*TRANSDUCTION AND THE CLUSTERING OF GENES IN FLUORESCENT PSEUDOMONADS**

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This paper describes a genetic system for a group of fluorescent pseudomonads that oxidize an exceptionally wide variety of substrates. We have directed our studies of the versatility of these organisms to the question of specificity and to the mechanism of oxygen reduction. These studies have identified a number of multimeric monoxygenases. Optimum progress in resolution of these problems is dependent on genetic, in addition to chemical, study of their regulation and cellular organization. Our chemical studies can now be enhanced by concomitant use of genetic analyses.[1]

Although there was no evidence of genetic interaction among strains of *Pseudomonas putida*, and little of bacteriophages parasitic upon members of the group, we considered transduction a likely enough method of gene transfer to merit investigation. The data reported below concern interstrain gene transfer in *Pseudomonas putida* via a transducing phage, pf16, whose characteristics are reported elsewhere.[2] Prior to this work, a genetic system had been found in *Pseudomonas aeruginosa*,[3] a species which contains a homogenous group of strains frequently associated with mammalian infection and which has not proved readily adaptable to metabolic studies.

Two colony variants, opaque (op) and translucent (tr), which differ in ability to adsorb bacteriophages, occur in the camphor-oxidizing strain C1.[4, 2] The translucent variant of strain A3.12, employed by Stanier and his colleagues,[5, 6] serves as both donor and recipient in interstrain gene transfer with a host-range mutant of pf16; this adsorption type differs from C1 and is designated A3tr.

Materials and Methods.—Organisms: The two bacterial strains employed were characterized as *Pseudomonas putida*, biotype A, by Stanier *et al.*[7] Strain C1 was isolated by (+)-camphor enrichment by Bradshaw[4] and A3.12 on lactate by Stanier.[7] The former has been used in our studies of oxygenases and biological specificity,[4, 8] the latter by Stanier in studies of enzyme induction and oxidation of aromatic acids.[9, 10] Strain C1 has several colony types of which op (ATCC 17,453) and tr (ATCC 23,287), which differ in phage adsorption properties,[2] concern us here. Strain A3.12, colony type A3tr (ATCC 12,633), resembles C1-tr but differs in phage-adsorption pattern; in transduction it serves as both donor and recipient with phage pf16 host-range mutant h2.

Mutants of these bacterial strains, prepared spontaneously (S) by UV irradiation (UV) and by nitrosoguanidine (NG) treatment, are listed in Table 1. The NG mutagenesis was essentially by the procedure of Fargie and Holloway.[3] The description of phenotypes and genotypes follows Demerec *et al.*,[11] with all fluorescent pseudomonads considered as one group, as indicated by Gunsalus *et al.*[12]

Mutants spontaneously resistant to streptomycin sulfate (Str) and *p*-fluorophenylalanine (Fpa) were prepared by streaking approximately 10^9 cells on Vogel-Bonner (VB)[13] agar containing 1 mg/ml of either Str or Fpa. The respective mutation frequencies were *ca.* 10^8 and 5×10^8. After 48 hr at 30°, selected colonies were streaked on minimal VB agar. Single colonies picked from these were restreaked on VB plus 1 mg/ml Str

TABLE 1. *Properties of Pseudomonas putida strains.*

Strain*			Colony	Derivation		
Designation	Genotype	Phenotype	type	Parent	By	Reference
PUG1	—	wt	op†	—	—	Bradshaw (1959)
PUG2	—	wt	tr	PUG1	S†	Bertland (1964)[21]
PRS1	—	wt	A3tr	—	—	Stanier (1966)
PUG282	*trpA1*	AS⁻†	tr	PUG2	NG	CG* (S1)
299	*A601*	"	op	PUG1	"	
280	*B11*	PRT⁻	tr	PUG2	"	CG (S11)
363	*B11,E41*	" , TS-A⁻	"	PUG280	S	" (S11i1)
302	*C22*	PRAI⁻	"	PUG2	NG	" (S22)
362	*D31*	InGPS⁻	"	"	"	" (S31)
327	*D631*	"	op	PUG1	"	
317	*D632*	"	"	"	"	
754	*E41*	TS-A⁻	tr	(363 × 310)†	T	
310	*F52*	TS-B⁻	"	PUG2	NG	CG (S52)
368	*F61*	TS-B⁻	"	"	"	CG (S61)
675	*fpa-1*	Fpa^r	"	PUG2	S	
674	*str-1*	Str^r	"	"	"	
461	*cam-1*	Cam⁻	"	PUG2	NG	
436	*cam-2*	"	"	"	"	
PRS3	*mdlB1002*	MDH⁻	A3tr	PRS1	UV	Hegeman (1966)[9]
PUG628	*ASmdlB1002*	"	tr	PRS3 × PUG2†	T	

Former names and references for strains: PUG1, C1B, Bradshaw, *J. Am. Chem. Soc.*, **81**, 5507 (1959); PUG2, C1S, Gunsalus *et al.*, *Arch. Mikrobiol.*, **59**, 113 (1967); PRS1, 90 (A3.12), Stanier *et al.*, *J. Gen. Microbiol.*, **43**, 159 (1966); PRS3, md⁻ (A3.12), Hegeman, *J. Bacteriol.*, **91**, 1155 (1966).

Transductions: First number is donor, second, recipient. PUG754 is a transductant selected on indole plates which lacks only tryptophan synthase A. *AS*, genes transferred from A3.12 (PRS1) to C1S (PUG2).

Enzymes: MDH, mandelate dehydrogenase; Cam⁻, inability to grow on (+)-camphor. *Tryptophan synthesis:* AS, anthranilate synthase; PRT, phosphoribosyl transferase; PRAI, phosphoribosyl anthranilate isomerase; InGPS, indoleglycerolphosphate synthase; TS-A and -B, tryptophan synthase subunits A and B.

* CG = Crawford and Gunsalus (1966).

† Abbreviations: Mutants: S, spontaneous; NG, N-methyl-N′-nitro-N-nitrosoguanidine; T, transduction; op, opaque; tr, translucent clone (PUG2) strain C1; A3tr, translucent strain A3.12 (PRS1). PUG363 is spontaneous fast-growing mutant selected on indole—termed S11i1 in ref. 16.

or Fpa to ensure selection of resistant mutants. Doubly resistant mutants were obtained from Fpa-resistant mutants linked to the tryptophan gene cluster (*trpABD*) by streaking on VB Str agar and selecting clones which were restreaked on VB agar and then on VB containing 1 mg each Fpa and Str.

Phages: Pseudomonas putida, strain C1, was used as host for isolation of phage from sewage. From about 25 phages,[2] only pf16, isolated on the opaque (op) colony type, has yielded a reproducible generalized transducing capacity as indicated by its ability to transduce a variety of unlinked markers such as *his*, *ilv*, *met*, and *trp*.[1] At 5×10^8 cells/ml, phage pf16 is adsorbed by op cells at greater than 90% in 10 min at 30° but only poorly by the tr-type cells of C1 or A3. Transduction in these strains is accomplished by two host-range mutants, pf16h1 and pf16h2. The C1 tr cells absorb pf16h1 less than 50% in 30 min at 30° with shaking. The op-type cells can serve as recipient for these mutant phages, adsorbing them almost as well as pf16, with cotransduction frequencies comparable to C1 tr, but without plaque formation.

Media: The composition of all media used is detailed elsewhere.[2]

Transduction: Detailed conditions for transduction with the three cell types using phage pf16 and its host-range mutants are tabulated in Table 2; transduction frequencies are shown in Figure 1*A* and *B*. For phage propagation, the double-layer technique of

TABLE 2. *Pseudomonas putida transducing systems with phage pf16.*

Phage*	Cell type transduced (clone no.)	Medium: Grow cells	Medium: Phage production	Transducing Conditions: Cells/ml	Transducing Conditions: Phage (MOI)	Transducing Conditions: Incubation (min, 30°)
pf16	op (PUG1)	L-Broth	T·agar	$5 \cdot 10^9$	0.5	20
pf16h1	tr (PUG2)	VB† "	"	"	0.5	30
pf16h2	A3tr (PRS1)	VB "	"	"	0.5	30

* With pf16 only, inactivate 3 log PFU by UV; with all three phage after adsorption, collect cells by centrifugation and resuspend in saline containing anti-pf16 serum K $\sim$ 10. Plate 0.1 ml; if dilute, use saline containing anti-pf16 serum at K $\sim$ 10.

† Enhances phage adsorption to C1tr and A3tr cells. The usual controls for sterility of phage lysates and antiserum and for reversion of recipients are run routinely.

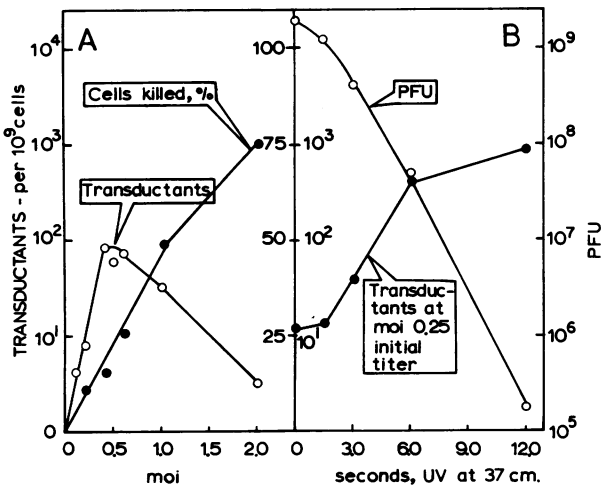

FIG. 1.—*P. putida* strain PUG1 transduction with phage pf16:

(*A*) Effect of multiplicity on transduction frequency and cell killing. Recipient *trpD601;* donor phage pf16 from confluent lysis plate of the prototroph. Transduction: 4-hr L-broth culture $5 \cdot 10^9$ cells/ml, 0.9 ml + 0.1 ml phage; MOI as indicated. Shaken 10 min, 30°; >90% phage adsorbed. Centrifuge, suspend cells in saline, spread 10^9 per duplicate plates.

(*B*) Effect of ultraviolet irradiation on transduction frequency. Seven ml phage in 10-cm diameter Falcon Petri dish at titer 10^{11} in PM medium, irradiated at 37 cm from 15-watt GE sterilamp. Transduction as in (*A*), adsorption and plating in dark.

Adams[14] was used with tryptone agar in both layers. Lysates titer at 10^{10} to 10^{11} plaques/ml. Ninety-sec irradiation in PM medium at 37 cm from a 15-watt GE Sterilamp decreases the titer about 3 log.

Donor phenotype selection and measurement of cotransduction frequency: Cotransduction frequencies (CTF) were measured as suggested by Yanofsky and Lennox.[15] Phages were grown on the donor cells to give an increase in phage titer of 10^6 or more. The recipients were plated on minimal medium with supplements to allow phenotype expression of the donor but not of the recipient. The transductants were scored, then restreaked on minimal and supplemented media to verify their properties.

Results.—Three types of transduction experiments have been carried out in order to confirm the existence of genetic transfer in these fluorescent pseudomonads and to establish the generality of the mechanism for interstrain gene transfer. These are: (1) analysis of the tryptophan biosynthetic pathway,

(2) studies of the linkage of camphor catabolic pathway loci, and (3) interstrain transfer of the genes of the mandelate degradative pathway.

Transduction of tryptophan genes: The tryptophan biosynthetic pathway, ascribed to *E. coli* as well as to other organisms,[15] has been shown in our earlier experiments[16] with *Pseudomonas putida* to consist of at least six separately measurable enzyme activities. Since it seems likely that all of the chemical steps have now been identified, with the possible exception of the conversion of chorismate to anthranilate, the structural genes of *Pseudomonas putida* have been labeled in accordance with the suggestion of Demerec *et al.*[11] beginning with anthranilic synthase and continuing to the tryptophan synthase B subunit. The genetic experiments described here demonstrate at least six loci for this pathway. The mutants used and their phenotypic properties are listed in Table 1.

The data in Table 3 indicate three linkage groups for the six known genes cod-

TABLE 3. *Linkages of tryptophan genes in P. putida transduction with phage pf16h1.*

Donor	Recipient	Transductants* (mutant/total)	Cotransfer frequency	Linkage
trpA1	*trpB11*	462/480	0.96	*trpAB*
"	*C22*	0/256	0	
"	*D31*	285/307	0.93	*AD*
"	*E41*	0/85	0	
"	*F61*	0/173	0	
trpB11	"	0/197	0	
C22	"	0/108	0	
D31	"	0/106	0	
E41	"	52/97	0.53	*EF*

* Selection with *trpA* donor on anthranilate, others on indole. Self-crosses yield rate of reversion.

ing for the proteins functional in tryptophan biosynthesis. Cotransduction frequencies for the *A*, *B*, and *D* loci indicate close linkage. The frequencies are comparable to those reported in *E. coli*,[15] where the loci are clustered in one segment of the chromosome. The two genes coding for tryptophan synthase A and B subunits are also closely linked; locus *C* does not cotransduce with any of the other five loci.

The chromosome of *Pseudomonas aeruginosa* has been shown by Fargie and Holloway[3] to contain *trp* loci that are not cotransduced. One of these, the gene coding for phosphoribosyl transferase, may be linked to resistance markers for streptomycin or *p*-fluorophenylalanine. To determine if a similar chromosomal arrangement is present in *P. putida*, linkage relationships between several *fpa* and *str* mutants and the *trpABD* were examined. These data are shown in Table 4. Both *fpa-1* and *str-1* mutations are cotransducible with *trpABD* but not with *trpC* or *trpEF*. In addition to *fpa-1*, other Fpa markers have been found which are not cotransducible with the *trpABD* cluster. All of the Str mutants mapped cotransduce at about 20 per cent with individual loci of *trpA*, *B*, or *D*. Further data and the fine structure mapping of the tryptophan genes will appear in a subsequent publication.

Gene linkage in the camphor degradative pathway:[17] The early steps of the camphor catabolic pathway are shown in Figure 2. The enzymes required for these

TABLE 4. *Linkage of streptomycin and fluorophenylalanine resistance loci to tryptophan cluster 1 (trpABD).*

Donor	Recipient	Clone type	Cotransfer frequency*	Linkage
trpA1	*trpB11*	*tr*	0.97	*trpAB*
	D31	"	0.93	*AD*
	D631	op†	0.88	*AD*
	D632	"	0.80	*AD*
pa-1	*trpA1*	tr	0.40	*A·fpa*
	A601	op†	0.55	*A·fpa*
	B11	tr	0.38	*B·fpa*
	C22	tr	0	
	F61	"	0	
str-1	*trpA1*	tr	0.21	*A·str*
	B11	"	0.20	*B·str*
	D31	"	0.16	*D·str*

* Selection for donor pheotype, with *trpA* on anthranilate, resistance markers on minimal (for *trp*+).

† Donor loci from tr cells with phage pf16h1; with this phage, opaque (op) colony serves as recipient only.

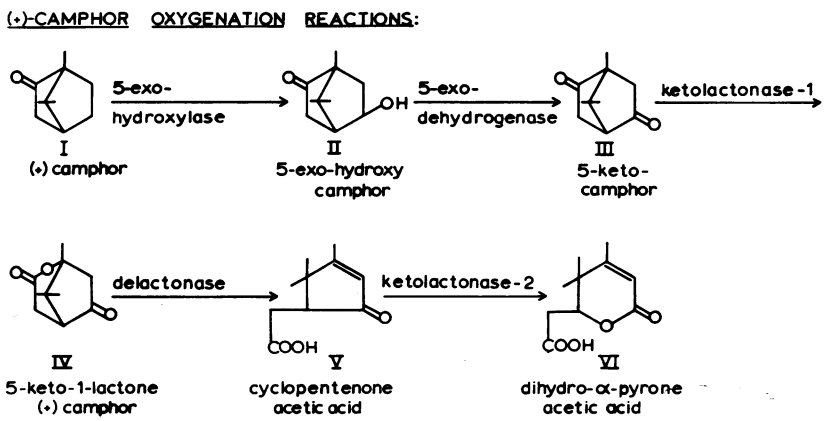

FIG. 2.—Pathway of (+)-camphor oxidation in *P. putida*.

five reactions are induced by all six of the substrates shown, several in a coordinate manner.[8] Mutants in this pathway, prepared by nitrosoguanidine mutagenesis, are transducible with the prototrophic C1tr-grown phage as donor. Two mutants, *cam-1* and *cam-2*, blocked, respectively, before and at some point beyond the fifth step, have been employed to confirm the possibility of analyzing the organization of this peripheral oxidation pathway by transduction. Very close linkage is observed with *cam-1* as donor and *cam-2*[17] as recipient and selecting for the wild-type allele of *cam-2* on plates containing compound V, Figure 2, as carbon source. Further, the Fpa markers known to be cotransduction with the tryptophan gene cluster (*trpABD*) show a low but definite cotransduction frequency of 8–9 per cent to *cam-2* and a lower but significant one to *cam-1* (Table 5). One of the Fpa markers which does not cotransduce with *trpABD* does not show linkage to either of the two *cam* markers.

TABLE 5. *Linkage of camphor oxidation and fluorophenylalanine resistance loci.*

Donor	Recipient	Select	Transductants (nonsel+/ total)	Cotransfer frequency	Linkage
*cam-1**	*cam-2*†	Cam·2+	39/45	0.87	*cam-1·cam-2*
fpa-1	"	Cam+	6/74	0.08	*fpa-1·cam-2*
"	"	Fpar	6/64	0.09	" "
"	*cam-1*	Cam+	3/138	0.02	*fpa1·cam-1*

* *Cam-1* lacks hydroxylase activity, step 1, Fig. 3, thus does not grow on (+)-camphor, but grows on the 5-exo-alcohol (II), diketone (III), and cyclopentenoic acid (V).

† *Cam-2* does not grow on any of the above compounds but does grow on the ultimate products isobutyrate and acetate, thus lacks late enzyme(s).

Interstrain transfer of genes for mandelate oxidation: *Pseudomonas putida* strain A3.12 (stock PRS1) oxidizes mandelate, benzoate, and *p*-hydroxybenzoate.[5, 6] The regulation, especially of the β-ketoadipate pathway, has also been elucidated.[9, 10]

The host-range phage mutant h2 permitted us to examine the linkage patterns of these aromatic pathways and to attempt interstrain gene transfer between A3.12 and the camphor-degrading strain C1. Ornston[10] demonstrated that C1tr (PUG2, Table 1) lacks one enzyme in the *p*-hydroxybenzoate pathway, i.e., β-carboxy-*cis*-*cis*-muconic acid-lactonizing enzyme (CMLE), which converts muconate to γ-carboxymuconolactone (see Fig. 3). Strain PUG2 also will not grow on D- or L-mandelate nor on benzoyl formate, but does grow on benzoate.

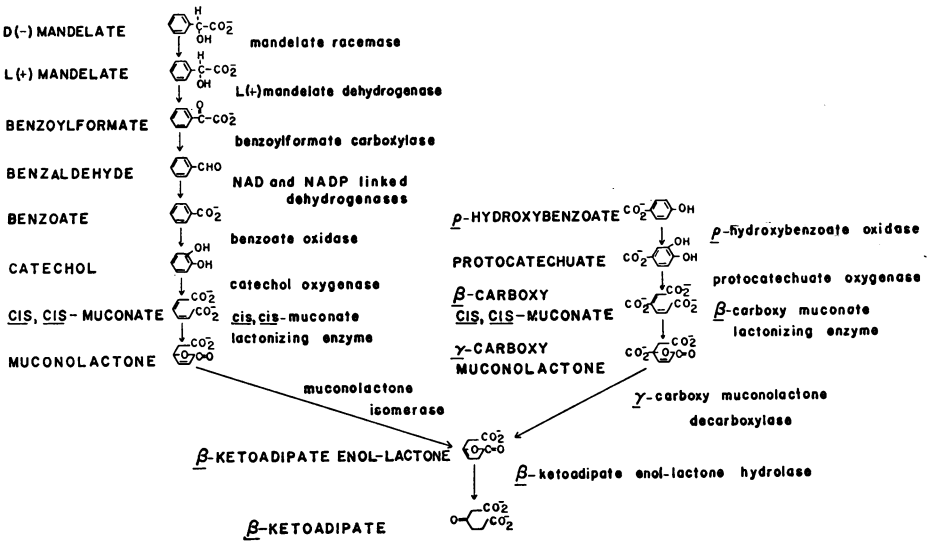

FIG. 3.—Coordinately induced enzymes of mandelate-benzoate pathway in *P. putida* (after Ingraham, *J. Bacteriol.*, 94, 1970 (1967)).

Phage pf16h2 grown on PRS1 was used to transfer these genes to PUG2, with selection on either DL-mandelate or on *p*-hydroxybenzoate (Table 6). Transductants were obtained in each case, none of which grew when tested on the substrate for the nonselected marker. Thus in strain PRS1, the genes for the

TABLE 6. *Interstrain transfer by phage pf16h2 of coordinately expressed mandelate genes.*

Strains		Marker		Transductants
Donor	Recipient	Select	Nonsel	(nonsel$^+$/total)
PRS1	PUG2	Mand*	POB$^+$	0/76
"	"	POB$^+$	Mand$^+$	0/118
"	*ASmd1B1002*†	"	"	0/56

* Mand$^+$ grows on L-mandelate; POB$^+$ grows on *p*-hydroxybenzoate.
† PUG628, see Table 1.

mandelate pathway are not closely linked to those for CMLE in the *p*-hydroxybenzoate pathway. These data show, in addition, that PUG2 is capable of accepting genetic material from an independently isolated heterologous fluorescent pseudomonad to which it is closely related.

The levels of the four enzymes in the mandelate cluster were analyzed in strains PRS1, PUG2, and in selected mutants and transductants (Table 7). Strain

TABLE 7. *Mandelate enzyme levels of transductants PRS1 (A3.12) to PUG2(C1S) by phage pf16h2.*

	Strain or Derivation				
	PRS1	PUG2	AS*	PRS3†	PUG628‡
Enzyme	(Units/mg protein: Unit, nmole min^{-1})				
Racemase	110	0	125	110	90
L-Mandelate DeH_2	2160	0	760	0	0
Benzoylformate $DeCO_2$	1680	0	1900	1700	3200
Benzaldehyde DeH_2	1400	0	400	1100	1600

* Transductant PRS1 × PUG2.
† PRS3, mandelate dehydrogenase mutant *md1B1002*.
‡ PUG628, transductant PRS3 × PUG2.

PUG2 was found to lack all four activities and the cross PRS1 × PUG2 to cotransfer all four. Not only are the mandelate enzymes absent from fully induced PUG2 cells, but also CRM was not detected with antibodies directed against two of the enzymes purified from PRS1.[18]

A mutant of PRS1, lacking the mandelate dehydrogenase function (PRS3, *md1B1002*) when crossed with PUG2 and transductants selected on benzoyl formate, gave cells containing the three other enzymes of the mandelate cluster (Table 7, columns 4 and 5). Thus we deduce that a rather large DNA fragment containing at least four genes functional in conversion of mandelate and benzoate is transferred from PRS1 → PUG2 in transduction. The linkage of mandelate genes and nonlinkage to the *CMLE* gene correlates with the finding of the separate regulatory control.[10] Nearly all the transductants from the cross PRS1 × PUG2 are inducible for the mandelate enzymes. Whether the regulatory genes are cotransduced with the structural genes or are already present in PUG2 is still not clear. Detailed results concerning genetic mapping of the mandelate and other aromatic pathways will be the subject of a future communication.

Precedence for cotransfer of as many as four genes is found in *E. coli* using phage P1.[19] In regulation, the *Shigella Sh* was shown to contain the i^+ gene for inducibility of β-galactosidase without carrying a functional *lac* gene.[20]

Summary.—A generalized transducing phage has been obtained for the soil and water pseudomonads now classed as *Pseudomonas putida*, biotype A. For the

tryptophan biosynthetic enzymes, preliminary genetic analysis has revealed three gene clusters corresponding to the regulatory groups we previously observed in this organism. Such organization is in contrast to the suggestion of scattered loci for *Pseudomonas aeruginosa* and a single operon observed in the enteric bacteria. Similarities in chromosomal organization of *Pseudomonas putida* and *Pseudomonas aeruginosa* have been observed for a limited number of loci. The bicyclic monoterpene oxidation system, a complex, specific, inducible pathway, has been shown to be susceptible to genetic analysis by transduction. Streptomycin and *p*-fluorophenylalanine resistance loci have been mapped adjacent to the *trpABD* cluster of the tryptophan biosynthetic pathway and the *fpa* marker found to be linked to two genes of the camphor pathway. Interstrain gene transfer of a coordinately induced set of four enzymes functioning in the degradation of mandelate has been achieved. These genes appear to be linked closely to form a cluster.

Of most general importance, these findings indicate that the large body of work on the chemistry and regulation of the complex inducible pathways of peripheral metabolism in the fluorescent pseudomonads may finally be subject to genetic analysis.

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[18] George Hegeman performed these immunological tests; we wish to express our appreciation to him for making the data available to us.

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