Comparative Complementation Mapping of *Methylophilus* spp. Using Cosmid Gene Libraries and Prime Plasmids

By B. R. LYON,¹ P. P. KEARNEY,² M. I. SINCLAIR¹ AND
B. W. HOLLOWAY¹*

¹Department of Genetics, Monash University, Clayton, Victoria 3168, Australia

²The Galton Laboratory, Department of Genetics and Biometry, University College London,
Wolfson House, 4 Stephenson Way, London NW1 2HE, UK

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Cosmid gene libraries of the obligate methylotrophs *Methylophilus viscogenes* and *Methylophilus methylotrophus* AS1 were generated by ligating *Sau*3A partial digests of chromosomal DNA into the broad-host-range cosmid vector pLA2917. Genetic linkage groups were identified for each organism by complementation following mobilization of the recombinant cosmids to a wide range of *Pseudomonas aeruginosa* and *Escherichia coli* auxotrophs. The linkage data thus obtained confirmed and extended those previously established by prime plasmid complementation, and the two methylotrophs showed very similar arrangements for four of the gene clusters studied. However, comparison of DNA sequences which complemented the same auxotrophic markers failed to demonstrate restriction fragment identity or substantial DNA–DNA homology between the two methylotrophs.

INTRODUCTION

The genetic analysis of bacteria has traditionally been dependent on the isolation of mutants followed by the characterization of the identified genes by DNA transfer and recombination. The isolation of mutants has proved difficult in methylotrophs, and de Vries (1986) has cited a number of reasons for this, including ineffectiveness of mutagenic treatments and enrichment procedures, and instability of mutants. Protocols for the isolation of both carbon-source and auxotrophic mutants of the facultative methylotroph *Methylobacterium* AM1 using transposon mutagenesis have recently been reported (Nunn & Lidstrom, 1986; Whitta *et al.*, 1985). While mapping of some genes has been achieved by conjugation in *Methylobacterium* AM1 (Tatra & Goodwin, 1985), the difficulty of mutant isolation, particularly with respect to auxotrophs, means that the usual bacterial genetic approaches are limited. The use of surrogate genetic techniques which employ clones, derived *in vivo* or *in vitro*, for the identification of markers by complementation of characterized mutants from other species is thus an attractive approach for the effective genetic analysis of methylotrophs. A study using prime plasmids from *Methylophilus methylotrophus* AS1 to complement *Pseudomonas aeruginosa* auxotrophs has allowed the assignment of 23 loci to the chromosome of this obligate methylotroph (Moore *et al.*, 1983). Randomly generated cosmid genome libraries of the facultative organisms *Methylobacterium* AM1 and *Methylobacterium organophilum* XX have also been used to complement carbon utilization mutants of methylotrophs (Allen & Hanson, 1985; Fulton *et al.*, 1984; Nunn & Lidstrom, 1986).

In this paper we report the construction of genomic libraries of the obligate methylotrophs *Methylophilus viscogenes* and *Methylophilus methylotrophus* AS1 in the wide-host-range cosmid pLA2917 (Allen & Hanson, 1985), and the use of these libraries for complementation mapping in *P. aeruginosa* and *Escherichia coli* auxotrophic recipients. *M. viscogenes* was isolated from the soil of a methanol synthesis plant in Texas. This organism grows on methanol and some

Table 1. *Strains used in this study*

In addition to the strains listed below, some PAO strains described by Moore *et al.* (1983) were also used. The PAO numbers of such strains are given in Table 3.

Strain	Genotype	Reference/ source*
<i>P. aeruginosa</i>		
PAO473	<i>trpF1</i>	(1)
PAO505	<i>met-9011 ami-200</i>	(1)
PAO660	<i>phe-2</i>	(1)
PAO705	<i>aro-1</i>	(1)
PAO706	<i>phe-3</i>	(1)
PAO716	<i>cys-54 rif-96</i>	(1)
PAO718	<i>cys-59</i>	(1)
PAO890	<i>trpB4</i>	(1)
PAO896	<i>pur-136</i>	(1)
PAO899	<i>met-28 str-1</i>	(1)
PAO905	<i>leu-10</i>	(1)
PAO913	<i>hisIIA68 rif-62</i>	(1)
PAO919	<i>hisV5035 rif-68</i>	(1)
PAO1818	<i>leu-9001 thr-9011 pur-9013</i>	(1)
PAO2196	<i>met-9020 catA1 nar-9011 chu-9002 trp-9029 lys-9015</i>	(1)
<i>M. methylotrophus</i>		
AS1	Prototroph	(8)
<i>M. viscogenes</i>		
CEL1555	Prototroph	(10)
<i>E. coli</i>		
2031	<i>metE70 lysA23 trpE38 purE42 proC32 leu-6 thi-1</i>	(11)
AB663	<i>argH1 purF1 xyl-7 supE44 λ⁻ Hfr</i>	(2)
AB2270	<i>ilvD188 argH1 purF1 xyl-7 supE44</i>	(2)
AT1325	<i>purB15 his-4 proA2 thi str</i>	(3)
AT2444	<i>metE68 thi-1 relA1 spoT1 λ⁻ Hfr</i>	(2)
AT2446	<i>metC69 thi-1 relA1 spoT1 λ⁻ Hfr</i>	(2)
AT2696	<i>hisG1 thyA3 lysA22 metB1 malA1(λ^R) xyl-7 mtl-2 rps-104 λ⁻ supE44?</i>	(2)
CV524	<i>leuD211 F⁺</i>	(2)
Hfr Cavalli	<i>metB1 relA1 spoT1 Hfr</i>	(2)
J53	<i>proA metF(λ)</i>	(6)
JC411	<i>argG6 his-1 leu-6 metB1 rps-104 sup-59 gal-6 malA1(λ^R) xyl-7 mtl-2 tonA2 lac λ⁻</i>	(3)
JM81A	<i>cysC</i>	(4)
JP2314	<i>trpE365 pyrF40</i>	(8)
K12517	<i>metA thi</i>	(5)
N134 Rif	<i>Δ(gpt-lac)5 relA1 spoT1 thi-1 argI68 λ Hfr rif</i>	(1)
PB576	<i>argH thi his pheA purF mtl xyl malA(λ^R) lacY1 or lacZ4 ton tsx str</i>	(8)
RH213	<i>leu-57 trp-76 his-204 argA76 mutH34 ilv-677 thi-1 lacZ332 xyl-7 mtl-2 malA1(λ^R) rpsL135</i>	(2)
RL84	<i>cysB63 galK35 his-68 malA1(λ^R) mtl-2 rpsL125 trp-1 tyrA2 xyl-7 thi-1 sup free</i>	(4)
RL88	<i>Δ(tonB-cysB)206 his-68 tyrA2 thi-1 galK2 malA1(λ^R) xyl-7 mtl-2 rpsL125 gyrA223 λ⁻</i>	(2)
SB3930	<i>hisB463 λ⁻</i>	(2)
SB3931	<i>hisF860 malA1(λ^R) xyl-5 mtl-1 rpsL145 λ⁻</i>	(2)
W3110 his ⁻ Δ(tonB trpAB)17	<i>his Δ(tonB trpAB)17</i>	(9)

* (1) Holloway Collection; (2) CGSC; (3) J. Pittard, personal communication; (4) R. Loughlin, personal communication; (5) N. Glansdorff, personal communication; (6) P. Barth, personal communication; (7) F. Gibson, personal communication; (8) Moore *et al.*, 1983; (9) C. Yanofsky, personal communication; (10) Holloway *et al.* (1987); (11) Naider & Becker (1975).

heterotrophic compounds including hexose sugars. It utilizes the ribulose monophosphate pathway of C₁ metabolism and shows general cultural and biochemical similarities to *M. methylotrophus* AS1 (L. M. Ryan Syintsakos & D. I. Stirling, personal communication).

Table 2. *Plasmids used in this study*

Plasmid	Characteristics	Parent	Chromosomal insert	Reference*
pLA2917	Km Tc cos	RK2	—	(1)
pMO172	Cb Km Tc Cma	R68	—	(2)
pMO574	Cb Km Tc, <i>CpyrE CtrpC CtrpD CtrpE Cmet-28</i> in <i>P. aeruginosa</i>	pMO172	<i>M. methylotrophus</i> AS1	(2)
pMO575	Cb Km Tc, <i>Cmet-9011 CtrpA CtrpB CargG CargF Cleu-10 CtrpF Cpur-66</i> in <i>P. aeruginosa</i>	pMO172		(2)
pMO995	Tc cos, <i>CtrpF CargG</i> in <i>P. aeruginosa</i>	pLA2917		
pMO996	Tc cos, <i>CpheA CtrpA CtrpB CpurF</i> in <i>E. coli</i>	pLA2917		
pMO1317	Cb Km Tc, <i>CpyrE CtrpE CtrpD CtrpC Cmet-28 Cphe-3</i> in <i>P. aeruginosa</i>	pMO172		
pMO1322	Cb Km Tc, <i>CargG CargF Cleu-10 CtrpB</i> in <i>P. aeruginosa</i>	pMO172		
pMO1335	Tc cos, <i>CtrpF CtrpA Cmet-9011 CargG CargF Cleu-10</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1336	Tc cos, <i>CtrpF CargG CargF Cleu-10</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1337	Tc cos, <i>CargG CargF</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1338	Tc cos, <i>CargG CargF Cleu-10</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1339	Tc cos, <i>CpyrE CtrpE CtrpC Cmet-28</i> in <i>P. aeruginosa</i>	pLA2917	<i>M. viscogenes</i> CEL1555	
pMO1340	Tc cos, <i>CtrpE CtrpC Cmet-28</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1341	Tc cos, <i>CtrpC Cmet-28</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1342	Tc cos, <i>CpyrB CproC</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1343	Tc cos, <i>CpyrD ChisV Cleu-8</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1344	Tc cos, <i>ChisV Cleu-8</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1345	Tc cos, <i>CproB Cpur-136</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1346	Tc cos, <i>ChisI ChisIII ChisIIB ChisIIA</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1347	Tc cos, <i>ChisIII ChisIIB ChisIIA</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1348	Tc cos, <i>ChisI ChisIII ChisIIB</i> in <i>P. aeruginosa</i>	pLA2917	<i>M. methylotrophus</i> AS1	
pMO1349	Tc cos, <i>ChisIII ChisIIB</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1350	Tc cos, <i>CpyrF Cphe-2</i> in <i>P. aeruginosa</i>	pLA2917		
MO1781	Tc cos, <i>CproB Cpur-136</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1784	Tc cos, <i>CmetC CmetB</i> in <i>E. coli</i> , <i>Cthr-48</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1785	Tc cos, <i>CmetE CmetC</i> in <i>E. coli</i>	pLA2917		
pMO1786	Tc cos, <i>CtrpC</i> in <i>P. aeruginosa</i> , <i>CtrpE</i> in <i>E. coli</i>	pLA2917		
pMO1787	Tc cos, <i>CtrpC</i> in <i>P. aeruginosa</i> , <i>CtrpE CmetA</i> in <i>E. coli</i>	pLA2917		
pMO1788	Tc cos, <i>CtrpE CmetA</i> in <i>E. coli</i>	pLA2917		
pMO1789	Tc cos, <i>CargA</i> in <i>E. coli</i> , <i>CargB</i> in <i>P. aeruginosa</i>	pLA2917		
pMO1888	Tc cos, <i>CtrpA CtrpB CpurF</i> in <i>E. coli</i> , <i>Cmet-9011</i> in <i>P. aeruginosa</i>	pLA2917		

* (1) Allen & Hanson (1985); (2) Moore *et al.* (1983); other plasmids, this paper.

METHODS

Bacterial strains and growth. *Methylophilus methylotrophus* AS1 was obtained from Imperial Chemical Industries and its general properties have been described by Anthony (1986). *M. viscogenes* was obtained from Dr D. I. Stirling, Celgene Corp., Warren, NJ, USA. Both methylotrophs were cultured in mineral salts medium containing 0.5% (v/v) methanol as sole carbon and energy source (Dalton & Whittenbury, 1976). *P. aeruginosa* and *E. coli* auxotrophs were grown in nutrient yeast broth (NYB) as described by Watson & Holloway (1976). The auxotrophs used have either been described previously (Moore *et al.*, 1983) or are listed in Table 1. Minimal medium (Vogel & Bonner, 1956) containing 0.25% (w/v) glucose as carbon and energy source plus appropriate growth factors was used for complementation testing of *P. aeruginosa* and *E. coli* auxotrophs.

Plasmids. The plasmids used in this study are listed in Table 2. Prime plasmids of *M. viscogenes* were isolated using the enhanced-chromosome-mobilizing (ECM) plasmid pMO172 as described by Moore *et al.*, (1983).

DNA isolation and manipulation. Chromosomal DNA was prepared by the method of Scott *et al.* (1981). Plasmid DNA was prepared by the method of Maniatis *et al.* (1982) and purified by CsCl equilibrium gradient

Table 3. *Markers of P. aeruginosa and E. coli complemented by the M. viscogenes and M. methylotrophus AS1 libraries*

For gene products relevant to individual markers see Bachmann (1983) and for information on *P. aeruginosa* markers see Holloway (1986). +, Complementation of auxotrophy; —, failure to complement; ND, not determined.

Marker	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>M. viscogenes</i>	<i>M. methylotrophus</i>
<i>argA</i>	PAO894	RH213	+	+
<i>argB</i>	PAO303		—	+
<i>argC</i>	PAO307		ND	+
<i>argF</i>	PAO904		+	—
		N134 Rif	—	—
<i>argG</i>	PAO305	JC411	+	+
<i>argH</i>	PAO362	AB663	+	—
<i>aro-1</i>	PAO705		—	—
<i>cys-54</i>	PAO716		—	+
<i>cys-59</i>	PAO718		—	+
<i>cysB</i>		RL84	—	+
<i>cysC</i>		JM81A	+	—
<i>hisI</i>	PAO903		+	+
<i>hisIIA</i>	PAO913		+	ND
<i>hisIIB</i>	PAO892		+	—
<i>hisIII</i>	PAO893		+	—
<i>hisIV</i>	PAO891		+	—
<i>hisV</i>	PAO919		+	ND
<i>hisB</i>		SB3930	—	+
<i>hisF</i>		SB3931	—	+
<i>hisG</i>		AT2696	—	—
<i>ilvB/C</i>	PAO900		—	+
<i>ilvD</i>	PAO898		+	+
		AB2270	—	—
<i>leu-8</i>	PAO897		+	+
<i>leu-10</i>	PAO905		+	+
<i>leuD</i>		CV524	—	—
<i>lysA</i>	PAO895		+	—
		AT2696	—	+
<i>lys-9015</i>	PAO2196		—	ND
<i>met-28</i>	PAO899		+	+
<i>met-9011</i>	PAO505		+	+
<i>metA</i>		KL2517	+	+
<i>metB</i>		Hfr Cavalli	—	+
<i>metC</i>		AT2446	+	+
<i>metE</i>		AT2444	+	+
<i>metF</i>		J53	—	+
<i>phe-2</i>	PAO660		+	—
<i>phe-3</i>	PAO706		—	—
<i>pheA</i>		PB576	+	+
<i>proA</i>	PAO907		+	+
		AT1325	—	—
<i>proB</i>	PAO888		+	+
		N134	—	—
<i>proC</i>	PAO906		+	ND
		2031	—	—
<i>pur-66</i>	PAO701		+	+
<i>pur-70</i>	PAO909		+	ND
<i>pur-136</i>	PAO896		+	+
<i>pur-9013</i>	PAO1818		—	ND
<i>purB</i>		AT1325	—	+
<i>purF</i>		AB663	—	+
<i>pyrB</i>	PAO129		+	+
<i>pyrD</i>	PAO114		+	+
<i>pyrE</i>	PAO483		+	—
<i>pyrF</i>	PAO161		+	—
		JP2314	—	+
<i>ser-3</i>	PAO2		—	—

Table 3. *contd.*

Marker	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>M. viscogenes</i>	<i>M. methylotrophus</i>
<i>thr-48</i>	PAO908		+	+
<i>thyA</i>		AT2696	—	+
<i>trpA</i>	PAO462		+	+
<i>trpB</i>	PAO890		ND	—
<i>trpAB</i>		W3110 <i>his</i> [−] Δ (<i>tonB trpAB17</i>)	—	+
<i>trpC</i>	PAO468		+	+
<i>trpE</i>	PAO472		+	+
		JP2314	ND	+
<i>trpF</i>	PAO473		+	+
<i>tyrA</i>		RL88	—	+

centrifugation. Restriction endonucleases (Biolabs) were used according to the conditions of Maniatis *et al.* (1982). T4 DNA ligase (BRESA) and calf alkaline phosphatase (Boehringer Mannheim) were used according to the manufacturers' instructions. Bacteriophage λ packaging kit was supplied by Boehringer Mannheim; *in vitro* packaging was done according to Maniatis *et al.* (1982) and the manufacturer's instructions. Electrophoretic analysis and DNA–DNA hybridization were done essentially as described by Lyon *et al.* (1987).

Construction of genomic libraries of M. viscogenes and M. methylotrophus. *Sau*3A partial digests of chromosomal DNA were ligated into the alkaline-phosphatase-treated *Bgl*II site of pLA2917. Size-fractionated DNA in the range 21–30 kb was isolated by centrifugation on sucrose gradients (*M. viscogenes*) or by electroelution from 0.5% agarose gels (*M. methylotrophus* AS1). Vector and insert DNA were mixed in the ratio of 2:1 at a final DNA concentration of 300 μ g ml^{−1}, the ligation mixture was packaged into λ and the resulting phage particles were used to infect *E. coli* S17-1, a strain possessing the transfer genes of RP4 integrated into the chromosome (Simon *et al.*, 1983).

Storage of recombinant cosmids. Packaged cosmids were transduced into *E. coli* S17-1 (Simon *et al.*, 1983) and plated onto L-agar containing 40 μ g tetracycline ml^{−1}. Gene libraries were prepared by picking single colonies into the cells of 96-well microtitre trays (Disposable Products), in a grid pattern of 50 clones per tray. The wells contained 0.2 ml of the storage medium described by Frey *et al.* (1983) supplemented with 40 μ g tetracycline ml^{−1}. Before these sets of trays were stored frozen at −70 °C, they were used to inoculate a working set of trays, the wells of which contained Y+T broth (0.2 g yeast extract l^{−1} plus 0.4 g tryptone l^{−1}) supplemented with 40 μ g tetracycline ml^{−1} and the mineral salts described by Isaac & Holloway (1972).

Complementation analysis. The ability of recombinant cosmids to complement auxotrophic mutants was tested by spot matings. Donor strains grown in microtitre trays were transferred using a 50-point multi-inoculator onto minimal agar plates containing tetracycline and appropriate growth factors which had been prespread with 0.1 ml of an overnight culture of the recipient. For complementation of *P. aeruginosa*, the various auxotrophic strains were grown at 43 °C to minimize host restriction (Holloway, 1965). Plates were scored for complementation after 48–72 h incubation at 37 °C.

RESULTS

Construction of genomic libraries of M. viscogenes and M. methylotrophus AS1

Genomic libraries of *M. viscogenes* (1000 clones) and *M. methylotrophus* AS1 (1500 clones) were constructed as described in Methods. An analysis of 12 cosmids from the *M. methylotrophus* AS1 gene library and 7 cosmids from the *M. viscogenes* gene library showed mean insert sizes of 22.7 ± 2.5 kb and 24.8 ± 2.5 kb respectively (data not shown). Given these mean insert sizes and a maximum genome size of 4000 kb, the minimum number of cosmids required for 99% assurance of a comprehensive gene library would be 809 for *M. methylotrophus* AS1 and 735 for *M. viscogenes* (Maniatis *et al.*, 1982).

Complementation of auxotrophic mutants

P. aeruginosa and *E. coli* strains carrying the auxotrophic markers listed in Table 1 or by Moore *et al.* (1983) were used as recipients in matings with donors from the *Methylophilus* spp. gene libraries. From the results shown in Table 3, the *M. viscogenes* gene library complemented 32 of the 41 *P. aeruginosa* markers tested (78%) but only 8 of the 27 *E. coli* markers (30%), while the *M. methylotrophus* gene library complemented 24 of the 37 *P. aeruginosa* markers (65%) and 19 of the 28 *E. coli* markers tested (68%). These figures confirm that both cosmid libraries are reasonably comprehensive and indicate that both *P. aeruginosa* and *E. coli* are effective

Table 4. *Genetic linkage groups in M. viscogenes and M. methylotrophus AS1*

Linkage was determined by complementation mapping using prime plasmids (**bold**) and cosmid banks, and *E. coli* and *P. aeruginosa* auxotrophs as recipients.

<i>M. viscogenes</i> CEL1555	<i>M. methylotrophus</i> AS1
CargG CargF Cleu-10 CtrpB	Cpur-66 CtrpF CargG CargF Cleu-10 - - - - CtrpB CtrpA - - - - Cmet-9011
<i>CtrpF CargG CargF Cleu-10 - - - - CtrpA Cmet-9011</i>	<i>CtrpF CargG - - - - - CpheA CtrpB CtrpA CpurF Cmet-9011</i>
CpyrE CtrpE CtrpD CtrpC - - - - Cmet-28 Cphe-3	CpyrE CtrpE CtrpD CtrpC - - - - Cmet-28
<i>CpyrE CtrpE - - - - CtrpC CmetA Cmet-28</i>	<i>CtrpE - - - - CtrpC CmetA Cmet-28</i>
<i>CpyrB CproC</i>	CpyrB CproC Cpur-66
<i>CproB Cpur-136</i>	Cleu-8 CproB Cpur-136
	<i>CproB Cpur-136</i>
<i>CpyrD ChisV Cleu-8</i>	
<i>CpyrF Cphe-2</i>	
<i>ChisI ChisIII ChisIIB ChisIIA</i>	
	<i>CargA CargB</i>
	<i>CmetE CmetC CmetB Cthr-48</i>

recipients and are useful in identifying methylotroph genes. Final proof of the existence of a particular gene, however, must ultimately depend on identification of the gene product.

Only those cosmids which complemented more than one marker are listed in Table 2.

Establishment of genetic linkage groups

Generation of prime plasmids resulted in the identification of four genetic linkage groups of *M. methylotrophus* AS1 (Moore *et al.*, 1983). However, similar experiments using *M. viscogenes* only succeeded in isolating primes comprising two linkage groups, represented by the plasmids pMO1317 and pMO1322 (Table 2). The six genetic linkage groups established using prime plasmids are presented in Table 4; the previously described nomenclature (Moore *et al.*, 1983) is used to identify the markers of these linkage groups, namely each locus carried by a prime or a cosmid is referred to by the allele of the recipient complemented preceded by the letter C.

Complementation of auxotrophs using the cosmid gene libraries confirmed all but one of the previously established linkage groups and led to the identification of five new *M. viscogenes* linkage groups and two new *M. methylotrophus* AS1 linkage groups (Table 4). Moreover, the smaller molecular size of the chromosomal inserts in the cosmids meant that linkage groups were often determined from a series of overlapping clones, hence the relative order of some genes could be ascertained. The analysis of complementation data using both cosmids and prime plasmids carrying *M. viscogenes* DNA enabled the following gene arrangements in that organism to be identified. The data for these conclusions are derived from Table 2, which lists the various cosmids and prime plasmids which have been characterized as being capable of complementing more than one marker, and from Table 3, which lists all the markers which can be complemented, most of them by cosmids which only complement one marker.

Markers of the cluster *CtrpF-CargG-CargF-Cleu-10-CtrpB-CtrpA-Cmet-9011* are found in various combinations in several *M. viscogenes* cosmids (pMO1335, pMO1336, pMO1338, pMO1337). Cosmids complementing *trpB* were not found, but linkage of this gene to *argG*, *argF* and *leu-10* was shown by means of the prime plasmid pMO1322. Similar arrangements were also found in *M. methylotrophus* AS1 primes (Moore *et al.*, 1983) and in cosmids pMO995, pMO996 and pMO1888. Strong similarities also are found with the marker cluster *CpyrE-CtrpE-CtrpC-Cmet-28* in both *Methylophilus* species.

The *CpyrB-CproC* linkage group is observed in both *Methylophilus* species, while the *Cleu-8-CproB-Cpur-136* cluster of *M. methylotrophus* AS1 suggests that the *CpyrD-ChisV-Cleu-8* and *CproB-Cpur-136* linkage groups of *M. viscogenes* may be adjacent to one another. The linkage groups *CpyrF-Cphe-2* and *ChisI-ChisIII-ChisIIB-ChisIIA* were only observed in *M. viscogenes*,

Fig. 1. (a) Agarose gel (1.2%, w/v) electrophoresis of purified prime plasmid and cosmid DNA cleaved with *Eco*RI and *Hind*III. Lanes contain: (A) λ phage DNA cleaved with *Hind*III; fragment sizes (kb) are shown on the left; (B) pMO1322; (C) pMO1335; (D) pMO1336; (E) pMO575; (F) pMO996; (G) pMO1888. (b, c) Autoradiographs following transfer of fragments from the gel in (a) to nylon filters and subsequent hybridization with 32 P-labelled pMO1322 (b) or pMO575 (c).

whilst the unique linkage groups *CargA*–*CargB* and *CmetE*–*CmetC*–*CmetB*–*Cthr-48* were seen only in *M. methylotrophus*. The order of genes in these clusters cannot be determined unambiguously with the available data.

Molecular comparison of prime plasmids and cosmids from M. viscogenes and M. methylotrophus AS1

Prime plasmids and cosmids from *M. viscogenes* and *M. methylotrophus* AS1 which were shown to complement equivalent amino acid biosynthetic functions in *P. aeruginosa* auxotrophs were selected for molecular analysis. It was hoped that such experiments would supplement the genetic linkage data, by demonstrating the relatedness of the chromosomal segments in prime plasmids and cosmids, and also give some insight into the genetic relatedness or otherwise of the two methylotrophs.

Prime plasmids and cosmids from both methylotrophs which were capable of complementing genetic markers within the two linkage groups *CtrpF*–*CargG*–*CargF*–*Cleu-10*–*CtrpB*–*CtrpA*–*Cmet-9011* and *CpyrE*–*CtrpE*–*CtrpC*–*Cmet-28* were examined. DNA from these prime plasmids and cosmids was digested with restriction endonucleases and analysed by agarose gel electrophoresis. The *Eco*RI/*Hind*III digest of the *M. viscogenes* prime (pMO1322) and cosmids (pMO1335 and pMO1336) and *M. methylotrophus* AS1 prime (pMO575) and cosmids (pMO996 and pMO1888) which complement markers in the first genetic cluster is shown in Fig. 1(a). Although many equivalent restriction fragments were observed in digests of the respective primes and cosmids from one strain, no restriction fragment identity was apparent between the DNA of the two methylotrophs. A similar result was obtained when comparing the *M. viscogenes* prime (pMO1317) and cosmids (pMO1339 and pMO1341) and *M. methylotrophus* AS1 prime (pMO574) and cosmids (pMO1787 and pMO1788) which complement markers in the second cluster (data not presented).

To confirm the equivalence of the prime plasmid and cosmid restriction fragments and to test for possible DNA homology between the *M. viscogenes* and *M. methylotrophus* AS1 chromosomal fragments, DNA was transferred to nylon filters and hybridized with radiolabelled DNA from either a *M. viscogenes* or a *M. methylotrophus* AS1 prime plasmid. In the example presented in Fig. 1(b), at least seven common fragments are observed in digests of the *M. viscogenes* prime plasmid and cosmids (lanes B, C and D), while only limited homology

was detected between the probe and chromosomal fragments from the prime plasmid and cosmids of *M. methylotrophus* AS1 (lanes E, F and G). It should be noted that two prime plasmid fragments and one cosmid fragment bear strong homology with the probe because the vectors contain regions which are homologous. A similar result was achieved when the *M. methylotrophus* prime was used as a probe (Fig. 1c). At least six prime plasmid and cosmid fragments were found to be common but very little DNA homology with the *M. viscogenes* sequences was observed.

DISCUSSION

Gene arrangement in *M. viscogenes* and *M. methylotrophus* AS1 was previously determined by complementation mapping using prime plasmids (M. I. Sinclair & B. W. Holloway, unpublished data; Moore *et al.*, 1983). The ability of prime plasmids to incorporate large segments of the chromosome makes them extremely useful in genetic linkage mapping; however, this same characteristic becomes a handicap when smaller segments of DNA are required to determine the relative gene order. Furthermore, it has proved impossible to isolate stable prime plasmids for all genetic markers of these two methylotrophs (P. P. Kearney, M. I. Sinclair & B. W. Holloway, unpublished data), hence, an alternative approach has been sought for the comprehensive mapping of methylotroph chromosomal genes through the use of cosmid libraries.

The random nature of a cosmid gene library as generated here for *M. viscogenes* and *M. methylotrophus* AS1 means that a comprehensive representation of the genome can be obtained with fewer than 1000 clones. The demonstrated ability of members of the cosmid libraries to complement various *P. aeruginosa* and *E. coli* auxotrophs has indicated their suitability for use in genetic mapping. Five of the six methylotroph linkage groups identified by prime plasmid complementation have been confirmed and two have been extended. *CtrpF*, *CtrpA* and *Cmet-9011* have been added to the *M. viscogenes* linkage group *CargG-CargF-Cleu-10-CtrpB*, while *CpheA* and *CpurF* have been added to the *M. methylotrophus* AS1 linkage group *Cpur-66-CtrpF-CargG-CargF-Cleu-10-CtrpB-CtrpA-Cmet-9011*. *CpurF* is not the same as *Cpur-66* since different cosmids complement each of these mutants.

Five additional *M. viscogenes* and two additional *M. methylotrophus* AS1 linkage groups have been found by using cosmids. The *CargA-CargB* and *CmetE-CmetC-CmetB-Cthr-48* clusters are new groups of linked markers in *M. methylotrophus* AS1, and together with *CmetA*, which has been added to the *CpyrE-CtrpE-CtrpC-Cmet-28* cluster of this methylotroph, emphasize the importance of using *E. coli*, which has a wide range of markers identified with respect to their biochemical lesion, as a complementation recipient. From the present data, it is not possible to determine if the methylotroph gene identified as *Cmet-28* by complementation in *P. aeruginosa* is the same as the gene which complements *metA* in *E. coli*.

The generally smaller insert size present in cosmids, compared to that of prime plasmids, has allowed the relative order of some markers within the larger linkage groups to be ascertained. For example, in the cluster of histidine markers in *M. viscogenes* the flanking markers are *ChisI* and *ChisIIA*, but the relative order of *ChisIII* and *ChisIIB* cannot be established. Also in *M. viscogenes* in the *Cmet-9011-Cleu-10-CargF-CargG-CtrpF-CtrpA* cluster, *Cmet-9011* and *CtrpA* are flanking markers and with *Cleu-10-CargF-CargG*, *CargF* is the middle marker. However, there remain a number of instances where the relative order of the markers is uncertain, and the resolution of these questions must await the complementation analysis of subclones of relevant cosmids. The physical joining of some of the linkage groups by chromosome walking seems feasible using cosmids. The *CpyrD-ChisV-Cleu-8* and *CproB-Cpur-136* clusters of *M. viscogenes* may be very close to one another and could be readily linked by such molecular techniques if the genetic maps are as similar as the complementation data suggest.

The absence of any means of recombinant analysis combined with the difficulty of getting mutants in these two methylotrophs makes the linkage data obtained by cosmid or prime complementation more significant. Further analysis of cosmids by transposon mutagenesis and subcloning should establish the precise linkage relationships of the genes identified above.

Whether the chromosomal analysis is undertaken using prime plasmids or cosmids, complementation mapping is based on a common assumption, namely, that no internal rearrangements have occurred in the chromosomal fragments carried. Comparison of the DNA from prime plasmids and cosmids which complemented equivalent markers revealed no evidence of molecular rearrangement and we have detected no instances of instability of the recombinant cosmids. The smaller size of the cosmids (about 40–50 kb) compared to that of prime plasmids (about 65–140 kb), together with the low copy number of the vector, result in increased stability and thereby increase the usefulness of the cosmid libraries for mapping studies.

Restriction and hybridization analysis of *M. viscogenes* and *M. methylotrophus* AS1 revealed the considerable differences which appear to exist between these organisms at the molecular level. The fact that no restriction identity and very little DNA–DNA homology was detected in these bacteria between genes with apparently the same function suggests a high degree of genetic divergence. The differing abilities of genes from the two species to complement auxotrophic mutants in either *P. aeruginosa* or *E. coli* may be a further measure of this divergence. These results are in contrast to the genetic linkage data, which indicate that the arrangement of genes on the chromosomes of these two methylotrophs has been highly conserved. It is surprising that so many auxotrophic markers can be demonstrated to be linked in both these organisms by complementation mapping. In view of the complementation linkage data obtained, it seems likely that there are one or a few auxotroph-rich regions of the methylotroph chromosome, a marker distribution pattern which has been shown to occur in pseudomonads (Holloway & Morgan, 1986).

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