Transposon Tagging to Detect a Latent Virus in *Myxococcus xanthus*

Abstract. *Transposon mutagenesis of the bacterium* Myxococcus xanthus *with the transposon Tn5 revealed a special class of bacterial mutants that transduced the transposon through culture supernatant fluids. Virus-like particles copurified with transducing activity. Transposon tagging for detecting these virus-like particles may be generally useful in isolating endogenous viral agents capable of transferring genetic information between cells.*

TODD STARICH
PAUL CORDES
JAMES ZISSLER
Department of Microbiology, University of Minnesota Medical School, Minneapolis 55455

Viruses traditionally have been discovered by observing their deleterious effects on host organisms. Assays for plaques, pocks, or other cytopathic effects on sensitive cells are important for detecting and purifying viral agents in the laboratory. These tests rely on the assumption that the virus has cytocidal activity on a particular indicator strain. Virus-like particles may frequently appear in culture supernatants of both prokaryotic and eukaryotic cell strains as determined by electron microscopy. However, it is often difficult to demonstrate any biological activity for these particles. This suggests that traditional assays may be inappropriate for detecting certain classes of virus-like elements.

We describe an alternative method for isolating virus-like particles that does not rely on the ability of the virus to kill sensitive cells, but rather on its ability to transfer genetic information between cells. This strategy involves first marking or tagging a putative viral genome by inserting within it a transposon that encodes a selectable genetic marker and then assaying for virus particles capable of mediating specialized transduction of this marker. This method may be generally useful in uncovering latent viruses (or other mobile genetic elements) that are not cytocidal or that kill only a special subset of cells present at low levels within a population. The principle involved in this strategy may be equally applicable to prokaryotic and eukaryotic systems, provided the proper genetic tools are available.

We applied this method to the soil bacterium *Myxococcus xanthus*, strain YS (*1*), which displays a developmental life cycle culminating in the production of spores (*2*). No prophage or plasmid endogenous to this strain has previously been isolated.

The transposon Tn5, which encodes resistance to the antibiotic kanamycin (*3*), provided the selectable marker in our virus assay. Tn5 can be introduced into *M. xanthus* from *Escherichia coli* by the phage vector P1::Tn5, which adsorbs to *M. xanthus* and successfully injects its DNA (*4, 5*). The P1 genome cannot establish stable residence in this foreign host, either by replicating to form a plasmid or by integrating to form a prophage. The transposon within P1::Tn5 DNA can rescue itself, however, by transposing from P1 DNA to sites within the *Myxococcus* chromosome. As Tn5 will transpose to apparently random sites (*5*), a Tn5 insertion may be found near a specific target gene if a sufficiently large number of independent insertions is screened.

To increase the chances of detecting a transposon insertion within a target prophage, we first picked 100 to 200 bacterial colonies that carried independent transposon insertions derived from infection with P1::Tn5. We pooled these to create a collection of independent kanamycin-resistant strains. After lysing cells from the pooled strains with chloroform and pelleting cell debris by centrifugation, we assayed the supernatant fluid for transducing activity. When 0.1 ml of supernatant was mixed with 10^8 kanamycin-sensitive cells (strain YS), 10 to 100 kanamycin-resistant colonies could be obtained. There were no viable cells in the donor supernatant fluid itself. The activity in the culture supernatant was not due to transformation by naked DNA, as treatment of supernatant with deoxyribonuclease (600 Kunitz units per milliliter of supernatant, 30 minutes,

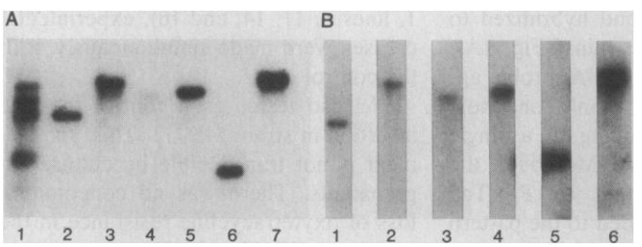

Fig. 1. Autoradiograph of hybridization of probe DNA to Southern blots of DNA from reference strains of *M. xanthus* containing transducible Tn5 insertions. The presence of a dark band shows homology in host DNA to Tn5 sequences in the probe DNA. (A) Phage P1::Tn5 probe DNA. Lane 1, P1::Tn5; lane 2, JZ120; lane 3, JZ118; lane 4, JZ116; lane 5, JZ114; lane 6, JZ113; and lane 7, JZ111. (B) Plasmid pBR322::Tn5 probe DNA. Lane 1, JZ120; lane 2, JZ118; lane 3, JZ116; lane 4, JZ114; lane 5, JZ113; and lane 6, JZ111.

Table 1. Crosses mapping Tn5 insertions.

Line	Donor strain		Recipient strain		Marker in transductants		Co-transduction (%)*
	Designation	Tn5 insertion site	Designation	Tn5 insertion site	Km^r	Tc^s	
1	MD412	Ω412	JZ122	Ω112	250	0	0
2	JZ112	Ω112	JZ122	Ω112	200	134	*
3	JZ113	Ω113	JZ122	Ω112	250	0	0
4	JZ114	Ω114	JZ122	Ω112	250	31	19
5	JZ115	Ω115	JZ122	Ω112	50	3	9.0
6	JZ116	Ω116	JZ122	Ω112	42	6	21
7	JZ118	Ω118	JZ122	Ω112	100	8	12
8	JZ114	Ω114	JZ124	Ω114	300	104	*
9	JZ112	Ω112	JZ124	Ω114	200	7	10
10	JZ117	Ω117	JZ124	Ω114	150	17	33
11	JZ114	Ω114	JZ124	Ω114	300	149	*
12	JZ113	Ω113	JZ124	Ω114	150	38	51
13	JZ119	Ω119	JZ124	Ω114	178	2	2.2
14	JZ114	Ω114	JZ124	Ω114	300	154	*
15	JZ111	Ω111	JZ124	Ω114	116	23	39
16	JZ114	Ω114	JZ124	Ω114	300	149	*
17	JZ120	Ω120	JZ124	Ω114	46	14	61

*The donor strain initially contained Tn5-Km; the recipient strain initially contained Tn5-TC. Cotransduction frequency is for cotransduction of Km^r and Tc^s markers originally located in donor DNA. The cross marked with an asterisk represents 100 percent for that set of data.

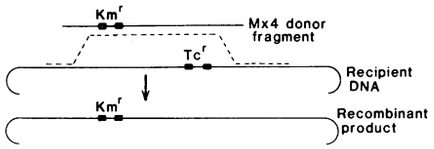

Fig. 2. Diagram of crosses for mapping Tn5 insertions in reference *M. xanthus* strains.

37°C) did not reduce transfer of kanamycin resistance.

One or two transductants from each pool were grown in culture and assayed for production of transducing activity in a new serial transduction assay. Each strain transduced recipient kanamycin-sensitive cells to produce approximately 10^2 kanamycin-resistant colonies per 0.1 ml of donor supernatant tested. Genetic strains from different pools were purified and stored as reference strains corresponding to independent Tn5 insertions (Table 1; strains JZ111 to JZ120 and Tn5 insertion sites Ω111 to Ω120 in Table 1).

To rule out the possibility that some form of phage P1 DNA might be maintained in donor cells, P1::Tn5 DNA was radioactively labeled and hybridized to DNA from reference strains (Fig. 1A). For each strain, the DNA probe appeared to hybridize to only one host DNA band, corresponding to a single restriction fragment size. Moreover, the pattern of hybridization to P1::Tn5 probe DNA corresponded to the pattern seen for plasmid pBR322:Tn5 probe DNA (Fig. 1B). Therefore, we detected hybridization to Tn5 sequences, and P1 DNA was not present in these strains. There was no detectable hybridization between cell DNA and known myxophage DNA probes (Mx4, Mx8, and Mx1).

Transducible Tn5 insertions in different reference strains were found on DNA restriction fragments of different sizes, suggesting that these insertions map at different chromosomal positions. To determine if transducible Tn5 insertions map within a common DNA region, we measured genetic linkage between different Tn5 insertions by a method described by Sodergren *et al.* (*6*). Figure 2 shows the cross for the mapping experiment, which is based on cotransduction of genetic markers with Mx4, a generalized transducing phage (*7*).

In a prototype cross between two Tn5 insertions, the donor DNA fragment bears a Tn5 element encoding kanamycin resistance (Tn5-Km), while the recipient chromosome harbors a Tn5 element encoding oxytetracycline resistance (Tn5-Tc). Tn5-Tc can be introduced into a site originally occupied by Tn5-Km through a genetic replacement technique (*8*). The Tn5-Tc replacement occupies the original site defined by Tn5-Km. Acquisition of kanamycin resistance by the recipient with concomitant loss of oxytetracycline resistance is a measure of linkage between the two insertions in the cross.

As expected in the control cross where the Tn5 insertion in both the donor and recipient occupies the same map position [JZ112, Ω112 × JZ122, Ω112 (Table 1)], we observed that the recipient lost oxytetracycline resistance at high frequency. In the experimental crosses, the recipient also lost oxytetracycline resistance at relatively high frequency, which indicates genetic linkage between the two Tn5 elements tested in the cross. Although some inserts did not appear to be clearly linked to Ω112 (for example, Ω113 in Table 1, line 3), they could be linked to Ω114 (for example, Ω113 in Table 1, line 12), which in turn is linked to Ω112 (Table 1, line 4). The data link all ten transducible inserts to each other. Because the values for control crosses may vary within a reference strain (Table 1, lines 8, 11, 14, and 16), experimental crosses were made simultaneously with the control cross.

We also tested as a control the Tn5 insertion in strain MD412. This Tn5 element is not transducible by culture supernatants. There was no concomitant loss of oxytetracycline resistance in the recipient (JZ122, Ω112) when strain MD412 was used as a donor (Table 1).

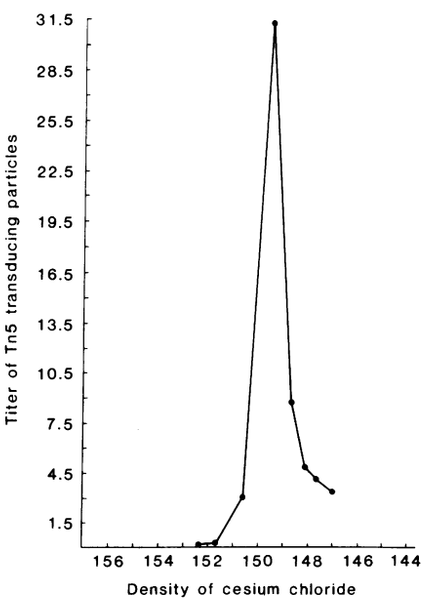

Fig. 3. Purification of Tn5 transducing particles from *M. xanthus* strain JZ119 by isopycnic centrifugation in cesium chloride. The titer of Tn5 transducing particles in the peak fraction at density 1.495 corresponds to 3.2×10^6 particles per milliliter.

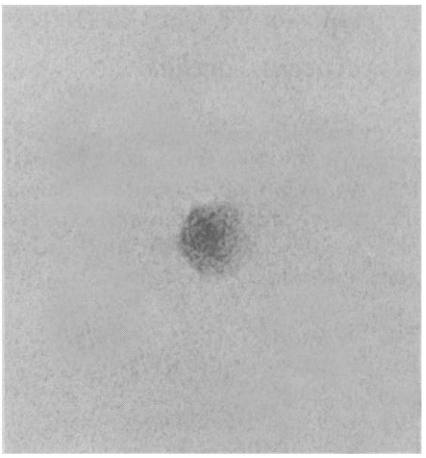

Fig. 4. Electron micrograph of a Tn5 transducing particle purified from *M. xanthus* strain JZ119 by isopycnic centrifugation in cesium chloride. The particle is negatively stained with 1 percent uranyl acetate. The diameter of the particle is 35 nm.

There was also no loss of resistance in control crosses between MD412, Ω412 and Ω114, Ω116, and Ω120. This suggests that the Tn5 element in strain MD412 is not linked to the transducible Tn5 insertions.

To characterize the nature of transducing activity, we concentrated material from supernatants and partially purified it by velocity centrifugation through a cesium chloride block gradient with three density layers. We extracted the middle layer (containing transducing activity) and banded it in a cesium chloride gradient by isopycnic density centrifugation (Fig. 3). Transducing activity appeared in one major peak with a density of 1.495 g/cm^3.

Electron micrographs of the gradient fractions with the highest transducing activity revealed a single type of particle (Fig. 4). The particles appeared to have icosahedral symmetry and differed from all of the known myxophages in their relatively small size and apparent lack of tails. Particles purified from three strains representing three different reference sites of Tn5 insertion (Ω115, Ω119, and Ω120) appeared morphologically similar.

Transducing particles resembled bacteriophage in several ways: they were capable of mediating transduction, they had an average density consistent with a structure containing nucleic acid associated with protein, they were resistant to deoxyribonuclease, and they were inactivated by heat treatment (70°C, 1 hour).

Our experiments illustrate the potential for using transposons to detect endogenous agents capable of transferring genetic material intercellularly. We assume that the transposon has inserted within a viral genome and is packaged

into particles during viral morphogenesis. A plausible model for this is specialized transduction of Tn5. Alternatively, one might imagine a mechanism of generalized transduction involving the packaging of random host DNA fragments into virus particles. However, the fact that ten Tn5 insertions selected independently as transducible all map to a common DNA region argues against this model.

Because transducing particles transfer genes between cells, they must interact with the surface of the recipient cell and deliver genetic material into the cell. Nevertheless, we did not detect a lytic cycle when testing for plaques on an agar overlay of vegetative YS cells. Although insertion of Tn5 within a particle may render it defective, the supernatant from normal YS bacteria lacking Tn5 also failed to produce plaques on an overlay of vegetative YS cells. We have tested, in addition to strain YS, other wild-type *Myxococcus* strains for sensitivity, including strains of *M. xanthus*, *M. virescens*, and *M. fulvus*. The reason that these particles fail to form plaques is unknown. It is possible that vegetative cells are immune or that these particles may not cause cell death or cell lysis in cells that produce them.

Our assay for particles is based on genetic selection of a drug resistance marker and therefore has the sensitivity to detect very low levels of particles in culture supernatants. Unlike classical schemes for detecting latent viruses based on plaque formation, our assay may detect viruses that never cause cell death. This approach could lead to the isolation of new viral-like elements that move horizontally between cells within a population. The potential importance of such elements remains to be explored, particularly in developmental systems where only a rare subset of cells within the population may be sensitive to viral infection.

References and Notes

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Enzymatic Removal of Bilirubin from Blood: A Potential Treatment for Neonatal Jaundice

Abstract. *Current treatments for severe jaundice can result in major complications. Neonatal jaundice is caused by excessive accumulation of bilirubin in the blood. A small blood filter containing immobilized bilirubin oxidase was developed to reduce serum bilirubin concentrations. When human or rat blood was passed through the enzyme filter, more than 90 percent of the bilirubin was degraded in a single pass. This procedure may have important applications in the clinical treatment of neonatal jaundice.*

ARTHUR LAVIN
Division of Newborn Medicine, Children's Hospital, Boston, Massachusetts 02115; Harvard Medical School, Boston 02115; and *Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge 02139*
CYNTHIA SUNG
Department of Chemical Engineering and *Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology*
ALEXANDER M. KLIBANOV
Department of Applied Biological Sciences, Massachusetts Institute of Technology
ROBERT LANGER*
Department of Applied Biological Sciences, Whitaker College of Health Sciences, and *Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology,* and *Department of Surgery, Children's Hospital*

* To whom correspondence should be addressed at the Massachusetts Institute of Technology.

All human newborns accumulate bilirubin to levels greater than those in adults, and 20 percent accumulate enough to stain their skin, resulting in jaundice (*1*). Bilirubin binds to cellular and mitochondrial membranes, causing cell death in a variety of tissues; clinically, bilirubin toxicity may lead to mental retardation, cerebral palsy, deafness, seizures, or death (*1*).

The two most common treatments for jaundiced infants are phototherapy and exchange transfusion (*1, 2*). In phototherapy, the infant is exposed to blue light that converts bilirubin to a less toxic photoisomer (*2*). Since only 15 percent of total body bilirubin can be photoisomerized through the skin, phototherapy does not control cases of severe jaundice (*2*). In those cases, infants undergo exchange transfusion, which requires staged removal of the infant's blood and its replacement with bilirubin-free adult blood. This procedure may result in hypoglycemia, hypocalcemia, acidosis, coagulopathies, graft-versus-host disease, transmission of infectious diseases (for example, hepatitis or acquired immune deficiency syndrome), or death (*1*). Although these treatments have reduced the occurrence of bilirubin toxicity, the risks associated with exchange transfusion remain a serious problem. Chromatography has been tested as a technique to replace exchange transfusions. Numerous resins have been used to adsorb bilirubin (*3*); however, they removed not only bilirubin but also essential compounds such as thyroxine, cortisol, and aldosterone (*3*).

Here, we report the use of a highly specific enzyme to remove bilirubin from the bloodstream. With this method, a small reactor containing an immobilized bilirubin-detoxifying enzyme is placed in an extracorporeal circuit. A prototype device markedly reduced serum bilirubin concentrations both in human blood and in genetically jaundiced rats.

Bilirubin oxidase from *Myrothecium verrucaria*, which catalyzes the oxidation of bilirubin with O_2, was used in this study (*4*). Results from electronic absorption spectroscopy (*5*) and thin-layer chromatography (*4, 6*) have shown that the initial product of the enzymatic reaction appears to be biliverdin, which is much less toxic than bilirubin (*7*). Bilirubin oxidase further oxidizes biliverdin to as yet unidentified products. We tested biliverdin and its enzymatic oxidation products for teratogenic (*8*), cytotoxic, and mutagenic (*9*) effects; none of these was detected, even at concentrations 60 times greater than the maximum physiological concentration.

Kinetic parameters of bilirubin oxidase were determined with solutions of bilirubin in 0.05*M* phosphate buffer (*10*). Enzymatic oxidation of bilirubin may be described by the Michaelis-Menten model with a Michaelis constant, K_m, of 50 μM. Bilirubin forms a noncovalent complex with human serum albumin (HSA), which has at least two binding sites for bilirubin, a primary site with an association constant K_a of $5.5 \times 10^7 M^{-1}$ (in defatted HSA at *p*H 7.4 and 37°C) and a secondary site with a K_a of 4.4×10^6 M^{-1} (*11*). Measuring enzymatic oxida-