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Bacteriophage-Associated Gene Transfer in Pneumococcus: Transduction or Pseudotransduction?

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Lysates of pneumococcal phage PG24 transferred genes from one host to another in a process with many of the properties of generalized transduction, in that the host genes were packaged in DNase-resistant particles that closely resembled infectious phage in physical properties, adsorbed to the recipient cells like phage, and were inhibited by antisera to the phage and by trypsin. However, phage processes did not complete the transfer of host DNA as they did phage DNA. Instead, gene transfer required development of competence and entry of the host DNA by the endonuclease-dependent pathway used for transforming and transfecting DNA. This process often occurred on the assay plate hours after adsorption of the particles to the cells, and the transfer was DNase sensitive if challenged at this time. Phenotypic expression was therefore also delayed. The product of entry was like that in transformation, a single strand of DNA that integrates by formation of a *hex*-sensitive donor-recipient heteroduplex. Whether this gene transfer process is unique to this system or is only the first one described is not clear. The term "pseudotransduction" may be useful in calling attention to its unexpected features. The DNA of PG24 phage has anomalous physical properties reflecting unusual bases.

We report a phage-associated gene transfer system for pneumococcus (*Streptococcus pneumoniae*) that has many of the properties of generalized transduction. However, its mechanism differs from that expected on the assumption that the phage facilitates entry of cell DNA as it does phage DNA. As will be shown, the cell DNA to be transferred is packaged in phage structures and adsorbs to recipient cells in this form, but does not enter them by the phage DNA entry process. Instead, it remains attached to the cells in a DNase-resistant state until they become competent and take up the donor DNA by the DNase-sensitive transformation pathway, leading to intracellular single strands that recombine by formation of heteroduplexes that are subject to action of the *hex*$^+$-dependent mismatch correction system (9, 10).

Whether or not this process should be called transduction depends on whether or not one requires the phage vector, the defining feature of transduction (16), to complete the transfer process by its own mechanisms, and the question has not heretofore arisen. However, the only direct evidence on intermediates in any generalized transduction system is for P22 in *Salmonella typhimurium*, for which it is reported that at least some of the recombination products represent integration of 4- to 6-megadalton (Mdal) duplex donor DNA segments (6).

With the system described here, the repertory of genetic assays in pneumococcus has been expanded from transformation alone to include phage recombination (17), transfection (18), and a form of generalized transduction. Parts of this work have been presented at Transformation Meetings (1976, 1978) and at the 3rd European Meeting on Transformation and Transfection, Granada, 1976. Further details may be found in R. D. Porter (Ph.D. thesis, Duke University, Durham, N.C., 1976 [University Microfilms, Ann Arbor, Mich.]).

MATERIALS AND METHODS

Bacteria and markers. Strains are listed with their relevant phenotypes in Table 1. With respect to markers, all except *thy-19* behave as point mutations of nearly equal efficiency for transformation of *hex* recipients. In *hex*$^+$ recipients *nov*-r1 is low efficiency and *ery*-r2 is intermediate efficiency; *str*-r1 is high efficiency in DP1600 and low efficiency in DP2000, whereas *str*-r41 has the converse properties low efficiency in DP1600 and high efficiency in DP2000 (9). (This is a good illustration of the specificity of *hex* action for the donor-recipient pairing rather than for the marker itself.) The *thy-19* marker is a deletion

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estimated to be larger than 15 kilobases (kb) extending into the thymidylate synthetase gene (N. B. Shoemaker, unpublished data). The protocol for scoring *thy* transformants will be described elsewhere (J. E. Wagstaff, N. B. Shoemaker, and W. R. Guild, manuscript in preparation). Scoring for drug resistance markers has been described (10).

The isolation and characterization of strains DP1219 and DP1771-1776, which are deficient in endonuclease, transformation, and transfection, have been described (18).

Transformation. Media and methods for preparation, storage at −85°C, thawing, and transformation of competent cells have been described (10). A relevant fact is that our competent cells are typically at a density near 2×10^7 colony-forming units (CFU) (4×10^7 cells) per ml when thawed. In some experiments these cells were concentrated after thawing by centrifugation at 0°C for 5 min at $6,000 \times g$ and suspension in 1/5 volume of their supernatant.

Phage. The growth, purification, plaque assay, and properties of ω3 and ω8 have been described, as have the media and a storage buffer (TMF) in which they are stable (17). Relevant properties are that they have latent periods of 55 to 60 min at low cell density, lengthening to 80 to 100 min at high cell density, and morphologies very similar to lambda or T1, except for a longer tail and tail fiber, and that they contain linear duplex DNA originally estimated from sedimentation velocity and electron micrographs to be 33 Mdal (17), using 6.0 Mdal as the reference size for PM2 phage DNA. However, PM2 is now reported to be 6.58 ± 0.18 Mdal (21), in which case it is probable that ω3 and PG24 have DNAs nearer 36 Mdal (L. A. Goscin, personal communication). Phage DNA was prepared from mature phage and from infected cells as described (17, 18).

Isolation of new phage strains. The method of Tiraby et al. (23) for isolation of new phage strains was used with modifications. Throat swabs from apparently healthy medical students were immersed in 2 ml of TMF, from which drops were spotted on a plate seeded with about 10^7 streptomycin-resistant pneumococcus cells, in the presence of 100 μg of streptomycin per ml. Of 81 samples, 29 showed a clear zone after overnight incubation, and from these, phage were purified through serial single-plaque isolation. Stocks of each isolate were prepared. Not all isolates grew well in liquid, and only a few have so far been studied in any detail. The one of immediate interest has been designated PG24.

Growth and purification of transducing lysates. Phage were grown on drug-resistant hosts by the successive dilution of small lysates into larger volumes of fresh cells as described (17). For PG24 the substitution of potassium for sodium did not have the large influence on yield that it did for ω3 and ω8 (17). The final lysate received 1 mg of pancreatic DNase I (Worthington) per liter and, after 10 to 20 min at 37°C, was clarified by low-speed centrifugation and concentrated by the polyethylene glycol (PEG) method as described (17).

Density gradient centrifugation. All runs were in polyallomer tubes in an SW-39 rotor in a Beckman L1 centrifuge. For equilibrium CsCl runs, 3.00 g of dry CsCl was added to 4.00 g of TMF containing PEG-purified phage (42.9% CsCl, density 1.46 g/ml). The input sample for sedimentation velocity runs was concentrated from the peak guanine-thymine (GT)-containing fraction from a CsCl gradient by centrifugation and resuspension in 0.25 ml of TMF, to which was added 10 μl of a reference stock of T7 phage, before layering the entire volume on a 5 to 20% gradient of sucrose dissolved in a solution of 0.1 M NaCl, 0.1 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.5), 2 mM $MgSO_4$, and 0.7 mM ethylenediaminetetraacetic acid. (Recovery of activity was poorer in sucrose made up in TMF. It was also poor when stocks not purified in CsCl were used. There was a suggestion that residual PEG may have been responsible.) Fractions were drop-collected from the bottom, diluted, and assayed. The temperature of a blank tube was measured after each run.

TABLE 1. *Strains of Pneumococcus*

Strain	Relevant properties	Origin (reference)
Rx1	*hex*	Ravin (see 20)
Derivatives of Rx1		
DP1147	*hex thy-7*	J. E. Wagstaff, this laboratory
DP1162	*hex thy-19*	Shoemaker
DP1219	*hex noz-19 (end)*	(18)
DP1600	*hex*$^+$	(20)
DP1602	*hex*$^+$ *str*-r1 *nov*-r1, *ery*-r2	(20)
DP1617	*thy*$^+$ *ery*-r2 (among others)	Shoemaker
DP1771 to DP1776	*hex end* transformation deficient	(18)
DP1800	*hex* (isogenic to DP1600)	Shoemaker
R6 strains		
DP2000	R6 *hex*$^+$	H. Bernheimer (see 10)
DP2300	R6 *str*-r41 *nov*-r1 *ery*-r2 *brya*^{r} *opt*^{r}	Formerly called 8M (see 20)

UV irradiation, electron microscopy, and antiserum preparation. UV irradiation, electron microscopy, and antiserum preparation were as described (17, 18). For early experiments anti-ω3 serum was used, because it shows high cross-reactivity to all our phages. An anti-PG24 serum prepared later gave equivalent results.

Assay for phage-associated gene transfer. Significant aspects of the assay procedures for phage-associated gene transfer are described in Results. In most cases a phage stock diluted to 2×10^8 to 3×10^8 plaque-forming units (PFU)/ml was mixed with an equal volume of 100 μg of DNase per ml in 0.1 M $MgSO_4$ and added to 9 volumes of growing cells at a density near 3×10^8/ml (estimated by turbidity). After adsorption (usually 40 min at 37°C), 0.1-ml samples were added to 4 ml of soft agar (17) containing 0.1 to 0.2 ml of antiserum and plated on 100-mm petri dishes containing 20 to 22 ml of Na-CAT agar buffered with Tris (17), which appeared to be necessary for the procedure to work. After time at 37°C for phenotypic

expression (see below), selective drugs were added in an 8-ml agar overlay. The plates were then incubated at 37°C for 20 to 24 h before counting. A parallel sample of the adsorption mixture was diluted and plated for PFU and/or CFU determinations in the absence of antiserum.

RESULTS

To look for presumptive transduction, phage grown on a host resistant to several drugs were concentrated and used to infect about 3×10^8 Rx1 cells per ml at multiplicities of infection of 0.1 to 0.2 in the presence of 10 μg of pancreatic DNase to prevent transformation by free DNA. After 40 min for adsorption of phage, several 0.1-ml samples were plated in agar in the presence of antiserum to suppress phage growth, and a sample was diluted and plated to assay for PFU in the absence of antiserum. Selective drugs were added after 2 h in initial experiments, on the basis of experience in transformation where phenotypic expression is usually complete in 70 to 80 min, and colonies were counted after 20 to 24 h of additional incubation at 37°C. Under these conditions there were about 10^9 cells per plate at the time of drug addition, and control plates without phage showed 0 to 5 spontaneous mutants for each drug. ω3 and ω8 gave numbers not clearly above the controlled level.

Of 29 new phage isolates obtained from throat swabs of medical students, 8 eventually grew well enough to yield stocks of $\geq 5 \times 10^{10}$ PFU/ml. These were first tested for presence of bacterial genes by using a detergent lysate of the phage as donor in transformation. By this test ω3, ω8, and several of the new isolates had a maximum of 10^{-4} parts of cell DNA per phage equivalent, and under our conditions this level would yield transductants at or below the spontaneous mutation frequency. Three isolates, however, gave transformation suggesting that 0.5 to 2% of the particles might carry bacterial DNA and, on testing for transduction as above, one gave a positive result. This isolate was named PG24 and has been examined further. We first briefly summarize a series of observations in the approximate order in which they were made, deferring in some cases the documentation and detailed discussion to present more critical tests developed on the basis of later findings.

The following results implied a typical generalized transduction system: (i) every lysate of PG24 transferred any of several unlinked cell markers from one host to the other at levels far above controls; (ii) under the assay conditions described above, neither DNase nor competing free DNA (Table 2) affected the yield of gene transfer (GT); (iii) the GT activity copurified with infectious phage through PEG and CsCl step gradients (see above); and (iv) the GT activity was inhibited by phage antisera (Fig. 1) and by trypsin (Table 3), though not to the same extent as were PFU. An unusual feature was that the ratio of GT to PFU varied widely among preparations, from about 10^{-3} to 10^{-6} Str^r/PFU. However, PG24 is unstable with respect to PFU titer and more difficult than ω3 to grow to high titers. Analysis of the results from many preparations suggests that the yield of GT per milliliter of original lysate is more constant than is

TABLE 2. *Lack of competition by free DNA for gene transfer*[a]

μg of DNA/ml of cells	Str^r/ml
50	623
5	630
0.5	750
None	656

[a] DNA was mixed with PG24 phage and Rx1 cells were added. DNase was added after the 40-min adsorption period in this experiment.

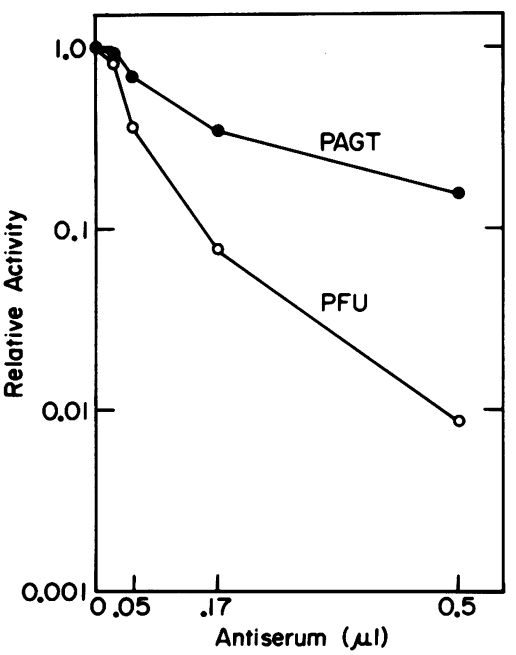

FIG. 1. *Sensitivity of phage-associated gene transfer and PFU to rabbit anti-PG24 serum. The indicated volume of antiserum in 50 μl of growth medium was added to 50 μl of a dilution of PG24/1602 in TMF. Cells (0.5 ml) and DNase (0.05 ml) were added immediately. After 40 min at 37°C, the mixture was plated by the standard methods, including further antibody in the GT assay plates. Reconstruction experiments showed that the residual antiserum in the PFU plates did not inhibit plaque formation.*

the yield of PFU and that GT is more stable than PFU, so that the variability seems to reflect more the denominator than the numerator.

Other tests showed that adsorption of GT particles continued for 40 to 60 min, comparable to the slow adsorption of PFU at these cell densities (17), and was essentially complete in that no further GT activity could be detected by addition of fresh cells to a supernatant taken after 60 min (data not shown). The half time for adsorption was about 15 min in the usual assay system. Therefore it was significant to find that phenotypic expression of newly transferred drug resistances took 1 to 2 h longer than that for transformants (Table 4), and the standard assay was revised to allow 3 h for expression.

***hex* dependence.** Table 5 shows results that were not expected on the basis of assuming injection of duplex cell DNA by the phage, in that marker ratios changed with the status of the *hex*-dependent mismatch correction system of the host. The ratio of *nov*-r1 to *str*-r1 fell 8- to 14-fold from *hex* to *hex*$^+$ cells as in transformation (9), and in *hex*$^+$ cells the low-efficiency *nov*^{r} marker was hypersensitive to UV irradiation of the phage stock, just as it is in transformation (9, 12). Because discrimination by the *hex* system occurs after formation of donor-recipient heteroduplex intermediates in transformation (20) and depends on specific kinds of donor-recipient mismatches (7, 11), these results strongly imply that essentially all donor GT markers are integrated by formation of heteroduplex, rather than by duplex-duplex recombination as reported for P22 in *Salmonella* (6). The lack of GT to the R6 strain DP2000 will be commented on later, as will the results in experiment 2C.

In experiment 2B the survival of PFU was less than 10^{-7}, or e^{-16}, implying a D_{37} (dose at which there is 37% survival) ≤ 37 J/m^2, consistent with the UV sensitivities of ω3 and ω8 (17). GT per input multiplicity of infection was down only 10- to 20-fold, a result consistent with other transduction systems. However, in other experiments at lower UV doses there was no stimulation of GT (data not shown), a result like transformation (9) and unlike transduction in most (2, 4) but not all (5) systems.

TABLE 3. *Effect of trypsin on PFU and gene transfer*[a]

Trypsin exposure (min)	Str^r (%)	PFU/ml (%)
0	515 (100)	1.3×10^7 (100)
1	342 (66)	5.2×10^6 (40)
10	125 (24)	8.2×10^5 (6.3)
20	93 (18)	5.4×10^5 (4.2)

[a] A phage solution was made 100 μg/ml of trypsin and incubated at 37°C; at the indicated times, samples were pipetted into an excess of soybean trypsin inhibitor and incubation was continued at 37°C. When all samples had been placed in the inhibitor, DNase and cells were added, adsorption was allowed for 40 min, and platings were made as usual.

TABLE 4. *Slow phenotypic expression of transferred genes*

Time[a] (h)	Expt 1			Expt 2		
	No. of transductants/plate			No. of transductants/plate		No. of transformants/plate[b]
	Str^r	Ery^r	Nov^r	Str^r	Ery^r	Str^r
1.5	5	3	1	0	—[c]	172
2.0	6	7	90	4	—	191
2.5	37	100	134	39	—	184
3.0	135	91	208	93	101	203
3.5	135	192	143	97	—	173
4.0	132	169	284	69	—	227

[a] Rx1 cells were exposed to PG24/1602 for 40 min before 0.1-ml samples were plated and incubated for the indicated additional times at 37°C before addition of drug (see text). DNase was added before phage addition. Numbers shown are colonies counted after 24 h of further incubation. Blank controls were also challenged at 3 h. They gave two Str^r colonies in experiment 1 and seven in experiment 2. No Ery or Nov colonies appeared.

[b] In experiment 2, a parallel sample of cells was exposed to a marked donor DNA and DNase addition was at 40 min. Though well past their optimum competence, the cells still transformed enough to give the results shown.

[c] —, Not determined.

Endonuclease dependence. Cells deficient in transformation and transfection because of a mutation in the membrane-bound endonuclease essential for entry of free DNA (13, 18) were also deficient in accepting GT to about the same extent as they were deficient in transformation (Table 6), although phage infection and recombination were normal in these strains (17).

Because the *hex* and *end* results suggested single-strand entry of the DNA, the assumption that the gene transfer activity was carried in phage particles was reexamined.

Sensitivity to antiserum and to enzymes. The results in Fig. 1 show that although GT is inhibited by antiserum, it is much less affected than is infectivity; Table 3 shows comparable results for the initial rate of inactivation by trypsin. Because it was possible that during lysis of infected cells some DNA might be trapped in membrane vesicles that would at least partially copurify with phage particles, we examined the

TABLE 5. *Effect of hex and of UV irradiation on gene transfer*

Expt	Condition	Recipient	Colonies[a]			Nov/Str	PFU/ml
			Str^r	Nov^r	Ery^r		
1	No UV	Rx1 *hex*	392	611	595	1.56	—[b]
	MOI, 0.1	DP1600 *hex*$^+$	272	29	335	0.11	—[b]
2A	No UV	Rx1 *hex*	355	682	399	1.92	2.9×10^7
	MOI, 0.1	DP1600 *hex*$^+$	209	53	264	0.25	3.0×10^7
		DP2000 *hex*$^+$	0	0	0	—	1.9×10^7
2B	UV[c]	Rx1 *hex*	173	255	168	1.48	<20
	MOI, 1	DP1600 *hex*$^+$	191	2[d]	196	0.01	<20
2C[e]	No UV	Rx1 *hex*	309	232	254	—	1.6×10^7
	MOI, 0.1	DP1600 *hex*$^+$	2	4	3	—	1.8×10^7

[a] In experiment 1, one 0.1-ml sample was scored per drug. In experiment 2, the data are the totals from 3 plates with 0.1 ml each.

[b] —, Not determined.

[c] The PG24/1602 stock was irradiated with 600 J/m^2 and exposed to cells at 10 times the concentration used for the nonirradiated control experiment.

[d] There were zero Nov^r colonies on three plates of blank controls.

[e] The donor in experiment 2C was PG24/2300, in contrast to PG24/1602 in the other experiments in this Table (see text).

TABLE 6. *Endonuclease and gene transfer*

Recipient[a]	Gene transfer,[b] Str^r/0.1 ml (%)	Transformability[c] (%)
Rx1	463 (100)	100
1771	13 (3)[d]	3–9
1772	96 (21)	30
1773	66 (14)	10
1774	5 (1)[d]	18
1775	101 (22)	10–30
1776	50 (11)	10–30
Blank (in Rx1)	10	
1219[e]	0–3 (0)	0.5

[a] 1771–1776 and 1219 are endonuclease-deficient strains. See Table 1 and reference 18.

[b] Numbers in parentheses are percentages.

[c] Highest levels ever observed for each mutant strain, at peak of competence cycle, relative to typical Rx1 level.

[d] Not significantly above blanks. Retests showed the same results.

[e] In several experiments done separately from the above, no GT above the level of blank controls has ever been detected in strain 1219.

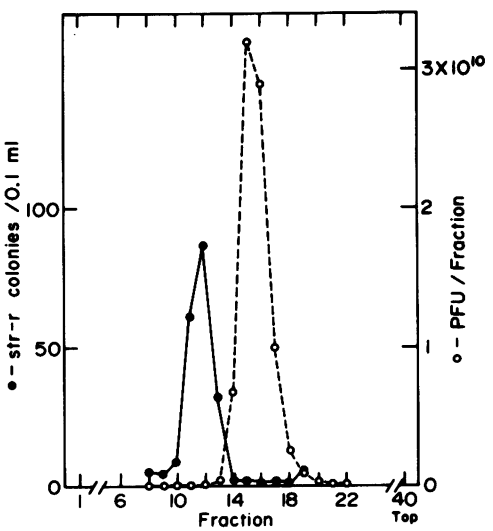

FIG. 2. *Gene transfer and PFU in an equilibrium CsCl gradient. PG24/1602 was centrifuged for 22 h at 33,000 rpm, 20°C, in CsCl as described in the text. A total of 40 three-drop fractions were collected from the bottom, diluted 10-fold in TMF, and assayed for GT and PFU determinations. For GT the equivalent of 5 μl of CsCl was used per ml of culture because controls showed that higher levels were inhibitory. PFU were assayed on further dilutions of the fractions. Fractions 14–19 were UV irradiated with 100 J/m^2 to reduce interference by phage infection (see Table 7 and text).*

effect of preincubation for 30 min with 0.5 U of phospholipase C (prepared from *Bacillus cereus* by R. M. Bell), followed by addition of 10 μg of DNase. No effect was seen on either PFU or GT (data not shown), whereas membrane vesicles would have been destroyed rapidly (J. Reynolds, personal communication).

Equilibrium CsCl banding. On long-term centrifuging to equilibrium in CsCl, GT activity banded reproducibly at densities about 27 ± 3 mg/ml greater than that of the main PFU peak (Fig. 2). In these experiments it was particularly important to ensure that, in the fractions containing large numbers of PFU, the potential GT activity was not being obscured by excess infectious phage, either on initial infection or by multiplication on the plate. Controls used in a

number of the critical experiments here have shown that the antiserum in the plate may fail to inhibit multiplication when more than 10^7 infected cells are put on one plate. Also, the initial multiplicity of infection has to be low enough so that the majority of potential transductants do not also receive a killing particle. Titering the CFU with and without phage controls the latter problem; the former was controlled by reconstruction experiments in which a known number of drug-resistant CFU were added to duplicate gene transfer assay plates to see if they survived.

In the CsCl gradient of Fig. 2, the PFU titer in the peak fractions exceeded the above limits. Therefore fraction 15 was irradiated with 100 J/m^2 of UV, estimated to reduce PFU titer 20-fold or more and gene transfer by 30%, based on the data of Table 5 where 600 J/m^2 reduced it 15- to 20-fold. The data in Table 7 show that 35 to 40% of the CFU survived initial infection and that few or no drug-resistant cells were lost by phage multiplication on the plates. From these data, 25 to 30% of any GT activity in fraction 15 should have been observed, and the lack of significant numbers of Str^r colonies therefore implies that there was little if any such activity at the buoyancy of the infectious particles.

Phage densities. By analytical ultracentrifugation in CsCl, PG24 was 15 mg/ml less dense than ω3, and on overloaded samples a small peak was observed about 19 mg/ml denser than the main PG24 peak (Fig. 3), consistent with the density expected for heads without tails (26). Electron micrographs of fractions from preparative gradients showed a large number of filled phage heads in this region, increasing from a proportion of less than 1 per 100 intact particles at the PFU peak to 1 head per 3 intact particles at the GT peak and to a majority of heads at higher densities (Fig. 4). For a time we suspected that the GT activity may reside solely in these filled phage heads, inasmuch as this could account for the different susceptibilities of GT and PFU to antisera and trypsin. However, velocity sedimentation in sucrose gradients (Fig. 5) showed that the majority of the GT activity cosedimented with the PFU, whereas heads without tails should sediment about 1.6 times faster (25). Some excess GT activity does appear at this position, however, and this may imply that both kinds of particle are active. The fast-sedimenting material of both kinds probably reflects some aggregates which occur in these preparations (17).

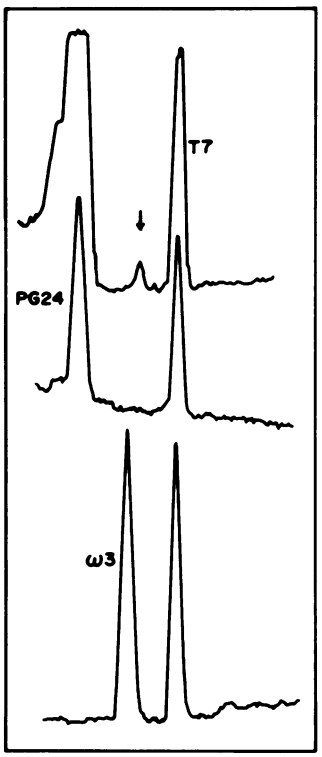

FIG. 3. *Buoyant densities of phages in CsCl. PG24 or ω3 phage were banded with T7 phage in CsCl in TMF, mean density near 1.48 g/ml, in the analytical ultracentrifuge. Densitometer tracings shown are from photographs taken at 265 nm after 18 to 25 h at 44,770 rpm, 25°C. Density increases to the right. Density differences were calculated as* $\Delta\rho = K\omega^2 r\Delta r$, *with* $K = 7.9 \times 10^{-10}$ *(3). Top, PG24 in excess. Arrow indicates a reproducible peak present in PG24 and 11 mg/ml less dense than T7. Middle, PG24 at 1/20 the amount above. PG24 is 30 mg/ml less dense than T7. Bottom, ω3 and T7. Density difference is 15 mg/ml.*

TABLE 7. *Control experiments for gene transfer assay*[a]

Fraction[b]	Survival during infection[c] (CFU/ml × 10^6)	Survival on plates[d]	
		−CFU	+CFU
Control	550	15	1,134
12	526	276	—
15	205	4	1,034

[a] See text.

[b] Fractions 12 and 15 are from the CsCl gradient shown in Fig. 2; fraction 15 received 100 J/m^2 of UV irradiation. The control represents uninfected cells.

[c] CFU in the assay tube after 40 min of adsorption.

[d] Str^r colonies per 0.2 ml of culture plated. Approximately 10^3 Str^r CFU per 0.2 ml was added to duplicate platings to test effectiveness of antiserum in suppressing phage killing on the plates. —, Not determined.

DNA. Examination of PG24 DNA showed it to be highly anomalous, with a buoyant density

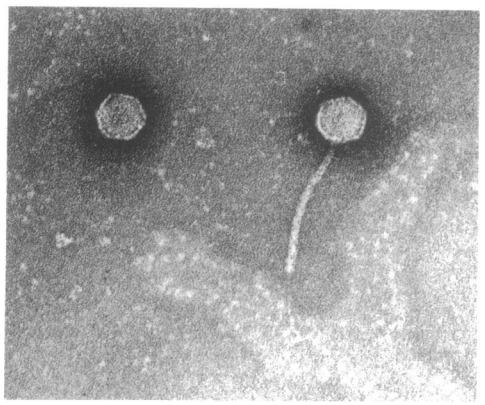

FIG. 4. *Electron micrograph of particles in the gene transfer peak of a preparative CsCl gradient of PG24/1602 (see text). A freshly glow-discharged Formvar-carbon grid was touched to the fraction material for 15 s, rinsed with TMF for 30 s, stained for 2 min with 2% aqueous uranyl acetate, dried, and examined in a JEOL JEM100C electron microscope. ×108,000.*

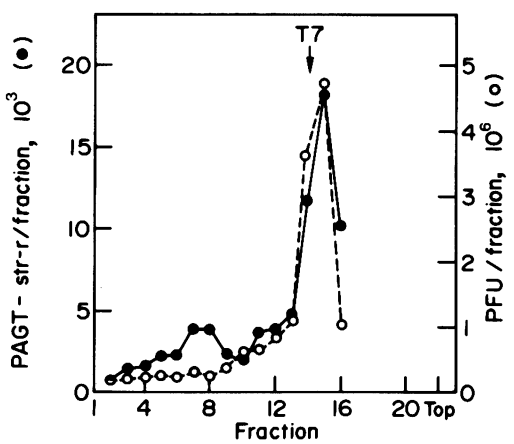

FIG. 5. *Sedimentation of GT and PFU in a neutral sucrose gradient. PG24/1602 mixed with T7 phage were sedimented as described in the text. After 50 min at 14,300 rpm, 20.4°C, 22 fractions (~0.23 ml) were collected and assayed for GT and PFU on RxI and for T7 on E. coli W3110. Assays of the top six fractions were lost, but other gradients showed no significant activity in this region and generally similar distributions, with poorer statistics, in the rest of the gradient. Recovery of input GT was 30% in this gradient; recovery of PFU was near 5%.*

of 1.671 g/ml on the scale where *Escherichia coli* DNA is 1.710 (Fig. 6). This implies an apparent composition of 11% guanine-cytosine (GC) base pairs (19). However, its T_m implies 52 to 53% GC, and its absorbance spectrum is anomalous, having a maximum at 260 nm and a minimum at 234 nm. This is intermediate between normal DNAs and ω3 or ω8 DNAs, which have maxima at 261.5 nm and minima at 237 nm (17). These properties suggest unusual bases, the presence of which in ω3 and PG24 has been confirmed by M. Mandel (personal communication).

The low density of PG24 DNA is consistent with finding GT more dense than PFU, though the degree of shift raises questions. On the explicit and unproven assumption that PG24, GT, and ω3 are isomorphous particles of the same protein content and DNA length, interpolation between the densities of PG24 and ω3 predicts GT to be 13 mg/ml denser than PFU. PG24 heads with 1.0 U of cell DNA should be at least 32 mg/ml denser than PFU. Alternatively, using the relations of Bellett et al. (3), an intact particle filled with 1.12 U of cell DNA would be 27 mg/ml denser, as observed in Fig. 2 and several similar gradients. The information available is insufficient to distinguish among the alternatives at this time.

However, although it will take further study to clarify the precise nature of the particles carrying gene transfer activity, the density is near that expected. These and the other results to this point implied that the cell DNA was in a phage head, usually or perhaps always with a

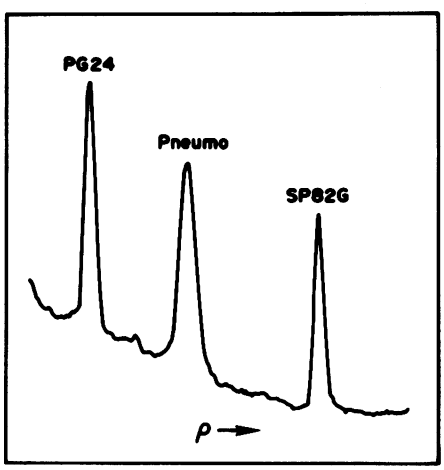

FIG. 6. *Distribution of PG24, pneumococcal, and SP82G DNAs in CsCl, after 20 h at 44,770 rpm, 25°C, in a model E analytical ultracentrifuge. Density differences were calculated as* $\Delta\rho = 8.4 \times 10^{-10}\ \omega^2 r \Delta r$ *(8): PG24 to pneumococcus, 29.1 mg/ml; PG24 to SP82G, 70.6 mg/ml; pneumococcus to SP82G, 41.5 mg/ml. For comparison, ω3 DNA is about 6.5 mg/ml more dense than pneumococcal DNA (17), which has a density of 1.700 g/ml on the scale where E. coli DNA is 1.710 g/ml (19). The low peaks in this tracing are due to noise and do not represent DNA species.*

tail attached, but that it was not injected by the usual phage mechanism. Instead, it had to find its way out of the phage and through the cell wall and membrane, apparently using the endonuclease pathway that yields intracellular single-strand intermediates (13). The delayed expression suggested that this might be a slow process. The difference in effects of antisera and trypsin on GT and PFU are consistent with some specificity for the phage injection mechanism as well as for attachment. This interpretation is also consistent with the greater stability of GT than PFU and can readily account for the variable GT/PFU ratios.

DNase sensitivity. The above arguments were strongly reinforced by experiments that examined more rigorously the question of sensitivity to DNase. Because of the need to exclude transformation by adventitious free DNA, we had always used cells well past their peak of competence, purified the phage through DNase, and usually had DNase present at 10 μg/ml during adsorption. Among the control experiments, free DNA was added to a DNase-free PG24/Rx1 stock and allowed to stand overnight at 4°C before the stock was exposed to Rx1 cells with or without DNase and assayed as usual for gene transfer. The added DNA gave transformants in the absence of DNase but none in its presence. However, in an experiment designed to compare transduction and transformation in the same recipients, concentrated competent cells were used for both assays. They gave four- to fivefold more GT activity than expected, but most of it was now prevented by DNase. Though our first reaction was that the donor phage preparation had been damaged, the same result was found with other preparations. Experiments of the following kind showed that completion of GT requires competence, either preexisting or developed later on the assay plate.

Table 8 summarizes one of several experiments on this phenomenon. It shows 4.3-fold more gene transfer to competent than to noncompetent cells. Taking the results for no additions to the system as 100%, 100 μg of DNase per ml added at zero time reduced GT about 10-fold in each case, and the surviving colonies were very small. Addition of DNase to the plating agar had no effect on transformation and only a little effect on gene transfer to competent cells, but reduced GT to noncompetent cells 11-fold. The surviving colonies were large. In other experiments (not shown), addition of 10 μg of DNase per ml at zero time sometimes reduced GT to noncompetent cells as much as twofold, but addition at the time of plating had as much effect as addition at zero time. Often, however, addition of 10 μg of DNase per ml at zero time had no obvious effect on GT to noncompetent cells. Addition of DNase both at zero time and at time of plating sometimes destroyed all GT activity (data not shown). The variability of these results suggests that the DNase added early was often inactive when diluted into the plating mixture.

A further result shown in Table 8 is that competitor DNA added to the adsorption mix had no effect on GT to noncompetent cells (as in Table 2), but reduced GT to competent cells fourfold. This factor is significant but much less so than expected if the GT DNA were merely released into the medium. Transformation by a quantity of DNA greater than that in the phage particles was reduced 100-fold if one counts only the large colonies. In this experiment a back-

TABLE 8. *Effects of DNase and competence on gene transfer by PG24*[a]

Additions	Transformation by 0.01 μg of DNA/ml of cells[b]		Gene transfer by PG24	
	Noncompetent	Competent	Noncompetent	Competent
None	1.6×10^4	6.1×10^5	1,600 (100%) (variable)	6,900 (100%) (90% large)
100 μg of DNase/ml added at zero time	<10	~20	160 (10%) (small)	780 (11%) (small)
50 μg of DNase/ml added to plating agar at 50 min	2.2×10^4	6.2×10^5	140 (9%) (large)	6,300 (92%) (large)
60 μg of competitor DNA/ml added at zero time; no DNase before or after plating	260 (1.6%) (large colonies)[c]	4,000 (0.6%) (large colonies)[c]	1,600 (100%) (variable)	1,700 (24%) (large)

[a] Data are transformants or transductants per milliliter of culture. Remarks in parentheses indicate colony sizes observed after 18 to 20 h of incubation.

[b] Cells were DP1800 (see Table 1). Noncompetent cells were 1.4×10^8 CFU/ml. Competent cells were concentrated fivefold after thawing by centrifugation and resuspension in 1/5 volume of supernatant (1.0×10^8 CFU/ml).

[c] In each case there were also 2,000 to 3,000 transformants per milliliter represented by much smaller colonies.

ground of smaller colonies implied that some transformation had occurred on the plate after dilution of the competitor DNA. The fact that the GT to competent cells gave large colonies suggests that the phage-associated donor DNA entered early and therefore had a strong but not absolute advantage over the free DNA competing for entry sites. In another experiment, 30 μg of competitor DNA per ml of plating agar had little effect on GT to either type of cell (not shown), again implying that the GT DNA has a strong advantage over free DNA and therefore is rarely if ever simply released to the medium.

Finally, these results helped clarify what had been a disturbing phenomenon, namely, that although GT activity was proportional to the amount of donor used in the standard assay, dilutions of the recipient culture did not give the expected numbers of colonies. The experiments in Table 9 showed that this was due to delayed expression of drug resistance when the cell density on the plate was reduced. Prior assays of this GT stock, as in experiments 1 to 3 of this table, had shown that at the standard concentration of cells there was quantitative adsorption of all the GT added to different absolute volumes of cells, and led to the expectation that in experiment 4, where 20-fold fewer cells were plated (in an attempt to reduce the spontaneous background), there should have been about 200 colonies per plate. They did not appear at 3 h but did appear if 5 h of incubation was allowed before challenge with the drug, by which time the cell density was again near 10^9 per plate and the spontaneous background reappeared (see Table 9, footnote *d*). In these and similar experiments, the few colonies appearing at the standard 3-h time were often in small patches on the plate rather than randomly spread. On hindsight, this may have reflected waves of competence spreading across the plate as a minimum cell density was reached. In any event, we interpret the delayed expression to be due to delayed entry. Related to this may be the overall lower yield of GT when competence must develop on the plate compared to that with already competent cells (Table 8). One interpretation consistent with the results to date is that the majority of the cells do not become competent soon enough to take in the GT DNA and express it before the plates are too heavily overgrown. Alternatively, nucleases released in the developing microcolony might interfere.

Summary of conclusions on the entry of GT DNA. To recapitulate our interpretation of the experiments described in the preceding section, the results all imply (i) that entry of GT cell DNA from PG24 requires the recipient cell to become competent; (ii) that if not already competent, the recipient cell often becomes so on the plate within 1 to 2 h if plated at high cell density and later at low cell density; (iii) that the DNA remains in a protected state in the phage head until the cell becomes competent; and (iv) that the localized position of the DNA gives it an advantage over free DNA at this time. The requirements for competence and for the membrane-bound endonuclease, plus the *hex* sensitivity of the recombinant products, imply that the DNA enters as a single strand by the transformation pathway, after having adsorbed to the cell in a phagelike package. The variable effects of DNase clearly reflect the concentration and the stability of the DNase present at the

TABLE 9. *Delayed expression of gene transfer at low cell density on plate*

Expt	Adsorption mixture (ml)			Drug at (h)[c]	No. of colonies/plate[d]	No. of Str^r in adsorption mix	No. of Str^r/ml of GT
	GT[a]	Total	Plated[b]				
1	0.005	0.55	0.1	3	337, 410	2,050	4.1×10^5
2	0.010	1.2	0.1	3	322, 364	4,130	4.1×10^5
3	0.050	1.1	0.1	3	1,613	17,700	3.5×10^5
4	0.020	0.22	0.0055	1.5	16, 17	660	0.3×10^5
				2	13, 15	560	0.3×10^5
				3	18, 24	1,040	0.5×10^5
				4	11, 15	520	0.3×10^5
				5	214, 222	8,700	4.4×10^5

[a] The peak fraction of GT from a CsCl gradient like that of Fig. 2. Initial volume was 0.10 ml (three drops), diluted to 1.0 ml with TMF and dialyzed to TMF. Cells were Rx1 at about 3×10^8/ml. Experiment 3 had 10 μg of DNase added; the others did not.

[b] Volume put on one plate with antiserum after 40 min at 37°C for adsorption. In experiment 4, after adsorption, the mix was diluted to 4.0 ml, from which 10 0.1-ml samples were plated.

[c] Time of incubation at 37°C after plating before addition of drug overlay.

[d] In experiment 4, Ery^r and Nov^r were also scored with similar results. Control plates without GT gave 2 to 5 Str^r colonies in experiments 1 to 3. In experiment 4, controls gave no colonies until 4 h, when 1 Ery^r and 3 Nov^r colonies appeared. At 5 h, control plates showed 0 and 4 Str^r, 3 and 3 Ery^r, and 2 and 9 Nov^r colonies.

time when the cells are ready to unpackage and take in the DNA. Therefore, the gene transfer particle is DNase resistant and closely resembles a normal phage structure, but the gene transfer process is DNase sensitive if challenged at the appropriate time.

Strain dependence. With the above insights into the nature of the PG24 gene transfer system, we can comment on results showing strain specificities. One is that we have yet to succeed in using noncompetent R6 cells as recipients for GT even though they are infected normally, as exemplified for strain DP2000 in Table 5, experiment 2A. We suspect that the apparent failure of R6 as recipient reflects the very high cell density needed at the time of drug challenge because we have previously described plating artifacts that eliminate R6 transformants when the initial cell density exceeds 10^8/plate (10). The resistant cells were probably killed by the products of lysis of the sensitive cells after drug addition.

A second parameter that seems to affect yields from nonisogenic donors is *hex*. A donor preparation grown in DP2300, an R6 derivative that is highly nonisogenic to Rx derivatives (9), donates GT markers well to Rx1 (*hex*) and to DP1800, a *hex* isogenic derivative of DP1600 (*hex*$^+$), but much less well to DP1600 itself, although in parallel tubes the same culture of DP1600 accepts high-efficiency markers well from PG24/DP1602, to which it is nearly isogenic (Table 5). We suspect that the effect is due to linkage of markers that are high efficiency, when assayed by transformation, to *hex*-sensitive mismatches sufficiently far from the markers themselves to rarely affect their efficiency in transformation, where the median length of entering DNA is 6 to 7 kb (15). If the phage-packaged DNA is localized on the cell surface such that it does not receive excess cuts by contact with several other binding sites, as it would when free in solution at an equivalent concentration (14–15), then much longer strands could enter the cell, be integrated into a single heteroduplex, and make the marker subject to rejection by *hex* action at remote sites (10).

Data confirming that longer strands enter the cell from PG24 particles than from transformation by purified DNA preparations come from an experiment comparing transduction and transformation of a deletion. In this situation wild-type donor strands must be long enough to bridge the deletion in the recipient, whereas the reciprocal conversion of wild type to deletion can succeed with short strands (1, 11). Transduction was much more efficient at donating the wild-type *thy*$^+$ strand to the deletion recipient *thy-19* than was transformation by a donor DNA preparation in which the median single-strand size was 16 kb (Table 10). Though expected for a transducing system in which the phage apparatus injects the cell DNA, this result need not have been true in this system, where the DNA passes through a DNase-sensitive state during entry. It lends support to the above interpretation of *hex* effects between nonisogenic strains, which is, however, still only a working hypothesis to explain a reproducible set of strain specificities.

Does PG24 act alone in producing GT? As initially isolated, PG24 and several other phages grew poorly, at least as reflected in PFU yield. The efficiency of plating has never approached the 50% range observed for ω3 and ω8 (17) and often was less than 1% as estimated from PFU per unit of phage DNA. Therefore, even though PG24 had been through two single-plaque isolation steps, we examined the possibility that there were two kinds of phage particles, of which only one transduced. A stock that had exhibited low and variable yields was subcultured continuously over a period of weeks, during which its yield rose 100-fold for unknown reasons. From this a replicating phage DNA preparation (18) was used to transfect cells at a very low DNA concentration where only 10^{-7} of the cells were transfected, in a process that is single-hit for collision kinetics (18). From a plate with three plaques, one was picked and regrown. This phage stock, temporarily called PG24*, has all the properties of the parent PG24, including DNA buoyant density and the ability to induce gene transfer when grown on any of several distantly related hosts. Therefore GT is associated with a single infecting phage type. However, we cannot exclude the possibility that the true transducing particle is a defective phage present in all our strains and induced only by infection with PG24, although there is no evidence to support such a hypothesis.

TABLE 10. *Evidence for longer intracellular strands from PG24 GT than from transformation with purified DNA*

Recipient[a]	Donor[b]	*thy*$^+$/*ery*
DP1162, *thy-19*	DNA	0.011
	PG24	0.14
DP1147, *thy-7*	DNA	1.23
	PG24	0.51

[a] *thy-19* is a deletion; *thy-7* and *ery*-r2 are point mutations (Table 1).

[b] A purified DNA from DP1617 (*thy*$^+$ *ery*-r2), of a median single-strand length of 16 kb (from alkaline sedimentation velocity), was used at 2 μg/ml in concentrated competent cells used simultaneously to assay GT from PG24/2300 (*thy*$^+$ *ery*-r2).

DISCUSSION

The process of generalized transduction in most systems is thought to involve the packaging of a segment of duplex bacterial DNA into otherwise normal phage particles which then inject it into recipient cells essentially intact (16). If it recombines with the recipient chromosome, a stable transductant is obtained; if not integrated, it often remains in the cell in a form which does not replicate but is transcribed and phenotypically expressed in an abortive transductant (16). Little more is known of the mechanism of the latter process, and the only direct evidence relevant to the entry mechanism in stable transduction is the report by Ebel-Tsipis et al. (6) that 4- to 6-Mdal segments of density-labeled donor DNA were found integrated as duplex in the chromosome of recipient *S. typhimurium* when transduced by P22. They pointed out that their data did not exclude additional integration of donor as donor-recipient heteroduplex. Other evidence on mechanisms of transduction is indirect, based on requirements for rec^+ gene products, such as in P1, which needs the *recA, recB,* and *recC* products (26).

The system described here for gene transfer in pneumococcus by lysates of phage PG24 fits the definition of generalized transduction by the following criteria: several unlinked genes (all those tested) are transferred to recipient cells at comparable low frequencies after having been packaged into DNase-resistant structures which copurify with infectious phage though several steps, band in equilibrium CsCl gradients close to the PFU (Fig. 2), mostly cosediment with PFU (Fig. 5), adsorb quantitatively to cells in a DNase-resistant form with kinetics like those of PFU, and are inhibited by antisera to the phage and by trypsin, but not by phospholipase C. These results are strong evidence that the transducing DNA is packaged in phagelike structures, most of which are complete with tails, although there is a suggestion that filled heads without tails may also be able to transfer cell genes (Fig. 5).

However, on examining the product of gene transfer, it is evident that essentially all recombination occurs by integration of a single strand of donor DNA into a donor-recipient heteroduplex. This conclusion follows from the dependence of marker ratios on the state of the *hex* gene, whose product acts on specific types of donor-recipient mismatches only after the formation of heteroduplex (20), and on the hex^+-dependent hypersensitivity of low-efficiency markers to UV irradiation (Table 5). The further finding (Table 6) that gene transfer requires the endonuclease essential for entry of transforming DNA as single strands in pneumococcus (18) suggested an entry pathway that would lead to the heteroduplexes implied by the *hex* dependence. The endonuclease is membrane bound and required for a process in which extracellular duplex donor DNA is converted from a bound and nicked state to equal masses of free extracellular oligonucleotides and intracellular single strands (13–15). During transformation of gram-positive organisms, the competent state is one in which the cell wall appears to develop "pores" that allow access of free DNA to binding sites at or near the membrane endonuclease (13).

However, it was still possible that in gene transfer the phage injected the cell DNA, which then was converted to single strands by the endonuclease after entry, perhaps slowly enough to account for the delayed phenotypic expression. This possibility was ruled out by the finding that the transfer process is DNase sensitive and that it requires development of competence on the plate if the cells are not already competent. The DNA remains protected, however, until the cells become competent, implying that unpackaging of the DNA is promoted by some as yet unknown feature of the competent state.

The question arises as to whether the phage DNA in this system also enters by the transformation pathway. For transfection, the answer is yes (18), but for infection, it appears to be no. The strongest arguments are (i) the latent period is only 60 to 70 min for PG24-infecting noncompetent cells (not shown); (ii) PFU titers are the same for noncompetent and competent cells and independent of the presence of 100 μg of DNase/ml (e.g., in the experiments of Table 8, data not shown there); and (iii) PFU titers are normal in the endonuclease-deficient strains of Table 6 (data not shown; 17). These results strongly imply a rapid phage-mediated entry process for those of the phage particles that are infectious. A possible interpretation, then, is that the phage DNA is recognized differently by some aspect of the entry process. However, an alternate possibility is raised by the low efficiency of plating of these phage. If only 1% or less of the particles can inject DNA at all, perhaps independently of the kind of DNA, and the other particles simply remain on the cell surface, then the single-strand transformation pathway could act on their DNA as it leaks out of the particle following development of competence. Because transfection requires multiple entry events (18), it is unlikely that this path would add significantly to infection, but it would be very efficient for GT. This interpretation cannot be excluded at this time.

Whichever interpretation proves correct, very little of the GT observed here can be due to entry of duplex DNA. An upper limit is set by the survival of the *hex*-sensitive UV-irradiated *nov*-r1 marker in Table 5, of the order of 1% at most. Therefore, the entry process and the product of integration are quite different from those expected on the assumption that the transducing DNA enters as does the infectious phage DNA.

Whether the PG24 gene transfer system is unique to itself or to pneumococcus, or is only the first such case described, must await further critical study. Although it is clearly a form of generalized transduction by the definition of a viral vector for host genes, the virus does not complete the transfer; the term pseudotransduction may be useful in calling attention to its unusual features. Another unusual gene transfer system has been described in *Rhodopseudomonas capsulata,* where the agent is a 70S particle of density 1.37 g/ml, not yet obviously associated with a phage (22). These and other transduction systems are discussed in a review to be published (K. B. Low and R. D. Porter, Annu. Rev. Genet., in press).

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