Genetic Mapping in *Methylophilus methylotrophus* AS1

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(Received 27 July 1982; revised 28 September 1982)

Prime plasmids have been isolated from *Methylophilus methylotrophus* AS1 using the IncP-1 plasmid pMO172. Such plasmids can complement mutant function when transferred to appropriate strains of *Pseudomonas aeruginosa* PAO. From the range of particular functions complemented by each prime plasmid, a preliminary map of the *M. methylotrophus* AS1 genome with four groups of linked markers was obtained. Physical examination of two of these prime plasmids showed that each had acquired an additional DNA segment. Southern hybridization experiments showed there was sequence homology between the additional DNA segments and *M. methylotrophus* AS1 DNA, confirming that these prime plasmids carried a segment of the *M. methylotrophus* AS1 genome. Mapping by complementation may be applicable to other bacteria in which mutants suitable for selecting recombinants are not readily available.

INTRODUCTION

There is an increasing interest in genetic studies of bacteria other than those traditionally used (Levinthal, 1974), because of the availability of plasmids with chromosome-mobilizing ability in a variety of species (Holloway, 1979) and the need for genetic manipulation of bacteria used in biotechnology. The development of a conventional genetic recombination system in bacteria involves the use of an effective chromosome mobilization agent, the construction of multiply marked strains and a means of selecting rare recombinants. *Methylophilus methylotrophus* AS1 is a Gram-negative bacillus capable of growth on methanol as sole carbon source. It is used by Imperial Chemical Industries for the commercial production of 'Pruteen', a dried protein, animal feed additive. Our attempts to isolate an adequate range of auxotrophically marked strains of *M. methylotrophus* were unsuccessful, in agreement with the results of O'Connor & Hanson (1978) who found that auxotrophs of methylotrophs are difficult to isolate.

Enhanced chromosome mobilizing (ECM) plasmids such as R68.45 are able to generate plasmid primes in a variety of bacterial genera (reviewed by Holloway, 1979). Primes carrying fragments of chromosomal DNA from *Pseudomonas putida*, *Escherichia coli* and *Rhizobium* spp. can complement the phenotype of *P. aeruginosa* mutants to produce a wild-type phenotype (Holloway *et al.*, 1980; Johnston *et al.*, 1978; Nagahari *et al.*, 1977; Morgan, 1982; J. Barton & A. Morgan, unpublished). Although ECM plasmids such as R68.45 can promote chromosome transfer in *M. methylotrophus* AS1, the frequency of recombinants and the range of suitable selective mutants are inadequate for an effective mapping system to be established (Holloway, 1981). Accordingly we set out to establish an alternative system of mapping, which we have called complementation mapping. An ECM plasmid was used to generate a library of prime plasmids carrying fragments of the *M. methylotrophus* AS1 genome. The genes carried by these primes were identified by complementation after transfer to suitably marked *P. aeruginosa* PAO strains. The prime plasmids were further tested for their ability to complement a range of *P. aeruginosa* mutants to indicate which genes were carried by an individual prime and hence linked on the *M. methylotrophus* genome. A preliminary account of this work was presented at the Fourth International Symposium on Genetics of Industrial Microorganisms (in Kyoto in 1982).

Table 1. *Strains used in this study*

All *P. aeruginosa* strains were derived from PAO1 (Holloway, 1969). The full genotypes of strains referred to in Table 3 are given below. Details of other strains listed in Table 2 and others used in this investigation are available from the authors.

Strain	Genotype/Phenotype*	Derivation†	Reference
<i>Pseudomonas aeruginosa</i>			
PAO5	<i>trp-54 rif-5 fon-1</i>	Spontaneous rifampicin resistant derivative of PAO3	Holloway Collection
PAO12	<i>pur-136 leu-8 chl-3</i>	<i>pur leu</i> derivative of PAO1, intermediate strain lost, NG	Pemberton & Holloway (1972)
PAO90	<i>his-67 str-9</i>	Spontaneous streptomycin-resistant derivative of a histidine auxotroph, strain since lost, NG	Holloway Collection
PAO114	<i>pyrD48 chl-2</i>	<i>pyr</i> derivative of PAO1, NG	Issac & Holloway (1968)
PAO129	<i>pyrB52 chl-2</i>	<i>pyr</i> derivative of PAO1, NG	Isaac & Holloway (1968)
PAO286	<i>met-28 trp-6 chl-2</i>	<i>met trp</i> derivative of PAO1, MC	Holloway Collection
PAO305	<i>argG27 chl-2</i>	<i>arg</i> derivative of PAO1, NG	Isaac & Holloway (1972)
PAO325	<i>argB18 lys-57 chl-2</i>	<i>lys</i> derivative of PAO303	Holloway Collection
PAO462	<i>trpA1</i>	<i>trpE1</i> (old terminology) derivative of PAO1	Calhoun <i>et al.</i> (1973)
PAO470	<i>trpD1</i>	<i>trpB1</i> (old terminology) derivative of PAO1	Calhoun <i>et al.</i> (1973)
PAO472	<i>trpE1</i>	<i>trpA1</i> (old terminology) derivative of PAO1	Calhoun <i>et al.</i> (1973)
PAO503	<i>met-9011</i>	Transductant (E79 <i>tv-2</i>) of PAO1817	Holloway Collection
PAO896	<i>pur-136 chl-3</i>	<i>leu-8</i> ⁺ transductant (E79 <i>tv-2</i>) of PAO12	Morgan (1982)
PAO897	<i>leu-8 chl-3</i>	<i>pur-136</i> ⁺ transductant (E79 <i>tv-2</i>) of PAO12	Morgan (1982)
PAO898	<i>ilv-202 str-1</i>	<i>met-28</i> ⁺ transductant (E79 <i>tv-2</i>) of PAO8	Morgan (1982)
PAO899	<i>met-28 str-1</i>	<i>ilv-202</i> ⁺ transductant (E79 <i>tv-2</i>) of PAO8	Holloway Collection
PAO900	<i>ilvB/C208</i>	<i>trpB4</i> ⁺ transductant (E79 <i>tv-2</i>) of PAO422	Morgan (1982)
PAO904	<i>argF10</i>	<i>leu-10</i> ⁺ transductant (E79 <i>tv-2</i>) of PAO25	Morgan (1982)
PAO907	<i>proA54</i>	<i>cys-5605</i> ⁺ <i>his-5075</i> ⁺ transductant (E79 <i>tv-2</i>)	Morgan (1982)
PAO1086	<i>proB64 str-93</i>	Spontaneous streptomycin resistant mutant of PAO888	Holloway Collection
<i>Pseudomonas putida</i>			
PPN1089	<i>cys-401 trp-405</i>	<i>cys-401 trp-405 arg</i> ⁺ derivative of PPN1088	A. Boronin & A. Morgan, unpublished
<i>Escherichia coli</i>			
JP2314	<i>trpE365 pyrF40 F</i> ⁻		J. Pittard, unpublished
<i>Methylophilus methylotrophus</i>			
AS1	Wild type		ICI Strain Collection
<i>Plasmids</i>			
R68.45	IncP-1 Cb ^r Km ^r Tc ^r Cma ⁺		Haas & Holloway (1976)
pMO61	IncP-1 Cb ^r Km ^r Tc ^r Cma ⁺		Riess <i>et al.</i> (1980)
pMO172	IncP-1 Cb ^r Km ^r Tc ^r Cma ⁺		Holloway Collection
<i>Bacteriophages</i>			
F116L	General transducing phage		Krishnapillai (1971)
G101	General transducing phage		Holloway & van de Putte (1968)
E79 <i>tv-2</i>	General transducing phage	Mutant of virulent phage E79	Morgan (1979)

* The following designations have been used. Anabolic markers: *arg*, arginine; *cys*, cysteine; *his*, histidine; *ilv*, isoleucine, valine; *leu*, leucine; *lys*, lysine; *met*, methionine; *pro*, proline; *pur*, purine; *pyr*, pyrimidine; *ser*, serine; *thr*, threonine; and *trp*, tryptophan. Resistance markers: *chl*, chloramphenicol; *fon*, phage F116L; *rif*, rifampicin; *str*, streptomycin. Plasmid characters: Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Cma, chromosome-mobilizing ability.

† Unless otherwise stated, all mutants were obtained after treatment of the parent strain indicated with ethyl methanesulphonate. NG indicates mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MC indicates mutagenesis with manganous chloride. In cases where the strain has been previously published, further details of isolation can be obtained from the cited reference.

Table 2. Strains of *P. aeruginosa* PAO used to select plasmid primes and to detect complementation by plasmid primes derived from *M. methylotrophus* AS1

Marker	Map location (min)†	Enzyme (where known)	<i>P. aeruginosa</i> PAO strain no.	Rifampicin-sensitive isogenic strain no.
*argA	18	N-Acetylglutamate synthase	894	952
*argB	21	N-Acetylglutamate 5-phosphotransferase	916	303
argC	35	N-Acetylglutamate 5-semialdehyde dehydrogenase	679	307
argF	45	Anabolic ornithine carbamoyltransferase	686	904
argG	45	Argininosuccinate synthetase	685	305
*argH	19	Argininosuccinase	373	362
cys-54	79	Unknown (requires cysteine for growth)	1155	944
*cys-59	94	Unknown (requires cysteine for growth)	1156	949
hisI	94	Histidinol dehydrogenase	675	903
*hisIIA	17	Unknown (requires histidine for growth)	913	GMA121
*hisIIB	17	Unknown (requires histidine for growth)	914	892
*hisIII	16	Unknown (requires histidine for growth)	915	893
*hisIV	14	Unknown (requires histidine for growth)	1152	891
hisV	30	Unknown (requires histidine for growth)	919	GMA035
ilvB/C	7	ilvB lacks acetohydroxy synthetase, ilvC lacks reductoisomerase	930	900
ilvD	31	Dehydroxy acid dehydratase	920	898
leu-8	42	Unknown (requires leucine for growth)	925	897
leu-10	53	Unknown (requires leucine for growth)	687	905
lys-12	20	Unknown (requires lysine for growth)	895	372
met-28	31	Unknown (requires methionine for growth)	680	286
met-9011	53	Unknown (requires methionine for growth)	926	503
proA	39	Glutamate 5-phosphotransferase	682	907
proB	1	Glutamyl 5-phosphate reductase	911	888
proC	32	Lacks ability to convert Δ^1 -pyrroline 5-carboxylate to proline	679	906
pur-66	42	Unknown (requires adenine for growth)	684	901
pur-70	90	Unknown (requires adenine for growth)	688	909
pur-136	25	Unknown (requires adenine for growth)	917	12
pyrB	32	Aspartate transcarbamylase	681	129
pyrD	57	Dihydroorotic acid dehydrogenase	928	114
pyrE	22	Orotidyl acid pyrophosphorylase	486	483
*pyrF	59	Orotidine monophosphate pyrophosphorylase	929	161
*ser-3	27	Unknown (requires serine for growth)	678	2
*thr-48	43	Unknown (requires threonine for growth)	683	908
trpA	27	Tryptophan synthase α -activity	918	462
trpB	27	Tryptophan synthase β -activity	677	467
trpC	34	Indoleglycerolphosphate synthase	921	468
trpD	34	Anthranelate phosphoribosyltransferase	922	470
trpE	34	Anthranelate synthase	923	472
trpF	54	Phosphoribosyl anthranilate isomerase	927	474

* No plasmid primes were isolated which complemented these markers.

† The map locations are those given by Holloway & Crockett (1982).

METHODS

Strains. The strains of *Methylophilus methylotrophus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Pseudomonas putida* are listed in Table 1, as are the plasmids and bacteriophages used. Table 2 lists the *P. aeruginosa* mutations used.

Media. The media for *P. aeruginosa* were as described previously (Holloway, 1978; Pemberton & Holloway, 1973; Royle & Holloway, 1980). For *M. methylotrophus* the C2 medium of Windass *et al.* (1980) was used.

Isolation of plasmid primes from *M. methylotrophus*. The ECM plasmid used was pMO172, a derivative of pMO61 (Riess *et al.*, 1980). pMO61 is a derivative of R68 with enhanced chromosome-mobilizing ability. pMO61 was transferred to *P. putida* and used to seek prime plasmids. pMO172 was one such derivative which originally demonstrated prime plasmid properties in *P. putida*, although these properties have since been lost (A. Morgan & A. Boronin, unpublished). pMO172 was transferred to *M. methylotrophus* AS1.

A 24 h culture of *M. methylotrophus* AS1(pMO172) was grown in liquid C2 medium, while a selected *P. aeruginosa* PAO strain was grown overnight at 43 °C in nutrient broth containing 0.4% KNO₃. Growth of such recipients at 43 °C produces a restriction-deficient phenotype which enhances foreign DNA transfer into *P. aeruginosa* PAO (Holloway, 1965). A 5 ml sample of each culture was filtered through a Gelman Metrical membrane filter (47 mm diameter, pore size 0.45 µm) and the filter bearing the cells was placed on a warm (37 °C) C2 agar plate plus 0.4% glucose. The membranes were then incubated for 6 h at 37 °C after which the bacteria were washed off in 2.5 ml warm saline and vortexed vigorously to suspend the cells evenly. Samples were plated on appropriate selective media to identify transconjugants which had acquired a plasmid prime capable of complementing the auxotrophic mutation in the recipient PAO strain. The selective plates were incubated at 37 °C for 3 d and any candidate colonies tested for the presence of plasmid primes carrying fragments of AS1 chromosome.

Preparation of plasmid DNA. Plasmid DNA was prepared as described by Nayudu & Holloway (1981). R68 and R68.45 were isolated from *E. coli* K12 strain JP2314. The ECM plasmids pMO61 and pMO172 were isolated from *P. aeruginosa* PAO8 and *P. putida* PPN1089. The prime plasmids pMO551 and pMO564 were isolated from *P. aeruginosa* PAO5 and PAO305 respectively.

Preparation of total bacterial DNA. Bacterial DNA was isolated from *M. methylotrophus* AS1 by the technique of Willetts *et al.* (1981).

Restriction enzyme and gel electrophoresis techniques. The techniques used were as described by Nayudu & Holloway (1981) except that λ HindIII and λ HindIII/EcoRI fragments (New England Biolabs) were used as standards with the maximum error estimated to be ± 0.15 kb.

Southern hybridization techniques. The Southern hybridization technique (Southern, 1975) was used with modifications as described by Willetts *et al.* (1981).

RESULTS

Isolation and genetic characterization of prime plasmids

Membrane matings were carried out to isolate prime plasmids of pMO172 carrying different fragments of AS1 chromosome, using different *P. aeruginosa* PAO auxotrophs as recipients and *M. methylotrophus* AS1(pMO172) as donor. The range of such primes isolated is listed below (Table 3). Exconjugant colonies which could result from the formation of prime plasmids were found at a frequency of about 10^{-8} per donor cell. As an example of a typical plasmid prime, let us take the isolation and characterization of pMO555. *Methylotrophus methylotrophus* AS1(pMO172) was membrane mated to *P. aeruginosa* PAO286 (*met-28 trpC*) and the mating mixture subsequently plated on to minimal medium plus tryptophan. One colony was shown to be able to transfer Met⁺ and carbenicillin resistance when mated to appropriate recipients. The transfer frequency of Met⁺ was shown to be 3.0×10^{-2} per donor cell. Selection for carbenicillin resistance showed a transfer frequency of this plasmid marker of 4.7×10^{-3} per donor cell and all the exconjugants had also transferred the Met⁺ phenotype. Kanamycin resistance and tetracycline resistance were also shown to be transferable. Three other auxotrophic functions were shown to be transferable, *trpC* (2.5×10^{-2} per donor cell), *trpD* (8.8×10^{-3}) and *trpE* (1.8×10^{-2}). None of the other markers listed in Table 2 could be transferred at this frequency, but some were transferred at frequencies of 10^{-5} – 10^{-6} per donor cell. This is presumed to be due to mobilization of chromosomal markers carried by PAO286 by the plasmid prime.

Both the *trpC* and *met-28* mutant alleles were shown by transduction to be still present in PAO286(pMO555). With bacteriophage F116L grown on PAO286(pMO555), selection was made for *argC*⁺ (using PAO307 as the recipient) on minimal medium plus tryptophan, and using PAO898 as a recipient, selection was made for *ilvD*⁺ on minimal medium plus methionine. Transduction frequency was about 10^{-6} per recipient cell: 40% of the *argC*⁺ transductants were Trp⁻ and 11% of the *ilvD*⁺ transductants were Met⁻. This indicated that in PAO286(pMO555) both the original auxotrophic alleles were still present and therefore that methionine and tryptophan synthesis was being accomplished by genetic material carried by the prime plasmid.

Thus from its origins and properties pMO555 is a prime plasmid carrying a segment of *M. methylotrophus* AS1 genome which includes a gene for methionine synthesis and three genes for tryptophan synthesis. Hence these four genes are linked on the AS1 genome. There is a problem concerning the nomenclature of the markers identified by the complementation procedure.

Strictly speaking, all we can identify by this method is the presence of a region in a prime plasmid which can change the phenotype of a given *P. aeruginosa* strain carrying a known marker. We suggest that such regions of the AS1 chromosome are best identified by symbols which do not imply a genotypic identification, but rather a phenotypic characterization. We propose to identify such AS1 markers by a prefix C (for complementation) followed by the gene symbol of the *P. aeruginosa* marker complemented.

A range of primes has been isolated using selection for different *P. aeruginosa* PAO markers. The principles of the procedures used were the same as those used to isolate pMO555. The properties of these primes are listed in Table 3.

Several different prime plasmids derived from pMO172 carried in *M. methylotrophus* AS1 have been transferred to *P. aeruginosa*, where each prime can complement either a single auxotrophic function or a range of auxotrophic functions (Table 3). The simplest explanation of these results is that different regions of the AS1 chromosome are being picked up by different prime plasmids and that the range of functions complemented in *P. aeruginosa* is a direct indication of which wild-type alleles are carried by each AS1 fragment. Thus, the pattern of complementation is a guide to the linkage relationships of AS1 genes. On this basis it is possible to construct linkage groups of the markers examined.

If the pattern of complementation of primes is to be effective as a procedure for mapping a number of conditions must be met.

(i) The plasmid generating the primes must be capable of forming primes which carry a wide variety of chromosomal fragments. This aspect of IncP-1 ECM plasmids has not been widely studied but data from *P. aeruginosa* (B. Holloway, unpublished; J. Hill and B. Holloway, unpublished; Morgan, 1982) show that a wide range of different primes can be isolated. The data in Table 3 support the view that a range of primes can be formed in *M. methylotrophus* AS1.

(ii) The recipient bacterium (*P. aeruginosa* PAO in this case) must be capable of allowing the expression of the incoming bacterial functions carried by the prime plasmid. We have discussed this aspect of *P. aeruginosa* in a previous paper (Holloway *et al.*, 1979) and there is evidence that a range of functions from *E. coli*, *P. putida* and now *M. methylotrophus* AS1 can be expressed in *P. aeruginosa*. Evidence to show that some functions are not expressed may be more difficult to obtain.

(iii) Auxotrophs play an important part in the selection procedures for the plasmid primes, although other markers can be used. For auxotrophic markers, it must be assumed that the biosynthetic pathways in the organism from which the primes are derived and those in the recipient organism are very similar. In at least one case minor differences are not of consequence, as shown with the tryptophan pathway. A derivative of R68.45 carrying the entire tryptophan operon of *E. coli* can complement all six functions (*trpA-F*) of *P. aeruginosa* (Holloway *et al.*, 1980).

(iv) No internal rearrangements occur in the chromosomal fragments carried by prime plasmids. Any deletions, inversions or translocations would reduce the effectiveness of mapping data obtained by this method. There is no evidence that such rearrangements occur in the formation of F prime plasmids in *E. coli* (Low, 1972) nor that any such rearrangements occur in the formation of R primes derived from R68.45 in *P. aeruginosa* (Holloway, 1978; Morgan, 1982; B. Holloway, unpublished).

Construction of linkage groups

1. *Cmet-28*, *CtrpC*, *CtrpD*, *CtrpE*, *CpyrE*. pMO551 complemented *trpC* only, whereas pMO590 (selection for *CtrpD*⁺) complemented *trpC* and *trpE* and pMO592 (selection for *CtrpE*⁺) complemented *trpC* and *trpD*. Thus *CtrpC*, *CtrpD* and *CtrpE* must be linked to each other. Primes where selection was made for *Cmet-28*⁺ (pMO552, pMO555 and pMO574) complemented all three *trp* genes, *trpC*, *trpD* and *trpE*, indicating that these four genes are clustered. pMO574 also showed the ability to complement *pyrE*. Thus we can identify a cluster of five markers *Cmet-28*, *CtrpC*, *CtrpD*, *CtrpE* and *CpyrE*, but it is not possible to identify their relative order.

Table 3. *Prime plasmids derived from pMO172 and carrying M. methylotrophus AS1 chromosomal fragments as indicated by complementation of P. aeruginosa markers*

The prime plasmids listed were isolated as described in the text. The marker selected was that of the recipient *P. aeruginosa* PAO strain used in the initial mating to isolate the prime plasmid. The marker transfer frequency for this marker was estimated by diluting the donor parent carrying the prime and plating measured samples on to minimal medium plus rifampicin, spread with a rifampicin-resistant mutant of the appropriate auxotrophic PAO recipient strain. The donor strain was tested against the range of strains listed in Table 2 to detect any other *P. aeruginosa* PAO auxotrophic markers which could be complemented. The transfer frequency of these other markers was tested in the same way as that for the initial marker used in the selection of the prime.

Prime plasmid and host strain	Marker selected	Transfer frequency of selected marker per donor cell	Additional markers carried (as listed in Table 2)	Transfer frequency of other markers per donor cell
PAO5(pMO551)	<i>trpC</i>	1.0×10^{-2}	none	
PAO286(pMO552)†	* <i>met-28</i>	2.4×10^{-2}	* <i>trpC</i>	3.3×10^{-2}
			<i>trpD</i>	2.3×10^{-2}
			<i>trpE</i>	8.5×10^{-3}
PAO12(pMO554)†	* <i>leu-8</i>	3.5×10^{-2}	none	
PAO286(pMO555)†	* <i>met-28</i>	3.0×10^{-2}	* <i>trpC</i>	2.5×10^{-2}
			<i>trpD</i>	8.8×10^{-3}
			<i>trpE</i>	1.8×10^{-2}
PAO898(pMO556)	<i>ilvD</i>	7.0×10^{-2}	none	
PAO898(pMO557)	<i>ilvD</i>	2.3×10^{-2}	none	
PAO1086(pMO562)	<i>proB</i>	2.9×10^{-2}	<i>pur-136</i>	1.2×10^{-1}
PAO907(pMO563)	<i>proA</i>	1.3×10^{-1}	none	
PAO305(pMO564)	<i>argG</i>	1.3×10^{-1}	<i>argF</i>	1.0×10^{-1}
			<i>trpA</i>	1.3×10^{-1}
			<i>trpB</i>	1.0×10^{-2}
			<i>trpF</i>	5.0×10^{-2}
			<i>leu-10</i>	8.6×10^{-2}
PAO896(pMO565)	<i>pur-136</i>	1.0×10^{-2}	<i>proB</i>	7.0×10^{-2}
PAO896(pMO566)	<i>pur-136</i>	6.7×10^{-2}	<i>proB</i>	5.1×10^{-2}
			<i>leu-8</i>	7.4×10^{-2}
PAO325(pMO571)	<i>lys-12</i>	4.9×10^{-2}	none	
PAO904(pMO572)	<i>argF</i>	3.1×10^{-1}	<i>trpA</i>	1.6×10^{-1}
			<i>trpB</i>	4.9×10^{-2}
			<i>trpF</i>	1.6×10^{-1}
			<i>argG</i>	2.5×10^{-1}
			<i>leu-10</i>	1.9×10^{-1}
PAO899(pMO574)	<i>met-28</i>	2.4×10^{-2}	<i>trpC</i>	9.5×10^{-3}
			<i>trpD</i>	1.9×10^{-2}
			<i>trpE</i>	4.8×10^{-3}
			<i>pyrE</i>	3.3×10^{-2}
PAO462(pMO575)	<i>trpA</i>	1.6×10^{-2}	<i>trpB</i>	3.0×10^{-3}
			<i>leu-10</i>	1.0×10^{-2}
			<i>pur-66</i>	2.0×10^{-2}
			<i>argG</i>	2.0×10^{-2}
			<i>trpF</i>	3.2×10^{-2}
			<i>argF</i>	5.0×10^{-3}
			<i>met-9011</i>	9.5×10^{-3}
PAO462(pMO576)	<i>trpA</i>	1.6×10^{-2}	<i>trpB</i>	2.1×10^{-3}
			<i>argF</i>	1.7×10^{-2}
			<i>argG</i>	2.5×10^{-2}
			<i>trpF</i>	5.8×10^{-2}
			<i>leu-10</i>	5.8×10^{-2}
			<i>met-9011</i>	4.2×10^{-3}
PAO90(pMO577)	<i>proC</i>	3.8×10^{-2}	<i>pyrB</i>	1.2×10^{-2}
			<i>pur-66</i>	1.5×10^{-2}
PAO503(pMO578)	<i>met-9011</i>	2.6×10^{-2}	<i>trpA</i>	8.4×10^{-2}
			<i>trpF</i>	5.3×10^{-3}
			<i>argG</i>	1.6×10^{-2}
			<i>leu-10</i>	5.8×10^{-2}
			<i>trpB</i>	4.6×10^{-3}
			<i>argF</i>	5.3×10^{-3}

Table 3 (continued)

Prime plasmid and host strain	Marker selected	Transfer frequency of selected marker per donor cell	Additional markers carried (as listed in Table 2)	Transfer frequency of other markers per donor cell
PAO897(pMO580)	<i>leu-8</i>	2.4×10^{-3}	<i>proB</i>	2.4×10^{-4}
PAO129(pMO581)	<i>pyrB</i>	1.1×10^{-2}	<i>proC</i>	1.4×10^{-2}
PAO129(pMO582)	<i>pyrB</i>	3.1×10^{-2}	<i>proC</i>	4.7×10^{-2}
PAO325(pMO583)	<i>lys-12</i>	1.7×10^{-1}	none	
PAO114(pMO585)	<i>pyrD</i>	5.9×10^{-3}	none	
PAO897(pMO586)	<i>pur-66</i>	2.9×10^{-1}	none	
PAO897(pMO587)	<i>leu-8</i>	3.5×10^{-1}	<i>proB</i>	2.5×10^{-2}
			<i>pur-136</i>	4.2×10^{-2}
PAO900(pMO589)	<i>ilvB/C</i>	1.3×10^{-2}	none	
PAO470(pMO590)	<i>trpD</i>	1.1×10^{-1}	<i>trpC</i>	1.7×10^{-1}
			<i>trpE</i>	1.5×10^{-1}
PAO472(pMO592)	<i>trpE</i>	5.3×10^{-2}	<i>trpC</i>	1.8×10^{-2}
			<i>trpD</i>	1.8×10^{-2}

† These bacterial strains were shown by transduction analysis to carry the *P. aeruginosa* mutant alleles marked *.

2. *CproB* – *Cpur-136* – *Cleu-8*. Selection for *CproB*⁺ (pMO562), *Cpur-136*⁺ (pMO565 and pMO566) and *Cleu-8*⁺ (pMO587 and pMO580) resulted in five primes which can complement unselected markers, *pur-136* in the case of pMO562, *proB* with pMO565, *proB* and *leu-8* with pMO566. pMO587 complements with *proB* and *pur-136* while pMO580 complements only *proB*. pMO554 (isolated by selection for *Cleu-8*⁺) resulted in a prime with no other detectable function cotransferred. From the pattern of markers complemented by these six plasmids the gene order is *Cleu-8* – *CproB* – *Cpur-136*.

3. *CpyrB* – *CproC* – *Cpur-66*. Four plasmids had the ability to complement these markers – pMO581 and pMO582 (selected for *CpyrB*⁺ and cotransfer of *CproC*⁺), pMO577 (selected for *CproC*⁺ and cotransfer of *CpyrB*⁺ and *pur-C66*⁺) and pMO586 (selected for *Cpur-66*⁺) which did not complement the other markers. Thus these three markers are linked but their order cannot be determined.

4. *Cpur-66* – *CtrpA* – *CtrpB* – *CtrpF* – *CargG* – *CargF* – *Cleu-10* – *Cmet-9011*. This is the largest cluster of linked markers. Five prime plasmids have been isolated which carried all or some of the markers – pMO564 (selected for *CargG*⁺ and cotransfer of *CargF*⁺, *CtrpA*⁺, *CtrpB*⁺, *CtrpF*⁺ and *Cleu-10*⁺), pMO578 (selected for *Cmet-9011*⁺ and cotransfer of *CtrpA*⁺, *CtrpB*⁺, *CtrpF*⁺, *CargG*⁺, *CargF*⁺ and *Cleu-10*⁺), pMO572 (selected for *CargF*⁺ with cotransfer of *CtrpA*⁺, *CtrpB*⁺, *CtrpF*⁺, *CargG*⁺ and *Cleu-10*⁺). pMO575 and pMO576 were isolated with selection for *CtrpA*⁺; both showed transfer of *Cleu-10*⁺, *CargG*⁺, *CargF*⁺, *CtrpB*⁺, *CtrpF*⁺ and *Cmet-9011*⁺, but pMO575 also cotransferred *Cpur-66*⁺. The gene *Cpur-66* must be at one end of this group with *Cmet-9011* at the other. The precise order of the intervening genes cannot be determined from the present data. On the basis solely of pMO575 which includes *Cpur-66*, this group of markers is linked to the previous group *CpyrB* – *CproC* – *Cpur-66*.

Several single markers have been shown to be carried by other primes. These include pMO563 *CproA*, pMO556 and pMO557 *CilvD*, pMO571 and pMO583 *Clys-12*, pMO585 *CpyrD* and pMO589 *CilvB* or *CilvC* (Table 3). Primes have been sought for other markers without success (Table 2).

Physical analysis of pMO172 and the prime plasmids

It was first shown by Jacob *et al.* (1977) that R68 and R68.45 differed in molecular structure in that the latter had acquired an additional 2.1–2.4 kb segment of DNA. This additional DNA segment is inserted near the determinant for kanamycin resistance (Riess *et al.*, 1980) and is a duplication of a pre-existing R68 DNA sequence called ISP (Riess *et al.*, 1980). Willetts *et al.* (1981) have shown that a portion of the tandem duplication is a transposable genetic element identified as IS21. The ECM plasmid pMO61 has been derived in a similar fashion to R68.45 and has essentially the same biological properties (Holloway *et al.*, 1979). Within the limits of restriction enzyme analysis pMO61 is identical to R68.45 in that it carries the same 2.1 kb direct

Fig. 1. Restriction enzyme map of the plasmid R68. The single *EcoRI* site is arbitrarily assigned as 0 kb and the cleavage sites of a number of restriction enzymes relevant to this study are indicated relative to this site. The *KpnI* and *PstI* sites at 36.0 kb and the *PstI* and *PvuII* sites at 36.8 kb are not ordered. The outer circle is a composite of all the cleavage sites, which are shown separately for each enzyme in the inner circles.

repeat in the same location (Riess *et al.*, 1980; Willetts *et al.*, 1981). Plasmid DNA preparations of R68 and R68.45 were made from derivatives of the *E. coli* K12 strain JP2314 and the ECM plasmids pMO61 and pMO172 were isolated from *P. aeruginosa* PAO8 and *P. putida* PPN1089. A comparison of the restriction enzyme pattern, after digestion with the restriction enzyme *PstI* (Fig. 3, tracks 6 and 7) indicates that R68.45 differs from R68 in having a unique 1.35 kb fragment. There is also an internal 0.75 kb R68 *PstI* fragment which is duplicated in R68.45 (Riess *et al.*, 1980). A comparison of pMO172 with R68.45 (Fig. 3, tracks 5 and 6) shows that they have identical *PstI* fragments. Digestion of pMO172 with the restriction enzymes *SmaI*, *KpnI*, *PvuII* and *HpaI* produced the 2.1 kb fragment characteristic of the duplication and there was no difference in restriction patterns when compared with R68.45. Thus within the limits of restriction enzyme analysis there was no detectable difference between pMO172, its parent pMO61, and R68.45.

The prime plasmids pMO551 and pMO564 were isolated from derivatives of *P. aeruginosa* PAO5 and PAO305. Digestion of pMO551 with the restriction enzymes *EcoRI* and *HindIII*, both of which have a single restriction enzyme site on pMO172 (Fig. 1), showed that pMO551 has acquired two additional *HindIII* and *EcoRI* sites (data not shown). This indicates that there has been an addition of DNA in pMO551 compared with its parent pMO172. The additional DNA is inserted into the smallest 6.5 kb *HindIII*-*SalI* fragment since this was absent in a *HindIII*/*SalI* double digest. The additional DNA was further localized to the 2.1 kb *HpaI* fragment of pMO172 since this was lost in an *HpaI* digest of pMO551 (Fig. 3, tracks 3 and 4). Since the characteristic 2.1 kb *SmaI*, *PvuII*, *HpaI* and *KpnI* fragments were also lost in pMO551, compared with its parent pMO172, the chromosomal segment is inserted somewhere between the *HpaI* site of the left-hand copy of the duplicated portion and the *SmaI* site of the right-hand copy (Fig. 2). Leemans *et al.* (1980) have suggested that the chromosomal segment of a prime plasmid is inserted between the two copies of the direct repeat. However, this explanation cannot hold for pMO551 since in a *PstI* digestion of pMO551 there was a loss of the 1.35 kb

Fig. 2. Restriction map of the 6.5 kb *Hind*III–*Sal*I region of R68.45 containing the DNA duplication. The order of the *Pst*I and *Pvu*II sites at 36.8 and 38.9 kb and the *Pst*I and *Kpn*I sites at 36.0 and 38.1 kb could not be determined. The bar lines indicate the restriction sites of the duplicate segments.

unique fragment as well as two new fragments of 2.2 kb and 1.0 kb in addition to an increase in the molecular weight of *Pst*I fragment C. Similarly in a *Sma*I digestion of pMO551 there was a loss of the characteristic 2.1 kb fragment, generation of a new fragment of 3.4 kb and an increase in the molecular weight of *Sma*I fragment D. Thus the area adjoining the direct repeat, covered by *Pst*I fragment C and *Sma*I fragment D (Fig. 1), has also been altered by the insertion. A double insertion event can be ruled out since in a *Kpn*I digestion of pMO551 there was a loss of the characteristic 2.1 kb fragment, and an increase in the molecular weight of *Kpn*I fragment C, but no new *Kpn*I fragments were generated. The simplest explanation of these results is that the chromosomal segment has inserted into the duplicated region and in this process, or subsequently, there has been a deletion of part or all of one of the copies of the duplicated segment. There is a precedent for this hypothesis since it has been observed in other ECM plasmids that a single copy of the duplicated segment can excise precisely and leave behind the other copy of the sequence (W. Schurter & B. W. Holloway, unpublished). Assuming there has been a precise deletion of one copy of the duplicated segment in pMO551, the size of the *M. methylotrophus* DNA insertion would be 4.8 kb.

Digestion of pMO564 with the restriction enzymes *Hind*III and *Eco*RI indicated that it had acquired a minimum of eight new *Eco*RI and 14 new *Hind*III restriction enzyme sites. The acquisition of a large number of restriction enzyme sites is indicative of a DNA insertion in

Fig. 3. Restriction enzyme cleavage of R68, R68.45, pMO172, pMO551 and pMO564. Tracks 1 and 8 contain λ HindIII and λ HindIII/EcoRI fragments used for size standards; the other tracks contain *Hpa*I digestion of pMO564 (track 2), pMO172 (track 3) and pMO551 (track 4), and *Pst*I digestion of pMO172 (track 5), R68.45 (track 6) and R68 (track 7).

pMO564, compared with its parent pMO172. Due to the large size of the DNA addition it was only possible to estimate its size approximately. Comparison of the *Hpa*I fragments of pMO564 with those of its parent pMO172 (Fig. 3, tracks 2 and 3) indicated that the size of the DNA addition was between 75 and 100 kb. The large size of the insertion also led to the generation of a large number of restriction fragments when the plasmid was digested with a range of restriction enzymes, so it was not possible to determine the location of the insertion. The relative size of the DNA additions in pMO551 and pMO564 correlates well with the pattern of markers of *P. aeruginosa* complemented by each plasmid; pMO551 carries a small DNA addition of approximately 4.8 kb and complements one *P. aeruginosa* marker while pMO564 carries a much larger DNA addition and can complement five *P. aeruginosa* markers (Table 3).

Fig. 4. (a) Restriction enzyme digests of *M. methylotrophus* AS1 total bacterial DNA with *Hpa*I (track 2), *Eco*RI (track 4) and *Hind*III (track 6). Tracks 1, 3 and 5 contain 0.01 µg pMO551 digested with *Hpa*I (track 1), *Eco*RI (track 3) or *Hind*III (track 5), and track 7 contains 0.01 µg pMO172 digested with *Pst*I. (b) Autoradiogram of hybridization of the probe pMO551 to the tracks described in (a).

Southern hybridization to determine the origin of the additional DNA in the prime plasmids

Southern hybridization experiments were used to provide information on the origin of the additional DNA segments of pMO551 and pMO564. Total bacterial DNA was isolated from *M. methylotrophus* AS1 then digested separately with *Hpa*I, *Eco*RI or *Hind*III and examined by the procedure of Southern (1975). The plasmid DNA used for this experiment was isolated from *P. aeruginosa* or *P. putida* to avoid any contamination with *M. methylotrophus* AS1 chromosomal DNA.

When the pMO172 probe was hybridized to *M. methylotrophus* AS1 chromosomal DNA fragments, no evidence of homology was observed even after prolonged exposure of the

Fig. 5. (a) Restriction enzyme digests of *M. methylotrophus* AS1 total bacterial DNA with *Hpa*I (track 2), *Eco*RI (track 4) and *Hind*III (track 6). Tracks 1, 3 and 5 contain 0.01 μ g pMO564 digested with *Hpa*I (track 1), *Eco*RI (track 3) or *Hind*III (track 5), and track 7 contains 0.01 μ g pMO172 digested with *Pst*I. (b) Autoradiogram of hybridization of the probe pMO564 to the tracks described in (a).

autoradiogram. A control track containing 0.01 μ g pMO172 DNA digested with *Pst*I showed strong hybridization (results not shown). There are thus no common sequences between pMO172 and the *M. methylotrophus* AS1 genome. However, hybridization of the pMO551 (Fig. 4b, tracks 2, 4 and 6) and pMO564 (Fig. 5b, tracks 2, 4 and 6) plasmid DNA probes to the *M. methylotrophus* AS1 DNA fragments showed that regions of homology exist. Since pMO172 has no sequence homology with *M. methylotrophus* AS1 DNA, it can be concluded that the prime plasmids pMO551 and pMO564 carry a segment of the *M. methylotrophus* AS1 genome. In the case of pMO551 there is a 1.1 kb internal *Hpa*I fragment (Fig. 4b, tracks 1 and 2), a 2.65 kb internal *Eco*RI fragment (Fig. 4b, tracks 3 and 4) and a 2.0 kb internal *Hind*III fragment (Fig.

4b, tracks 5 and 6) which are unchanged in the DNA segment carried by the prime plasmid, compared with the *M. methylotrophus* AS1 chromosome. pMO564 has a minimum of three internal *HpaI* fragments (Fig. 5b, tracks 1 and 2), four internal *EcoRI* fragments (Fig. 5b, tracks 3 and 4) and eight internal *HindIII* fragments (Fig. 5b, tracks 5 and 6) which are unchanged in the DNA segment carried by it, compared with the *M. methylotrophus* AS1 genome. The comparison of such internal fragments shows there is identity between the segment carried by the prime plasmid and the *M. methylotrophus* AS1 DNA. In a number of cases where hybridization was carried out to *M. methylotrophus* AS1 DNA fragments (Fig. 4b, track 4 and Fig. 5b, tracks 4 and 6) weaker bands of hybridization, especially to larger molecular weight fragments, were observed. These are most likely to be partial digest fragments and were even observed after prolonged incubation (minimum of 8 h) of the restriction enzyme digestion.

DISCUSSION

We have isolated a variety of prime plasmids which carry sections of *Methylophilus methylotrophus* AS1 chromosome. The evidence for this view can be summarized as follows. (i) When the prime plasmid is transferred to an appropriate *P. aeruginosa* PAO strain it can complement a specific range of auxotrophic markers ranging, for different prime plasmids, from one to eight auxotrophic functions. This complementation ability and the antibiotic resistance markers carried by the plasmid are transferred at similar frequencies. (ii) Physical analysis of two of these prime plasmids showed they had acquired an additional DNA segment and Southern hybridization experiments demonstrated that this additional DNA was a segment of *M. methylotrophus* AS1 DNA.

The isolation of this type of plasmid from *M. methylotrophus* AS1 has an added interest because it provides a means of mapping the genes of that organism. Mapping of *M. methylotrophus* AS1 is intrinsically difficult because auxotrophic mutations of this organism are very difficult to isolate. Furthermore, mobilization of chromosomal genes by ECM plasmids occurs at a low frequency (Holloway, 1981) and as yet no transducing bacteriophage has been found for any methylotrophic bacterium.

The essential features of our mapping procedure are thus the formation of primes carrying *M. methylotrophus* AS1 DNA; the need for a wide range of such primes, covering most, if not all of the genome; the ability of such prime plasmids to transfer to *P. aeruginosa*; and the expression of the encoded functions in that bacterium. This last feature is detected by complementation of *P. aeruginosa* PAO functions absent from mutant strains. To date only auxotrophic markers have been complemented but there is no reason why other functions should not be complemented. Indeed this may be a procedure for identifying metabolic functions of *M. methylotrophus* AS1 for which mutants cannot, for one reason or another, be isolated. It should be possible to use recipients other than *P. aeruginosa* for detecting such functions.

It can be concluded from the range of primes isolated that there is variation in the size of DNA fragment incorporated into the prime plasmids. This is an advantage to the method in that such variations help to identify adjoining genes in the same way that deletions of different length permit deletion mapping.

Pseudomonas aeruginosa has a variety of features which make it an attractive recipient for this type of mapping. Firstly, there is increasing evidence that DNA from other bacteria is expressed readily in *P. aeruginosa*. Secondly, the lack of clustering of auxotrophic markers in *P. aeruginosa*, compared with that found in enteric bacteria, means that the chance of detecting cotransferred functions is higher. There are now over 100 genes mapped in *P. aeruginosa* PAO and the number is increasing. Finally, the so-called '43 °C effect' by which restriction endonuclease action can be reduced to aid the entry of foreign DNA greatly increases the chance of successful prime plasmid establishment.

The choice of pMO172 as the generator of *M. methylotrophus* AS1 plasmid primes was to some extent fortuitous and we have not examined the prime-forming ability of other ECM plasmids. Preliminary experiments (B. Holloway & W. Schurter, unpublished) have shown that different ECM plasmids differ in the ease with which they can form prime plasmids. The possibility exists

that ECM plasmids can be isolated which can form primes more frequently than those already tested for this ability, such as R68.45 and pMO172. As yet little is known of the mechanism by which prime plasmids are derived from ECM plasmids. There is some evidence (Leemans *et al.*, 1980; N. Willetts & C. Crowther, unpublished) that the single copies of IS2/ flank the inserted piece of chromosomal DNA, but more data are needed to substantiate this.

Further experiments need to be done to expand the present findings. These include the demonstration of *M. methylotrophus* AS1 enzyme activity in the complemented *P. aeruginosa* recipient and an examination of the regulatory pattern of such enzymes. It will be interesting to define the range of bacteria which can complement *M. methylotrophus* functions effectively. Identification of a range of functions of *M. methylotrophus* in this manner could be of considerable interest in demonstrating the metabolic pathways which occur in that organism and establishing whether any metabolic functions are dormant or repressed.

This work was supported by Imperial Chemical Industries. The technical assistance of Robyn Bray is gratefully acknowledged.

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