Genet. Res., Camb. (1974), **23**, *pp.* 335–359
With 4 text-figures
Printed in Great Britain

Genetic analysis of amidase mutants of *Pseudomonas aeruginosa*

BY JOAN L. BETZ, JANE E. BROWN,
PATRICIA H. CLARKE AND MARTIN DAY
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT

(*Received* 14 *February* 1974)

SUMMARY

Mutants of *Pseudomonas aeruginosa*, which differed in amide growth phenotype from the wild-type strain, were subjected to genetic analysis using the generalized transducing phage F116. The map order of some mutational sites was determined by 3-factor crosses in which a mutation in the linked regulator gene *amiR* was used as the outside marker to determine the relative order of mutations in the amidase structural gene *amiE*. Acetamide-positive transductants were recovered in crosses between amidase-negative strains and strains PhB3 (PAC377), V2 (PAC353) and V5 (PAC356) producing mutant amidases which hydrolyse phenylacetamide and valeramide but not acetamide. Some recombinants carried the mutation *amiE*16 determining the properties of the mutant B amidase produced by strain B6 (PAC351) from which both PhB and V class mutants were derived, while other recombinants produced A amidase determined by the wild-type *amiE* gene.

1. INTRODUCTION

Pseudomonas aeruginosa is able to grow in a minimal salt medium containing acetamide as the sole source of carbon and nitrogen by producing an inducible aliphatic amidase (Kelly & Clarke, 1962). The properties of the enzyme are determined by a single amidase structural gene and its synthesis is controlled by a linked regulator gene. The amidase-positive character can be transduced into an amidase-negative recipient by bacteriophage F116 isolated by Holloway, Egan & Monk (1960). When constitutive amidase-positive strains are used to prepare the phage lysates the amidase-positive transductants are found to acquire the constitutivity marker at frequencies of 80–100% (Brammar, Clarke & Skinner, 1967). Other mutations in the regulator gene alter the inducer specificity and whereas the wild-type strain is induced by acetamide and propionamide and only very weakly induced by formamide, it is possible to isolate regulator mutants which are induced by formamide at a higher rate than by acetamide (Brammar *et al.* 1967). Butyramide is not an inducer for the wild-type strain but behaves as a competitive repressor and also strongly represses amidase synthesis in some constitutive strains (Brown & Clarke, 1970).

The amide substrate specificity of the wild-type enzyme is relatively narrow.

Acetamide and propionamide are the best substrates and are also the best inducers. These are the only two aliphatic amides which support growth of the wild-type strain. Formamide is hydrolysed by the wild-type enzyme at about 10% of the acetamide rate and butyramide at 2% but neither of these amides can be used as growth substrates. Acetamide-negative mutants were isolated as minute shadowy colonies on acetamide plates and the ones which were acetamide-negative (others were acetate-negative) were also propionamide-negative (Skinner & Clarke, 1968). These mutants were used in genetic crosses by straightforward selection of recombinants on acetamide plates. We have also isolated mutants which are able to grow on a wider range of amides than the parent strain, *P. aeruginosa* PACI. These include classes of mutants able to utilize (*a*) butyramide, (*b*) valeramide, (*c*) phenylacetamide and (*d*) acetanilide. We have shown that in most instances the change of phenotype is due to the production of altered amidase proteins (Betz *et al.* 1974).

Brown, Brown & Clarke (1969) isolated certain butyramide-utilizing mutants which produce an amidase with altered substrate specificity, hydrolysing butyramide at about 30% of the acetamide rate. These mutants grow on acetamide, propionamide or butyramide and are totally devoid of the wild-type enzyme so that it could be concluded that the mutation occurred in a single structural gene determining the amidase protein. Further mutations in one of these butyramide-utilizing strains produced valeramide-utilizing mutants and the amidases of some of these mutants were changed in substrate specificity to such an extent that they were unable to grow on acetamide. Betz & Clarke (1972) isolated phenylacetamide-utilizing mutants by several different routes and some of these were unable to grow on acetamide but grew well on butyramide and valeramide. The growth characteristics of these strains gave a unique opportunity for carrying out genetic crosses between amidase-positive mutants, which were acetamide-negative. The close linkage of the amidase structural and regulator genes made it possible to use a mutation in the regulator gene as the outside marker in three-factors crosses to determine the map orders of the mutations in the structural gene in much the same way as the early fine structure mapping of the gene for β-galactosidase used mutations in the linked regulator gene (Jacob & Wollman, 1961).

Previous transductional analysis of *Pseudomonas aeruginosa* has been mainly concerned with gene linkage and has shown that there is far less clustering of genes for related biosynthetic enzymes than occurs in the Enterobacteriacae (Holloway, Krishnapillai & Stanisich, 1971). The genes for the histidine biosynthetic pathway, for example, belong to at least five separate linkage groups in *P. aeruginosa* compared with the single operon for the histidine genes of *Escherichia coli*. Mee & Lee (1969) showed that one of the histidine genes, *his*-1, was cotransduced with a gene required for cysteine biosynthesis at a frequency of 70–90% and were able to use this as the basis for mapping mutations in the *his*-1 region by three-factor crosses using the transducing bacteriophage F116. This is the only previous attempt to carry out fine structure genetic analysis in *P. aeruginosa* by mapping mutations in a single gene. The amidase mutants were isolated as part of

a programme on directed evolution of an enzyme and we hoped that it would be possible to correlate the results of intragenic crosses with the studies we have made on the structure and function of the wild-type and mutant amidases.

2. METHODS

(i) *Organisms.* The bacterial strains were all derived from the wild-type strain of *P. aeruginosa* PACI and are listed in Table 1. The series numbers denote the class of amidase mutant and its derivation. Since most of the strains described have mutations in one or both of only two genes, but have a range of different phenotypes, it is convenient to have a shorthand nomenclature which can give an idea of their relationship to one another and indicate the possible recombinant phenotypes which could arise. This is discussed in more detail later. Table 1 also gives the amide growth phenotypes of the strains used and the types of amidase produced. The transducing bacteriophage F116 was kindly provided by Professor B. W. Holloway.

(ii) *Media and growth conditions.* The mutants were lyophilized as soon as possible after isolation. Working cultures were maintained on nutrient agar slopes at 4 °C and subcultured monthly. The amide growth media were prepared as described by Betz & Clarke (1972).

(iii) *Preparation of phage lysates.* The plate method of Adams (1959) was used to produce lysates of F116 with 10^{10}–10^{11} pfu/ml. The lysate was cleared of bacteria by adding a few drops of chloroform, incubating at 37 °C for 1 h and centrifuging. The supernatant was decanted from the chloroform and stored at 4 °C. Phage F116 is sensitive to chloroform but by keeping this contact minimal it was possible to destroy the bacteria and retain a high phage titre. All phage preparations for transduction experiments were propagated twice in the donor strain.

(iv) *Transduction.* The method was based on that of Holloway *et al.* (1962). Recipient bacteria were grown overnight in nutrient broth and resuspended in dilution buffer to give 1 to 4×10^9 bacteria/ml. Samples were mixed with an equal volume of phage lysate at a multiplicity of infection of 10. The mixture was incubated at 37 °C for 1 h, centrifuged, and the bacteria resuspended in an equal volume of dilution buffer. Samples of 0·1 ml of the bacterial suspension, or suitable dilutions to give not more than 100 transductant colonies per plate, were spread on the amide selective media. Samples of the donor bacteria and the phage lysate were plated on the same media (as controls for reversion) and the phage lysate was also plated on nutrient agar to test for bacterial contamination. Plates were incubated at 37 °C for 2–3 days and for a few of the crosses in which recombinants were selected on amide media which supported slow growth, the plates were re-examined after 5–6 days.

(v) *Replica plating.* The method of Lederberg & Lederberg (1952) was used. The recombinant colonies to be analysed were first streaked to obtain single colonies except for those crosses where the recipient phenotype could not obscure the subsequent analysis. Colonies were patched on appropriate minimal medium (usually succinate or acetamide as carbon source) using a sterile toothpick or flamed loop. About

Table 1. *Strains of* Pseudomonas aeruginosa

Strain no.	Series no.	Parent	Mutagen	Relevant genotype*	Amide growth phenotype†	*Amidase* – type of enzyme‡	References
PAC1	Wild-type			*amiR*+ *amiE*+	AM+ Ind	Ami A	Kelly & Clarke (1962)
PAC111	C11	PAC1	spont.	*amiR*11 *amiE*+	AM+ Con	Ami A	Brammar *et al.* (1967)
PAC142	L10	PAC1	spont.	*amiR*33 *amiE*+	AM+ Con Crp −r	Ami A	Clarke (1970)
PAC128	CB4	PAC111	spont.	*amiR*11, 37 *amiE*+	AM+ B+ Con	Ami A	Brown & Clarke (1970)
PAC303	Am3	PAC1	NMG	*amiR*+ *amiE*3	AM− Ind	CRM−	Skinner & Clarke (1968)
PAC304	Am4	PAC1	NMG	*amiR*+ *amiE*4	AM− Ind	CRM−	Skinner & Clarke (1968)
PAC306	Am6	PAC1	NMG	*amiR*+ *amiE*6	AM− Ind	CRM−	Skinner & Clarke (1968)
PAC307	Am7	PAC1	NMG	*amiR*+ *amiE*7	AM− Ind	CRM+	Skinner & Clarke (1968)
PAC308	Am8	PAC1	NMG	*amiR*+ *amiE*8	AM− Ind	CRM+	Skinner & Clarke (1968)
PAC309	Am9	PAC1	NMG	*amiR*+ *amiE*9	AM− Ind	CRM+	Skinner & Clarke (1968)
PAC310	Am10	PAC1	NMG	*amiR*+ *amiE*10	AM− Ind	CRM−	Skinner & Clarke (1968)
PAC322	CAm2	PAC111	NMG	*amiR*+ *amiE*18	AM− Ind	CRM−	J. E. Brown (1969)
PAC321	CAm1	PAC111	NMG	*amiR*11 *amiE*17	AM− Con	CRM−	J. E. Brown (1969)
PAC323	CAm3	PAC111	NMG	*amiR*11 *amiE*19	AM− Con	CRM−	J. E. Brown (1969)
PAC324	CAm4	PAC111	NMG	*amiR*11 *amiE*20	AM− Con	CRM−	J. E. Brown (1969)
PAC325	CAm5	PAC111	NMG	*amiR*11 *amiE*21	AM− Con	CRM−	J. E. Brown (1969)
PAC326	LAm1	PAC142	NMG	*amiR*33 *amiE*34	AM− Con Crp −r	Ami−d	P. R. Brown (1969)
PAC351	B6	PAC111	NMG	*amiR*11 *amiE*16	AM+ B+ Con	Ami B	Brown *et al.* (1969)
PAC353	V2	PAC351	EMS	*amiR*11 *amiE*16, 23	AM+ B+ V+ Con	Ami V	Brown *et al.* (1969)
PAC356	V5	PAC351	EMS	*amiR*11 *amiE*16, 26	AM− B+ V+ Con	Ami V	Brown *et al.* (1969)

Table 1 (*cont.*)

Strain no.	Series no.	Parent	Mutagen	Relevant genotype*	Amide growth phenotype	*Amidase* – type of enzyme‡	References
PAC375	PhB1	PAC351	EMS	*amiR*11 *amiE*16, 65	AM−, B+, V+, Ph+,	Ami PhB	Betz & Clarke (1972)
PAC376	PhB2	PAC351	EMS	*amiR*11 *amiE*16, 66			
PAC377	PhB3	PAC351	EMS	*amiR*11 *amiE*16, 67			
PAC378	PhB4	PAC351	EMS	*amiR*11 *amiE*16, 68			
PAC379	PhB5	PAC351	NMG	*amiR*11 *amiE*16, 69			
PAC380	PhB6	PAC351	EMS	*amiR*11 *amiE*16, 70			
PAC382	PhB9	PAC351	spont.	*amiR*11 *amiE*16, 71			
PAC383	PhB11	PAC351	NMG	*amiR*11 *amiE*16, 72			
PAC384	PhB12	PAC351	NMG	*amiR*11 *amiE*16, 73			
PAC385	PhB13	PAC351	spont.	*amiR*11 *amiE*16, 74			
PAC386	PhB14	PAC351	NMG	*amiR*11 *amiE*16, 75			
PAC387	PhB16	PAC351	UV	*amiR*11 *amiE*16, 76			
PAC392	PhF1	PAC128	UV	*amiR*11, 37*amiE*82	AM(+), B+, V+, Ph+,	Ami PhF	Betz & Clarke (1972)

* Genotype abbreviations: *amiR* = amidase regulator gene; *amiE* = amidase structural gene.

† Amide growth phenotype: Ind = inducible; Con = constitutive. Growth on minimal agar containing as carbon source + or −; AM acetamide; B = butyramide; V = valeramide; Ph = phenylacetamide used as nitrogen source only.

‡ Type of amidase: A = wild-type enzyme; B = B enzyme with specificity as PAC351; V = V enzyme as produced by V series of mutants; PhB = enzyme produced by PAC377; PhF = enzyme produced by PAC392. CRM = cross-reacting material; Ami−d = defective.

33 colonies including relevant control strains were patched on each plate. After overnight growth at 37 °C the master plate was replicated with sterile nylon velvet on the required range of amide selective media. Plates were incubated for 1–2 days at 37 °C.

(vi) *Analysis of recombinants for constitutivity.* Strains producing wild-type amidase (A enzyme) or the altered enzyme of the butyramide-utilizing mutants of the B series (B enzyme) are able to grow on S/F plates (succinate/formamide) if they produce the amidase constitutively. Recombinant colonies from growth on succinate or acetamide plates could be replicated on S/F plates to test for constitutivity. For some experiments direct enzyme assays were carried out by a micromethod derived from the amidase transferase assay of Brammar & Clarke (1964). Single colonies, or some of the bacteria from patches of growth on succinate plates, were picked off with a loop and suspended in 0·1 ml tris buffer 0·1 M pH 7·2; 0·2 ml sample of amide substrate mixture (containing 0·2 M acetamide, 0·5 M hydroxylamine hydrochloride and 0·5 M tris buffer) was added and after 30 min incubation at room temperature the reaction was stopped by the addition of 0·4 ml ferric chloride reagent. Constitutive strains gave a deep red-black colour while strains which did not form enzyme on this medium gave the same yellow colour as the substrate control.

(vii) *Analysis of recombinations for A or B amidase.* Some of the acetamide-positive recombinants produced wild-type A amidase; others produced mutant B amidase; transductants belonging to either of these classes of recombinant could be inducible or constitutive. The preliminary test for constitutivity was carried out by replicating to S/F plates. From the master plate each colony was inoculated into 5 ml pyruvate minimal medium in universal bottles with 10 mM lactamide added as inducer for the inducible strains. After overnight growth the cultures were centrifuged and the bacterial pellet resuspended in 0·5 ml tris buffer. Each culture was assayed by the transferase reaction using 0·02 ml of the bacterial suspension with 1 ml of the acetamide substrate mixture (as above) and 0·2 ml of the bacterial suspension with 1 ml of the butyramide substrate mixture (0·2 M butyramide). After 10 min incubation at 37 °C the reaction was stopped by the addition of 2 ml ferric chloride reagent. The A enzyme under these conditions gives a red-black colour with acetamide (strong reaction) and a yellow colour with butyramide (no reaction); the B enzyme gives a red-black colour with both acetamide and butyramide.

3. RESULTS

(i) *Isolation and characterization of mutants* (Table 1)

The acetamide-negative mutants of the Am series, strains PAC301 to PAC310, were isolated directly from the wild-type strain and were totally lacking in amidase activity (Skinner & Clarke, 1968). The spontaneous rate of reversion of these mutants was $< 10^{-8}$ but in all cases acetamide-positive revertants were obtained in the presence of EMS (ethyl methane sulphonate) (Table 2). The revertants were

inducible and the acetamide-positive transductants obtained in crosses between Am strains were also all inducible. It was concluded that members of the Am series of mutants had point mutations in the amidase structural gene *amiE* and retained the inducible wild-type regulator gene. All the Am strains could be used as recipients in transduction but Am7 and Am10 could not be used as donors since they were unable to propagate phage F116. Am7 produced colonies which had a dry and wrinkled appearance and the inability to propagate the bacteriophage may have been due to additional mutations affecting the bacterial surface.

Table 2. *Reversion rates of acetamide-negative mutants (number of revertants obtained from 10^8 bacteria)*

Strain no.	Series no.	Revertants Spont.	Revertants EMS	Strain no.	Series no.	Revertants Spont.	Revertants EMS
PAC307	Am3	< 1	15–30	PAC353	V2	< 1	ND
PAC304	Am4	< 1	15–30	PAC356	V5	< 1	ND
PAC306	Am6	< 1	15–30	PAC375	PhB1	< 1	2
PAC307	Am7	< 1	15–30	PAC376	PhB2	< 1	5
PAC308	Am8	< 1	15–30	PAC377	PhB3	< 1	3
PAC309	Am9	< 1	15–30	PAC378	PhB4	< 1	5
PAC310	Am10	< 1	15–30	PAC379	PhB5	< 1	2
PAC321	CAm1	42	ND	PAC380	PhB6	< 1	3
PAC322	CAm2	< 1	15–30	PAC381	PhB9	< 1	6
*PAC323	CAm3	0	0	PAC385	PhB13	< 1	4
PAC324	CAm4	2	15–30	PAC386	PhB14	< 1	4
PAC325	CAm5	< 1	15–30	PAC326	LAm1	< 1	2

EMS = ethyl methanesulphonate, ND = not determined.
* PAC323 has never reverted.

The CAm mutants were isolated from the constitutive strain PAC111 and were expected to be potentially constitutive. Strains PAC321 (CAm1), PAC324 (CAm4) and PAC325 (CAm5) gave only constitutive acetamide-positive revertants and there were no anomalous results in crosses between the three CAm mutants or between these CAm mutants and those of the Am series. It was concluded that these three mutants had point mutations in the *amiE* gene and retained the original regulator gene mutation *amiR*11. Strain PAC323 (CAm3) did not revert, either spontaneously or after treatment with EMS, NMG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) or the acridine compound ICR 191A, which suggested that it might be a deletion mutant. Strain PAC322 (CAm2) gave revertants which themselves were all inducible and produced only inducible transductants in crosses with the Am series and it was concluded that the regulator gene of this strain had reverted to the wild type. CAm2 was therefore treated as a potentially inducible acetamide-negative mutant (*amiR*$^+$ *amiE*18). All the CAm series of strains could be used as donors in transduction and all but CAm1, which had too high a reversion rate, could be used as recipients (Table 2).

Strain PAC326 (LAm1) was isolated from a constitutive mutant PAC142 (L10) which produces very high levels of wild-type A amidase in all media. Strain L10 is

resistant to catabolite repression by succinate and although the constitutivity character of L10 was cotransduced with the amidase-positive character at the same high frequency as that of strain C11, there was no cotransduction of the resistance to catabolite repression. Strain L10 belongs to the group of butyramide-positive constitutive mutants which are resistant to butyramide represssion (Brown & Clarke, 1970). It was therefore possible to isolate butyramide-negative mutants of this strain and the LAm series of mutants were derived in this way. Some of the butyramide-negative LAm mutants were also unable to grow on acetamide and all were thought to have mutations in the *amiE* gene. LAm1 produced a thin film of growth on acetamide after several days and although it could be used as a donor it was not satisfactory as a recipient in transduction. Betz & Clarke (1972) used LAm1 as the parent strain in the isolation of one of the phenylacetamide-utilizing strains.

The butyramide-utilizing mutants isolated by Brown *et al.* (1969) were acetamide-positive and produced B amidase which differs in substrate profile and electrophoretic mobility from A amidase. Strain PAC351 (B6) was derived by a single mutational step from C11 and the properties of the B amidase indicate that it is unlikely that more than one amino acid change had occurred in the enzyme protein. Strain B6 is therefore thought to have the genotype *amiR*11 *amiE*16. It was used as the parent strain to derive the valeramide-utilizing mutants of the V series which are therefore expected to have the *amiR*11 mutation, and the *amiE*16 mutation of strain B6 and at least one more mutation in the amidase structural gene. Two of the V series of mutants PAC353 (V2) and PAC356 (V5) were acetamide-negative and could be used as donor or recipient in transduction and recombinants could be selected on acetamide plates.

Betz & Clarke (1972) isolated the phenylacetamide-utilizing mutant PAC377 (PhB3) by a single mutational step from strain B6. The amidase produced by PhB3 differed in substrate profile from the A, B and V amidases and the derivation of this mutant made it likely that PhB3 possessed the *amiR*11 and *amiE*16 mutations of strain B6 and at least one additional mutation in the structural gene (*amiR*11 *amiE*16, 67). Mutant PhB3 was able to grow on butyramide, valeramide and phenylacetamide but not on acetamide. It could be used as donor or recipient in transduction and recombinants could be selected on acetamide plates.

Constitutive mutants of the CB series all produce A amidase but are able to grow on butyramide since they are resistant to butyramide repression (Brown & Clarke, 1970). These were derived either from the wild type, or from strain PAC111, by selection on butyramide plates and the butyramide-resistant character was cotransduced with the constitutivity and amidase-positive characters with the same high frequency as the constitutivity marker *amiR*11. It is thought that the CB mutants produce regulator gene proteins with a reduced affinity for amide analogue repressors (Brown & Clarke, 1970) and strain PAC128 (CB4) was successfully used as the parent strain for the isolation of the phenylacetamide-utilizing mutant PAC388 (PhF1). Only a single mutational step intervened between the wild-type A amidase and the mutant amidase of PhF1 and it was provisionally

assumed that this mutant had only one mutation in the structural gene but two mutations in the regulator gene (*amiR*11, 37 *amiE*82). Mutant PhF1 differs from PhB3 in that it grows on acetamide although very feebly. It could be used satisfactorily as donor in transductions and the background growth on acetamide plates was sufficiently low for it to be used as recipient in some crosses where rapidly growing acetamide-positive transductants were to be selected.

(ii) *Recombination between acetamide-negative mutants*

The acetamide-negative mutants belonged to several different phenotypic classes. The Am and CAm series of mutants were conventional negative mutants and the recovery of acetamide-positive transductants in crosses between any two of them was taken to indicate that the two mutations were at different sites. The same considerations were applied to crosses involving the V, PhB and PhF mutants although these classes of mutants had a positive growth phenotype on some of the higher amides.

Table 3. *Selection of donor and recombinant phenotype in transduction (number of transductants/10^9 recipient bacteria)*

Donor	PAC377 (PhB3) *amiR*11*amiE*16,67			PAC1 *amiR*+ *amiE*+
Recipient	AM*	B	S/Ph	AM
PAC307 (Am7) *amiR*+ *amiE*7	10	4000	4500	4000
PACI *amiR*+ *amiE*+	np	4500	5000	np
PAC351 (B6) *amiR*11 *amiE*16	np	np	3000	np
PAC377 (PhB3) *amiR*11 *amiE*16, 67	0	np	np	3100

* Selection of transductants on amide media. AM = acetamide 0·1 % (w/v); B = butyramide 0·1 %; S/Ph = succinate 1·0 % (w/v) + phenylacetamide 0·1 % (w/v). np = not possible to do this cross since the recipient grows on the medium.

Under the standard conditions the number of acetamide-positive transductants recovered in transductional crosses carried out on nine separate occasions, with the same phage preparation from the wild-type as donor and the acetamide-negative mutant PAC307 as recipient, ranged from 2000 to 3600 per 10^9 recipient bacteria (mean value 2500). Similar values were obtained by Skinner (1967) for the transduction of other markers but there was a greater variation in the recovery of transductants for different genes. In crosses between acetamide-negative mutants the number of transductants recovered was very much lower, as would be predicted for intragenic recombination, ranging from 1 to 100 per 10^9 recipients (Tables 3, 4).

Reciprocal crosses between the Am series mutants PAC303, PAC304, PAC306 and PAC308 were unproductive and suggested that the mutations were either at the same site or in close proximity. Crosses between Am7, Am8, Am9 and Am10 all gave recombinants and it was concluded that mutations *amiE*7, 8, 9 and 10

Table 4. *Crosses between acetamide-negative mutants: recovery of acetamide-positive transductants*

(Numbers of recombinants selected on acetamide plates/10^9 recipient bacteria.)

DONOR PAC...	307*	308	309	310*	321†	322	323	324	325	326†	353	356	377	392	1
Series	Am7	Am8	Am9	Am10	CAm1	CAm2	CAm3	CAm4	CAm5	LAm1	V2	V5	PhB3	PhF1	Wild type
RECIPIENT															
Am7*	·	34	44	·	25	1	58	80	28	22	6	14	10	8	3×10^3
Am8	·	0	20	·	6	4	20	35	79	1	35	0	20	41	3×10^3
Am9	·	2	0	·	8	8	8	·	·	1	6	2	5	11	·
Am10*	·	8	8	·	16	2	9	·	·	30	2	7	8	10	·
CAm1†	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
CAm2	·	5	15	·	4	0	·	·	·	3	0	7	3	·	$2{\cdot}5 \times 10^3$
CAm3	·	0	·	·	0	·	·	·	·	0	·	·	·	·	$2{\cdot}5 \times 10^3$
CAm4	·	30	·	·	·	·	20	0	28	·	·	·	·	·	·
CAm5	·	39	·	·	·	·	6	16	0	·	·	·	·	·	$2{\cdot}5 \times 10^3$
LAm1†	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
V2	·	13	9	·	11	11	41	·	·	9	0	52	0	·	$2{\cdot}5 \times 10^3$
V5	·	1	3	·	8	·	8	·	·	·	100	0	23	·	·
PhB3	·	12	12	·	100	5	3	·	·	0	0	60	0	·	3×10^3
PhF1	·	9	17	·	·	·	·	·	·	·	·	·	·	0	·
amiE mutations	7	8	9	10	17	18	19	20	21	34	16, 23	16, 26	16, 67	82	

* Strains PAC307 (Am7) and PAC310 (Am10) could not be used as donors.

† Strains PAC321 (CAm1) and PAC326 (LAm1) could not be used as recipients.

. No crosses carried out.

were at independent sites. Strains Am7 and Am8 were used as recipients in all crosses involving other acetamide-negative strains as the first step in determining uniqueness of mutational sites.

Strain PAC322, which was considered to resemble the Am series in genotype, gave recombinants with Am8, Am9, and Am10 but very few with Am7. The CAm series mutants, PAC321, PAC323, PAC324 and PAC325, all gave recombinants in crosses with Am7 and Am8 and with one of the other CAm mutants indicating that mutations *amiE*17, 18, 19, 20 and 21 are also at independent sites.

It was important to establish that the donor phenotype could be recovered at the same high frequency when one of the mutants producing altered enzyme was used as donor as when the wild-type strain was used. With a phenylacetamide-utilizing mutant of the PhB group as donor, and an Am series mutant as recipient, the transductants which had acquired the donor phenotype would be able to grow on phenylacetamide and butyramide but not on acetamide. The wild-type strain could be used as recipient with a PhB mutant as donor and transductants acquiring the donor phenotype could be selected on phenylacetamide and butyramide plates. With a butyramide-utilizing mutant of the B series as recipient and a PhB mutant as donor, the donor phenotype could be selected on phenylacetamide. Table 3 gives the results of these crosses with PhB3 as donor and it can be seen that, when the tranductants are selected on the appropriate medium for the recovery of the donor phenotype, the numbers of amidase-positive transductants obtained are comparable with those recovered from crosses with the wild type as donor and acetamide-negative strains as recipients. The numbers of transductants acquiring the amide growth phenotype of the donor strain ranged from 3000 to 5000 per 10^9 recipient bacteria. On the other hand, if selection was made for the acetamide-positive transductants, which could arise only by a recombination event, then the numbers recovered were very low. With PhB3 as donor and Am7 as recipient, ten acetamide-positive transductants were recovered and this cross is therefore comparable to a cross between two Am series mutants in which selection is made for amidase-positive transductants. These results showed that in spite of the double mutation in the amidase structural gene of PhB3 it behaved normally as donor and recipient and indicated that it would be possible to select for recombination between the altered enzyme mutants provided that they were acetamide-negative. Similar checks on recovery of donor phenotype were carried out with the V mutants as donors and Am mutants as recipients and again gave large numbers of amidase-positive transductants when the donor phenotype was selected and small numbers when selection was made for the recombinant transductants on acetamide plates. The recovery of recombinants which are acetamide-positive does not necessarily imply that they all possess the wild-type *amiE*$^+$, since it is known that some of the mutants producing altered enzymes, e.g. the B series and some of the V series mutants, can also grow on acetamide. The results were used in the first instance to decide whether or not the mutations determining the altered enzymes were at different sites in the amidase structural gene.

Crosses carried out with PhB3 as donor and 11 other PhB mutants (Table 1)

as recipients did not give any transductants on acetamide plates. Although these strains had been derived from B6 by a variety of mutagenic treatments (Table 1) they had identical amide growth phenotypes and the amidase substrate profiles were very similar (Betz & Clarke, 1972). This had suggested that the amidases produced by the strains might be identical with each other and the absence of recombination between these strains indicates that the mutations of the PhB series are at the same site in the *amiE* gene. No acetamide-positive transductants were obtained in the reciprocal crosses between PhB3 (*amiR*11 *amiE*16, 67) and the V series mutant V2 (*amiR*11 *amiE*16, 23). These two mutants were derived from the same parent by single mutational steps and the absence of recombinants in this cross suggests that *amiE*23 may be at the same site as *amiE*67 although these two mutations determine amidase proteins with different substrate specificities. On the other hand, reciprocal crosses between the two V series mutants V2 and V5 gave 50–100 acetamide-positive transductants and the cross between V5 and PhB3 gave 60 acetamide-positive transductants indicating that the *amiE*26 mutation of V5 was at a different site from *amiE*23 and *amiE*67. The other mutants listed in Table 4 were examined in less detail so that the evidence is not unequivocal from these genetic experiments that they had mutations at unique sites.

The values given in Table 4 are for the numbers of transductants which appeared on acetamide plates calculated with respect to 10^9 recipient bacteria. Each cross was carried out at least twice and some were carried out on more than five separate occasions. The figures were corrected if necessary for revertants appearing on the control plates but these were few in number (see Table 2) and often none were present. Little importance is attached to the actual numbers recovered in the crosses between the acetamide-negative mutants since they were not standardized against a donor marker of known efficiency of transfer. When any of the mutants were used as both donor and recipient the crosses were invariably unproductive and it was considered significant, and indicative of either the same or nearby sites of mutation, when crosses between two different mutants did not produce any acetamide-positive transductants. For the altered enzyme mutants V2, V5, PhB3 and PhF1, the main significance of these results was that these amidase-positive mutants could be used in the same way as the amidase-negative mutants and that comparable numbers of the acetamide-positive transductants were recovered.

Only the crosses between the Am series of mutants were simple two-factor crosses. In all the other cases the strains carried additional mutations in the regulator gene or the structural gene or in both. The crosses between strains carrying three or more mutations were intended to give information on map order as well as uniqueness of mutational sites. The transductants from these crosses were analysed in detail with respect to regulator phenotype and the class of amidase protein produced.

(iii) *Three-factor crosses between Am, CAm and LAm mutants*

The first method used to determine the map order of some of the mutations in the structural gene *amiE* depended on using a mutation in the closely linked regulator gene *amiR* as the outside marker in crosses between two amidase-negative mutants. Crosses were carried out between mutants of the Am series, which were potentially inducible for amidase, and mutants of the CAm and LAm series, which were potentially constitutive, and the ratios of inducible and constitutive amidase-positive transductants determined. Most of the crosses were carried out with the mutation *amiR*11 carried by the CAm strains as the outside marker but a few crosses were made with LAm1 which carries *amiR*33 from the constitutive mutant PAC142 (L10). Since these two *amiR* mutations result in different phenotypes it is not unreasonable to assume that they are at different sites. Both *amiR*11 and *amiR*33 are cotransduced with the *amiE* gene (J. E. Brown, 1969) and appear to determine modified regulator proteins.

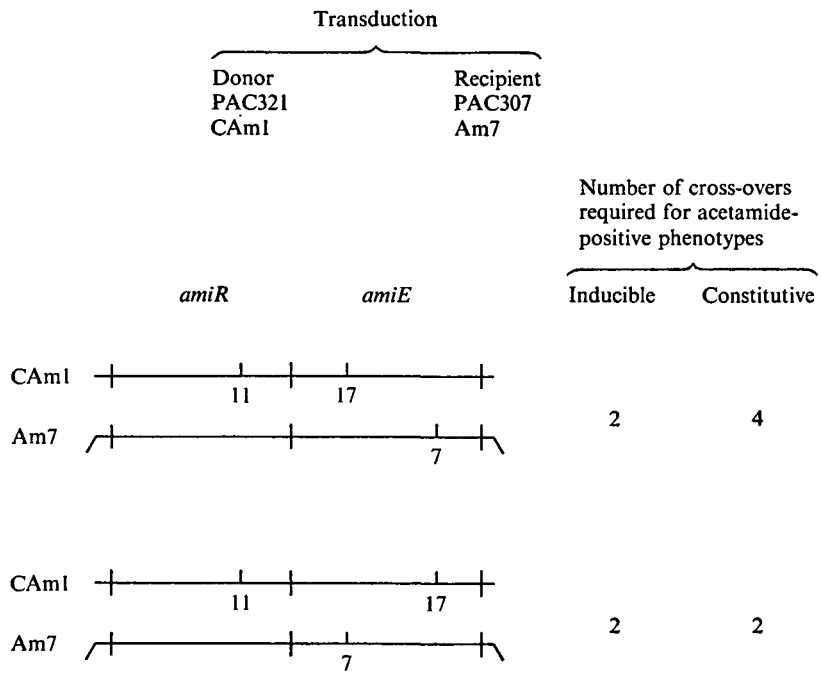

Fig. 1. Possible orders of *amiE* mutations from three-factor crosses between Am, CAm and LAm strains. Details in Table 5. *amiR*$^+$ *amiE*$^+$: inducible, A amidase. *ami*R11 *ami*E$^+$: constitutive, A amidase.

Fig. 1 shows the two possible orders of the mutations *amiR*11, *amiE*17, *amiE*7 and the number of crossovers which would be required to give inducible or constitutive amidase-positive transductants in the cross CAm1 × Am7. In this cross most of the recombinants were inducible (Table 5) which fits best with the order *amiR*11 – *amiE*17-7. In the cross CAm1 × Am8, inducible and constitutive trans-

https://doi.org/10.1017/S001667230001497X Published online by Cambridge University Press

ductants were obtained in the ratio of 1 : 1 which fits best with the mutational order *amiR*11-*amiE*8-17. If the Am mutation is between the *amiE* mutation of the CAm mutant and the *amiR* gene it requires two crossovers to generate both inducible and constitutive recombinants but if the *amiE* mutation is distal it requires two crossovers to generate inducible recombinants and four crossovers to generate constitutive recombinants. The actual numbers of inducible and constitutive recombinants will be affected by the probability of a crossover occurring at all, and the main factor determining this will be the distance apart of the mutational sites. Crossovers occurring outside the amidase genes will be more likely than between the mutational sites within the genes. However, when the mutational sites are very closely linked there may be high negative interference. Table 5 summarizes the results obtained and the most probable orders of the mutational sites.

(iv) *Crosses between the phenylacetamide mutant PhF1 and Am mutants*

The phenylacetamide-utilizing mutant PAC392 (PhF1) was derived by a single mutational step from strain PAC128 (CB4) (*amiR*11, 37 *amiE*+). The respective positions of the two *amiR* mutations are not known but it is assumed that two different sites within the *amiR* gene are concerned. The genotype of PhF1 is therefore *amiR*11,37 *amiE*82. This mutant grows well on butyramide and phenylacetamide but very poorly on acetamide, producing small colonies after 7 days at 37 °C, and this made it possible to use it both as donor and recipient in crosses with Am strains. When PhF1 was used as the recipient the acetamide-positive recombinant transductants appeared as large colonies in a hazy background of growth. This was quite satisfactory for two-factor crosses but difficult for the analysis of recombinants obtained in three-factor crosses. When PhF1 was used as donor the recombinant transductants appeared as large colonies after 2 days while the transductants which had acquired the entire PhF1 mutant amidase genes, conferring donor phenotype, did not appear until 7 days.

Recombination between PhF1 and Am7 would be expected to generate the acetamide-positive genotypes, *amiR*+ *amiE*+, *amiR*11 *amiE*+, *amiR*37 *amiE*+ and *amiR*11, 37 *amiE*+. All these classes would produce A amidase. *amiR*+ recombinants would be inducible; *amiR*11 recombinants would be constitutive and butyramide-negative; *amiR*11, 37 recombinants would be constitutive and butyramide-positive; the phenotype of *amiR*37 is not known. In practice it proved possible to discriminate easily between constitutive and inducible transductants in these crosses but replica plating to butyramide plates did not give clear-cut discrimination between the two expected butyramide phenotypes. In Table 6 the transductants are grouped as inducible or constitutive without separation into butyramide-positive or butyramide-negative recombinants. The chance of *amiR*37 conferring butyramide-inducibility is low since among many regulator mutants growing on butyramide none has been found with an altered inducer specificity allowing induction by butyramide. The results of these crosses have therefore been treated as three-factor crosses with two *amiE* mutations and the *amiR* mutations regarded as a single outside marker (Fig. 2).

Table 5. *Three-factor crosses between acetamide-negative mutants of Am, CAm and LAm series*

Donor	Genotype	Recipient	Genotype	No. of transductants analysed	Inducible (%)	Constitutive (%)	Map order
PAC321, CAm1	*amiR*11 *amiE*17	PAC307	*amiE*7	96	95	5	R-17-7
		PAC308	*amiE*8	112	59	41	R-8-17
		PAC309	*amiE*9	145	59	41	R-9-17
		PAC310	*amiE*10	184	41	59	R-10-17
		PAC322	*amiE*18	13	0	100	R-18-17
PAC323, CAm3	*amiR*11 *amiE*19	PAC 307	*amiE*7	15	100	0	R-19-7
PAC324	*amiR*11 *amiE*20	PAC307	*amiE*7	40	90	10	R-20-7
		PAC308	*amiE*8	20	90	10	R-20-8
PAC325, CAm5	*amiR*11 *amiE*21	PAC307	*amiE*7	28	100	0	R-21-7
		PAC308	*amiE*8	41	100	0	R-21-8
PAC326, LAm1	*amiR*33 *amiE*34	PAC307	*amiE*7	77	97	3	R-34-7
		PAC308	*amiE*8	96	96	4	R-34-8
		PAC309	*amiE*9	7	100	0	R-34-9
		PAC310	*amiE*10	46	94	6	R-34-10

Recombinant transductants selected on AM plates, 0·1 % (w/v) acetamide in minimal agar.
Constitutive recombinants identified by replica plating to S/F plates, 1 % (w/v) succinate + 0·1 % (v/v) formamide in minimal agar.

https://doi.org/10.1017/S001667230001497X Published online by Cambridge University Press

With PhF1 as donor most of the acetamide-positive transductants were inducible in crosses with Am7, Am8, Am9 and Am10 as recipients. A reciprocal cross, with Am8 as the donor and PhF1 as the recipient, gave a ratio of constitutive to inducible acetamide-positive transductants of 2:1 (Table 6). The tentative map order deduced from these results was *amiR–amiE*82–(8, 9, 10)–7; the relative positions of *amiE*8 and *amiE*7 being taken from the results of the previous crosses involving these strains.

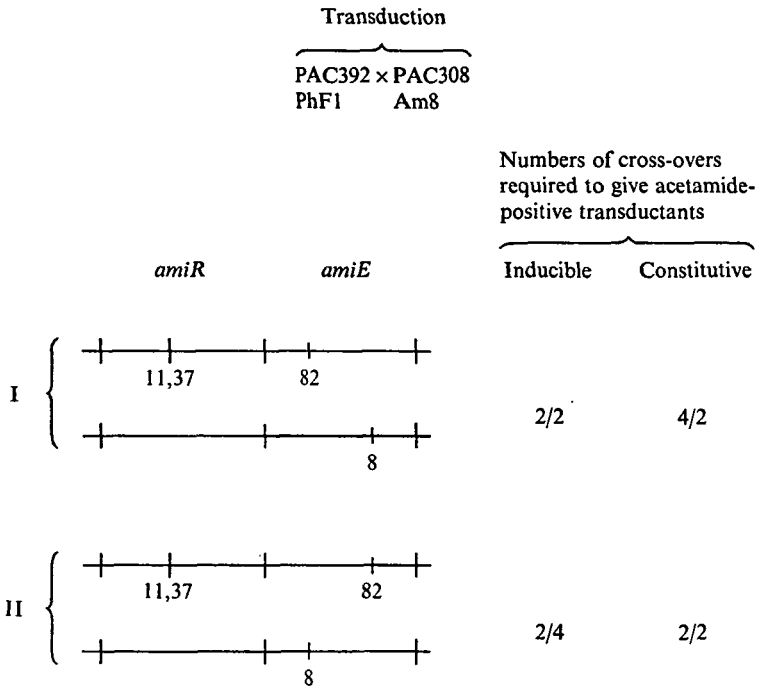

Fig. 2. Possible orders of *amiE* mutations from crosses between PhF1 and Am strains. Details in Table 6. Inducible, *amiR*+ *amiE*+. Constitutive, *amiR*11 *amiE*+, *amiR*11, 37 *amiE*+. Not known, *amiR*37 *amiE*+.

(v) *Crosses between PhB3 and the V and CAm series of mutants*

Crosses between some of the mutants producing altered enzymes were expected to generate recombinants which would be acetamide-positive but producing amidases other than the wild-type A amidase. If the two strains carried the same constitutive mutation in the regulator gene and mutations in the structural gene which could generate the genotype for B amidase as well as A amidase, it was thought that the ratio of B amidase-type to A amidase-type transductants could be used to order the *amiE* mutations.

The mutant strains CAm1, V5 and PhB3 were all derived from the constitutive acetamide-positive strain C11 (PAC111) and are thus expected to have retained the regulator mutation *amiR*11 conferring constitutivity. All acetamide-positive recombinants between these strains would be expected to be constitutive. PhB3, V2 and V5 were derived from strain B6 (PAC351), a butyramide-utilizing strain

carrying the *amiE*16 mutation, so that it was predicted that the acetamide-positive recombinants would be of two classes; those with the genotype *amiR*11 *amiE*$^+$ producing wild-type enzyme and those with the genotype *amiR*11 *amiE*16 producing the mutant B amidase. It was assumed in the first instance that the recombinant genotypes *amiE*16, 17, *amiE*17, 67, *amiE*16, 17, 67 and *amiE*7 which could arise in the cross between CAm1 and PhB3 would be likely to be acetamide-negative. If this assumption is made the six possible orders of the mutations *amiE*16, 17 and 67 with respect to the *amiR* gene and the number of crossovers required to generate *amiE*$^+$ and *amiE*16 will be those given in Fig. 3. Analysis of the number of acetamide-positive transductants producing A or B amidase will not give a unique order for these mutations but was expected to assist in reducing the number of possible orders. The analysis of the acetamide-positive transductants from the crosses between CAm1 and V2 and V5 was based on similar assumptions.

Table 6. *Analysis of crosses between the phenylacetamide mutant PhF1 and amidase-negative mutants of Am series*

Donor	Recipient	No. of transductants analysed	Inducible (%)	Constitutive (%)*
PAC392 PhF1, *amiR*11, 37*amiE*82	PAC307	68	87	13
	PAC308	115	65	35
	PAC309	52	65	35
	PAC310	44	75	25
PAC308, Am8, *AmiR*$^+$ *amiE*8	PAC392	21	33	66

* The constitutive group includes all recombinant with *amiE*$^+$ which may be *amiR*11, *amiR*37 or *amiR*11, 37.

It was particularly important in these crosses to obtain transductant colonies completely free from recipient bacteria since PhB3, V2 and V5 were capable of growth on butyramide as well as the higher amides and this could have obscured the results. The A and B amidases were identified by the mini-assay transferase method from transductant cultures grown overnight in lactate medium and all tests gave unambiguous results. Table 7 shows that the acetamide-positive transductants from the CAm1 × PhB3 cross were predominantly of the constitutive B amidase phenotype; only 1 transductant of the 66 analysed produced A amidase. This result would fit best with map orders I and II of Fig. 3. However, it was realized that the constitutive B amidase phenotype could have been produced by recombinants with genotypes other than *amiR*11 *amiE*16 and the analysis was repeated in more detail. The 65 which had grown on butyramide were retested for amide growth phenotype and it was confirmed that all grew well on acetamide and succinate/formamide plates. When they were retested on butyramide it was found that although they were able to grow on this amide only 33 of them grew as well as B6 and the other 32 grew rather slowly. Six transductants of each class

were picked off, grown in lactate medium single colonies reisolated and retested on the selective amide media. This confirmed the occurrence of normal and slow-growing constitutive butyramide-positive recombinants. Assays of washed suspensions of both types of recombinant gave butyramide/acetamide ratios for amidase activity which appeared to correspond with those for the normal B enzyme. It was thought that if the recombinants with slow growth on butyramide had retained

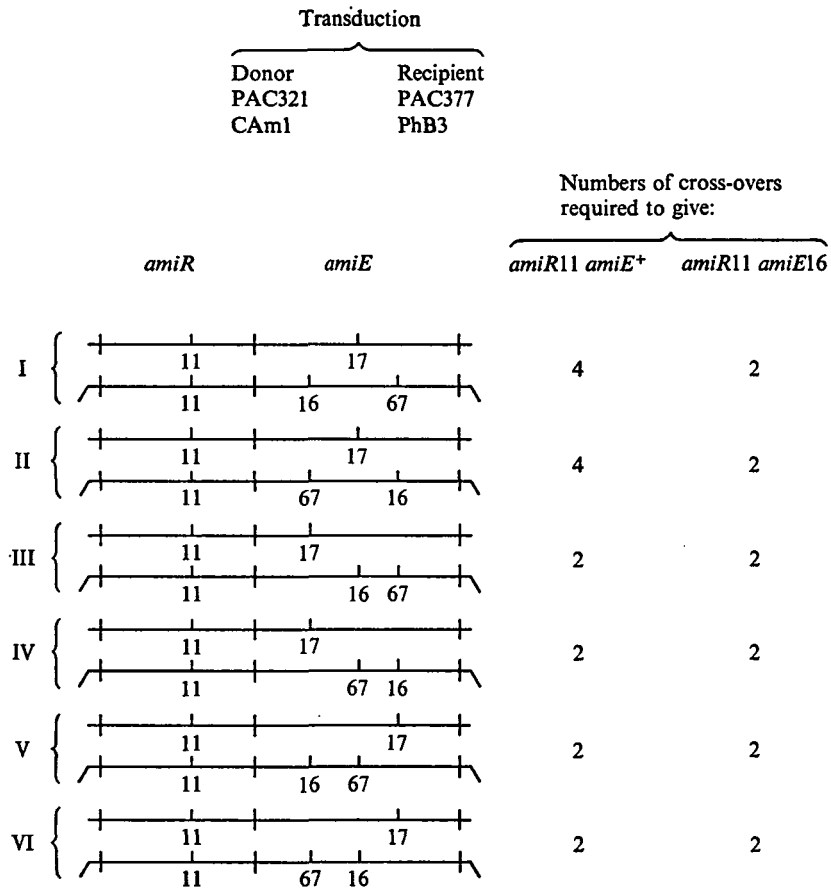

Fig. 3. Possible orders of *amiE* mutations from crosses between CAm1 and PhB3 and the V series mutants. Details in Table 7. *amiR*11 *amiE*+, constitutive, A amidase. *amiR*11 *amiE*16, constitutive, B amidase.

the *amiE*67 mutation it would be possible to rescue the PhB3 genotype by using B6 as donor and selecting for recombinants on phenylacetamide. Table 7 shows that while both of the recombinant types were able to act normally as recipients in crosses with PhB3 and acquire the donor phenotype at the usual frequency there were no phenylacetamide-positive recombinants when B6 was used as the donor. It therefore seemed unlikely that the slow growth on butyramide was due to the genotypes *amiR*11 *amiE*16, 17, 67, *amiR*11 *amiE*6, 67 or *amiR*11 *amiE*67. The anomalous results of this cross were not resolved so that it could not contribute

to the final map order. Crosses between CAm1 and V2 and V5 also gave predominantly constitutive B amidase recombinants, but it was thought that in view of the complexities which had been revealed in the PhB3 cross these results should not be used to calculate the order of the mutational sites.

Table 7. (*a*) *Analysis of acetamide-positive recombinant transductants from crosses between CAm1 and PhB3, V2 and V5*

Donor	Recipient	Total no. analysed	No. of recombinants* Ind A	Ind B	Con A	Con B
CAml, *amiR*11 *amiE*17	PhB3, *amiR*11 *amiE*16, 67	66	0	0	1	65†
	V2, *amiR*11 *amiE*16, 23	23	0	0	1	22
	V5, *amiR*11 *ami*E16, 26	15	0	0	0	15

(*b*) *Crosses between B6, PhB3 and Con B+ and Con B-s recombinants (number of phenylacetamide-positive transductants recovered 10^9 recipients)*

Recipient	Donor B6	PhB3
B6, *amiR*11 *amiE*16	0	$1{\cdot}8 \times 10^3$
Con B+	0	$2{\cdot}6 \times 10^3$
Con B-s	0	$4{\cdot}3 \times 10^3$

* Ind = inducible; Con = constitutive; A = amidase with A amidase substrate specificity; B = amidase with B amidase substrate specificity.

† Twenty three grew at normal B6 rate on butyramide (Con B+) and 22 grew more slowly (Con B−s).

(vi) *Four-factor analysis of acetamide-positive transductants from crosses between Am series mutants and PhB3 and V mutants*

Crosses between the potentially inducible amidase-negative mutants of the Am series and the constitutive altered enzyme mutants, were expected to generate inducible as well as constitutive acetamide-positive recombinants. In the cross between Am8 and PhB3, the four expected phenotypes were with respect to type of amidase and its synthesis; inducible, A amidase; inducible, B amidase; constitutive, A amidase; constitutive, B amidase (Table 8). These could be related to the wild-type genes *amiR*+ and *amiE*+ and the two mutations whose phenotype was known *amiR*11 and *amiE*16. The phenotypes of the possible recombinants carrying the structural gene mutations *amiE*16, 67, 8, *amiE*16, 8, or *amiE*67 are not known. Three of the four possible phenotypes could be identified by replica plating to acetamide, butyramide and succinate/formamide plates. The inducible A amidase recombinant would grow on acetamide only, the constitutive A amidase recombinant would grow on acetamide and succinate/formamide and the constitutive B amidase recombinant would grow on all three media. However, an inducible recombinant producing B amidase would not be able to grow on butyramide since

butyramide is not an inducer for a strain with a wild-type regulator gene. As before, it was more satisfactory to determine the type of amidase produced by direct enzyme assay (Table 8).

Table 8. *Analysis of acetamide-positive transductants from crosses between PhB3, V2, V5 and amidase-negative mutants of Am series*

Donor	Recipient	No. of transductants analysed	No. of recombinants*			
			Ind A	Ind B	Con A	Con B
PhB3	Am7	75	7	62	0	6
	Am8	82	72	2	7	1
	Am9	78	51	3	22	2
	Am10	117	101	7	5	4
	CAm2	5	1	3	1	0
V2	Am7	12	2	8	0	2
	Am8	53	46	0	5	2
	Am9	21	17	0	3	1
	Am10	6	5	1	0	0
V5	Am7	27	3	14	2	9
	Am10	12	9	0	3	0
Am8	PhB3	79	46	1	28	4
Am9	PhB3	72	21	2	39	10
	V2	26	5	0	16	5
	V5	7	1	0	1	5
CAm2	PhB3	20	3	8	4	5

* Ind = inducible; Con = constitutive; A = amidase A; B = Amidase B.

The two valeramide mutants could also give rise to the same classes of recombinants and the transductants from crosses with V2 and V5 as donors were analysed in the same way. Strains PAC377, PAC353 and PAC356 (PhB3, V2 and V5) could also be used as recipients so that reciprocal crosses were possible with the Am mutants as donors (Table 8).

It was clear that there were so many possible recombinant classes to be obtained in these crosses that it would not be possible to deduce a single map order. However, it was satisfactory to find that all four possible phenotypes were recovered. The inducible B amidase recombinant was particularly welcomed since there is no other obvious method of obtaining a mutant of this class. The wild-type enzyme has such low activity on butyramide that strains with *amiE*$^+$ can only grow on butyramide if they produce large amounts of the enzyme. The wild-type strain cannot be induced by butyramide and all the butyramide utilizing mutants we have isolated (both B and CB classes) are constitutive for amidase synthesis.

4. DISCUSSION

Our main objective in this investigation was to obtain the maximum amount of information from transductional crosses between the mutants at our disposal. The physiological properties of the mutants producing altered enzymes were well known from previous studies and it was hoped that it would be possible to establish whether any or all of these mutations were at independent sites. It was also hoped that it might be possible to establish a map order for these mutations which could be related at a later date to amino acid substitutions in the enzyme protein. Several of the altered enzyme mutants had been obtained by successive single site mutations and for these it was intended to devise tests to determine whether or not the earlier mutations had been conserved during the acquisition of the new phenotype.

The values for the total numbers of transductants obtained in the two-factor crosses are given in Table 4 but they were not used to calculate map distances since it was felt that the system was not sufficiently standardized to do this. The numbers of transductants obtained in many of the crosses were so low that standardization against an outside auxotrophic marker would not have improved the accuracy and resolution. It can be seen that anomalous results were obtained in several of the reciprocal crosses. This was most evident in crosses between strains carrying more than one mutation in the *amiE* gene. There are other reports of differences in the numbers of transductants obtained in reciprocal crosses in *P. aeruginosa*. Feary *et al.* (1969) found anomalies in reciprocal crosses between *arg* mutants as also did Mee & Lee (1967) in crosses between *his* mutants.

Unfortunately reciprocal crosses could not be carried out between some of the most interesting strains. Strain Am7 had acquired an additional mutation which did not allow propagation of the transducing bacteriophage so that crosses could be carried out in one direction only. However, many of the crosses involving this strain gave very asymmetric ratios for the third marker in the three-factor crosses so that these values could be used to determine the map order of the mutational sites. Crosses between most of the other amidase negative mutants of the Am and CAm series could be carried out in both directions but not all of these were analysed in detail. The amidase negative mutants did not exhibit any particularly interesting characteristics and were mainly of interest in their relationships to the altered enzyme mutants. Reciprocal crosses were carried out between amidase-negative and altered enzyme mutants whenever possible but some of these presented other difficulties since the recombinants obtained from a cross in one direction could be more readily analysed than those obtained in the reciprocal cross. When the altered enzyme mutant PhF1 was used as donor, in a cross with the amidase-negative mutant Am8, the recombinants could be analysed by replica plating or by picking off and streaking on selective media for the continuous marker. However, the recombinants in the reciprocal cross with Am8 (or any other mutant) as donor and PhF1 as recipient, appeared among an appreciable amount of background growth. Very careful purification to single colonies was required before the regulator

phenotype could be tested. Purification to single colonies was also necessary with crosses with PhB3 and V2 and V5 as recipients but with these mutants no background growth was visible so that the purification procedure was less tedious and gave more reliable results.

The map orders which we deduced from the three-factor crosses were as follows: *R–amiE* (10, 9) 8-17-7; *R–amiE* 18-17-7; *R–amiE* 82-8-7; *R–amiE* 34-8-7; *R–amiE* (19, 20, 21)-8-7, (Tables 5, 6). For some of the crosses between CAm1 and Am8, 9 and 10 the ratios of inducible and constitutive recombinants were around 60:40 and these values are less reliable in establishing map order because of negative interference which can result from a high frequency of clustered exchanges over a short gene region. With these reservations in mind we have combined all the data from the three-factor and four-factor crosses to construct a tentative map of the *amiE* gene which will provide us with the basis for further studies on the genetics of this system (Fig. 4).

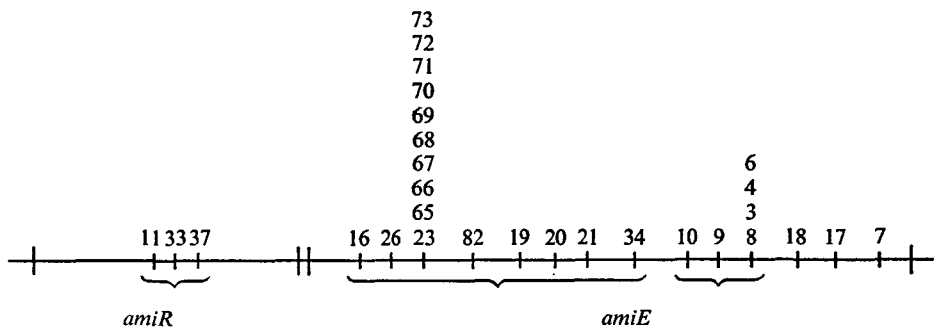

Fig. 4. Map order of mutations in the *amiE* gene derived from the crosses shown in Figs. 1–3 and Tables 4–7. Mutations shown grouped within brackets were not ordered with respect to each other. The scale is arbitrary and does not indicate map distances.

It could be safely assumed that the mutations conferring altered substrate specificity were in the amidase structural gene but it could not be ruled out that some of the amidase-negative mutants carried only mutations in the regulator gene. However, it seemed most likely from the properties of the Am and CAm mutants and of the recombinants obtained in the various crosses, that all the acetamide-negative mutants examined here had structural gene mutations. We have subsequently isolated mutants which appear to be promotor mutants (Smythe & Clarke, 1972) and some temperature-sensitive mutants which may be regulator mutants (Farin & Clarke, 1974). The mutations have not yet been mapped with respect to the mutations used in this study.

Crosses between the two V mutants and the phenylacetamide-utilizing mutant PhB3 gave clear-cut genetic evidence that the second mutation in strain V2 (*amiE*16, 23) is at a different site from the second mutation of strain V5 (*amiE*16, 26). Previously it had been concluded from the amide growth ranges, and the substrate profiles of washed suspensions, that the V mutants formed a heterogeneous group (Brown *et al.* 1969). Equally interesting was the finding that muta-

tion *amiE*23 of strain V2 could be at the same site as the *amiE*67 mutation of strain PhB3. If this is so, it indicates that two different mutations at the same site can result in two enzyme proteins with different substrate specificities. It should be emphasized that PhB3 utilizes both phenylacetamide and valeramide, while V2 utilizes valeramide only, so that it might not require a very marked difference in the amino acid substitutions to produce amidases with these two different substrate growth profiles.

Recovery of recombinants which appeared to produce wild-type A amidase is not conclusive evidence that the wild-type structural gene was recovered since these acetamide-positive recombinants could have produced pseudo-wild enzyme. However, when the recombinants from the various crosses were analysed the group which appeared to be producing A amidase behaved in a uniform way, and in so far as the tests went, it seemed most likely that they produced the wild-type enzyme and thus had undergone a recombination which generated the *amiE*+ gene. When one of the parents carried the *amiE*16 mutation it was predicted that recombination would give rise to progeny producing the B amidase characteristic of the B group of mutants. PhB3, and the two V mutants, gave some recombinants in crosses with Am and CAm mutants which produced amidases with the same amide substrate activities as strain B6, indicating that during the mutational events giving rise to the V and PhB phenotypes the original mutational *amiE*16 was conserved. However, there were some anomalies in the crosses between PhB3 and CAm1 in that some of the butyramide-utilizing recombinants grew slowly on butyramide. It is possible that one of the parent strains contained a mutation which had not been previously detected. Strain CAm1, for example, was obtained after NMG mutagenesis, which is known to give multiple closely linked mutations. However, the spontaneous revertants arising from CAm1 appeared to be identical with strain C11. Slow growth on butyramide could be due to a regulator gene mutation or to a pseudo-B amidase determined by one of the other mutations in the *amiE* gene of the parental strains (Table 7).

None of the mutations, with the possible exception of CAm3, behaved as deletion mutations. A further series of Am or CAm mutants carrying a range of deletions would be valuable to confirm the map order of mutations and to assign mutations to particular regions of the structural gene. Wheelis & Ornston (1972) were able to use deletion mutations in the gene *catB* to determine the map order of *catB* point mutations in *P. putida*. Their findings were confirmed with crosses carried out with a regulatory mutant carrying a mutation conferring constitutivity (Wu, Ornston & Ornston, 1972). The frequency of cotransduction of the constitutivity character with the ability to grow on benzoate ranged from 37 to 80% with different *catB* markers and thus the formula devised by Wu (1966) could be used to calculate the relative distances between the *catB* mutations and the mutation conferring constitutivity. This method could not be used with the amidase regulator and structural genes since the cotransduction frequency was high in all cases and varied only between 80 and 98% so that the differences observed were not likely to be significant.

It is possible that most of the mutations which we have examined are in a relatively short region of the *amiE* gene concerned with amide binding. Isolation of a larger number of amidase-negative mutants, including deletion mutants, will help to establish the genetic length of the amidase structural gene. Studies are being made on the amino acid differences between the wild-type and mutant amidase proteins and it is hoped that it will be possible to correlate these with the mutational sites.

We are grateful to Professor B. W. Holloway for valuable advice and very helpful discussion, J. L. B. is grateful to the National Science Foundation U.S.A. and M. D. to the Medical Research Council for research training grants.

REFERENCES

ADAMS, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers.

BETZ, J. L., BROWN, P. R., SMYTH, M. J. & CLARKE, P. H. (1974). *Nature* **247**, 261–264.

BETZ, J. L. & CLARKE, P. H. (1972). Selective evolution of phenylacetamide-utilizing strains of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **73**, 161–174.

BRAMMAR, W. J. & CLARKE, P. H. (1964). Induction and repression of *Pseudomonas aeruginosa* amidase. *Journal of General Microbiology* **37**, 307–319.

BRAMMAR, W. J., CLARKE, P. H. & SKINNER, A. J. (1967). Biochemical and genetic studies with regulator mutants of the *Pseudomonas aeruginosa* 8602 amidase system. *Journal of General Microbiology* **47**, 87–102.

BROWN, J. E. (1969). Regulator and structural gene mutants of *Pseudomonas aeruginosa* amidase. Ph.D. Thesis, University of London.

BROWN, J. E., BROWN, P. R. & CLARKE, P. H. (1969). Butyramide-utilizing mutants of *Pseudomonas aeruginosa* 8602 which produce an amidase with altered substrate specificity. *Journal of General Microbiology* **57**, 273–285.

BROWN, J. E. & CLARKE, P. H. (1970). Mutations in a regulator gene allowing *Pseudomonas aeruginosa* 8602 to grow on butyramide. *Journal of General Microbiology* **64**, 329–342.

BROWN, P. R. (1969). Studies on wild-type and mutant amidase proteins from *Pseudomonas aeruginosa*. Ph. D. Thesis, University of London.

CLARKE, P. H. (1970). *Advances in Microbial Physiology* **4**, 179–222.

FARIN, F. & CLARKE, P. H. (1974). Positive control of amidase synthesis in *Pseudomonas aeruginosa*. *Proceedings of the Society for General Microbiology* (in the Press).

FEARY, T. W., WILLIAMS, B., CALHOUN, D. & WALKER, T. A. (1969). An analysis of arginine requiring mutants in *Pseudomonas aeruginosa*. *Genetics* **62**, 673–686.

HOLLOWAY, B. W., EGAN, J. B. & MONK, M. (1960). Lysogeny in *Pseudomonas aeruginosa*. *Australian Journal of Experimental Biology and Medical Science* **38**, 321–330.

HOLLOWAY, B. W., KRISHNAPILLAI, V. & STANISICH, V. (1971). *Pseudomonas* genetics. *Annual Review of Genetics* **5**, 425–446.

HOLLOWAY, B. W., MONK, M., HODGINS, L. & FARGIE, B. (1962). Effects of radiation on transduction in *Pseudomonas aeruginosa*. *Virology* **18**, 89–94.

JACOB, F. & WOLLMAN, E. L. (1961). In *Sexuality and the Genetics of Bacteria*. London: Academic Press.

KELLY, M. & CLARKE, P. H. (1962). An inducible amidase produced by a strain of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **27**, 305–316.

LEDERBERG, J. & LEDERBERG, E. M. (1952). Replica plating and indirect selection of bacterial mutants. *Journal of Bacteriology* **63**, 399–406.

MEE, B. J. & LEE, B. T. O. (1967). An analysis of histidine requiring mutants in *Pseudomonas aeruginosa*. *Genetics* **55**, 709–722.

MEE, B. J. & LEE, B. T. O. (1969). A map order for His I, one of the genetic regions controllin. histidine biosynthesis in *Pseudomonas aeruginosa*, using the transducing phage F116 *Genetics* **62**, 687–696.

SKINNER, A. J. (1967). Biochemical and genetic studies with *Pseudomonas aeruginosa*. Ph.D. Thesis, University of London.

SKINNER, A. J. & CLARKE, P. H. (1968). Acetate and acetamide mutants of *Pseudomonas aeruginosa* 8602. *Journal of General Microbiology* **50**, 183–194.

SMYTH, P. F. & CLARKE, P. H. (1972). Catabolite repression of *Pseudomonas aeruginosa* amidase. *Journal of General Microbiology* **73**, ix.

WHEELIS, M. L. & ORNSTON, L. N. (1972). Genetic control of enzyme induction in the β-ketoadipate pathway of *Pseudomonas putida*: Deletion mapping of *cat* mutations. *Journal of Bacteriology* **109**, 790–795.

WU, C.-H., ORNSTON, M. K. & ORNSTON, L. N. (1972). Genetic control of enzyme induction in the β-ketoadipate pathway of *Pseudomonas putida*: Two point crosses with a regulatory mutant strain. *Journal of Bacteriology* **109**, 796–802.

WU, T. T. (1966). A model for the three-point analysis of random general transductions. *Genetics* **54**, 405–410.