complete genetic linkage. Linkage was verified by restriction mapping of purified DNA from a recombinant phage clone. We also demonstrate that insertion of the *npt*⁺ recombinant phages into the λ prophage can be readily distinguished from insertion into bacterial chromosomal sequences.

The physical map of the *Escherichia coli* genome constructed by Kohara and co-workers (22) is a major advance in bacterial genetics. The map was constructed from a series of ca. 20-kbp segments of *E. coli* DNA cloned into either one of two vectors, λ EMBL4 (11) or λ 2001 (18). A selection of 476 of these overlapping clones (called the miniset) covers essentially the entire *E. coli* chromosome with fine-resolution restriction data. Therefore, if the genetic map position of a given gene is known, a small number (e.g., 5 to 20) of these clones can be screened for the wild-type gene.

The λ vectors used by Kohara et al. (22) were chosen for their capacity (10 to 22 kbp) as cloning vectors and obligatory lytic growth. However, as pointed out by these workers, complementation of *E. coli* mutants with these phages is difficult. These phages are defective in both the repression and integration pathways of lysogeny, since they lack the *cI* repressor, integrase, and the phage chromosomal attachment (*att*) site. Thus, to complement a bacterial mutant, indirect means must be used. Coinfection of the host cells with both a Kohara phage and a helper phage proficient in lysogeny allows complementation analysis, as does infection of a λ lysogen of the mutant bacterial strain. However, the former approach requires that phage titers be accurately known, which becomes tedious when a number of Kohara phage clones must be screened. Additional difficulty arises in both methods when the Kohara phage fails to complement the bacterial defect. In this case the only means to establish whether the Kohara phage actually lysogenized the recipient strain is to induce each lysogen and look for release of both the Kohara phage and the helper phage. Since the two phages are genetically distinct, they can be differentiated by testing certain phage markers (e.g., *cI*, *red*, *gam*).

It occurred to us that if the Kohara phages could be tagged with a marker that was readily selectable in the bacterial host, the utility of these clones in complementation analysis could be increased. The addition of a selectable marker to

the Kohara phage would allow direct selection of lysogens, thus allowing complementation of highly revertable, poorly selectable, and nonselectable markers. In addition, marker introduction would eliminate the tedious labor necessary to resolve the ambiguity of bacterial defects that fail to be complemented.

Our aim was to use homologous recombination to replace a segment of λ DNA with an antibiotic resistance determinant. This replacement had to involve a segment of λ DNA not required for lytic growth and could not increase the length of the phage DNA (which would preclude packaging of certain phages). We chose to replace the λ DNA segment between the lysis genes and the right cohesive (*cos*) end because Young and co-workers (39) had previously shown that λ phages carrying Tn903 insertions in this region grew well. Moreover, this seemed the only nonessential large segment of λ DNA remaining in these phages. Our method (Fig. 1 and 2) replaces a 2.0-kbp segment of λ DNA with a 1.2-kbp segment of DNA encoding the Tn903 neomycin phosphotransferase, resulting in an 800-bp reduction in the size of the recombinant phages. Recombinant *npt*⁺ Kohara phages are readily isolated and used to select lysogens for complementation analysis.

MATERIALS AND METHODS

Media, bacterial strains, plasmids, and phages. For most genetic procedures, rich broth (RB) (5) was used for bacterial growth. When minimal medium was required, the bacteria were grown on medium E (29). Carbon (0.4%) sources and amino acid (concentrations as recommended by Davis et al. [6]) supplements were sterilized separately and added to the culture before inoculation. During manipulations with bacteriophage lambda, strains were propagated in TB Mg²⁺ (10 g of tryptone and 5 g of NaCl per liter with 10 mM MgSO₄) and then plated onto TB Mg²⁺ agar plates in TB Mg²⁺ top agar. Antibiotics (Sigma) were used at the following concentrations: neomycin, 25 μ g/ml; tetracycline, 10 μ g/ml; and ampicillin, 50 μ g/ml. The lactose (Lac) pheno-

* Corresponding author.

FIG. 1. Formation of a λ plasmid (monomer) recombinant molecule and subsequent packaging of npt^+ recombinant λ chromosomes. Plasmid pMFH49 sequences are represented in the cointegrate molecule as bold lines with an open rectangle for the plasmid *cos* site. The Kohara phage insert is denoted as a jagged line, and the phage *cos* site is shown as a solid rectangle. The locations of some λ genes are shown. We have designated λ as a circle because formation of linear concatamers is inhibited by host RecBCD nuclease activity when the phage is *red gam* (i.e., Kohara clones). The solid arrow within the cointegrate molecule represents a λ chromosome that can be packaged by the terminase enzyme, whereas the dashed arrow represents a λ chromosome that cannot be packaged. The terminase enzyme will not package the λ chromosome represented by the dashed arrow for two reasons: (i) the enzyme requires two different *cos* sites (one for initiation and the other for termination), and (ii) the addition of plasmid sequences to the phage DNA makes it too large to be packaged into the phage head. It should be noted that recombinant molecules formed with multimer plasmids will provide additional *cos* sites, but they will also make the molecule too large to be packaged. As a result, only npt^+ recombinant phage can be packaged from these molecules.

type was determined by the addition of both the inducer IPTG (isopropyl- β -D-thiogalactoside) and the indicator dye X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) to agar medium at a final concentration of 0.1 mM. The arabinose (Ara) phenotype was determined by using arabinose-MacConkey agar plates.

The bacterial strains, plasmids, and phages used in this study and their relevant properties are listed in Table 1. P1 transductions and plasmid transformations were performed by standard methods (26, 35). Strain MFH208 is a transformant of strain LE392 with pMFH49. Strain MFH210 was constructed by infecting strain MC1061 with P1vir grown on strain LCD69, followed by selection for resistance to tetracycline (Tc^r). The *polA1* and *zig-621::Tn10* markers are about 50% cotransducible, and the desired *polA1* transductants were identified by their sensitivity to UV light. Lyso-genization of this strain (MFH210) with λ CI857Jam gave strain MFH211. Strain MFH213, which carries a *lac* deletion, was constructed by transducing strain M4126(P2) to Tc^r with P1vir grown on strain MFH32. Lac^- transductants were identified by patching colonies onto media supplemented with X-gal. Strain CY801 was constructed by infecting the Hfr strain B8 with P1vir grown on strain CY687 and then selecting for resistance to chloramphenicol (Cm^r). Cm^r transductants were shown to be Bio^- by lack of growth in

the absence of biotin. Strain MFH226 was constructed by conjugation of $F' lacZ^+ c1857$ (28) into strain MFH210.

Plasmid pMFH49 was constructed as outlined in Fig. 3. Plasmid pJH2, which carries the lambda lysis gene region (λ coordinates 44,972 through 47,347) adjacent to a 1,800-bp sequence of Tn903, was constructed by Garrett et al. by subcloning a 4,175-bp *EcoRI* (λ coordinate 44,972)-*HindIII* (Tn903 sequence) restriction fragment from λ 363s7 into the *tet* gene of the multicopy plasmid pBH20 (13). Plasmid pCY121 is a pUC4K (38) derivative in which the *bla* gene was removed by partial digestion (two of three sites) with *Apa*LI. Plasmid pCY128, which carries the λ *cos* site, was constructed by subcloning a 414-bp *HincII* λ *cos* fragment (λ coordinates 48,298 through 199) carrying *Pst*I ends from pMUA-10 (27) into pHSG395 (37). The orientation of the *cos* site was determined by double digestion with *Dra*I and *Hind*III. Plasmid pMFH49 was formed by the simultaneous ligation of three restriction fragments: the *Acc*I restriction fragment of pCY121 carrying the *npt* gene, the *EcoRI*-*Cla*I (λ coordinates 44,972 through 46,439) restriction fragment of pJH2 carrying the λ lysis genes, and the *ori*-containing fragment of pCY128 obtained by *EcoRI* and *Acc*I digestion. All DNA manipulations were done as described previously (15).

Conjugational methods. Liquid matings were done essen-

FIG. 2. Diagram showing the in vivo method of replacing the DNA between the lysis genes and the right cohesive end of λ with the *npt* gene from Tn903. (A) λ -plasmid crosses and production of progeny phage. (B) Scoring progeny phage for *npt*⁺ recombinants.

tially as described by Miller (29), except for one modification. When either the recipient or the donor strain carried a λ prophage regulated by a temperature-sensitive repressor (*cI857*), matings and incubations were performed at 33°C. At this temperature we observed both efficient mating and repression of λ lytic functions. When neither strain was temperature sensitive for prophage induction, matings were carried out at 37°C.

Plasmid-Kohara phage crosses. Through previous use of our copy of the Kohara minibank, we found some clones to be contaminated with another phage (distinguished by a threefold greater plaque size). Therefore, the purity of phage lysates used in this study was ensured by propagating new stocks from isolated plaques on the host strain MC1061. The minibank clones were then converted to *npt*⁺ derivatives (via homologous recombination and packaging) during lytic growth on the plasmid containing strain MFH208. Both the plaque purification and plate lysis methods were essentially those of Maniatis et al. (26), except that TB Mg²⁺ was used as the complex medium. The addition of Mg²⁺ was found to increase the titer of all clones tested in this study (clones 10A6 [miniset clone no. 138], 8D2 [no. 106], 7H9 [no. 107], and 8H11 [no. 108]) ca. 100-fold, presumably due to stabilization of the phage capsids. The hydrolysis of X-gal (included with IPTG in the bottom agar) verified that the 10A6 clone carried the *lacZ* gene. All phage lysates were titrated on the host strain, MC1061.

Transduction and estimation of recombination frequencies. Strain MFH211 was grown overnight at 30°C in TB Mg²⁺ supplemented with tetracycline and 0.2% maltose. The cells

TABLE 1. Bacterial strains, plasmids, and phages employed

Strain, plasmid, or phage	Genotype or description	Source or reference
<i>E. coli</i> K-12		
MC1061	<i>araD139 Δ(araABC-leu)7697 ΔlacX74 mcrB galU galK hsdR thi</i>	4
LE392	<i>hsdR514 supE44 supF58 galK2 galT22 metB1 trpR55 lacY1</i>	2
ML17	LE392 <i>recA1</i> (λ cI857b2S7)	Laboratory strain
DH5 α	<i>supE44 ΔlacU169(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA36 thi-1 relA1</i>	14
LCD69	<i>ΔlacU169 araD139 thi non nalA rpsL metE polA1 zig-621::Tn10</i>	L. C. DeVeaux
M4126(P2)	W3110 <i>thrB trpR24</i> (P2)	12
V316	<i>supF58 pro thi argA::Tn10 mel-1 recC1001</i> (P2)	G. Smith
B8	Hfr <i>metB1 relA1 spoT</i> (P.O.118)	3
CY687	MC4100 <i>ΔbioC::cat</i>	32
CY801	B8 <i>ΔbioC::cat</i>	This work
MFH34	<i>metB relA nalA Δ(lacIPOZ)C29 zah::Tn10</i>	15
MFH208	LE392(pMFH49)	This work
MFH210	MC1061 <i>polA1 zig-621::Tn10</i>	This work
MFH211	MFH210 (λ cI857Jam)	This work
MFH213	M4126(P2) <i>Δ(lacIPOZ)C29 zah::Tn10</i>	This work
MFH226	MFH210/F' <i>lacZ⁺ cI857</i>	This work
Plasmids		
pBR322	General cloning vector, Ap ^r Tc ^r	1
pJH2	Lambda lysis gene region clone, Ap ^r	13
pHSG395	General cloning vector, Cm ^r	37
pUC4K	<i>npt</i> gene cartridge plasmid Neo ^r Ap ^r	38
F' <i>lacZ⁺ cI857</i>	F' <i>cro cI857 rex⁺ φ(N'-lacZ⁺)22 A4(Hyb) proA⁺B⁺</i>	28
pCY121	Ap ^s derivative of pUC4K	This work
pCY128	Lambda <i>cos</i> region cloned into pHSG395, Cm ^r	This work
pMFH49	Lambda lysis gene region and <i>npt</i> cloned into pCY128, Cm ^r Ap ^r	This work
Phages		
10A6 (<i>lac⁺</i>)	λ cI857Jam	J. Gardner
10A6 (<i>lac⁺</i>), 8H1, 8D2, and 7H9 (<i>ara⁺</i>)	<i>Sau</i> 3A inserts of W3110 chromosomal DNA cloned into λ EMBL4 vectors	22

FIG. 3. Schematic diagrams showing the derivation of plasmid pMFH49. Dashed arrows denote truncated genes, and bold lines denote the lambda lysis gene region.

were collected by centrifugation and suspended in an equal volume of 10 mM MgSO₄. Phage adsorption was facilitated by incubating 0.2 ml of cells with 0.1 ml of lysate (multiplicity of infection, 0.4) for 20 min at 30°C. The cells were collected by centrifugation, washed with 2 ml of medium E, and then suspended in 2 ml of TB (without Mg²⁺). After expression for 1 h at 30°C, the cells were concentrated by centrifugation, suspended in 0.1 ml of medium E, plated onto RB-neomycin plates, and incubated at 30°C for 1 to 2 days.

Complementation of the *lacZ* and *araABCD* mutations was scored by the formation of blue colonies on X-gal plates and red colonies on arabinose-MacConkey plates, respectively. Complementation of the *leu* mutation was scored by growth on minimal medium-glucose plates lacking leucine.

The recombination frequency between the λ clone carrying *lac* (10A6) and pMFH49 was determined by dividing the number of recombinant phage (LacZ⁺ Neo^r) transductants by the number of parental phage (LacZ⁺ Neo^s) transductants. The parental transductants were scored by plating serial dilutions of the infected cell mix onto minimal E medium plates supplemented with leucine and lactose. The percentage of recombinant transductants was obtained by replica plating onto the same plates supplemented with neomycin and X-gal. Neo^r transductants unable to hydrolyze X-gal represented double-crossover events in which only the *npt* sequence was inserted into the chromosome.

Recovery of *npt*⁺ Kohara phages. A culture grown over-

night at 30°C in RB supplemented with neomycin was diluted 50-fold in 15 ml of RB and shaken at 30°C to a cell density of 4×10^8 cells per ml. At this time the culture was heat shocked at 45°C for 15 min and then incubated at 38°C until lysis occurred (2.5 to 5.0 h). After the addition of 500 μ l of chloroform and a 30-min incubation, cell debris was removed by centrifugation.

Recombinant Kohara phages were purified from the helper phage by using Spi⁻ selection (24). The lysate was titrated on either strain MFH213 or strain V316 to obtain isolated plaques. These plaques were further purified by streaking onto a bottom agar plate and covered with soft agar containing the same host strain. An isolated plaque from the second plate was then used to prepare phage lysates. It should be noted that this method results in contamination of the Kohara λ stock with P2 phage (the result of spontaneous P2 phage release). Although P2 contamination was not a problem in our work, it can readily be avoided by selection of the Spi⁻ phenotype on a host strain that carries either an excision deficient P2 prophage (25) or a plasmid encoding the *old* gene of P2 (10). In addition, the phage yield will be increased if the host strain lacks the activity of the RecBCD enzyme.

Burst size of *npt*⁺ Kohara phages produced upon induction of a complemented lysogen. We developed an assay in which complemented lysogens could be screened for the production of Spi⁻ phage. To facilitate mass screening, cultures

were grown overnight in the wells of a sterile microtiter dish containing 500 μ l of RB. The dish was covered with pressure-sensitive film (Becton Dickinson) to prevent airborne contamination and then shaken at 30°C on a rotary platform (200 rpm). After overnight incubation, 20 μ l was removed from each well and inoculated into a second dish. This new dish was incubated at 30°C for 2 h, shifted to 42°C for 1 h, and then shifted to 37°C till lysis occurred (2 to 3 h). The lysates were diluted 100-fold (in a third microtiter dish), transferred with a prong device onto plates containing an overlay of indicator bacteria, dried for 5 min in a laminar-flow hood, and then incubated overnight at 37°C. The indicator plates were TB Mg²⁺ plates overlaid with 2.5 ml of TB Mg²⁺ top agar inoculated with 50 μ l of the P2 lysogenic strain V316. This strain was grown overnight in TB Mg²⁺ supplemented with tetracycline and 0.2% maltose, collected by centrifugation, and suspended in 0.5 volume of 10 mM MgSO₄. In this assay a zone of clearing was scored as a large burst of phage, whereas no zone of clearing was scored as either a low burst or no burst of phage. Undiluted lysates that produced no zone of clearing were scored as not containing any Spi⁻ phage.

RESULTS

Rationale of the method. Figures 1 and 2 diagram the method for isolating *npt*⁺ derivatives of the Kohara phages. Plasmid pMFH49 carries (i) the λ lysis region (λ coordinates 44,970 through 46,440) to allow RecA-mediated recombination of the plasmid with the infecting phage, (ii) the *npt* gene for placement into progeny phage, and (iii) a properly oriented λ *cos* site to allow packaging into phage capsids. Homologous recombination between the two lysis gene DNA segments of the monomeric plasmid and the phage results in a recombinant molecule that carries two λ *cos* sites; one is donated by the plasmid, and the second is that of the phage. We expected that when the λ -encoded packaging enzyme (terminase) encountered a *cos* site on this hybrid molecule, a cut would be made in the DNA and the chromosome would be packaged unidirectionally (Nu1 end to the R end) into the prohead until the terminal *cos* site was encountered (7). Because *cos* cutting is coupled to the capacity of the phage head, a terminal *cos* site is not recognized (cut) unless the phage head contains 78 to 105% (38 to 51 kbp) of a wild-type λ DNA molecule (8). As illustrated in Fig. 1, the substrate requirements for terminase-mediated packaging ensure that only initiation from the *cos* site residing in the λ phage yields a viable phage particle. Thus, only *npt*⁺ recombinant phages are packaged from the cointegrate molecule. In effect, the placement of the *cos* site on plasmid pMFH49 converts a single-crossover recombination event in the lysis gene region into a double-crossover event. It should be noted that a double-recombination event (both at the lysis genes and at *cos*) between pMFH49 and phage λ would also yield an *npt*⁺ recombinant phage, albeit at a low frequency (19).

The recombinant progeny phage were scored by transduction of a host strain (carrying a λ prophage, the *polA1* mutation, and [optionally] the mutation to be complemented) to Neo^r. The *polA1* (DNA polymerase I) mutation was present in the host strain to prevent the formation of Neo^r colonies via λ -mediated packaging of the plasmid. Cosmids such as pMFH49 are packaged into phage capsids despite their small size. The λ DNA replication machinery is thought to result in rolling-circle replication of the cosmid to produce linear cosmid multimers sufficiently long to be packaged into

λ particles (9, 30). Although the Kohara phages are *red gam* and thus deficient in rolling-circle replication, we found appreciable transduction of pMFH49 with these phages. Hence, in agreement with the results of Miwa and Matsubara (30), the Gam deficiency seems to decrease but not eliminate transduction of small plasmids. DNA polymerase I is required for replication of ColE1-type plasmids (21), and thus the *polA1* mutation prevented the replication and maintenance of transduced pMFH49 molecules and ensured that the Neo^r transductants resulted from insertion of the recombinant Kohara phage. The recipient host strain was also a λ lysogen (we used λ cIts857Jam) to provide both the *cI* repressor and the homology needed for insertion of the Kohara clone through recombination with the prophage.

Hollifield et al. (16) have reported that the yield of λ phage-plasmid recombination products is increased 100-fold by the presence of a gene called *rap*. This increase may be due either to an increase in recombination per se, as reported by Hollifield et al., or to increased survival of phages carrying plasmid sequences (36). In any case, the Kohara phages lack the *rap* sequences, and thus phage-plasmid recombination products may be reduced compared with those in *rap*-containing phages (16, 20).

Tests of the method. First, Kohara phage clone 10A6 carrying the *lac* operon was grown on a *recA*⁺ strain carrying pMFH49. The resulting lysate was then used to infect strain MFH211 (a *polA1* [λ cIts857Jam] derivative of the Δ *lac* strain MC1061), and the infected cells were plated on medium containing neomycin. All of the resulting colonies were Lac⁺ upon replica plating to medium containing X-gal, indicating that the *lac* and *npt* genes were genetically linked in the λ transducing phage. We measured the efficiency of recombination between Kohara phage 10A6 and plasmid pMFH49 by selection of recombinant phage (Lac⁺ Neo^r) transductants and parental phages (Lac⁺ Neo^s) on lactose minimal medium plus or minus neomycin. We found that the number of recombinant phages (Lac⁺ Neo^r) was ca. 1.4×10^{-4} (mean of two experiments) of the total number of Lac⁺ phages, a result similar to that reported by Hollifield et al. (16) for λ -plasmid single-crossover events (1,500 bp of plasmid homology) with *rap* phages.

Direct evidence for the recombination event depicted in Fig. 1 was obtained by recovery of the Lac⁺ *npt* phages from the complemented lysogen (see below) followed by restriction mapping of the purified λ DNA. The restriction maps of the recombinant λ phage and the original Kohara 10A6 phage obtained with *Hind*III, *Cla*I, and *Sma*I were completely consistent with the replacement of the λ sequences with the Tn903 *npt* gene (data not shown).

As a second test we inserted the Tn903 *npt* gene into Kohara phage 7H9, which carries the arabinose operon. Neo^r transductants of strain MFH211 [*araD* Δ (*araABC-leu*)] were found at a frequency similar to that of the *lac* phage, and all were found to be Ara⁺ upon replica plating onto MacConkey medium containing arabinose. As negative controls, two Kohara clones flanking the Ara⁺ clone (λ 8D2, λ 8H1) were converted to *npt*⁺; they did not complement the arabinose mutation. In addition, these clones did not complement the host leucine mutation.

Induction and purification of lysogenic recombinant bacteriophage lambda. Lysates from the Neo^r lysogens contained comparable numbers of Kohara phage particles and helper phage particles. The two phage types were distinguished by plating on a *recA* host strain and a host strain lysogenic for P2. Phages lacking *red gam* functions (e.g., the Kohara phage) plate efficiently on strains that are lysogenic for phage

P2 (the Spi^- phenotype) but are unable to form plaques on *recA* strains. *red*⁺ *gam*⁺ phages (the helper phage) have the opposite growth characteristics. Therefore, we used Spi^- selection as a means to purify the *npt*⁺ Kohara phage. In the case of the *npt* derivative of Kohara phage 10A6, we used a Δlac P2 lysogen as the plating bacterium on a medium containing X-gal and IPTG; we found that all plaques (1,000 scored) became blue.

It should be noted that recombination can occur between the *npt* Kohara phage and the helper phage during excision and replication. Such recombination could result in an *npt* Kohara phage and an *npt*⁺ helper phage. However, judging from results obtained with similarly spaced λ markers (*susA-susF* or *susF-susJ*) (34), such recombinants should compose only 1 to 2% of the total phage progeny. Since stringent selection against Spi^+ phage requires function of the phage *red* genes (24), recombination could not be blocked. The small fraction of lacking *npt* Kohara phages (indistinguishable from the original Kohara phages) arising on the P2 lysogens could be recognized by their inability to transduce a lysogen to Neo^r .

Sites of insertion of *npt*⁺ Kohara phages in the *E. coli* chromosome. At least five distinct sites in the bacterial chromosome can provide homology for recombination by the *npt*⁺ Kohara phages. These are (i) the λ prophage, (ii) the chromosomal segment homologous to the insert carried by the phage, and (iii) the three incomplete lambdoid prophages (Rac, Qsr', and Qin) present in the chromosome of most *E. coli* K-12 strains (23, 33). The predominant recombinational event is a single crossover resulting in insertion of the *npt*⁺ Kohara phage into one of these sites. However, double-crossover events consisting of recombination on both sides of the *npt* gene could result in insertion of only the *npt* sequences into either λ prophage, Qsr', or Qin sequences. In the case of phage clone 10A6 (*lacZ*⁺), this rare event (1% of transductants) was observed as Neo^r transductants that were unable to hydrolyze X-gal.

To verify that the Kohara phage integrated at the predicted locations, we selected for insertion at each specific chromosomal site by individually deleting all (or most) of the remaining insertion sites. Insertion of Kohara clone 10A6 (*lac*⁺) at the λ prophage site was favored by transduction of the lysogenic strain MFH211 (Δlac) to Neo^r . The *npt* gene was shown to have inserted at (or near) the site of prophage integration by mating with strain CY801 (HfrB8 $\Delta\text{bioC}::\text{cat}$). In all 10 transductants tested, selection for Cm^r resulted in the concurrent loss of the Neo^r phenotype, indicating close linkage between *bioC* and the inserted *npt* gene (*att* λ is adjacent to the *bio* operon). Insertion of the same λ clone into *lac* operon sequences was favored by transduction of strain MFH226 (a *polA1* F' *lacZ* cI857 derivative of the Δlac Δara strain MC1061) to Neo^r . This strain contains neither the chromosomal *lac* sequences nor a λ prophage, thereby precluding insertion at these sites. Instead, this strain carried an F' factor that provided λ repressor and *lac* sequences for homologous recombination with the Kohara phage clone. Although the F' factor carried the λ repressor, the only sequences homologous to the λ -phage sequences were small segments of the *N* and *cro* genes. Lysogens resulting from insertion into the *lac* sequences present on the F' factor were identified by mating with strain ML17 (*recA* λ^+) and selecting for Neo^r exconjugants. Insertions into the incomplete prophage sequences were detected by transduction of strain MFH226 to Neo^r with the *npt*⁺ Kohara clone 8H1 (*ara*⁺). Since this strain lacks all other regions of homology, inte-

gration should occur primarily at the incomplete prophage sites (illegitimate recombination events could also occur).

Insertion of *npt*⁺ Kohara phage into the λ prophage site was distinguished from insertions into all other chromosomal locations by the yield of Spi^- phages produced upon induction of the complemented lysogen. In our assay (described in Materials and Methods), only *npt*⁺ Kohara phage that integrated into the λ prophage yielded large phage bursts (10^8 PFU/ml) upon induction. The *npt*⁺ Kohara phages that integrated by homology with either sequences on the F' factor or the incomplete prophage sequences yielded small phage bursts (10^4 PFU/ml) upon induction. This variation is due to the different excision pathways available to these two classes of lysogens. Tandem prophages at the *att* site can be excised by action of the *int* and *xis* genes, giving dimeric λ circles that can be replicated by an efficient rolling circle mechanism and thus giving a large burst of phages. In contrast, Kohara phage that insert into either the bacterial chromosomal sequences or the incomplete prophage sequences can only exit the bacterial sequences via homologous recombination. Since homologous recombination is 10^3 - to 10^4 -fold (dependent on the amount of homology present) less efficient than λ *int* *xis*-mediated excision (35), the burst of Spi^- phages should be very low. As expected, no Spi^- phage were released after induction of Neo^r transductants resulting from double-crossover events.

In conclusion, *npt*⁺ Kohara phages inserted into a resident λ prophage can be recognized by efficient production of Spi^- phage. In most complementation experiments, either the site of insertion of the *npt*⁺ Kohara phage is irrelevant or the bacterial *att* site is preferred. However, there may be cases in which insertion of the *npt*⁺ Kohara phage into the bacterial chromosome sequence homologous to that carried by the Kohara phage or into an incomplete prophage site is preferred. Such insertions can be enriched by using a plasmid to provide a λ cI repressor and characterized by conjugational or transductional mapping.

DISCUSSION

Our method of tagging the Kohara phage clones with a neomycin resistance marker for detection in a lysogenic host requires only two simple manipulations. First, the Kohara phage clone is propagated on a *recA*⁺ host strain that harbors pMFH49 to yield a phage lysate. Next, *npt*⁺ recombinant phage are identified in this lysate by transduction of a suitable lysogenic host strain to neomycin resistance. For complementation analysis, the host strain can be either a derivative of strain MFH211 (*polA1* $\lambda\text{cI}857$) that carries the mutation to be complemented (introduced by transduction or conjugation) or a mutant strain of interest carrying both a λ lysogen and the *polA1* allele. The *polA1* allele is readily introduced into a given strain by cotransduction with a closely linked Tn10 transposon insertion followed by screening of transformants for sensitivity to UV light or methyl methanesulfonate (17). If complementation analysis cannot be carried out in a *polA1* background, the recombinant phage can be recovered from *npt*⁺ transductants of strain MFH211 by temperature induction and then transduced into the mutant background; the only requirement is the presence of a λ lysogen (or just a functional λ cI repressor). Complementation of temperature-sensitive host mutations precludes the use of cI857, and thus a cI⁺ lysogen would be needed and recombinant phages would then be recovered by UV induction. In addition, any *npt*⁺ Kohara phage that forms a tandem prophage at the λ *att* site can easily be recognized by

induction and analysis of the released phage. Our method can be applied to any λ phage derivative that carries the λ lysis genes and can also be adapted for use in microtiter dishes so that a large collection of clones can simultaneously be converted to npt^+ derivatives. We have not attempted to convert the entire Kohara bank to npt^+ derivatives, because we believe that this should be done with an original copy of the bank.

Our method increases the usefulness of the Kohara phage clone bank for the complementation of genes that lack a clearly selectable phenotype (i.e., *recD*) or have a high reversion rate. Previously, only one approach could be used for this type of complementation. Dilysogens were formed by coinfection of the Kohara phage and helper phage and then screened for complementation. However, given a negative complementation result, a dilysogen could only be confirmed by inducing lysis of single-colony isolates and assaying for release of two genetically distinct phages (i.e., clear versus turbid plaques).

Upon transduction of a strain to neomycin resistance with an npt^+ phage, it necessarily becomes diploid for the bacterial genes inserted in the phage. This allows the *in vivo* construction of gene fusions (i.e., Mud fusions) to study regulation of essential genes. For instance, if the Kohara phage clone carried the *rpsK* gene (30S ribosomal subunit protein S11), a gene or protein fusion could then be made of either gene copy without the lethal loss of gene function.

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