Development of a P1 phagemid system for the delivery of DNA into Gram-negative bacteria

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The inability to transform many clinically important Gram-negative bacteria has hampered genetic studies addressing the mechanism of bacterial pathogenesis. This report describes the development and construction of a delivery system utilizing the broad-host-range transducing bacteriophage P1. The phagemids used in this system contain a P1 pac initiation site to package the vector, a P1 lytic replicon to generate concatemeric DNA, a broad-host-range origin of replication and an antibiotic-resistance determinant to select bacterial clones containing the recircularized phagemid. Phagemid DNA was successfully introduced by infection and stably maintained in members of the families Enterobacteriaceae (Escherichia coli, Shigella flexneri, Shigella dysenteriae, Klebsiella pneumoniae and Citrobacter freundii) and Pseudomonadaceae (Pseudomonas aeruginosa). In addition to laboratory strains, these virions were used successfully to deliver phagemids to a number of strains isolated from patients. This ability to deliver genetic information to wild-type strains raises the potential for use in antimicrobial therapies and DNA vaccine development.

Keywords: delivery system, bacteriophage, gene transfer, transduction

INTRODUCTION

The ability to deliver genetic information to cells is an essential first step for many powerful investigative approaches. Only a limited number of bacteria (for example, Haemophilus influenzae, Streptococcus pneumoniae and Bacillus subtilis) can be transformed by natural competence (Lorenz & Wackernagel, 1994). A number of factors however, such as prolonged incubation with calcium chloride, treatment of bacteria with dimethyl sulfoxide, hexaminecobalt and dithiothreitol in the presence of cations, or addition of polyethylene glycol, can induce artificial competence (Hanahan et al., 1991). Genetic information for example can be delivered to Escherichia coli K-12 by transformation of chemically or electro-competent cells, phage transduction and conjugational mating (Benedik, 1989; Dower et al., 1988; Hanahan et al., 1991). However, many bacterial species of clinical, environmental and industrial importance cannot be made competent.

Recombinant DNA manipulations in bacteria typically involve initial cloning and molecular analyses in E. coli, followed by reintroduction of the cloned DNA into the original host genetic background for studies of gene expression and reverse genetics. Some species are recalcitrant to standard transformation techniques. Therefore, genetic analysis of these species is largely impaired. Most bacterial species possess restriction/modification systems that have evolved to protect the cell from foreign DNA (Bickle & Krüger, 1993). Modification of DNA can differ between species and among strains of the same species, creating barriers to gene transfer. To facilitate the movement of DNA some transformation protocols are limited to specific strains that are defective in one or more restriction systems (Novick, 1990; Takagi & Kisumi, 1985). Non-specific barriers such as high intra- or extracellular nuclease activity can also have profound effects on transformation efficiency (Omenn & Friedman, 1970; Shireen et al., 1990; Wu et al., 2001). Genetic exchange between mutated laboratory strains and clinical or environmental isolates can be hampered by the lack of alternative methods for the delivery of genes.

The ability to electroporate protoplasts, spheroplasts and intact cells has advanced microbiological studies in organisms where other transformation procedures have...
failed (Chassy et al., 1988). However, the generation of cells lacking cell walls can be labour-intensive, time-consuming and difficult to reproduce. In addition, these methods normally require optimization of numerous strain-dependent parameters for efficient transformation and regeneration. Transformation efficiencies of intact cells can be highly variable depending on the growth media, growth phase and final concentration of cells, composition of the electroporation medium, electric parameters and conditions used to select for transformants.

The bacteriophage P1 has been widely used in the construction of recombinant bacteria because of its ability to transduce chromosomal markers as well as episomes such as F and R plasmids (Arber, 1960; Lennox, 1955). Besides E. coli B, C and K, P1 can adsorb to and inject its DNA into 25 Gram-negative species (Yarmolinsky & Sternberg, 1988). Such a lifestyle has resulted in the evolution of an antirestriction function. P1 DNA is only weakly restricted when it infects a cell carrying type I restriction and modification systems even though DNA purified from P1 phage particles is a good substrate for type I restriction enzymes in vitro (Iida et al., 1987). This protection has been attributed to the presence of the defense against restriction proteins, DarA and DarB, within the phage head. The Dar proteins protect any DNA, including transduced DNA, from restriction (Iida et al., 1987).

In this communication, we describe the construction of a phagemid vector, P1pBHR-T, which can be used for cloning in E. coli or several Gram-negative hosts. We also describe the development of a P1 phage delivery system that will have great use for the movement of P1pBHR-T between a variety of clinically relevant Gram-negative species.

**METHODS**

**Bacterial strains, plasmids, phage and growth conditions.** The bacterial strains, plasmids and phage used in this study are listed in Table 1. Bacterial cells were grown in Luria–Bertani medium (LB), LC medium (LB containing 10 mM MgSO_4_ and 5 mM CaCl_2_ or LB agar. Selection for plasmids was accomplished by the addition of kanamycin (Kan, 50 µg ml⁻¹), ampicillin (Amp, 100 µg ml⁻¹) or carbenicillin (500 µg ml⁻¹) as needed. DNA manipulations were carried out by standard methods (Sambrook et al., 1989).

**Construction of P1 phagemid P1pBHR-T.** The rationale for the construction of P1pBHR-T is described in Results and the plasmid map is shown in Fig. 1. The elements necessary for P1 packaging, including the pac initiation site and lytic repilon, were inserted initially into the cloning vector pBluescript II SK. The P1 lytic repilon was generated by fusion of two PCR-amplified fragments, resulting in a 52% in-frame deletion of kilA. Primers 5'–ACCGTTCTTCGAGCACAGCAATGGAA-GGAGATTCTTTTCAGC-3' and 5'–GCACCGTTCTCAAGAGGTCAGACAGG-3' (restriction enzyme recognition sites in bold) were used to amplify a 1178 bp PCR fragment, corresponding to the C-terminus of kilA and the entire repI gene. The XhoI/HindIII-digested PCR product was cloned into the corresponding sites of pBluescript II SK, generating pCW1. The DNA fragment containing the P53 promoter, P53 antisense promoter and N-terminus of kilA was PCR amplified with primers 5'–GTCACACTTGGATGCTGACGAATGCT- TGG-3' and 5'–GTGGGACTCGAGGAACGAAAATAGCTTTTCTGCC-3'. The 403 bp XhoI-digested PCR product was cloned into the unique XhoI site of pCW1 to yield plasmid pCW2. The insert was cloned in the same orientation as the repI gene, thereby placing the lytic repilon under the transcriptional control of the C1 repressor-regulated promoter P53. The P1 packaging site (pac) was PCR-amplified with primers 5'–GACAGGCTCTCTAGAATAAGCCGATGCA-GGAACC-3' and 5'–CGTACCGGATCCAAAGGTTCTACGTTACGCC-3'. The 305 bp XbaI/BamHI-digested PCR product was cloned into the corresponding sites of pCW2. The P1 elements were then PCR-amplified as a cassette (1904 bp) using primers 5'–GTGACACCATGGCTGAGGCTTTTCTAGAGCATCG-3' and 5'–CGACCACCTTGCTCTCTAGAATAAGCCGATGCA-GGAACC-3'. The Ncol-digested PCR product was inserted into the unique Ncol site of the broad-host-range vector pBHR2 (MobITec) creating plasmid pCW3. The unique BamHI and SalI sites of pCW3 were treated with DNA polymerase I Klenow fragment to create blunt ends and transcriptional terminators, T1_{143} (Wright et al., 1992), were then cloned into these sites to generate P1pBHR-T. To facilitate detection of plasmid transduction in P. aeruginosa, the ampicillin-resistance gene including its putative promoter was amplified by PCR from pBluescript II SK using primers 5'–GGTCTACGAAATATGAGCGTACAGG-3' and 5'–AACTTGGTCTAGACAGG-3' and cloned into the DraI site of P1pBHR-T, thereby creating plasmid P1pBHR-bla.

**Phage-induced bacteriophage P1cM conts100 lyosen carrying plasmid P1pBHR-T.** The P1 lysogen, C600(P1), was transformed with plasmid P1pBHR-T, and Kan⁺ transformants were selected and grown at 32 °C in LB medium. Overnight cultures were diluted 100-fold and grown at 32 °C in LB medium until the OD₆₀₀ reached 1.0, at which time the culture was shifted to 42 °C and aerated in a gyrating water bath until lysis occurred (1 h). Chloroform (1%, v/v), DNase (10 µg ml⁻¹) and RNase (1 µg ml⁻¹) were added and incubation was continued for an additional 30 min at 37 °C. The phage stock was clarified by centrifugation at 2500 g for 15 min and passed through a 0.2 µm filter.

**Phage delivery and analysis.** An overnight culture of the host strain was diluted in LB and grown to mid-exponential phase (OD₆₀₀ 0.4). The cells were centrifuged at 2500 g for 10 min at 4 °C and concentrated to OD₆₀₀ 2.0 (10⁷ c.f.u. ml⁻¹) with LC medium. Phage (100 µl) was added at various multiplicity of infection (m.o.i.) and allowed to adsorb to the cells (100 µl) for 15 min at 32 °C. LC medium containing 10 mM sodium citrate was added (800 µl), and cells were incubated at 32 °C for 45 min or 90 min to allow expression of antibiotic-resistance genes (kanamycin and carbenicillin, respectively). The infected cells were centrifuged at 7000 g for 5 min and resuspended in 100 µl LC medium containing 10 mM sodium citrate. Transductants were detected by spotting 7.5 µl samples of a 10-fold serial dilution of the mixture onto LB agar plates containing appropriate antibiotic selection. Plates were scored following overnight incubation at 32 °C. No transductants were observed when 10² viable bacteria were assayed on selective media in the absence of phage lysis. P1pBHR-T was recovered from transduced cells by the alkaline lysis method (QIAprep miniprep kit, QIagen).
### Table 1. Characteristics and origins of bacteria, plasmids and phage used in this study

<table>
<thead>
<tr>
<th>Bacterium, plasmid or phage</th>
<th>Description or genotype*</th>
<th>Source or reference†</th>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C600</td>
<td><em>thi-1 thr-1 leuB6 lacY1 tonA21 supE44</em></td>
<td>Promega</td>
</tr>
<tr>
<td>C600(P1)</td>
<td>Cell carries a P1Cm cts.100 prophage</td>
<td>Rosner (1972)</td>
</tr>
<tr>
<td>JM101</td>
<td>supE thi Δ[lac–proAB] [F' traD36 proAB lacP'ZΔM15]</td>
<td>NEB</td>
</tr>
<tr>
<td>JM101(P1)</td>
<td>Cell carries a P1Cm cts.100 prophage</td>
<td>This study</td>
</tr>
<tr>
<td>DH5α</td>
<td>$\phi$80d lacZΔM15 recA1 endA1 gyrA96 thi-1 bsdR17 supE44 relA1 deoR Δ[lacZYA–argF]U169</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>JM109</td>
<td>endA1 recA1 gyrA96 thi bsdR17 relA1 supE44 Δ[lac–proAB] [F' traD36 proAB lacP'ZΔM15]</td>
<td>NEB</td>
</tr>
<tr>
<td>EC-1</td>
<td>Urine clinical isolate, AmpR²</td>
<td>MUSC</td>
</tr>
<tr>
<td>EC-2</td>
<td>Urine clinical isolate, AmpR²</td>
<td>MUSC</td>
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<tr>
<td><em>P. aeruginosa</em></td>
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<tr>
<td>PAO1</td>
<td>Clinical isolate</td>
<td>PGSC</td>
</tr>
<tr>
<td>PA-1</td>
<td>Clinical isolate</td>
<td>MUSC</td>
</tr>
<tr>
<td>S. flexneri</td>
<td>Serotype 2b</td>
<td>ATCC 12022</td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>60R</td>
<td>J. Butterton‡</td>
</tr>
<tr>
<td><em>C. freundii</em></td>
<td>Produces restriction endonuclease CfrA1</td>
<td>ATCC 8090</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>Wild-type</td>
<td>ATCC 10031</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pBluescript II SK</td>
<td>Cloning vector, ColE1 origin</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBBR122</td>
<td>Cloning vector, broad-host-range origin</td>
<td>MoBiTec</td>
</tr>
<tr>
<td><strong>Phage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1Cm cts.100</td>
<td>thermoinducible P1Cm</td>
<td>Rosner (1972)</td>
</tr>
</tbody>
</table>

*Cm, chloramphenicol marker.

† NEB, New England Biolabs; MUSC, Medical University of South Carolina, Department of Pathology and Laboratory Medicine; PGSC, Pseudomonas Genetic Stock Center, East Carolina University; ATCC, American Type Culture Collection.

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**RESULTS**

**Construction of the P1 phagemid**

To construct a single vector capable of delivering DNA to a wide range of bacterial species, we constructed a phagemid containing all the essential signals for P1 packaging, a selectable marker for transfer detection, and a broad-host-range origin of replication (P1pBHR-T, Fig. 1). The parent plasmid, pBBR122, has been shown to be capable of replicating at medium copy number in at least 26 Gram-negative species and was stably maintained by selective pressure in all Gram-negative organisms tested so far (Antoine & Locht, 1992; Elzer et al., 1995; Gliesche, 1997; Su et al., 2001). Incompatibility tests have shown that pBBR122 does not belong to the IncP, IncQ or IncW groups of broad-host-range plasmids (Antoine & Locht, 1992). The phagemid P1pBHR-T was also found to be compatible with plasmids containing the ColE1 or p15A origins of replication (data not shown). This is particularly relevant for transfer of the phagemid to clinical and environmental isolates since the majority of such strains may harbour native plasmids.
The elements necessary for packaging into P1 phage capsids were inserted into pBRR122. These elements included the P1 lytic replicon and minimal pac site. The lytic replicon consists of the CI repressor-controlled P53 promoter, the promoter P53 antisense and the kilA and repL genes. The KilA protein is not essential for replicon function but is lethal to the bacterial cell. The kilA gene was therefore inactivated by an in-frame deletion, resulting in a protein 52% of the original size. During the late stages of the phage life cycle the lytic replicon initiates a rolling-circle mode of replication that generates concatemeric DNA, which is the substrate for packaging. Packaging is initiated when phage-encoded proteins recognize and cleave the unique pac site. The DNA is then brought into the empty P1 phage head, and packaging proceeds unidirectionally until the head is full. Since the P1 phage head can package approximately 110–115 kb of DNA (Sternberg, 1990), fragments as large as 100 kb could potentially be cloned and delivered by this system. The ability to clone high-molecular-mass DNA fragments is important in the analysis of large genes or gene clusters that encode components of biochemical or signalling pathways.

**Production of phagemid-containing virions**

The phagemid was maintained in a P1 lysogen which provided all the replication factors needed to activate the lytic cycle and all the structural components to form mature viral particles. The P1 prophage carried a chloramphenicol-resistance marker and the c1.100 temperature-sensitive repressor mutation. This mutation enabled the P1 lytic cycle to be rapidly induced when the temperature of the exponential-phase lysogenic culture was shifted from 32 °C to 42 °C. Lysates typically contained approximately 80% P1 and 20% phagemid particles as determined by infecting *E. coli* C600 and C600(P1), respectively (data not shown).

**Delivery to multiple strains of *E. coli***

The ability to deliver the phagemid to multiple strains of bacteria was tested first with laboratory strains and clinical isolates of *E. coli*. Since the host recombination system may affect concatemer resolution and plasmid rearrangement, RecA+ (C600 and JM101) and RecA- (DH5x and JM109) strains were included. Increasing titres of phage were added to fixed numbers of bacterial cells and limited to a single round of infection by the addition of 10 mM sodium citrate. After infection, phagemid-containing transductants were selected by virtue of their ability to grow in the presence of antibiotics. As shown in Fig. 2(a), the total number of transductants increased progressively as the m.o.i. increased. Antibiotic-resistant colonies were not recovered if the phage lysate or cells were tested alone. Since the P1 prophage carried the c1.100 mutation, cells infected with this phage were rendered temperature sensitive. Therefore, to minimize induction of the P1 lytic cycle and maximize the number of transductants recovered all procedures were performed at 32 °C.

Following injection into the bacterial host, P1 DNA must be recircularized before it is rapidly degraded by cellular nucleases. The Cre–loxP site-specific recombination system plays an important role in the cyclization of P1 DNA (Hochman *et al*., 1983; Segev & Cohen, 1981; Sternberg *et al*., 1986). Only two components are...
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**Fig. 3.** Transduction of P1pBHR-bla to *P. aeruginosa*. (a) The ability of bacteriophage P1 to infect and transduce laboratory and clinical isolates of *P. aeruginosa* was determined by infecting cells at a m.o.i. of $10^{-1}$, $10^{-2}$ or $10^{-3}$. A 10-fold serial dilution of the infected culture was applied onto media containing carbenicillin at 500 µg ml$^{-1}$, starting with undiluted (from top to bottom). Successful delivery and replication of the phagemid can be visualized by acquisition of the antibiotic marker *bla*. (b) Restriction digest analysis of *P. aeruginosa* transductants. Plasmid DNA isolated from the parent strain and two representative carbenicillin-resistant colonies from each infection was digested with BamHI and analysed by agarose gel electrophoresis. Lane M, 1 kb DNA ladder; lanes 1 and 2, PAO1; lane 3, control DNA from parent strain; lanes 4 and 5, PA-1. Predicted DNA fragments generated following BamHI digestion are 7920 and 42 bp. Positions of molecular size markers are indicated on the left.

**Fig. 4.** Transduction of P1pBHR-T to *K. pneumoniae*, *C. freundii*, *S. flexneri* and *S. dysenteriae*. (a) Bacterial species were infected by P1 at a m.o.i. of $10^{-2}$, $10^{-3}$ and $10^{-4}$ and 10-fold serial dilutions of phage-infected cultures were applied onto media containing kanamycin at 50 µg ml$^{-1}$, starting with undiluted (from top to bottom). Presumptive transductants harbouring the phagemid P1pBHR-T were selected by virtue of their resistance to kanamycin. (b) Restriction digest analysis of *K. pneumoniae* and *C. freundii* transductants. Plasmid DNA isolated from the parent strain and two representative Kan$^R$ colonies from each infection were digested with HindIII and analysed by agarose gel electrophoresis. Lane M, 1 kb DNA ladder; lane 1, control DNA from parent strain; lanes 2 and 3, DNA isolated from Kan$^R$ transductants. Predicted DNA fragments generated following HindIII digestion are 3332 and 3951 bp. Positions of molecular size standards are indicated on the right. (c) Restriction digest analysis of *S. flexneri* and *S. dysenteriae* transductants. Control DNA or plasmid DNA isolated from Kan$^R$ colonies was digested with HindIII and analysed by agarose gel electrophoresis. Lane M, 1 kb DNA ladder; lane 1, control DNA isolated from parent strain; lanes 2 and 3, DNA isolated from Kan$^R$ transductants. Predicted DNA fragments generated following HindIII digestion are 3332 and 3951 bp. Positions of molecular size markers are indicated on the right.

required for this system: a *loxP* site on the P1 DNA and a P1-encoded recombinase protein, Cre (Sternberg & Hamilton, 1981). The phagemid must, however, utilize an alternative mechanism for cyclizing its DNA since it does not contain a *cre–loxP* region. It is assumed that the transduced plasmid multimers are resolved in the recipient to single copies of the plasmid or possibly circular multimers. Consequently, the number of recovered transductants is a function of not only the m.o.i. but also the efficiency of recircularization.
Successful delivery of P1pBHR-T was confirmed by extraction of this plasmid from representative isolates. Antibiotic-resistant transductants harboured plasmid DNA whose migration was identical to that originally seen in the parent strain (Fig. 2b). Restriction enzyme digestion demonstrated that gross deletions or genetic rearrangements in P1pBHR-T did not occur as a consequence of packaging or recircularization. Acquisition of P1pBHR-T did not result in displacement (incompatibility) of native plasmids in clinical isolates (data not shown).

The delivery vehicle can transduce many Gram-negative species

To demonstrate the utility of the delivery system, transduction of the phagemid was tested in various Gram-negative bacteria including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Shigella flexneri* and *Shigella dysenteriae*. All the bacteria tested were successfully transduced by the P1 delivery system (Figs 3a and 4a). The *P. aeruginosa* clinical isolate PA-1 was transduced at a lower efficiency than the laboratory strain PAO1 (Fig. 3a). It is noteworthy that a similar effect has been reported for electroporation of *P. aeruginosa* isolates from lung sputum of cystic fibrosis patients and wild-type strains isolated from different sources for other Gram-negative species (Diver et al., 1990; Wirth et al., 1989). Functionality of the pBBR122 origin of replication among the Gram-negative species was confirmed by extraction and analysis of P1pBHR-T from representative transductants (Figs 3b, 4b and 4c).

The majority of bacteria carry plasmids or lysogenized phage that protect their host by expressing potent toxins (for reviews, see Dinsmore & Klaenhammer, 1995; Snyder, 1995). This is particularly relevant for transduction of environmental *P. aeruginosa* strains since 40% of isolates recovered from natural ecosystems (lake water, sediment, soil and sewage) contain DNA sequences homologous to phage genomes (Ogunseitan et al., 1992). The P1 delivery system, however, does not appear to be under the constraints of superinfection exclusion since P1pBHR-T can be successfully delivered to a P1 lysogen (data not shown). The phagemid was also introduced by infection into *S. flexneri* and *S. dysenteriae* strains harbouring a natural resident plasmid (Fig. 4c).

DISCUSSION

In this study, phagemid DNA was readily introduced into a variety of Gram-negative bacteria, including *E. coli*, via a P1 phage delivery system. Phagemid P1pBHR-T is a relatively small plasmid (7-3 kb) containing one or two antibiotic-resistance determinants (KanR and/or AmpR). Both are readily selectable markers for Gram-negative bacteria. The ability to screen presumptive transductants for antibiotic resistance was a reliable and simple means of phenotypically confirming transduction of the phagemid to *E. coli* and other Gram-negative bacteria. The functionality of the pBBR122 origin of replication among Gram-negative bacteria was also extended further in this study.

Since the various Gram-negative bacteria accepted DNA packaged from another bacterial genus (*E. coli*), this suggested protection of the DNA by the P1 Dar proteins. Both the DarA and DarB proteins were provided by the P1 prophage and are thought to be incorporated into the phagemid-carrying P1 capsids during lysate production. These internal head proteins have been shown to bind phage DNA and are injected along with the infecting DNA into the recipient cell. Moreover, DNA binding is not specific for the P1 phage genome because any DNA packaged into a P1 phage head has been shown to be protected against restriction (Iida et al., 1987). Alternatively, the species tested may simply not possess an effective restriction/modification system or the transduced phagemid DNA may lack a restriction endonuclease recognition sequence recognized by these systems. The results obtained suggest that the delivery system may be applicable to the transduction of many different Gram-negative bacteria. There are additional bacterial species to which it should be possible to extend this P1 delivery system. The host range of P1 includes, for example, *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Salmonella typhimurium* (Lawton & Molnar, 1972; Murooka & Harada, 1979; Okada & Watanabe, 1968).

This procedure could be used for many genetic approaches, including the construction of strains, heterologous expression of genes and proteins, and analysis of endogenous gene expression. One important advantage of a phage delivery system is that, in contrast to transformation, phage infection normally occurs at high frequency in hosts competent for that phage. Low transformation efficiency of many bacteria has prevented the introduction of a gene library into these bacteria for direct complementation. In addition to using this procedure for the generation of recombinant bacteria, it should also be possible to construct genomic libraries in the phagemid vector. After obtaining transformants in *E. coli* the library could be pooled and infected en masse with P1 phage, generating an entire packaged library. This could be used to transduce any P1-sensitive host *in vitro* and *in vivo*. Bacterial transduction has been reported in environmental settings such as marine and freshwater aquatic habitats and in soil (Miller, 1998; Zeph et al., 1988). In this regard, the P1 delivery system could be helpful in addressing questions concerning the fate of genetically engineered vectors released into these environments and the transfer by transduction of DNA to indigenous organisms.

One of the challenges of current molecular therapy is how to deliver the therapeutic agent to the offending bacterium. Because of their importance as pathogens, effort was directed towards the development of delivery vectors and improvements of methods to introduce recombinant DNA molecules into Gram-negative bac-
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C. Westwater and others


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