

DEFECTIVE PHAGE AND CHROMOSOME MOBILIZATION IN *PSEUDOMONAS PUTIDA**

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Abstract.—The transfer of transducing phage DNA in association with the mandelate genetic region of *Pseudomonas putida* strain PRS1 (termed *pfdm*) has been achieved by growing together mandelate-positive PpG2 cells harboring *pfdm* as an extrachromosomal element and mandelate-deleted PpG1 strains. This transfer is analogous to sexual conjugation in the enterobacteria. The transfer of *pfdm* elements is always associated with chromosome mobilization and some rare recombinants acquire genetic donor ability. We have therefore concluded that the *pfdm* elements are responsible for initiation of chromosome mobilization in a manner not yet fully understood.

We have described recently¹ the inter-strain transfer of a cluster of genes governing the synthesis of the four enzymes which degrade D-mandelate to benzoate from *Pseudomonas putida* strain PRS1 to the mandelate-deleted strain PpG2.† The transfer is mediated by defective phage pf16h2 in which a portion of the genome is replaced by the bacterial mandelate cluster.² These hybrid phage-bacterial DNA complexes cannot replicate vegetatively by themselves and need superinfection by viable phages to give rise to transducing particles.² The defective phages have been termed *pfdm* and comprise two classes: class 1 retains the immunity-determining region of the phage genome, thereby producing immune transductants; class 2 transductants are phage sensitive, apparently due to absence of the immunity-determining region. The *pfdm* particles appear to be heterogeneous in content of DNA from phage and host as shown by a variability in buoyant density in a CsCl density gradient.² We have concluded that the *pfdm* particles multiply autonomously as extrachromosomal elements because in sensitive transductants they occasionally segregate spontaneously, can be cured by ultraviolet irradiation, and produce a satellite DNA band on sedimentation. We have avoided designating these particles episomes because we lack direct evidence of their association with the host chromosome.³

In this paper we present evidence that the presence of *pfdm* particles confers on some rare cells the ability to transfer to recipients, by a process analogous to mating in enteric bacteria, not only the *pfdm* element but also large segments of the bacterial chromosome. Some exceptional cells receiving *pfdm* elements and chromosomal segments in turn inherit the ability to transfer their chromosome to other recipients. Most of the recombinants are unstable diploids such as are typically obtained in *Escherichia coli* if the recipients carry *recA* mutations⁴ or are produced by intergeneric matings.⁵

Materials and Methods.—*Organisms and media:* The cultivation of PpG pseudomonad strains has been described earlier,¹ as has the composition of mandelate-succinate-tetrazolium chloride plates for distinguishing Mdl⁻ segregants.² The conditions for produc-

tion of HFT (high frequency transducing) lysates by cells harboring *pf_{dm}* have also been reported.²

Transduction with phage *pf16* and host range mutants has also been described.¹

Transfer of chromosomal segments and pf_{dm} particles by cellular contact: Donor strains harboring *pf_{dm}* as an extrachromosomal element and recipient auxotrophic strains prepared from PpG1 were grown overnight in tryptone broth with shaking at 30°. Logarithmic phase cultures containing $1-2 \times 10^9$ cells/ml were obtained by transfer of 0.1 ml of the overnight culture to 5 ml tryptone broth and continued incubation for about 3 hr. For mating experiments the cultures were mixed in equal proportions, incubated at 30° in stationary condition for 1 hr, or as specified. These cells were collected by centrifugation, suspended in saline, and plated. After 4 days incubation at 30°, recombinant clones were purified by restreaking on the selection medium and then analyzed for presence of unselected markers. A better yield of recombinants was usually obtained if the donor and recipient cells were mixed together at about 10^6 cells/ml and grown overnight in tryptone broth with mild shaking.

Results.—Gene transfer by conjugation has hitherto been unknown in saprophytic pseudomonad species, e.g., *P. putida* or *P. fluorescens*. Neither has genetic exchange been observed on mixing in pairs a variety of the auxotrophic mutants of strains PpG1 or PpG2 which do undergo recombination by phage transduction nor on mixing with heterologous *P. putida* strains. In contrast, by growing a mandelate-positive transductant of PpG822 together with a tryptophan auxotrophic mutant followed by plating the mixture on media which support neither strain alone, clones appeared suggesting that recombination had occurred. The strains used and their properties and designations are listed in Table 1; the results of three crosses are shown in Table 2. The first and second concern the mandelate gene cluster and two auxotrophic mutations in tryptophan genes of a single cluster. The third cross concerns mandelate and two tryptophan mutations in cistrons unlinked by transduction with phage *pf16*, i.e., *trpA* and *E* loci.⁶ We have also used PpG898, a tryptophan auxotroph (*trpD633*) carrying a streptomycin resistance locus (*str-601*) with selection on mandelate minimal medium

TABLE 1. *Pseudomonas putida* strains used.

Strain			Derivation		
Designation	Genotype	Phenotype	Parent	By	Ref.
PpG282	<i>trpA1</i>	AS ⁻	PpG2	NG	Chakrabarty <i>et al.</i> , 1968
299	<i>trpA601</i>	AS ⁻	1	NG	"
328	<i>trpD633</i>	InGPS ⁻	1	NG	"
898	<i>trpD633str-601</i>	InGPS ⁻ , Str ^r	328	S	
541	<i>trpA601E509</i>	AS ⁻ , TS-A ⁻	299	S	Gunsalus <i>et al.</i> , 1968
893	<i>trpA601E509</i> <i>str-602 pf1^r, 11^a</i>	AS ⁻ , TS-A ⁻ , Str ^r	541	S	
895	<i>trpA601E509pan-601</i> <i>pf11^r, 1^r, str^d</i>	AS ⁻ , TS-A ⁻ , Str ^d	893	S	
822	<i>AS mdl⁺</i>	D-Mdl ⁺ , pf16h2 ^a	PRS1 × PpG2	T(h2)	Chakrabarty and Gunsalus, 1968
896	<i>mdl⁺str*pf1^a, 11^a</i>	D-Mdl ⁺	822 × 895	*	
897	<i>mdl⁺str-601arg-634</i> <i>pf1^r, 11^a</i>	D-Mdl ⁺ , Arg ⁻	822 × 898	†	

NG = nitrosoguanidine; S = spontaneous, T = transduction.

* Conjugation, then back mutation Str^d → Str^a.

† Conjugation, then selection for Arg⁻.

TABLE 2. *Transfer of chromosome and pfdm markers in P. putida strain PpG mutants.*

Donor	Recipient	Transduction		Cell Contact	
		Transfer*	Cotransfer	Transfer†	Cotransfer‡
		(frequency)		(frequency)	
<i>AS mdl</i> ⁺	<i>trpD633</i>	$2 \cdot 10^{-6}$	0	$4 \cdot 10^{-9}$	1
	<i>A601</i>	$5 \cdot 10^{-6}$	0	$<10^{-9}$...
	<i>A601E509</i>	10^{-6}	0	$2 \cdot 10^{-9}$	1

Selected for *Mdl*⁺, nonselection for recipient phenotype (*Trp*⁻).

* Per adsorbed phage.

† Per acceptor cell (10^9 cells each parent used).

‡ Estimated from 50 colonies tested for nonselected marker.

supplemented with both streptomycin (1 mg/ml) and tryptophan (25 μ g/ml) and found cotransfer of the *trp*⁺ region of the donor. The recombinants differed from spontaneous *Str*^r mutants of the donor in other recipient phenotypes including phage-sensitivity, colony morphology, analog-resistance and carbon sources utilized. That is, the recombinants were clearly PpG1 recipients which had received *mdl* and *trp* markers from the donor *mdl*⁺PpG2 (strain PpG822).

The very low recombination frequencies led us to consider the possibility that gene transfer had occurred by transformation with naked DNA from lysed donor cells or even by transductions by phages released from them. Incubation of the donor-recipient cell mixture with DNase at a range of concentrations (1–50 μ g/ml) did not inhibit the gene transfer, suggesting that naked DNA is not involved. A treatment of the recipient cells with DNA isolated from donor cells also did not result in mandelate-positive transformants. The conclusion that the observed gene transfer is not phage-mediated derives from the following observations:

(1) Strain PpG822, *AS mdl*⁺, has not been found to produce either viable phage or transducing particles. Thus, when a 15 or a 48-hour culture was treated with chloroform, the cell debris removed by centrifugation, and the sterile supernatant tested for transducing activity and for plaque-forming particles, neither was observed.

(2) A 26-hour culture of an *AS mdl*⁺ strain was filtered through a 0.45- μ millipore filter and the cells and the filtrate tested separately for gene transfer potency; only the cells retained such ability.

(3) An antiserum treatment of the donor cells—anti-pf16, final K = 10 minutes⁻¹, 2 hours—prior to mixing with the recipients and subsequent plating did not abolish the transfer. In some cases the inclusion of antiserum seemed to have a stimulatory effect. Similar treatment of a phage preparation containing transducing particles with anti-pf16 serum reduced the number of recombinants by more than 90 per cent as compared to untreated controls.

(4) Loci for multiple auxotrophic mutations, unlinked by phage-mediated transduction,⁶ are repaired simultaneously with the acquisition of the mandelate cluster. Cotransduction of the other chromosomal markers used with mandelate genes was never observed, further indicating that chromosomal transfer in this cell contact system cannot result from transduction.

The time course of a typical conjugation experiment is shown in Table 3. The phage pf1 which replicates in the donor but does not attack the recipient because

TABLE 3. *Conjugation transfer of pfdm markers: Effect of pf1 and pf11 phages.*

Donor	Recipient	Phage	Time added (min)	Recombinants Mdl ⁺ , Trp ⁺ (per 10 ⁸ cells)
<i>AS mdl⁺</i>	<i>trpD633</i>	None		8
		pf1	-60*	0
			0	4
			60	9
		pf11	0	0
			120	0

* Donor treated for 60 min with pf1 before mixing with recipient. All phages added at moi = 2.

of poor adsorption, eliminates transfer of *pfdm* particles. Similarly, phage pf11 which lyses the recipient strain, PpG1, but does not adsorb on the donor⁷ abolishes the gene transfer. The elimination of transfer by phage pf1 of *pfdm* depends markedly upon the time of addition to the donor-recipient mixture. The inhibition is much greater if the phages are added 60 minutes before the recipient cells and is virtually unobservable when pf1 is added 60 minutes after donor-recipient contact. The genotype of all recombinants, *mdl⁺trp⁺*, requires the transfer of chromosomal markers from the donor.

Nature of recombinants: Some Mdl⁺Trp⁺Str^r recombinants, after an initial purification by streaking on mandelate plates supplemented with streptomycin and tryptophan were found to give rise to Mdl⁻ segregants as well as mandelate-variable sectorial colonies when streaked on mandelate-succinate-tetrazolium plates containing tryptophan. If the mandelate-succinate-tetrazolium plates lacked tryptophan, these segregants were conspicuously absent. The Mdl⁻ segregants, from a cross using a *trpA⁻E⁻* double mutant as recipient (Table 2), were found to be *trpA⁻E⁻*, whereas intermediate recombinant types were not found; that is, they behave exactly as do the recipients. On further restreaking some of the Mdl⁺Trp⁺ recombinants and all of the mandelate-variable ones again produce Mdl⁻Trp⁻ segregants and Mdl⁺Trp⁺ clones. Stable Trp⁺Mdl⁺ recombinants have also been isolated from unstable recombinants after three or four single colony isolations. They seem to be stable haploid cells since they do not segregate Mdl⁻Trp⁻ cells at an observable frequency and cannot be cured of *pfdm* particles by UV-irradiation—the donor *mdl⁺PpG2* cells were readily cured by UV-irradiation. We do not, however, have direct evidence that the *pfdm* particles are associated with the bacterial chromosome. The continued segregation of sectorial mandelate-variable clones, even after five or six colony isolations, with the formation of unstable Mdl⁺Trp⁺ clones plus Trp⁻Mdl⁻ segregants, suggests that these clones are heterozygous and exhibit partial diploidy in the *mdl* and *trp* regions. The stable Mdl⁺ recombinants form translucent colonies like the donor whereas the recipient forms opaque colonies on tryptone agar plates. The heterozygotes are clearly distinguishable from both donor and recipient clones as being translucent at the center with opaque sectors at the edges. Thus, the colony morphology is an additional chromosomal marker inherited by the recipient.

Infectious fertility in rare PpG1 recombinants: The formation of unstable merodiploids by crosses of PpG822 × PpG1 suggests that normal recombina-

tional events, with chromosomal breakage and reciprocal crossovers, are somehow retarded in the zygotes. We therefore wished to find if *mdl*⁺PpG1 recombinants from these crosses could transfer either chromosomal markers or *pfdm* (or both) to other PpG1 recipients. Since the *str*⁺ (*Str*^s) character is dominant over *Str*^r, the zygotes are sensitive to streptomycin. It is conceivable that the selection on a streptomycin medium might have eliminated perpetuation of a large number of zygotes which received the *str*⁺ allele of the donor. Therefore, auxotrophic mutants requiring a variety of amino acids were prepared from the donor *mdl*⁺PpG2 and *mdl*⁺PpG1 stable haploid recombinants. Crosses were then performed with these donors using PpG1 as recipient and the frequency of transfer of *pfdm* particles was noted. As shown in Table 4, the PpG1 recombinants were able to donate both *pfdm* and the chromosomal markers to other PpG1 cells, although at a low frequency. Selection of recombinants in absence of streptomycin using PpG897, an Arg⁻Mdl⁺PpG1 recombinant, as donor also did not enhance the frequency of transfer. The recombinants are again predominantly unstable diploids which on segregation lose the *pfdm* and chromosomal markers of the donor.

TABLE 4. *Transfer of chromosomal markers by recombinants.*

Donor		X		Recipient		Markers selected	Recombinants	Frequency
<i>mdl</i> ⁺ <i>str</i> ^s *				<i>trpA601E509str-602</i>		<i>trpA</i> ⁺ <i>E</i> ⁺ <i>str</i> ^r	<i>mdl</i> ⁺	10 ⁻⁸
<i>mdl</i> ⁺ <i>str-601</i>		<i>arg-634</i> †		<i>trpA601E509</i>		<i>mdl</i> ⁺	<i>trpA</i> ⁺ <i>E</i> ⁺	10 ⁻⁸

* 896 × 893.

† 897 × 541.

The donor and recipient suspensions in tryptone broth were mixed (1:1), incubated 90 min at 30°, and collected by centrifugation. After suspension in saline, aliquots were plated on glucose minimal + streptomycin or mandelate minimal + tryptophan plates.

Discussion.—The production of Mdl⁺ recombinants by growing together the cells of a *mdl*⁺PpG2 donor and an *mdl*-deleted PpG1 recipient provides a new and interesting case of gene transfer in the genus *Pseudomonas*. This system requires contact between donor and recipient cells and is not mediated either by naked DNA or by phage. Because PpG2 cells which do not harbor *pfdm* particles lack the ability to effect gene transfer and other differences are not known between PpG2 and *mdl*⁺PpG2, we have concluded that the *pfdm* elements promote fertility in these organisms. The fertility is infectious as shown by the ability of recipient PpG1 cells acquiring the *pfdm* elements to donate their chromosomes to appropriate recipients. In a large population of *mdl*⁺PpG1 recombinants, however, only rare cells act as genetic donors. The precise status of the *pfdm* particles which promote the fertility remains obscure. They are, however, known to contain DNA from both phage pf16 and PRS1 bacterial chromosome.²

In the enterobacteria the transmissible plasmids which are known to promote fertility are the F, or sex factor,⁸ the colicinogenic factors,^{9, 10} and the drug-resistance factors.¹¹ In *Pseudomonas aeruginosa* the sex factor FP is known to be freely transmissible.^{16, 17} There are several aspects in which the *pfdm* mobilization of chromosomal genes differs from the transfer by F¹² or drug-resistance factors.¹³ In the latter cases chromosomal mobilization is induced by integration,

whether stable or unstable, of the necessary factors with the donor chromosome. When integration does not occur the factors themselves can be transferred from donor to recipient and can confer maleness or related properties without transfer of chromosomal characters. In *Pseudomonas putida* we have not observed transfer of the *pfdm* element without cell chromosomal genes. The extremely low frequency of transfer of the chromosomal markers induced by *pfdm* (10^{-8}) is reminiscent of the behavior of the drug-resistance factors¹³ and the colicins,⁹ i.e., 10^{-8} as compared to *ca.* 10^{-8} for the R^+ and as low as 10^{-9} for the $colI^+$ population.¹³ In contrast, the F^+ transfer frequencies are in the order of 10^{-4} or 10^{-5} per donor. The low rate of recombination in cells harboring $colI$ or R factors has been ascribed to the production of repressor molecules which severely limit the conjugation process, whereas the high-frequency transfer of the F factor is attributed to a loss of the repressor synthetic function.¹³ Similarly, for *pfdm* the low-transfer potency may result from a rapid production of repressor molecules with consequent inhibition of the formation of mating substances in PpG2 and the lack of donor-recipient contact. It is also possible that in this bacterial species the low recombination frequency is the genetic trait; that is, the cells are poor recipients. This would be analogous to the low frequency of transfer of drug resistance factor by conjugation in *Salmonella* as compared to the high frequency in the other enteric bacteria such as *Serratia*, *Shigella*, and *Escherichia*.¹⁴ Thus, poor recipient characteristics in both *Salmonella* and *Pseudomonas* might be traced to inherent genotypic character.

The simultaneous segregation of *pfdm* and chromosomal markers implies that the transfer occurs from those rare cells in which the *pfdm* has integrated with the cellular chromosome. Thus, the low transfer frequency could result from integration at a low rate. We lack evidence whether *pfdm* has preferred sites of integration as, for example, have the transmissible episomes like F^8 and $ColV$ ¹⁵ in *E. coli*. The low frequency with which *mdl*⁺ recombinants act as genetic donors, even when the *pfdm* elements appear to be integrated with the cell chromosome, suggests that integration alone may not be the only criterion for *pfdm* induced fertility. It is conceivable that for the cell to act as genetic donor the *pfdm* must be attached at certain specific positions. One can thus visualize a system where in a given population of cells, the *pfdm* particles are loosely attached at different sites on the chromosome and are only rarely integrated transiently at certain preferred locations, thereby giving rise to the rare potent donor cells. The infectious fertility produced by interstrain transduction followed by cell contact transfer of chromosomal elements is proving useful in extending *Pseudomonas putida* genetics. Further work will be required to understand the precise mechanisms which so far remain obscure.

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† PpG denotes a *Pseudomonas putida* stock from the Gunsalus collection (formerly PUG) to permit species designation in the broadening genetic systems of this genus.

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