# FINE STRUCTURE MAPPING OF THE TRYPTOPHAN GENES IN *PSEUDOMONAS PUTIDA*[1]

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WE have used the tryptophan pathway to develop a gene transfer system via transducing bacteriophage in *Pseudomonas putida* (CHAKRABARTY, GUNSALUS and GUNSALUS 1967). The tryptophan pathway itself has proved interesting in the number and nature of its enzymes, their mode of regulation, and the organization of the genes in the bacterial chromosome. Enzymatic analysis of extracts of prototrophic and auxotrophic strains grown on limiting and excess tryptophan levels has demonstrated six enzymatic activities under three types of regulation (CRAWFORD and GUNSALUS 1966). The reactions and gene designations are shown in Figure 1. Assignation of letters for the individual genes follows the suggestion of DEMEREC, *et al.*, (1966).

Three early enzymes, anthranilate synthase (AS), phosphoribosyl transferase (PRT), and the condensing enzyme for indole ring closure, indole-glycerol phosphate synthase (InGPS), are under repression control; they are produced in increased amounts on exhaustion of tryptophan, though not necessarily in a coordinate manner. The AS is feedback inhibited by tryptophan (QUEENER and GUNSALUS 1968). The genes controlling the synthesis of these enzymes (*trpA* (AS), *trpB* (PRT), and *trpD* (InGPS), are closely linked by transduction (CHAKRABARTY, GUNSALUS and GUNSALUS 1967, 1968).

A first estimate of the size of the catalytically active proteins was obtained by molecular sieve filtration on Sephadex G-100 (ENATSU and CRAWFORD 1968). The PRT and InGPS activities were recovered in better than 80% yield with retention volumes indicating respective molecular weights of 64,000 and 32,000. The recovery of AS activity rarely exceeds 20% and has subsequently been shown to require two protein fractions, separable from the other tryptophan enzymatic activities, with molecular sizes of about 17,000 and 80,000 estimated from a Sephadex G-100 column (QUEENER and GUNSALUS 1968). Of ten AS mutants examined, all lacked activity for the larger component. There is evidence for an intermediate between chorismate and anthranilate, based in part on the activity of the two enzyme components when separated by a dialysis membrane. SOMERVILLE and ELFORD (1967) have also presented evidence for several

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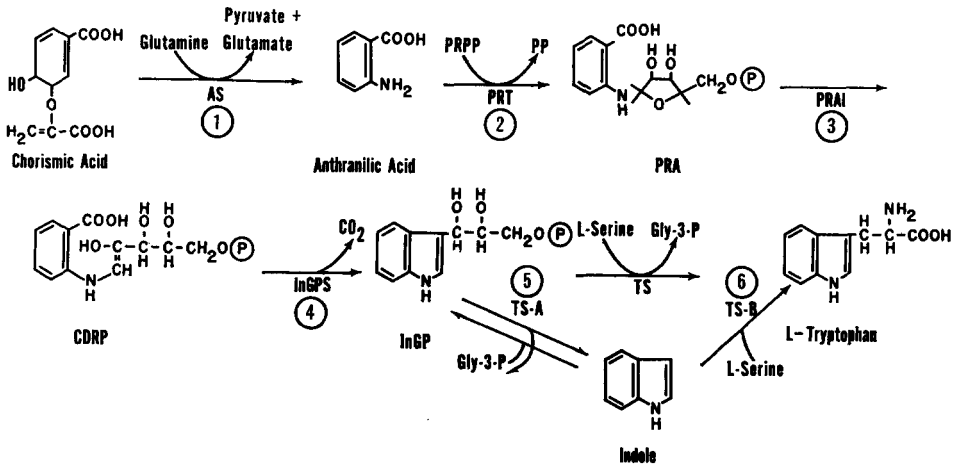


FIGURE 1.—Tryptophan biosynthetic pathway and gene designations in *Pseudomonas putida*. Chorismic acid is the last intermediate common to the synthesis of aromatic amino acids. Abbreviations: AS, anthranilate synthase (*trpA*); PRT, phosphoribosyl transferase (*trpB*); PRAI, phosphoribosyl anthranilate isomerase (*trpC*); InGPS, indoleglycerol phosphate synthase (*trpD*); TS-A, tryptophan synthase A protein (*trpE*); TS-B, tryptophan synthase B protein (*trpF*).

types of AS mutants and a reaction intermediate in *Escherichia coli*. Similarly, evidence is accumulating that the chorismate to p-aminobenzoate conversion in yeast and in *E. coli* is multistep (HENDLER and SRINIVASAN 1967; HUANG and PITTARD 1967). In the present manuscript, AS refers to the overall conversion indicated by step 1 in Figure 1; the AS mutants used lack activity for the larger component (QUEENER and GUNSALUS, 1968).

The enzyme for the third step shown in Figure 1, phosphoribosyl isomerase (PRAI) which converts anthranilate-N-riboside-5-phosphate to the deoxyribulotide, can be measured by the fluorescence assay reported earlier (CRAWFORD and GUNSALUS 1966). In the first experiments the levels of this activity were essentially invariant. By gel filtration the size of PRAI is about 39,000 (ENATSU and CRAWFORD 1968). The gene (*trpC*) controlling this enzyme is unlinked to other tryptophan loci in our transduction experiments (CHAKRABARTY *et al.* 1968).

The synthesis of tryptophan from indoleglycerol phosphate has been shown in *E. coli* to require two proteins, tryptophan synthase subunits A and B (TS-A; TS-B). The two subunits can be assayed by reactions 5 and 6, Figure 1. In *P. putida* also two proteins are required for tryptophan synthase activity; they resemble the *E. coli* subunits in size but are more readily dissociable (ENATSU and CRAWFORD 1968). The genes for the two proteins (*trpE* for component A and *trpF* for component B) are cotransducible with a high degree of linkage: they are not cotransduced with the other tryptophan structural genes (CHAKRABARTY, *et al.* 1968).

The regulation of the tryptophan synthase proteins was found to be unique,

for they are strongly induced by indoleglycerol phosphate and poorly or not at all by indole (CRAWFORD and GUNSALUS 1966). This mode of regulation is reflected in the lack of growth of most mutants on indole. Two mutant classes able to grow on indole have been recognized; one is defective in TS-A activity (step 5 in Figure 1) and the other is constitutive for the TS-A and TS-B activities (CRAWFORD and GUNSALUS 1966).

This paper demonstrates the suitability of the phage pf16-*P. putida* transducing system for fine structure mapping and assigns an order and spacing to the genes in the first tryptophan linkage group. The regulatory locus conferring constitutivity to TS-A and TS-B is shown to be cotransduced in high frequency with the gene for TS-A. Three non-tryptophan loci, resulting in requirements for methionine, adenine and leucine, are shown to be cotransduced with the PRAI (*trpC*) locus.

These gene positionings are very similar to those found in *Pseudomonas aeruginosa* (FARGIE and HOLLOWAY 1965; WALTHO and HOLLOWAY 1966); they are considered in relationship to the modes of regulation of enzyme formation found in this genus.

## MATERIALS AND METHODS

*Pseudomonas putida* strain C1 was isolated from a camphor enrichment culture (BRADSHAW *et al.* 1959), and has been assigned to biotype A of the species (STANIER, PALLERONI and DOUDOROFF 1966). The original isolate (ATCC 17453) forms opaque (op) colonies; a translucent colony variant C1tr (ATCC 23287) differs in phage sensitivity (CHAKRABARTY, NIBLACK and GUNSALUS 1967). Both variants are prototrophs. Phage pf16 can transduce markers within the C1op group; a host range variant, pf16h1, can transduce markers within the C1tr group or from a C1tr donor to a C1op recipient. Auxotrophic and resistance markers were isolated from these strains spontaneously or following mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (CRAWFORD and GUNSALUS 1966; CHAKRABARTY, *et al.* 1968). Table 1 presents the phenotypes and derivations of the strains used in this study along with their previous designations, if any. It should be noted that C1tr mutations are numbered below 500, C1op mutations above. Methods for determining the enzymatic defects in tryptophan auxotrophs were described earlier (CRAWFORD and GUNSALUS 1966).

*Phage production and transduction techniques:* The general methodology has been described (CHAKRABARTY, *et al.* 1968); care must be taken to prevent killing of the transduced cells by non-defective phage. For the transductions involving C1op donors and recipients, phage pf16 lysates were diluted to 2–5 $\times$ $10^9$ pfu/ml in PM medium (NIBLACK *et al.* 1968) and irradiated with a General Electric germicidal lamp to $10^{-3}$ survival. Equal amounts of irradiated lysate and an overnight (15 hr$\pm$) culture of recipient cells in L-broth (LENNOX 1955) were mixed, allowed to stand 20 min at room temperature, then centrifuged. Packed cells were resuspended to their original concentration in minimal medium (VOGEL and BONNER 1956) containing rabbit phage antiserum diluted to a K (first order velocity constant) of 8 to 10. Cells were spread immediately on minimal medium containing, where needed, the following supplements in $\mu$g/ml: L-tryptophan, 10; indole, 5; anthranilate, 5; L-methionine, 20; L-leucine, 40; dihydrostreptomycin, 1000, p-fluorophenylalanine hydrate, 1000.

*Determination of linkage by donor marker cotransfer:* In a transduction where donor and recipient were phenotypically distinguishable, cotransfer frequency (the proportion of recombinants showing the donor phenotype among the total transductants) was determined by replica plating. For example, phage grown on *met-601* were used to transduce various *trp* mutants for the ability to grow on methionine supplemented minimal agar. On replication to minimal agar,

TABLE 1

*Derivation and properties of* P. putida *strains used*

| Genotype | Phenotype[1] | Parent C1 | Derivation[2] |
|---|---|---|---|
| *trpA1* | AS⁻ | tr | NG on C1tr (previously S1)[4] |
| *A519, A521* | AS⁻ | op | NG on *met-601*; C1op × *met601trpA* |
| *A601, A602* | AS⁻ | op | NG on C1op |
| *B520, B611, B612, B613* | PRT⁻ | op | NG on *met-601*; C1op × *met-601trpB* |
| *C621, C622* | PRAI⁻ | op | NG on C1op |
| *D31, D32* | InGPS⁻ | tr | NG on C1tr (previously S31 and S32)[4] |
| *D518* | InGPS⁻ | op | NG on *met-601*; C1op × *met601trpD* |
| *D631, D632, D633, D634* | InGPS⁻ | op | NG on C1op |
| *E641* | TS-A⁻ | op | NG on C1op |
| *F651, F653, F661, F662* | TS-B⁻ | op | NG on C1op |
| *A1 trpX1, A1 trpX2* | AS⁻, TS const. | tr | S on *trpA1* (previously S1i4 and S1i5)[4] |
| *A601(trpX1), A601(trpX2)* | AS⁻, TS const. | op[3] | *trpA1trpX* × *trpA601trpE509* |
| *A601 trpB520 fpa-507* | AS⁻, PRT⁻, Fpa⁻ | op | *trpA601fpa507* × *trpB520* |
| *A601 trpD634(fpa-1)* | AS⁻, InGPS⁻, Fpa⁻ | op[3] | *trpA601(fpa-1)* × *trpD634* |
| *A601 trpE509* | AS⁻, TS-A⁻ | op | S on *trpA601* |
| *A601 fpa-507, trpA602fpa-507* | AS⁻, Fpa⁻ | op | *trpA* × *trpD633fpa-507* |
| *A601(fpa-1), trpA602(fpa-1)* | AS⁻, Fpa⁻ | op[3] | *fpa-1* × *trpA* |
| *D633 fpa-507* | InGPS⁻, Fpa⁻ | op | S on *trpD633* |
| *D634 fpa-507* | InGPS⁻, Fpa⁻ | op | *fpa-507* × *trpD634* |
| *D634(fpa-1)* | InGPS⁻, Fpa⁻ | op[3] | *fpa-1* × *trpD634* |
| *D31 fpa-1* | InGPS⁻, Fpa⁻ | tr | *fpa-1* × *trpD31* |
| *leu-501* | Leu⁻ | op | NG on *his-601; his-601leu-501* × *met-601trpC621* |
| *met-601* | Met⁻ | op | NG on C1op |
| *met-601 trpA519* | Met⁻, AS⁻ | op | NG on *met-601* |
| *met-601 trpA601, met-601trpA602* | Met⁻, AS⁻ | op | *trpA* × *met-601trpB520* |
| *met-601 trpB520* | Met⁻, PRT⁻ | op | NG on *met-601* |
| *met-601 trpD518* | Met⁻, InGPS⁻ | op | NG on *met-601* |
| *met-601 trpD633 fpa-507* | Met⁻, InGPS⁻, Fpa⁻ | op | *trpD633fpa-507* × *met-601* |
| *fpa-1* | Fpa⁻ | tr | S on C1tr |
| *fpa-507* | Fpa⁻ | op | *fpa-507str-503* × *trpD631* |
| *C1op(fpa-1)* | Fpa⁻ | op[3] | *fpa-1* × C1op |
| *fpa-1 str-1* | Fpa⁻, Str⁻ | tr | S on *fpa-1* |
| *fpa-507str-503* | Fpa⁻, Str⁻ | op | S on *trpD631; fpa-507* × *trpD631str-503* |

[1] Abbreviation: AS, anthranilate synthase, PRT, phosphoribosyl transferase; PRAI, phosphoribosyl-anthranilate isomerase; InGPS, indoleglycerol phosphate synthase; TS-A, tryptophan synthase A protein; TS-B, tryptophan synthase B protein; Leu, leucine; Met, methionine; Fpa⁻, p-fluorophenylalanine resistant (wild type is sensitive); Str⁻, dihydrostreptomycin resistant (wild type is sensitive).

[2] Mode of origin of mutations: S = spontaneous, NG = N-methyl-N′-nitro-N-nitrosoguanidine. Transductions: shown as donor × recipient.

[3] Hybrid containing chiefly the C1op genome; marker in parenthesis was transferred from C1tr along with an unknown amount of adjacent genetic material.

[4] Crawford and Gunsalus (1966) designation in biochemical studies.

methionine requiring recombinants were found only with *trpC* recipients. This method can be used when *trpA* (anthranilate utilizing) and *trpE* (indole utilizing) donors are transduced into other *trp* mutants; the extremely poor growth of *trpA*, *trpC* and *trpD* mutants on indole was previously reported (CRAWFORD and GUNSALUS 1966). In cotransfer experiments with *trp* mutants, prototrophs were scored by replication or by selection and streaking of transductant clones on supplemented and unsupplemented media to avoid the erroneous scoring caused by the accumulation of anthranilate by backgrounds of *trpB*, *C* and *D* cells and of indole by *trpF* cells. Such accumulations can obscure the requirements of transduced clones.

*Linkage estimation by reduction of frequency of prototrophic recombinants (prototroph depression method):* For greater precision in the estimation of distances between markers of the same phenotype, simultaneous transduction of a reference marker was used. For this purpose, a series of *trp* mutants was obtained in the *met*-601 background, and various *trp* mutants were used to transduce these double auxotrophs to methionine or to tryptophan independence. The ratio of $met^{-}trp^{+}$ to $met^{+}trp^{-}$ cells using wild type or unlinked *trp* donors was taken as unity; recombination distances between linked *trp* markers were expressed as a fraction of this unit. As with the first method, cross feeding between recipients and recombinants could simulate prototrophy; replication or selection and streaking was used in all questionable cases to avoid error. The method appeared to be most accurate with closely spaced markers (0.05 recombination units); analogous results were observed in similar experiments with *Escherichia coli* mutants (YANOFSKY *et al.* 1964).

*Positioning by three point tests:* Linked double mutant strains were prepared and conventional three point tests performed with markers which could be scored independently of the selection required to obtain recombinants. The frequency among the recombinants of the nonselected marker was used to assign an order to the three markers. The experiment was always done reciprocally and, whenever possible, in both coupling phases. When three linked markers could be scored independently, distances as well as order were ascertained by the method of WU (1966).

## RESULTS

The data presented in this paper extend our earlier demonstration of a genetic transducing system in *P. putida* to include fine structure mapping of the *trpABD* linkage group. In addition, several auxotrophic markers linked to the *trpC* locus are identified, and one class of regulatory mutations is shown to be linked to the *trpEF* gene cluster.

*The* trpABD *linkage group:* Table 2 shows cotransduction among the *trpABD* and *trpEF* mutations in the C1op background by use of the donor marker cotransfer method. Linkages of *trpA601* to *trpB520* and *trpD633* are somewhat lower (0.80 and 0.74) than previously reported for analogous markers in the C1tr system. CHAKRABARTY *et al.* (1968) found values of 0.96 and 0.93 for linkage of *trpA1* to *trpB11* and to *trpD31*. This difference may arise in part from methodology, for in the present system ultraviolet (UV) irradiation was used to decrease cell killing. The effect of this procedure on linkage estimation will be discussed more fully with the data in Table 3. The cotransfer of *trpE* with a *trpF* marker reported earlier in C1tr strains was 0.53. The data of Table 2 show a value of 0.62 by the replication method, but 0.92 when the transductants were picked, restreaked on indole-supplemented agar, and replicated to minimal agar. This discrepancy appears to arise from reversion to wild type among recipient cells being cross-fed on the transduction plates. Although additional adjustments in

TABLE 2

*Tryptophan gene linkages established by cotransfer*

| Recipient | Donor: *trpA601* Colonies[1] Total | Mutant | CTF[2] | Donor: *trpE641* Colonies Total | Mutant | CTF[2] |
|---|---|---|---|---|---|---|
| *trpA519* | ... | ... | ... | 57 | 0 | 0 |
| *B520* | 169 | 135 | 0.80 | 66 | 0 | 0 |
| *C621* | 25 | 0 | 0 | 66 | 0 | 0 |
| *D633* | 92 | 68 | 0.74 | 96 | 0 | 0 |
| *E641* | 26 | 0 | 0 | .. | . | .. |
| *F661* | 90 | 0 | 0 | 125 | 78 | 0.62 |
| *F661*[3] | ... | ... | ... | 100 | 92 | 0.92[3] |

[1] Selection with *trpA*⁻ donor on anthranilate, *trpE*⁻ on indole.
[2] CTF = cotransduction frequency.
[3] Transductants reselected on indole plates before testing; direct replication gives an erroneously low cotransfer frequency because of excessive feeding of background cells coupled with reversion.

technique may be required as experience is gained with this system, the data presented in Table 2 are representative of many similar experiments with other markers. We therefore consider these linkages, and their order of magnitude, established.

WALTHO and HOLLOWAY (1966) reported a linkage by transduction with phage F116 in *P. aeruginosa* strain 2 between a *trp* locus (the gene for PRT, which we have termed *trpB* in *P. putida*) and certain p-fluorophenylalanine and streptomycin resistant mutants. Our earlier paper (CHAKRABARTY, GUNSALUS and GUNSALUS 1968) demonstrated this linkage in *P. putida*; the *fpa* markers showed 0.5 and a *str* marker 0.2 cotransfer frequencies with *trpA* and *trpB* markers. Table 3 shows an extensive study of the linkage of two *fpa* markers to the *trpABD* region.

Three observations are pertinent when considering the effectiveness of our transducing system in linkage estimation. The *fpa-1* marker, originally isolated in C1tr, was transferred to the C1op cell type by transduction with pf16h1. When this marker, before or after transfer, is mapped along with the marker *fpa-507* isolated in the C1op cell type, no systematic difference in transfer frequencies is observed. Thus, the two cell types, opaque and translucent (see Table 1), appear to be genetically equivalent. Second, the linkage between *fpa* and *trp* depends upon the nature of the selection, whether for prototrophy or resistance. When selection is for *trp*⁺, as in our earlier study, a cotransfer of about 0.5 is observed, independent of the orientation of the non-selected marker. In contrast, selection for *fpa*⁻ (resistance) gives only about 0.15 cotransfer in one coupling phase and even less when wild type is transduced by phage grown on a double mutant. Several possible explanations for this result will be presented in the discussion. A third observation may be seen in the last section of Table 3. Two different

TABLE 3

*Linkage of* fpa *locus to the* trpABD *cluster*

| Parental strains | | Markers | | | | | |
|---|---|---|---|---|---|---|---|
| Donor | Recipient | selected *trp*+ | non-selected *fpa*- | CTF[1] | selected *fpa*- | non-selected *trp*+ | CTF[1] |
| *fpa-1* | *trpA601* | 278 | 115 | 0.41 | 154 | 17 | 0.11 |
| C1op(*fpa-1*)[2] | *trpA601* | 136 | 69 | 0.51 | 17 | 3 | 0.18 |
| *fpa-507* | *trpA601* | 204 | 87 | 0.43 | 135 | 20 | 0.15 |
| *fpa-1* | *trpD633* | 78 | 40 | 0.51 | 49 | 18 | 0.37 |
| C1op(*fpa-1*) | *trpD633* | 161 | 101 | 0.63 | 246 | 38 | 0.15 |
| *fpa-507* | *trpD633* | 62 | 36 | 0.58 | 56 | 7 | 0.13 |
| *fpa-1* | *trpD634* | 92 | 30 | 0.33 | 299 | 81 | 0.26 |
| C1op(*fpa-1*) | *trpD634* | 66 | 19 | 0.29 | 123 | 18 | 0.15 |
| *fpa-507* | *trpD634* | 85 | 34 | 0.40 | 226 | 29 | 0.13 |
| *fpa-1* | *trpD32* | 340 | 162 | 0.48 | 184 | 59 | 0.32 |
| | | *trp*+ | *fpa*+ | | *fpa*-[3] | *trp*- | |
| C1op | *trpA601(fpa-1)*[2] | 101 | 58 | 0.57 | 208 | 5 | 0.02 |
| C1op | *trpA602(fpa-1)*[2] | 98 | 46 | 0.47 | 95 | 5 | 0.05 |
| C1op | *trpA602fpa-507* | 101 | 50 | 0.50 | 131 | 2 | 0.02 |
| C1op | *trpD632(fpa-1)*[2] | 134 | 95 | 0.71 | 182 | 13 | 0.07 |
| C1op | *trpD634fpa-507* | ... | .. | ... | 71 | 5 | 0.07 |
| C1tr | *trpD31fpa-1* | 102 | 54 | 0.53 | 32 | 1 | 0.03 |
| | | *trp*+ | *fpa*+ | | *fpa*- | *trp*+ | |
| *fpa-1* | *trpD634* | 67[4] | 45 | 0.67[4] | ... | ... | ... |
| *fpa-1* | *trpD634* | 82 | 26 | 0.32 | ... | ... | ... |
| *fpa-507* | *trpD634* | ... | ... | ... | 175[4] | 35 | 0.20[4] |
| *fpa-507* | *trpD634* | ... | ... | ... | 2295 | 280 | 0.12 |

[1] CTF = cotransfer frequency.
[2] Hybrid strain; marker in parenthesis was transferred from tr to op by transduction.
[3] Donor and recipient reversed for these experiments.
[4] Phage not subjected to ultraviolet inactivation before transduction.

transductions were compared with and without prior UV irradiation of the phage lysate. In both instances the cotransfer frequency was reduced about 50% by UV irradiation. Similar effects of UV irradiation on cotransduction frequencies have been seen in other systems, e.g. BENZINGER and HARTMAN (1962).

*Three point tests for gene order in the* trpABD *region:* Ordering of the p-fluorophenylalanine resistance markers *fpa-1* and *fpa-507* relative to the *trpA* and *D* cistrons is shown in Table 4. Streptomycin resistance is considered in Table 5, and the gene order among the *trpABD* cistrons is determined in Table 6. All of the results indicate the order *fpa—trpA—trpB—trpD—str.*

Although *fpa* appears to be too far from *trpA, B,* and *D* for optimal three point tests, the experiments in Table 4 are consistent only with the order *fpa-trpA-trpD.* We observed no significant difference between *fpa-1* and *fpa-507.* The result from cross 5 in Table 4 suggests the order *fpa—trpA519—trpA601.* The second portion of the table shows two experiments which allowed complete scor-

TABLE 4

*Three point tests with* fpa *and* trpABD

| Cross No. | Strain I | Strain II | I as Donor: Markers selected *trp*+ | I as Donor: Markers non-selected *fpa*+ | I as Donor: Frequency of donor allele | II as Donor: Markers selected *trp*+ | II as Donor: Markers non-selected *fpa*− | II as Donor: Frequency of donor allele |
|---|---|---|---|---|---|---|---|---|
| 1 | *trpD633* | *trpA601 fpa-507* | 71 | 17 | 0.24 | 179 | 22 | 0.12 |
| 2 | *D634* | *trpA601(fpa-1)*[1] | 424 | 105 | 0.25 | 102 | 4 | 0.04 |
| 3 | *D632* | *trpA601 fpa-507* | 125 | 34 | 0.27 | . . . | . . | . . . |
| 4 | *A601* | *trpD633 fpa-507* | 51 | 6 | 0.12 | 171 | 37 | 0.22 |
| 5 | *A519* | *trpA601 fpa-507* | 66 | 6 | 0.09 | . . . | . . | . . . |

| | | | II as Donor: Selected | II as Donor: Non-selected | | | |
|---|---|---|---|---|---|---|---|
| 6 | *trpD634* | *trpA601(fpa-1)*[1] | *fpa*− 846 | *trpD*− 782[2] | *trpA*− 29 | *trpA*−*trpD*− 26[2] | wt 9 |
| 7 | C1op | *trpA601 trpD634(fpa-1)*[1] | *fpa*− 1447 | wt 1404 | *trpA*−*trpD*− 27 | *trpA*− 15 | *trpD*− 1 |

[1] Hybrid strain; marker in parenthesis was transferred from tr to op by transduction.
[2] Calculated from an analysis of a random sample of 62 of the 808 anthranilate non-utilizers, 60 of which accumulated anthranilate.

ing of recombinant genotypes. These conform to the Wu (1966) case of selection for an outside marker and confirm the order suggested by the *trp*+ selection experiments.

Two streptomycin resistance markers linked to the *trpABD* region, *str-1* from C1tr and *str-503* from C1op, occupy positions well removed from these *trp* genes and on the opposite side from *fpa* (Table 5). The infrequent cotransfer of *str* and *fpa* is apparent with both center and outside marker selection. The two experiments selecting *fpa*− conform to the Wu case of outside marker selection, establishing the order *fpa—trpAD—str*. These results are consistent with our earlier cotransfer experiments (Chakrabarty *et al.* 1968) and with several other unpublished three point tests. Again, experiments in the C1tr and C1op cell types give comparable map distances and ordering. We have calculated the values $\alpha$ and $\beta$ (Wu 1966) for the experiments of Table 5. The distance $\alpha$ (the proportion of the average transducing segment lying between *fpa* and *trp*) was 0.25 for

TABLE 5

*Three point tests with* str, fpa *and* trpABD

| Donor | Recipient | Selected *trp*+ | Markers Non-selected *fpa*+*str*+ | *fpa*−*str*+ | *fpa*+*str*− | *fpa*−*str*− |
|---|---|---|---|---|---|---|
| *fpa-1 str-1* | *trpA1* | 511 | 146 | 254 | 97 | 4 |
| *fpa-507 str-503* | *D631* | 425 | 269 | 126 | 26 | 4 |
| | | *fpa*− | *trp*−*str*+ | *trp*+*str*+ | *trp*+*str*− | *trp*−*str*− |
| *fpa-1 str-1* | *trpA601* | 82 | 61 | 19 | 2 | 0 |
| *fpa-507 str-503* | *D631* | 195 | 160 | 34 | 1 | 0 |

TABLE 6

*Three point tests within the* trpABD *cluster*

| Strain I | Strain II | I as Donor Markers Selected ($B^+D^+$) | I as Donor Markers Non-selected $A^+$ | I as Donor Frequency of donor allele | II as Donor Markers Selected ($B^+D^+$) | II as Donor Markers Non-selected $A^+$ | II as Donor Frequency of donor allele |
|---|---|---|---|---|---|---|---|
| *trpD634* | *trpA601 trpB520 fpa-507* | 17[1] | 15 | 0.88 | 34[2] | 26[2] | 0.23 |
| *D631* | *trpA601 trpB520 fpa-507* | 13 | 13 | 1.00 | 11 | 11 | 0 |
| *D633* | *trpA601 trpB520 fpa-507* | 54 | 54 | 1.00 | 39 | 38 | 0.03 |
| *D518* | *trpA601 trpB520 fpa-507* | 48 | 48 | 1.00 | . . | . . | . . . |
| *trpB520* | *trpA601 trpD634(fpa-1)* | 176[3] | 55 | 0.30 | 19[4] | 3[4] | 0.84 |

[1] Data in this column corrected for 96 reversions to *trpA601 trpB*+ on a total of 52 plates in the four experiments.

[2] Data in this column corrected for 6, 20 and 76 reversions to wild type on the 6, 5 and 12 plates in each experiment.

[3] Corrected for 12 *trpA601 trpB*+ revertants on 8 plates.

[4] Corrected for 4 wild-type revertants on 4 plates.

*trp*$^+$ selection and 0.41 for *fpa*$^-$ selection. The $\beta$ parameter (corresponding to the *trp—str* distance) was calculated to be 0.48 for *trp*$^+$ selection and 0.36 for *fpa*$^-$ selection. These values indicate that *fpa* and *str* lie near the ends of any transducing segment carrying both markers.

Two double auxotrophs were constructed for the purpose of distinguishing unambiguously between the order *trpABD* and *trpADB*. The data shown in Table 6 established the order *trpABD*.

*Fine structure mapping of the* trpABD *genes:* Typical values obtained by measuring prototrophic recombinant frequencies in the *trpA*, *B*, and *D* cistrons are shown in Table 7. Double mutants, *met-601 trp*$^-$, were used as recipients for phage grown in 12 *trpA*, *B*, or *D* strains. All values were corrected for the ratio of *trp*$^+$ to *met*$^+$ transductants observed with the wild-type donor. From these experiments we have constructed the linkage map shown in Figure 2. Additivity of map distances between the four *trpA* mutants is quite satisfactory; that between the *trpB* and *trpD* strains is less so and is complicated in some instances by unexpectedly low recombination values. In view of this, we consider the order we have assigned to markers within the *trpB* and *D* cistrons to be tentative. Several of the map distances between *trpA* and *trpB* or *D* mutants are also shown in Figure 2. The twelve mutants tested appear to form two subclusters, one for the four *trpA* mutants and one for the *trpB* and *trpD* mutants; the distance between the two sub-clusters is great enough to evoke inconsistencies in the data (see METHODS). It seems possible that a segment of unmarked genetic material lies between the *trpA* and *trpB* cistrons. If such a segment exists, it is not clear whether tryptophan genes would be involved, or because of the type of chromosomal organization in this organism, whether other unrelated genes could be enclosed. One possible candidate, for which no evidence is yet available, would be the smaller component of anthranilate synthase observed by QUEENER and GUNSALUS (1968).

TABLE 7

*Linkage estimation in the* trpABD *cluster*

| Donor | Recipient: *met601trp⁻* *A602* | *A519* | *A601* | *B520* | *D518* | *D633* |
|---|---|---|---|---|---|---|
| C1op[1] | 35/61 (.57) | 569/678 (.84) | 563/820 (.69) | 1117/1597 (.70) | 400/548 (.73) | 512/765 (.67) |
| *trpA602* | 0/198 0 | 80/1595 .06 | 78/1593 .07 | 214/1210 .25 | 45/370 .17 | 96/649 .22 |
| *A521* | 2/120 .03 | 13/310 .05 | 14/323 .06 | 41/373 .16 | 25/199 .17 | 55/337 .24 |
| *A519* | 10/248 .07 | 0/869 0 | 23/1054 .03 | 140/1549 .13 | 37/491 .10 | 75/518 .22 |
| *A601* | 3/47 .11 | 4/210 .02 | 0/272 0 | 132/1056 .19 | 98/419 .32 | 13/93 .21 |
| *trpB520* | 8/66 .21 | 228/774 .34 | 152/680 .32 | 0/560 0 | 54/602 .12 | 14/661 .03 |
| *B611* | .. .. | 64/401 .16 | 36/357 .15 | 10/588 .02 | 53/500 .15 | 8/459 .03 |
| *B612* | .. .. | 63/346 .21 | 48/280 .25 | 4/417 .01 | 37/366 .14 | 3/402 .01 |
| *B613* | .. .. | 24/278 .10 | 27/267 .15 | 7/266 .04 | 26/422 .08 | 2/203 .01 |
| *trpD518* | 11/137 .14 | 17/306 .07 | 177/2498 .10 | 20/9754 .003 | 0/916 0 | 0/983 0 |
| *D633* | 26/128 .35 | 162/547 .35 | 145/729 .29 | 73/2095 .05 | 3/628 .007 | 0/571 0 |
| *D631* | 15/91 .29 | 87/273 .37 | 112/562 .29 | 55/1325 .06 | 26/951 .04 | 0/288 0 |
| *D634* | 4/35 .20 | 38/209 .21 | 83/309 .39 | 95/1310 .10 | 3/406 .01 | 0/306 0 |

[1] Data presented as the ratio of *trp*+ to *met*+ transductants.
[2] In each experiment with a *trp*⁻ donor, the *trp*+ to *met*+ ratio is followed by a mapping function corrected for the experiment with wild-type donor.

*The* trpC *linkage region:* The gene coding for PRAI (*trpC*) is unlinked to other tryptophan loci as shown both in earlier experiments (CHAKRABARTY *et al.* 1967, 1968) and in Table 2. FARGIE and HOLLOWAY (1965) showed linkage in *P. aeruginosa* between the locus for PRAI and methionine, adenine and leucine loci. In *P. putida, met-601* and *leu-501* are linked to the *trpC* locus (Table 8) but not

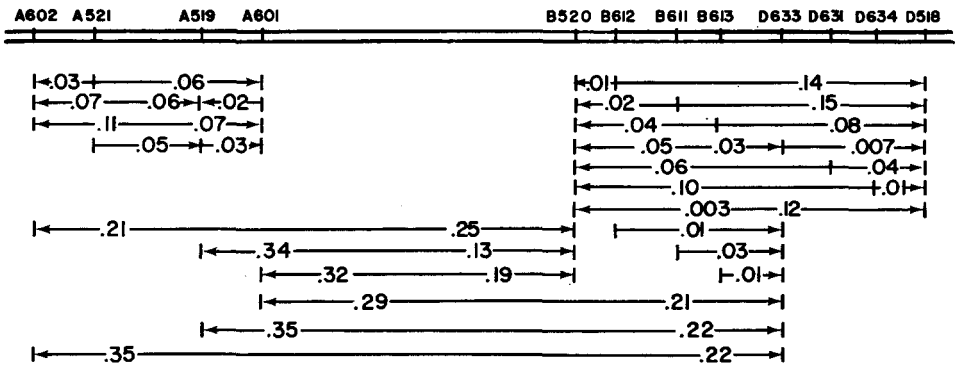


FIGURE 2.—Genetic map of the *trpABD* linkage group. Recipients, indicated by direction of arrow, contain the additional reference marker *met-601*. In reciprocal experiments the number nearest the arrowhead represents the measurement with that strain as recipient.

to other *trp* genes. Recently we have found an adenine requiring mutant (*ade-601*) which shows 50% cotransfer with *trpC621* and more than 90% cotransfer with *met-601*.

*The tryptophan synthase (*trpEF*) linkage group:* The linkage of the *trpE* and *F* cistrons by donor marker cotransfer, and the scoring problems encountered, were discussed in connection with Table 2. A related problem besets fine structure mapping by the prototrophic recombinant frequency technique, since *trpF* recipient cells excrete enough indole to feed *trpE* clones on minimal media. By streaking clones from transduction plates on indole plates before replication, this scoring problem was overcome. The prototrophic recombinant frequency method confirmed the close linkage of *trpE* and *F* markers shown by the last line of Table 2.

Our earlier studies demonstrated two ways in which a mutant blocked early in the pathway, such as *trpA1*, could mutate to indole utilization; i.e., by a structural mutation in *trpE*, the gene for TS-A, and by a regulatory mutation resulting in constitutive over-production of both subunits of tryptophan synthase. Two

TABLE 8

*Cotransfer of tryptophan genes with other auxotrophic markers*

| Donor | Recipient: *met-601* Markers Selected | Non-selected | CTF[1] | Recipient: *leu-501* Markers Selected | Non-selected | CTF[1] |
|---|---|---|---|---|---|---|
| | *met*+ | *trp*− | | *leu*+ | *trp*− | |
| *trpC621* | 39 | 29 | 0.74 | 139 | 106 | 0.76 |
| *C622* | 53 | 37 | 0.70 | ... | ... | ... |
| *D631* | 18 | 0 | 0 | 344 | 0 | 0 |
| *E641* | 60 | 0 | 0 | ... | ... | ... |
| *F653* | 358 | 0 | 0 | 200 | 0 | 0 |

[1] CTF = co-transfer frequency.

TABLE 9

*Cotransfer of* trpX *markers with the* trpEF *cluster*

| Donor | Recipient: *trpA601 E509* Recombinants[1] Indole utilizers | Total | CTF[2] |
|---|---|---|---|
| *trpA601(trpX1)* | 26 | 35 | 0.74 |
| *trpA601(trpX2)* | 34 | 41 | 0.83 |
| C1op | 0 | 20 | 0 |

[1] Recombinants selected on anthranilate supplemented plates.
[2] CTF = cotransfer frequency.

of the regulatory mutants studied earlier (CRAWFORD and GUNSALUS 1966), have been redesignated *trpX1* and *trpX2* (see Table 1). These mutations do not affect the ability of the strains containing them to grow on anthranilate or minimal medium, provided they are otherwise able to do so. Evidence for the linkage of *trpX1* and *X2* to the *trpEF* region of the chromosome was obtained in two ways. CHAKRABARTY and S. F. QUEENER used a modification of the whole cell assay of ITO and CRAWFORD (1965) to measure the indole to tryptophan reaction in transductants of strains *trpF651* and *F662*. Using *trpX1* and *X2* donors, the ratios of constitutive to total transductants were, respectively, 0.95 and 1 for *trpF651*, and 0.70 and 0.96 for *trpF662*. Neither the recipients nor spontaneous revertants showed constitutive activity levels. A more extensive experiment with conclusive evidence of linkage resulted from the transduction of a double mutant, *trpA601 trpE509*, to anthranilate utilization with phage pf16h1 grown on *trpA1 trpX1* and *trpA1 trpX2* cells. Thirty-five of 36 recombinants grew on indole as well as on anthranilate, showing that the *trpE* locus had been replaced by a chromosomal segment bearing the *trpX1* or *X2* marker. Table 9 shows a second transfer of the regulatory loci using as donors the *trpA601 (trpX1)* and *trpA601 (trpX2)* strains synthesized in the previous experiment. The two experiments, one involving *trpA1* and the other *trpA601*, differ only in the use of the h1 host range phage mutant in the first and pf16 phage with UV irradiation of the transducing lysate in the second. To avoid scoring confusion, the recombinants were purified by streaking on anthranilate-containing medium, then replicated to indole, anthranilate, and minimal plates. No colonies able to grow on minimal agar appeared. Controls without phage contained no *trpA601 trpE*$^+$ revertants. Indole-utilizing clones did not appear when phage grown on wild type was the donor. The results again demonstrate the close genetic homology between C1tr and C1op cells and confirm the close approximation of the *trpX* markers to the *trpEF* gene cluster. They do not show clearly whether *trpX* is genetically distinct from the structural genes *trpE* and *trpF*.

## DISCUSSION

Three advances in understanding the genetics of the fluorescent Pseudomonads,

their chromosomal organization, and the mode of regulation of their enzymes have been made in this study.

Transduction with phage pf16 has been shown to be usable in fine structure mapping, and an essential colinearity of the opaque and translucent cell types of *P. putida* C1, formerly called B and S (CHAKRABARTY, NIBLACK and GUNSALUS 1967), has been demonstrated.

Three linkage regions of genes for enzymes of the tryptophan synthetic pathway have been mapped. The group of genes (*trpA*, *B* and *D*) for the early enzymes, AS, PRT, and InGPS, is repressible; the group (*trpE* and *F*) for the late enzymes, tryptophan synthase A and B subunits, is inducible. A locus for constitutive production of these subunits is also closely linked to this gene cluster. A third group of loci, including the *trpC* gene for the isomerase (PRAI) which converts the N-ribotide of anthranilate to the deoxyribulotide, a *met* and a *leu* mutation, is unlinked to the areas concerned with the early or late enzymes. In early studies the levels of this enzyme were almost invariant and remained at a high level. More extensive studies of the regulation of this gene may provide clues to the advantages enjoyed by an organism which can dispose the genes of a single pathway into several linkage groups.

A close relationship in the chromosomal organization of *P. aeruginosa* and *P. putida* can be predicted from the linkage similarities observed so far. Admittedly, our selection of auxotrophs for mapping has been influenced by the earlier publications of HOLLOWAY and coworkers concerning linkages in *P. aeruginosa*. Improvement in the methodology for donor phenotype selection has reconciled one previous point of difference—the linkage of the genes for the tryptophan synthase subunits occurs in both species.

Preliminary studies of the size of the cell genome by $P^{32}$ labeling by the method of HERSHEY and MELECHEN (1957) suggest an amount of DNA per cell equal to or slightly larger than that of *E. coli*. Thus, a genome of 2 to 3 $\times$ $10^9$ daltons is indicated for *P. putida*. The chromosome of phage pf16 contains approximately $10^8$ daltons of double-stranded DNA (NIBLACK, *et al.* 1968). Assuming that a generalized transducing phage particle contains a continuous fragment of DNA, the maximum size of the transduced piece could be 3 to 5% of the total cell genome. Thus, about 30 linkage groups of the size of the *fpa—trpABD—str* segment whose mapping is reported in this paper might close the anticipated circular chromosome and place the linkage groups in a single continuous unit.

Evidence is accumulating that defective phages mediate in transduction with phage pf16, particularly in interstrain gene transfer. Segregating heterogenotes have been observed in the transfer of the mandelate gene cluster from *P. putida* strain A3.12 (PRS1) to the C1 strain (PUG2) (CHAKRABARTY, *et al.* 1968; CHAKRABARTY, MITTON and GUNSALUS 1968). Other mechanisms of gene transfer within this group of fluorescent Pseudomonads undoubtedly exist and will be validated. At present, however, it is clear that phage pf16 has sufficient capacity for effective genetic studies.

A striking discrepancy exists between the cotransfer values observed with *fpa*

and *trpABD* markers depending on the marker selected. Several explanations may be suggested for this phenomenon. The distribution of the markers in the transducing phage population may be non-random. For instance, many particles may carry *fpa* but not *trp*, whereas nearly all *trp*-containing particles also carry *fpa*. OZEKI's (1960) postulate of a preferential break point for the host chromosome might occur. In this case, if *fpa* were located near the end of the transducing fragment and *trp* near the middle, recombinants receiving *trp* would be less likely to obtain *fpa* than *vice versa*. Perhaps a more likely explanation is an alteration of the recombinational event in recipients exposed to p-fluorophenylalanine. The recipients are placed on selective media within 20 minutes of their exposure to phage. MUNIER and COHEN (1959) showed the incorporation of p-fluorophenylalanine into proteins in place of phenylalanine. Until the resistant phenotype develops, the recipient cells are likely to exhibit physiological abnormalities. A final decision among hypotheses must await the appearance of additional markers linked to *fpa* and the construction of suitable strains for genetic analysis.

Chromosomal organization in the fluorescent Pseudomonads clearly corresponds more closely to the situation in fungi than in enteric bacteria. Extensive single linkage groups, similar to those for the tryptophan synthetic pathway in *E. coli* (YANOFSKY and LENNOX 1959) and *Salmonella typhimurium* (BLUME and BALBINDER 1966) and the histidine pathway in *S. typhimurium* (AMES and HARTMAN 1962) have not thus far been observed in either *P. aeruginosa* or *P. putida* (FARGIE and HOLLOWAY 1965; MEE and LEE 1967; CHAKRARARTY, GUNSALUS 1968; and data presented here). Although *Neurospora crassa* (BARRATT *et al.* 1954) and *Aspergillus nidulans* (DORN 1967) also do not show marked clustering of the genes for biosynthetic pathways, their distribution of the genes controlling tryptophan synthetic enzymes does not correspond to that of the Pseudomonads. In Neurospora, Aspergillus and yeast, though the loci for AS, PRT and InGPS are not linked (AHMAD and CATCHESIDE 1960; ROBERTS 1967, MORTIMER and HAWTHORNE 1966), the active enzyme is an aggregate of polypeptide chains formed by two unlinked loci (DEMOSS and WEGMAN 1965; HÜTTER and DEMOSS 1967).

In the case of *P. putida*, our studies indicate three types of regulation of the synthesis of enzymes of the tryptophan pathway. Those enzymes sharing a common mode of regulation are clustered, possibly in operons. Our observations therefore extend the earlier ones of HOLLOWAY and coworkers in *P. aeruginosa*. Although the genes controlling an unbranched pathway are not all clustered in a single linkage group, neither are they scattered completely at random.

The pattern that we have described for the tryptophan genes in *P. putida* may be a general one for Pseudomonads. An extension of the mapping in *P. aeruginosa* by MEE and LEE (1967) indicates at least five gene clusters in the histidine pathway. Their preliminary evidence suggests the occurrence of two clusters, each containing genes for at least two reactions. A similar type of organization is suggested by PEARCE and LOUTIT's (1965) study of the isoleucine-valine pathway in

the same organism. Our early studies of the chromosomal organization of genes in degradative pathways (CHAKRABARTY, *et al.* 1967, 1968) also suggest clustering of genes for certain enzymes sharing a common regulatory mode, usually coordinate induction. Other enzymes involved in the same pathway, such as flavoproteins which play a role in electron transport, appear to be regulated differently and to be unlinked to the inducible clusters.

While it is too early to construct a detailed hypothesis of gene organization and regulation, the fluorescent Pseudomonads have shown that both induction and repression play a role in both catabolic and biosynthetic processes (GUNSALUS *et al.* 1967) and that some form of feedback inhibition of pre-formed enzymes also occurs. A systematic study of the chromosomal regions devoted to peripheral and essential catabolic processes in different strains and to the regulation of those genes for biosynthetic pathways which seem to be isolated within presumed regions for other biosynthetic pathways is now needed.

We wish to acknowledge the valuable technical help of VICKY HOULETTE in the isolation and characterization of mutants and the preparation of antisera used in these investigations.

## SUMMARY

We have investigated the chromosomal organization of *Pseudomonas putida* using transducing phage pf16 in experiments involving both cotransfer of specific markers and depression of prototrophic frequency. Six genes affecting tryptophan synthetic enzymes have been mapped in three linkage groups; these correspond to the regulatory groups established earlier by biochemical studies. The genes in the first group, *trpABD*, specifying AS, PRT, and InGPS, have been shown to occur in that order, with contiguity between *trpB* and *D* but a short distance separating the *trpA* and *B* markers. Loci for p-fluorophenylalanine and streptomycin resistance are linked to the *trpABD* cluster. The *fpa* cistron lies on the *trpA* side with about 50% cotransfer; *str* lies on the *trpD* side with about 20% cotransfer.—A second linkage group contains the gene for PRAI, *trpC*, surrounded by *met*, *ade*, and *leu* loci. The third linkage group contains the loci *trpE* and *F* for the A and B subunits of tryptophan synthase; these are closely linked, and a locus conferring constitutivity upon them also maps very near.—A considerable similarity exists in chromosomal organization between *P. putida* and *P. aeruginosa*. We confirm the findings of HOLLOWAY and coworkers that in these fluorescent Pseudomonads the genes for a given biosynthetic pathway are not contiguous. Our additional data do not, however, confirm HOLLOWAY's suggestion that the genes are randomly scattered; rather, we find them occurring in small clusters which correspond to the regulatory mechanisms observed for those portions of the biosynthetic pathway.

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