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Absence of clustering of functionally related genes in *Pseudomonas aeruginosa**

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1. INTRODUCTION

The system of sexual recombination which has been described for *Pseudomonas aeruginosa* (Holloway, 1955, 1956; Holloway & Fargie, 1960) has not proved particularly useful for detailed mapping purposes. This has been mainly due to the failure to find stable and usable Hfr forms, which have proved so valuable in genetic studies on *Escherichia coli*. However, transduction has been shown to be a delicate and precise tool for mapping bacterial genes, particularly in *Salmonella typhimurium* (see review by Demerec & Hartman, 1959). With any transduction system an important factor affecting its usefulness for mapping purposes is the length of chromosome transferred and this varies for different phages. Lennox (1955) using phage P1 in *E. coli* was able to demonstrate co-transduction of up to four markers, *thr*, *ara*, *leu* and *az*, and Yanofsky & Lennox (1959) used the co-transducing properties of this phage in their fine structure study of the genes concerned with tryptophan biosynthesis in *E. coli*.

Transduction has been shown to occur in *P. aeruginosa* with phages B3, B110 and F116 (Holloway & Monk, 1959; Holloway *et al.*, 1962). The response to radiation of the temperate *Pseudomonas* phage F116 suggested that it might be able to co-transduce linked bacterial markers at measurable frequencies and this was subsequently confirmed (Holloway *et al.*, 1962, 1963). These results indicated the suitability of this phage for a fine structure study of the *P. aeruginosa* chromosome. A feature of chromosome maps of other bacteria, namely *E. coli*, *S. typhimurium* and *Bacillus subtilis*, is the clustering of genes affecting sequential steps of certain biosynthetic or degradative pathways. This finding has been of great importance for recent theories on the genetic control of enzyme synthesis. Such a gene arrangement does not seem to exist in *P. aeruginosa*; genes concerned with related steps of biosynthetic pathways have not been found to show close linkage. Preliminary data for this view has already been published (Holloway *et al.*, 1963).

2. METHODS

Media: Nutrient Broth (NB): 1% beef extract, 1% peptone, 0·8% NaCl. Nutrient Agar (NA): NB solidified with 1·2% agar (Davis). Minimal Medium

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(MM): that of Vogel & Bonner (1956) solidified where necessary with 1·5% Difco agar. Enriched Minimal Medium (EMM): MM enriched by the addition of 2·5% NB.

General cultural procedures: The same as were used previously (Holloway, 1955; Holloway *et al.*, 1962).

Bacterial strains: all mutants were derived from *P. aeruginosa* strain 2 (originally LIII-3bi of Don & van den Ende, 1950).

Bacteriophage: F116: (Holloway, Egan & Monk, 1960).

Propagation of F116 was by the layer agar technique; phage assay and other techniques followed those of Adams (1959).

Bacterial auxotrophic mutants: these were produced using manganous chloride, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) or ethyl methane sulphonate (EMS) as mutagens. The procedure for manganous chloride has been described previously (Holloway, 1955). For MNNG, the bacteria as washed cell suspensions in citrate buffer pH 6·0, were exposed to 20μg./ml. MNNG for 1–2 hours at 37°C., after which the cells were centrifuged, resuspended in nutrient broth and grown overnight. With EMS, an overnight broth culture was exposed to 0·4% EMS for 1 hour at 37°C., then diluted 1/10 in fresh broth and grown overnight. In each case selection for mutants was made by penicillin treatment (Lederberg, 1950) and replica plating on complete and minimal medium. Mutants were first characterized for stability, and any showing a high rate of back mutation to prototrophy were discarded. Those remaining were characterized for their primary growth requirement. This was done by first adding solutions of amino acid, purine or pyrimidine mixtures dropwise to the surface of a minimal agar plate spread with a washed suspension of the unknown mutant. Precise growth requirements were then determined in a similar manner. In all, nearly 200 mutants were used and Table 1 shows the classification of the mutants on the basis of their primary growth requirement into fourteen groups. It can be seen that further classification into thirty-two loci was possible on the basis of biochemical and transduction tests. The biochemical tests employed were growth responses to known or suspected intermediates, cross-feeding tests, identification of accumulated products and enzymic studies. The method of growth response testing was that employed for the primary identification of mutant requirements, described above. Cross-feeding tests between mutants were done by seeding melted EMM held at 45°C. with a washed suspension of one mutant, and pouring a plate with this mixture. Drops of washed suspensions of the other mutants to be tested were then placed on the surface of the seeded agar. In this way, feeding of one mutant by another could be detected as growth of either the surface cultures or the embedded culture. Methods employed in accumulation and enzymic studies are described in the sections below dealing with particular mutant groups. The methods used in transduction tests are given below.

The mutant numbers given in Tables 2–11 refer to the acquisition numbers given to the mutants when isolated and are given here to facilitate reference in subsequent publications. Where such testing has enabled us to identify the precise

site of the mutant lesion in a biosynthetic pathway we have used the nomenclature of the current literature. Where such positioning was not possible, we have given arabic numbers to the various subgroups involved.

It is desirable to guard against the isolation and use of sibling auxotrophic mutants. This can normally be overcome by the use of the Adelberg & Meyers (1953) technique in which independently arising clones of mutants can be isolated by immobilizing them within an agar medium. This technique cannot be used for

Table 1. *Loci identified amongst* 176 *auxotrophic mutants of* P. aeruginosa *strain* 2

Group requirement	Loci	Basis for differentiation of loci
Arginine	*arg 1, arg 2, arg 3*	Transduction, growth requirements
Histidine	*his 1, his 2, his 3*	Transduction
Isoleucine + valine	*ilva 1, ilva 2*	Transduction
Leucine	*leu 1, leu 2*	Transduction
Methionine	*met 1, met 2a, met 2b*	Transduction, growth requirements, cross-feeding
Cysteine	*cys 1, cys 2*	Transduction, cross-feeding
Proline	*pro 1, pro 2, pro 3*	Transduction, cross-feeding
Tryptophan	*try 1, try 2, try 3bi, try 3bii*	Transduction, cross-feeding, growth requirements, enzymic studies
Adenine	*ade 1, ade 2, ade 3*	Transduction
Uracil	*ura 1, ura 2, ura 3*	Transduction
Serine	*ser*	
Lysine	*lys*	
Homoserine	*hom*	
Threonine	*thr*	

P. aeruginosa owing to the high concentrations of penicillin needed (15,000 units/ml.). To minimize the use of sibling mutants, the auxotrophs have been isolated from a number of independent experiments with different mutagens.

Transduction tests

In view of the large number of recombinant experiments to be carried out, a simplified transduction test was used. Phage F116 was propagated on one auxotrophic mutant and a preparation free from viable bacterial cells was obtained by chloroform treatment. The recipient auxotrophic bacterial strain was grown in broth overnight and 0·1 ml. spread evenly over the surface of an enriched minimal plate. A measured drop of the phage preparation was placed on the surface of the inoculated plate and allowed to dry without spreading. Twelve drops could be applied to the surface of one plate. If transduction occurred, up to 300 colonies appeared after 2 days' incubation at 37°C. in the area where the drop was applied. A comparison could be made on any one plate of the transduction frequencies of up to twelve different phage preparations with one recipient. The transduction frequency of any one phage preparation for a variety of receptor strains could also be compared on different plates inoculated with these receptor strains. It was found that the different classes of mutants showed characteristic and reproducible

frequencies of transduction. For example, *ilva* mutants had very high transduction frequencies, the *ade* mutants much lower.

Maximum transduction frequency of the recipient auxotrophs occurs with phage propagated on the prototrophic parent. If phage propagated on any mutants gives a transduction frequency similar to that given by the wild-type when spotted onto any given recipient, then it can be assumed that the two mutated sites concerned are not closely situated on the chromosome. If, on the other hand, there is a marked reduction of prototrophic formation when the phage propagated on one mutant is spotted on a recipient mutant, then this is evidence for co-transduction, and hence linkage, of the two markers concerned.

In some cases this test may not reveal existing linkages. Co-transduction at a low frequency may not result in sufficiently obvious reductions in prototroph production. Furthermore if, as Ozeki (1960) has shown, transduced chromosome fragments have predetermined ends, then obligatory breakage of the chromosome between two closely linked markers will mean that they cannot be co-transduced. We may thus need to distinguish between linkage of two markers on the chromosome and linkage as indicated by inclusion of the two markers in the same transduction piece.

Conclusive evidence for linkage comes from the donor phenotype selection test, as described by Clowes (1958), where co-transduction can be directly demonstrated. Such a test confirms the linkages established with the simplified transduction test, and thereby establishes the validity of the evidence for lack of linkage in the latter test. Confirmatory evidence for linkage can also come from sexual recombination studies.

It is obvious that where we are dealing with a donor and recipient having a mutation at the same locus we expect to observe greatly reduced or no transduction and thus we can identify different loci on the basis of this transduction test. The occurrence of low-frequency transduction between mutants of the same locus indicates that different sites within the locus are represented, and that mutants of one locus are not all siblings.

3. RESULTS

Our aim has been to distinguish differences in the various auxotrophic mutants isolated and to determine their linkage relationships. As described above, two main criteria have been used to recognize differences—biochemical, determined by the various tests outlined above, and genetic, as determined by transduction tests.

Two aspects of linkage have been investigated: firstly, the genetic relationships of the various markers of particular biosynthetic pathways and secondly, the grouping of markers of unrelated pathways.

I. *Linkage relationships of markers affecting related biosynthetic steps*

The various classes of mutants were taken individually and within each class the mutants were recombined with each other using transduction, each mutant acting

in turn as donor and recipient. In most cases it was immediately apparent from the results that different loci existed within each group. In the present study we have not been concerned with the nature of different sites within each locus as has been done with many loci in *S. typhimurium*.

(*a*) *Methionine* (met)

By testing growth responses, the sixteen mutants having a primary requirement of methionine were divided into two groups.

1. Growth with methionine only.
2. Growth with methionine, homocysteine or cystathionine.

From the description of methionine biosynthesis given for *E. coli* by Umbarger & Davis (1962), we can say that group 1 mutants are unable to convert homocysteine to methionine; this is supported by the fact that none of the other mutants could cross-feed this group. The second group must be blocked at some point prior to

Table 2. *Linkage relationships of* met *loci in* P. aeruginosa *strain* 2. *Phage F*116 *was grown on each of the sixteen methionine-requiring mutants. Drops of these phage preparations were placed on plates of EMM spread with a washed suspension of each of the mutants in turn. The plates were incubated at* 37°*C. for* 2 *days, then each spot examined for prototrophic transduced colonies*

+, transduction to prototrophy at wild-type frequency; ±, transduction to prototrophy at 0–5% of wild-type frequency; 0, no prototrophs formed. A similar procedure and terminology for expression of results holds for Tables 3–11.

Recipient	Donor		
	met 1	*met 2a*	*met 2b*
met 1			
360	0	+	+
met 2a			
375, 410, 466, 469, 532, 554, 593	+	±	+
met 2b			
301, 307, 313, 327, 331, 379, 493, 501	+	+	±

cystathionine formation. Two subgroups can be distinguished in this group on the basis of cross-feeding, 2b mutants cross-feeding 2a mutants.

Transduction tests with these mutants are shown in Table 2.

It is seen that the transduction results fully confirm the growth requirement and cross-feeding studies in identifying three groups of methionine mutants. Furthermore, where each group acts in turn as recipient, the frequency of transduction to prototrophy of each group as donor is not observably different from that obtained where phage propagated on the wild-type prototroph is used as donor. This would indicate that these three loci are not linked. This result is confirmed in a subsequent section where the linkage of unrelated markers is considered and *met 2b* but not *met 2a* or *met 1* is shown to be linked to loci of other pathways.

(*b*) *Cysteine* (cys)

Four mutants were found which responded to cysteine. These were divided into two groups by cross-feeding tests, *cys 1* cross-feeding *cys 2*. Transduction tests, see Table 3, confirmed this division. These two loci show no linkage to one another with the simplified transduction test.

Table 3. *Linkage relationships of* cys *loci in* P. aeruginosa *strain* 2

	Donor	
Recipient	*cys 1*	*cys 2*
cys 1		
341, 353, 374	±	+
cys 2		
430	+	0

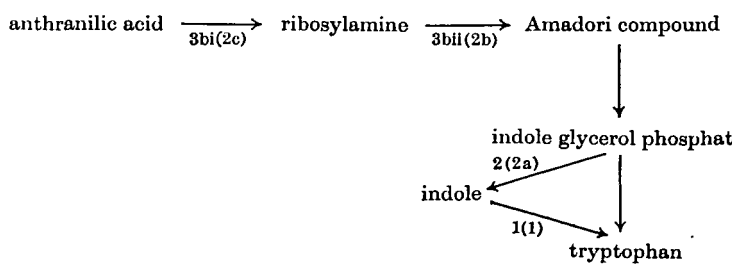
(*c*) *Tryptophan* (try)

Among the thirty-nine mutants examined, two classes of tryptophan mutants could be distinguished by growth responses.

1. Growth with tryptophan.
2. Growth with tryptophan or indole.

The group 1 mutants cross-feed all other mutants; they accumulate indole, as shown by the Erlich–Böhme test.

Group 2 can be subdivided into three groups by accumulation and enzyme studies. Mutants of group 2a accumulate indole glycerol as indicated by the Salkowski test (Yanofsky, 1956), and ultra-violet absorption spectrophotometry, where absorption peaks characteristic of indole glycerol are formed. Both 2b and 2c mutants accumulate anthranilic acid, as indicated by the characteristic ultra-violet absorption spectrum. Enzyme studies using cell-free extracts of representative mutants of 2b and 2c (Doy, 1964) show that 2b mutants cannot convert N-*o*-carboxyphenylribosylamine-5-phosphate to 1-(*o*-carboxyphenylamino)-1-deoxyribulose-5-phosphate and 2c mutants cannot convert anthranilic acid to the ribosylamine. There is no suggestion that the pathway of tryptophan biosynthesis in *P. aeruginosa* differs from that found in *E. coli*. In the pathway of tryptophan biosynthesis established for *E. coli* as given below, the points of blockage of the



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Pseudomonas mutant types are indicated in brackets, together with their relationship to the mutants of *E. coli* described by Yanofsky & Lennox (1959) and Doy *et al.* (1961).

The transduction results supported these groupings; the results of reciprocal transduction tests between the thirty-nine mutants are shown in Table 4.

Table 4. *Linkage relationships of* try *loci in* P. aeruginosa *strain* 2

Recipient	Donor *try 1*	*try 2*	*try 3bi*	*try 3bii*
try 1				
305, 314, 315, 317, 321, 322, 329, 349, 352, 408, 413, 415, 421, 455, 471, 475, 492, 516, 520, 523, 531, 537	±	+	+	+
try 2				
330, 445, 535	±	±	+	+
try 3bi				
458, 464, 483, 518	+	+	±	+
try 3bii				
302, 312, 334, 446, 447, 502, 507	+	+	+	±

As with the *met* markers, these transduction tests seem to indicate that *try 1*, *try 3bi* and *try 3bii* are not linked. Confirmation of the lack of clustering of the *try* markers is provided by the linkage of *try 3bi* and *try 3bii* to other markers (see below). *Try 1* and *try 2* show an anomalous situation; when *try 2* is carried by the donor and *try 1* by the recipient the transduction frequency achieved is that obtained with the wild-type donor. When *try 2* is carried by the recipient and *try 1* by the donor, a drastic reduction in transduction frequency occurs. One hypothesis to explain these facts is that *try 1* and *try 2* are linked, and the variation in reciprocal crosses stems from their relative positions on a transduction fragment with fixed ends (Ozeki, 1960).

(*d*) *Leucine* (leu)

The thirty-eight mutants requiring leucine were tested for growth responses. While all responded to α-keto isocaproate none responded to α-keto isovalerate. Other intermediates were not tested and none of the mutants cross-fed each other.

Transduction tests were carried out as for other mutants. The results shown in Table 5 clearly indicate the existence of two groups of *leu* mutants. The frequency

Table 5. *Linkage relationships of* leu *loci in* P. aeruginosa *strain* 2

Recipient	Donor *leu 1*	*leu 2*
leu 1		
485, 515	0	+
leu 2		
409, 418, 427, 432, 444, 449, 453, 457, 460, 467, 476, 477, 484, 486, 491, 505, 508, 511, 512, 513, 526, 527, 528, 539, 540, 545, 550, 553, 555, 563, 570, 572, 574, 577, 584	+	±

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of transductions to prototrophy was not observably different for each group from that obtained with wild-type phage, which would indicate that these two loci are not linked. This is confirmed by the linkage of *leu 2* but not *leu 1* to other loci.

(*e*) *Isoleucine and valine* (ilva)

Twenty-one mutants having the double requirement of isoleucine and valine were examined for their biochemical and linkage relationships. Cross-feeding did not occur between any of the mutants; no further biochemical investigations were made. The twenty-one mutants very clearly fell into two transduction groups (Table 6). The wild-type frequencies of prototrophic transductants found in the

Table 6. *Linkage relationships of* ilva *loci in* P. aeruginosa *strain* 2

	Donor	
Recipient	*ilva 1*	*ilva 2*
ilva 1		
3, 181, 308, 407, 423, 425, 463, 470, 543, 566, 571, 576, 579, 594	±	+
ilva 2		
2, 402, 416, 494, 541, 562, 567, 590	+	±

ilva 1 × *ilva 2* crosses would seem to indicate that they are not linked. The fact that no alternative growth requirements were available for this class of mutants means that we cannot directly show the co-transduction of any two different *ilva* loci by means of the donor phenotype selection test. However, evidence for the lack of close linkage of these two *ilva* markers comes from sexual crosses between strain 1 (FP−) and strain 2 (FP+). Strain 2-308 (*ilva 1*) and 2-2 (*ilva 2*) were crossed to an *ilva* mutant of strain 1. No recombinants were formed in the cross with 2-308, but in the second cross prototrophic recombinants were formed with a frequency of $8{\cdot}0 \times 10^{-7}$. This result would not be expected if *ilva 1* and *ilva 2* showed close linkage, thus the sexual recombination data confirm the results obtained with transduction.

(*f*) *Histidine* (his)

Twenty-one mutants requiring histidine were examined genetically and biochemically. None of the established intermediates of histidine biosynthesis in *E. coli* were available to us. However, cross-feeding tests established that the

Table 7. *Linkage relationships of* his *loci in* P. aeruginosa *strain* 2

	Donor		
Recipient	*his 1*	*his 2*	*his 3*
his 1			
304, 340, 387, 431, 437, 400, 529	±	+	+
his 2			
306, 354, 404, 406, 439, 474, 498, 507, 510, 561, 588, 591	+	±	+
his 3			
480, 582	+	+	0

twenty-one mutants could be divided into three groups; group 1 was cross-fed by groups 2 or 3, group 2 was cross-fed by group 3 only, and group 3 was not cross-fed by either groups 1 or 2. Transduction tests clearly confirmed the separate identity of these groups (Table 7). The wild-type frequency of prototrophy production in *his* × *his* crosses would indicate lack of close linkage between the three groups, but as no alternative growth requirements were available for these histidine mutants this conclusion cannot be definitely tested by the donor phenotype selection test.

(*g*) *Proline* (pro)

Five proline mutants were isolated. They could be divided into two groups by cross-feeding tests; transduction tests confirmed this division and further showed that the group which was cross-fed could be subdivided into two groups (*pro 1* and *pro 2*). None of the three groups showed linkage to each other by the spotting transduction procedure and this is indicated in Table 8.

Table 8. *Linkage relationships of* pro *loci in* P. aeruginosa *strain* 2

	Donor		
Recipient	*pro 1*	*pro 2*	*pro 3*
pro 1			
61, 674	0	+	+
pro 2			
694	+	0	+
pro 3			
546, 782	+	+	0

(*h*) *Arginine* (arg)

Eight arginine mutants were isolated. Testing growth responses showed three groups; group 1 responded to arginine only, group 2 responded to arginine and argininosuccinate, group 3 responded to arginine, argininosuccinate, citrulline and ornithine. No cross-feeding was observed between any of the mutants. Transduction tests showed that the three groups of arginine mutants were not linked to each other (Table 9).

Table 9. *Linkage relationships of* arg *loci in* P. aeruginosa *strain* 2

	Donor		
Recipient	*arg 1*	*arg 2*	*arg 3*
arg 1			
303, 479, 559	±	+	+
arg 2			
318, 380, 791, 799	+	±	+
arg 3			
771	+	+	0

A donor phenotype selection experiment was carried out using 2-303 (*arg 1*) as recipient and F116 propagated on 2-771 (*arg 3*), with selection of the recombinants on ornithine-supplemented agar. Of 951 colonies selected, none showed the donor

phenotype (ability to grow on ornithine) indicating that *arg 1* and *arg 2* are not closely linked and confirming the result of the prototroph selection transduction test.

(*i*) *Adenine* (ade)

All the mutants of this class grow with adenine or hypoxanthine. By transduction tests they can be classified into three unlinked groups (Table 10), as confirmed by the linkage of *ade 2* but not *ade 1* or *ade 3* to other unrelated markers (described below).

Table 10. *Linkage relationships of* ade *loci in* P. aeruginosa *strain* 2

	Donor		
Recipient	*ade 1*	*ade 2*	*ade 3*
ade 1			
316, 378, 495, 573, 586	±	+	+
ade 2			
370, 420, 500, 522, 551	+	±	+
ade 3			
371, 452, 524	+	+	±

(*j*) *Uracil* (ura)

Five uracil-requiring mutants were found. No cross-feeding responses were found between them and no further biochemical investigations were made. By transduction tests they could be divided into three groups (Table 11). The transduction frequencies observed indicate that the three uracil loci are not clustered.

Table 11. *Linkage relationships of* ura *loci in* P. aeruginosa *strain* 2

	Donor		
Recipient	*ura 1*	*ura 2*	*ura 3*
ura 1			
631, 644	±	+	+
ura 2			
635, 732	+	±	+
ura 3			
767	+	+	0

(*k*) *Glycine or serine* (ser)

Six mutants were isolated whose growth requirements were satisfied by either glycine or serine. Cross-feeding tests did not differentiate between these mutants; no further biochemical tests were carried out. By transduction tests they all appeared to be at the same locus. One mutant was isolated which responded to serine only, and this appeared to be located at the same locus as the glycine–serine class of mutants.

(*l*) *Homoserine* (hom) *and threonine* (thr)

Three mutants requiring homoserine or a mixture of methionine and threonine were isolated. Transduction tests failed to differentiate them and all showed linkage, as indicated by a reduction in prototroph formation, to each of seven threonine-requiring mutants. These seven are all apparently at the same locus, as indicated by their linkage to *hom* and their lack of differentiation by intra-group transduction tests. Donor phenotype selection experiments confirmed the close linkage of *hom* and *thr*. Phage F116 propagated on 2-326 (*thr*) was used to transduce into 2-366 (*hom*), on threonine supplemented minimal plates. The transduced colonies were then scored for the co-transduction of *thr* and *hom*$^+$ and it was found that 971/1018 colonies tested (96%) showed co-transduction of the two markers. This then is an example of clustering of related loci, as these two markers have biosynthetic relationships.

II. *Linkage between markers of unrelated pathways*

A search for linkage between unrelated markers by means of the co-transduction test can potentially give three types of information.

1. By establishing linkage it shows that the transduction method does demonstrate existing linkage, which is important in view of the results of section I where related markers in a number of biosynthetic pathways failed to show linkage to each other in most cases.
2. Any linkage demonstrated between unrelated markers can confirm the lack of linkage shown by related markers.
3. The linkage patterns of unrelated markers may have significance.

Reciprocal transduction tests were made between all thirty-two different loci identified. The great majority of these transductions showed no evidence of co-transduction, i.e. the frequency of prototroph formation was the same as that with phage propagated on wild-type bacteria. However some combinations of markers did show a reduction in prototroph formation. It was found that four apparently unrelated loci are grouped together on the chromosome. The four loci concerned are *met 2b*, *ade 2*, *try 3bii* and *leu 2*. The results of transduction tests between all the loci of the tryptophan, methionine, adenine and leucine pathways are shown in Table 12.

These tests were carried out using the simplified transduction test. They were all confirmed by donor phenotype selection transductions where phage propagated on one mutant infected bacterial cells of the second mutant and selection for transductants was made on minimal agar supplemented with the requirement of the donor strain. The colonies appearing were then replicated onto minimal medium and scored for their ability to grow. By this means the actual frequency of co-transduction of the two markers was determined. By carrying out a series of such experiments with the markers of the cluster it was possible to determine their relative positions. The data so obtained and the relative positions of the markers are shown in Fig. 1.

Table 12. *Linkage relationships by transduction between methionine, tryptophan, adenine and leucine requiring mutants of* P. aeruginosa *strain* 2

+ + = production of prototrophs at wild-type frequency; 0 = no prototrophs formed; + = reduction in prototroph formation to 5–20% of wild-type frequency; ± = reduction in prototroph formation to 0–5% of wild-type frequency.

Recipient \ Donor	*met 1*	*met 2a*	*met 2b*	*ade 1*	*ade 2*	*ade 3*	*try 1*	*try 2*	*try 3bii*	*try 3bi*	*leu 1*	*leu 2*
met 1	0	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
met 2a	+ +	0	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
met 2b	+ +	+ +	0	+ +	+	+ +	+ +	+ +	+	+ +	+ +	+
ade 1	+ +	+ +	+ +	0	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
ade 2	+ +	+ +	+	+ +	0	+ +	+ +	+ +	+	+ +	+ +	+
ade 3	+ +	+ +	+ +	+ +	+ +	0	+ +	+ +	+ +	+ +	+ +	+ +
try 1	+ +	+ +	+ +	+ +	+ +	+ +	0	+ +	+ +	+ +	+ +	+ +
try 2	+ +	+ +	+ +	+ +	+ +	+ +	±	0	+ +	+ +	+ +	+ +
try 3bii	+ +	+ +	+	+ +	+	+ +	+ +	+ +	0	+ +	+ +	+
try 3bi	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	0	+ +	+ +
leu 1	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	0	+ +
leu 2	+ +	+ +	+	+ +	+	+ +	+ +	+ +	+	+ +	+ +	0

It is seen that, while there is not complete additivity, the relative order of the markers as given in Fig. 1 is the most likely.

Linkage of two unrelated genes was described in an earlier paper (Holloway *et al.*, 1962)—the linkage of the gene controlling streptomycin resistance (*str*) to *try 3bi*. No linkage of *str* to any of the other *try* markers described in the present paper has been found.

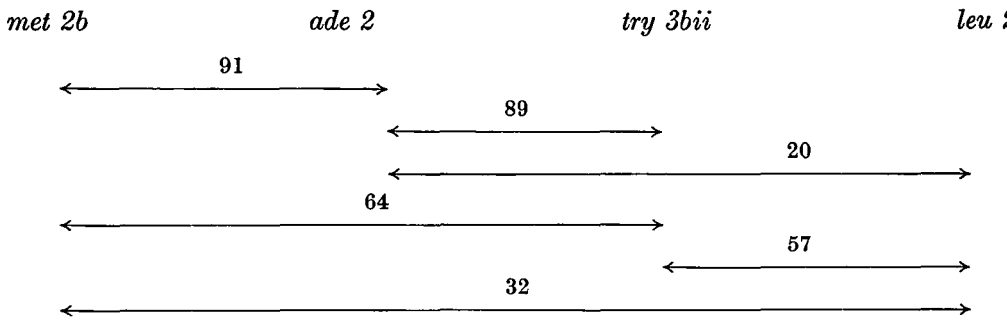

Fig. 1. Linkage relationships of *met 2b*, *ade 2*, *try 3bii* and *leu 2*. One representative strain of each locus was used. Figures given are percent co-transduction.

The linkage relationships shown in Table 12 and Fig. 1 confirm completely the non-clustering of markers for the methionine, leucine, adenine and tryptophan pathways, and they indicate the adequacy of the F116 transduction system for detecting close linkage. These results support the view that the transduction results obtained with the proline, uracil, isoleucine plus valine, arginine, cysteine and histidine pathways are strongly indicative of a non-clustering of the markers tested within those pathways.

4. DISCUSSION

The linkage relationships of the related biosynthetic markers studied here in *P. aeruginosa* indicate quite a different situation from that occurring in other bacteria. Demerec (1964) has recently summarized the linkage data for *S. typhimurium* and concludes that with eighty-seven auxotrophic mutant loci belonging to eighteen phenotypically distinct groups, over 70% are arranged in clusters of two or more on the chromosome. For *E. coli* such clustering has been shown for at least nine phenotypic groups, and four clusters of related markers are known for *Bacillus subtilis* (Anagnostopoulos *et al.*, 1964; Nestor, Schafer & Lederberg, 1963; Ephrati-Elizur *et al.*, 1961). Only one clear example of clustering has been shown in the *P. aeruginosa* mutants examined, two related mutants in the homoserine–threonine pathway being closely linked.

The important conclusion to be drawn from this result concerns the nature of genetic control of enzyme synthesis in *Pseudomonas*. The operon (Jacob & Monod, 1961) is clearly the unit of control where genes are clustered together but as yet there is no clear picture of how this theory could be modified for genes which are

scattered. There is at present no exception to the finding that related genes which are clustered on the chromosome show co-ordinate repression or induction (Ames *et al.*, 1963; Beckwith *et al.*, 1962). It will thus be of interest to investigate the nature of control mechanisms in *P. aeruginosa* for those pathways which are clustered in *E. coli* or *S. typhimurium* and seem to have the characteristic operon type of control such as leucine (Margolin, 1963), tryptophan (Matsushiro *et al.* 1963), and histidine (Ames *et al.*, 1963).

A comparison of the mechanisms of control of valine and isoleucine metabolism in *P. aeruginosa* and *E. coli* (Horvath *et al.*, 1964) has shown that for *P. aeruginosa* but not for *E. coli*, leucine shows an end-product induction effect on the α-aceto-lactic acid-forming enzyme. A similar end-product induction has been observed by Gorini (1960) and Gorini & Gunderson (1961) in arginine biosynthesis in *E. coli*. The markers for arginine biosynthesis in *E. coli* are not all clustered and this behaviour may well be characteristic of biosynthetic pathways with non-clustered markers.

The evolutionary significance of clustering of markers is at present not clear. Examples of linkage of markers with related function are known in many genetic systems (for review, see Bodmer & Parsons, 1962) but the phenomenon seems to occur more frequently in micro-organisms.

It would be very desirable to have an estimate of the size of the piece of bacterial chromosome transduced by F116. Lee (unpublished) has shown that for F116 the density of the transducing particle as measured in a cesium chloride gradient is only slightly different from that for the plaque-forming particle. From sedimentation studies (Friefelder, Holloway & Davison, unpublished) it has been found that the molecular weight of the DNA of F116 is between 4×10^7 and 5×10^7. Assuming a molecular weight of 2×10^9 for the DNA of *P. aeruginosa* this gives an absolute upper limit of 2·5% of the bacterial chromosome, which can be transduced in one piece. As yet we have insufficient data to state whether the transduced pieces are of predetermined size or have fixed ends, but genetic stocks for examining this question are being constructed.

A comparison of co-transduction and sexual recombination data has been possible in one case. The loci *try 3bi* and *str* are linked in sexual crosses, showing 14% recombination in strain 2 × strain 2 crosses (Holloway, 1956), while co-transduction of *str* and *try* with F116 occurs at a frequency of 11% in strain 2 (Holloway *et al.*, 1962). However, further data of this kind should be obtained and we do not wish to place too much reliance in this one comparison, as preliminary evidence has shown a possible excess of multiple crossovers in the *str–try 3bi* region, with both transduction data (Waltho, unpublished) and sexual recombination data.

SUMMARY

Phage F116 transduces sufficiently large fragments of the *Pseudomonas aeruginosa* chromosome for co-transduction of linked markers to occur. The linkage relationships of 176 auxotrophic mutants have been examined by means of this technique,

following characterization of the mutants for their nutritional requirements and by other biochemical means. Thirty-two different loci from fourteen biosynthetic pathways have been differentiated by such combined means. Unlike other bacteria, the clustering of related loci of a biosynthetic pathway appears to be rare. This situation was confirmed for the leucine, methionine, adenine, isoleucine plus valine and arginine pathways. No evidence for linkage of markers within the histidine, proline, cysteine and uracil pathways has been found but further data is needed to completely confirm this conclusion for these groups of markers. The importance of these findings in relation to the genetic control of enzyme biosynthesis in *P. aeruginosa* is discussed. For markers of the tryptophan pathway, two are possibly linked but the other three are definitely not linked. The only confirmed linkage of related markers was between a homoserine locus and a threonine locus.

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