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Rapid confirmation of single copy lambda prophage integration by PCR

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Unnatural gene dosages associated with the use of high copy number cloning plasmids can significantly affect the interpretation of some genetic experiments such as reporter gene assays, genetic complementation tests, and functional investigations of engineered mutations. Although medium copy plasmids are occasionally sufficient to reduce such problems, unit copy vectors are generally preferred for avoiding dosage artifacts. Near-unit copy plasmids such as mini F (1) or mini P1 (2) may serve this purpose, but the bacteriophage lambda (λ) is advantageous because a single prophage is stably maintained without selection and can be used simultaneously with plasmids of any incompatibility group. As a vector, λ is easy to handle, versatile and generally familiar (3,4). The development of λ transducing phages and cloning plasmids with homologous DNAs for *in vivo* recombination has been helpful, and of particular utility are systems for constructing single copy fusions to the reporter gene *lacZ* [e.g., λRZ5 developed by R.Zagursky (unpublished); 5,6,7]. Integration of the λ vector into the chromosome by recombination between the bacterial attachment site, *attB*, and the phage attachment site, *attP*, is usually desired so as to maintain integrity of the cloned element. This phage-mediated recombination creates two hybrid sites, *attL* and *attR*, bordering the prophage, while an intact *attP* site is retained only by the insertion of multiple prophages (8). The assay introduced here discriminates between single- and polylysogens by detecting the presence of the *attL* and *attP* structures.

Several methods have been applied in the past to screen lysogens for single prophage integration. These include: solution hybridization between lysogen and λ DNAs, Southern hybridization of lysogen DNA, yield of prophage during superinfection by heteroimmune phage, or quantitation of a phenotypic marker carried by the prophage. Solution hybridization measures the kinetics of reannealing labeled, denatured λ DNA in the presence of unlabeled total DNA extracted from the lysogenic strain (9). The reannealing rate of a single lysogen falls between those of nonlysogen and dilysogen. Southern hybridization detects the structure of enzyme-restricted DNA extracted from the lysogen using labeled probes of complete λ DNA (10), or DNA fragments containing, for example, *attP* (11). Assays for prophage yield during superinfection by an heteroimmune phage, generally called 'Ter' tests (12,13), are based on the ability to package tandem lysogens by a vegetatively replicating heteroimmune phage that is defective for integration. Lytic growth of the superinfecting phage provides the means, employing its *ter*minase enzyme, for packaging DNA from tandem prophages because of their multiple *cos* sites. Thus, the phage burst produced by superinfecting a single copy lysogen yields a relatively low titer of progeny having the genotype (e.g., immunity) of the resident prophage. Quantification of the level of cI-repressor can also be used to indicate prophage number in a different superinfection test. In this case, the degree of

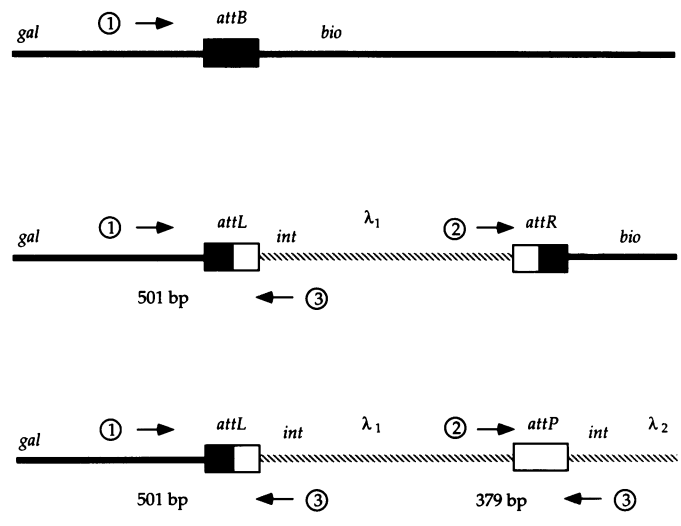

Figure 1. Diagram of DNA fragments amplified by single- and multilysogens. Represented are the 4 different *att* sites occurring in a nonlysogen (top) and lysogens of single (middle) and multiple (bottom) prophage copy number. Lines depict bacterial DNA (solid) and lambda DNA (hatched), with *att* sites (boxes) and relevant genes named above. The respective locations and orietntations for hybridization of PCR primers numbered 1, 2 and 3 (see text) are indicated by arrow heads above and below the DNA lines. Fragment sizes produced by productive priming are given in base pairs below.

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resistance to superinfection by the virulent mutant λcI90c17 depends directly on the amount of repressor present in the host cell which increases proportionally with additional prophage. Hence, only single lysogens are sensitive to superinfection by this mutant phage (14). Finally, instead of testing properties of λ, it is often possible to quantify a phenotypic marker carried on the prophage. Relative levels of β-galactosidase and β-lactamase produced by λlacZ$^+$bla$^+$ lysogens have been measured for this purpose (5). The above mentioned techniques have the disadvantages of being either laborious, wasteful or simply inferential. We have refined an alternative method based on the use of polymerase chain reaction (PCR) to amplify fragments primed from three locations flanking the bacterial and prophage attachment sites. The strategy is diagrammed in Figure 1. This technique is definitive, rapid, easy and requires only the acquisition of three oligonucleotide primers with the assumption that the equipment and reagents for PCR amplification are available.

Single copy number λ integrants were confirmed by PCR using an equimolar mix of the following three oligonucleotide primers (Fig. 1):

1) top strand primer to the left of *E.coli attB*:
   5′-gaggtaccagcgcggtttgatc-3′;
2) top strand primer to the left of λ attP:
   5′-tttaatatattgatatttatatcattttacgtttctcgttc- 3′;
3) bottom strand primer within the λ*int* gene:
   5′-actcgtcgcgaaccgctttc-3′.

Freshly grown lysogenic colonies were picked separately into 500 μl of sterile water using the disposable tip of a pipetteman. Cells were well suspended by vortexing followed by careful removal of the supernatant after a 1 minute spin in a microfuge. After a total of three such washings the cell pellet was thoroughly resuspended in a final volume of 100 μl of sterile water, and then used promptly for PCR or stored at −20°C for testing later. While we also tested TE buffer (10 mM Tris−HCl, 1mM Na_2EDTA, pH 8) or 50 mM sodium phosphate (pH 7) as cell diluent/wash buffers, they produced results that were no better than those with water. Reactions were performed with 20 μl of the cell suspensions as template and an equimolar mix of all three primers at 500 mM final concentration, with addition of deoxynucleotides, buffer, magnesium salt, and enzyme as specified by the polymerase manufacturer. We have employed Taq (Life Technologies Inc.), Pfu (Stratagene) and ULTma (Roche Molecular Systems Inc.) polymerase enzymes with similar results and suspect that any thermally stable DNA-dependent DNA polymerase should suffice. The following thermal cycling program was used: 95°C for 1′ followed by 25 × [95°C for 1′; 55°C for 1′; 72°C for 1′] then 72°C for 5′. The amplified DNA fragments were visualized after electrophoresis of 6 μl of the PCR samples using a 1.5% agarose minisubmarine gel (data submitted, not shown). As is clearly evident, a strain without a prophage produced no band (lane 1), and a strain with a normally integrated single prophage produced a single fragment of 501 base pairs (bp) primed from the λ *int* and *attB* primers. A strain with two or more prophages produced this fragment plus one of 379 bp primed from the λ *int* and *attP* primers. Note that in this case, as well as all other cases of multilysogens tested, the lower band is more intense than the upper band. λ phage lysate control produced only the 379 bp *attP* fragment.

A potential problem with this assay is that spontaneous induction of λ, replication, and cell lysis occurs in a subpopulation of lysogenic cells during their growth in colonies on solid medium. This yields free phage with an *attP* site which generates a 379 bp fragment in the PCR reaction. However, by thorough washing during colony suspension to remove most of the free phage, this spurious PCR product can be eliminated altogether or reduced to a nearly undetectable level.

We propose that the PCR assay presented here is usually preferable to other methods for detecting single copy λ lysogens because it is rapid, simple and conclusive. Several candidate lysogens may be screened simultaneously and definitive results are obtainable in a few hours. A practical limitation to this assay derives from the fact that primer hybridization requires the tested phage to contain *int* sequence and integrate by *attP* X *attB* recombination. For most cases in which λ is used as a vector to study bacterial gene expression we recommend this method to verify single copy number integration.

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