R-Plasmid-mediated Chromosome Mobilization in the Facultative Methylophilic *Pseudomonas* AM1

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Chromosomal genes of the facultative methylophilic, *Pseudomonas* AM1, have been mobilized using the broad host-range plasmid, R68.45. Genes coding for C₁ metabolism were transferred at a frequency of 10⁻⁵ to 10⁻⁴ per donor cell. The genes coding for resistance to cycloserine, phosphonomycin and streptomycin are linked to the methanol dehydrogenase gene, while the marker for thiamin auxotrophy is not.

INTRODUCTION

The genetics of methylophilic bacteria have not been extensively studied because there are few methods available for transferring chromosomal genes between genetically marked strains of these organisms. O'Connor & Hanson (1978) used transformation to study linkage of C₁ utilization genes in *Methylobacterium organophilum*. Two investigations of the genetics of methylophilic bacteria have made use of broad host-range plasmids. Gautier & Bonewald (1980) cloned the wild-type methanol dehydrogenase gene of *Pseudomonas* AM1 in *Escherichia coli* using plasmid R1162. The methanol dehydrogenase gene was then transferred to a methanol dehydrogenase mutant of *Pseudomonas* AM1, using RP4 to mobilize the hybrid plasmid. Moore *et al.* (1983) developed a method of 'complementation mapping' in *Methylophilus methylotrophus* using R' derivatives of pM1072.

Some broad host-range plasmids can mobilize the chromosomes of many Gram-negative bacteria (Holloway, 1979). One such plasmid, R68.45 (Haas & Holloway, 1976), which codes for resistance to kanamycin, tetracycline and β -lactam antibiotics, has been used to map the chromosomes of several genera (Berlinger & Hopwood, 1976; Sistrom, 1977; Casadesus & Olivares, 1979; Hamada *et al.*, 1979; Megias *et al.*, 1982). This plasmid has been transferred to several methylophilic bacteria (Jeyaseelan & Guest, 1979; Warner *et al.*, 1980), but chromosome mobilization was not demonstrated in these studies.

In this paper we demonstrate that R68.45 can mobilize the chromosome of the facultative, serine-pathway methylophilic *Pseudomonas* AM1, and present some preliminary data on gene linkage in this organism.

METHODS

Bacterial strains. The bacterial strains used are shown in Table 1.

Media and growth of organisms. *Pseudomonas aeruginosa* PAO8 carrying R68.45 was grown in nutrient broth (Oxoid no. 2) containing 0.5% (w/v) yeast extract and kanamycin (500 μ g ml⁻¹), and was maintained on agar slopes of the same medium. Mutant strains of *Pseudomonas* AM1 were grown in minimal salts medium (MacLennan *et al.*, 1971) with either 0.2% (w/v) sodium succinate or 0.2% (v/v) methanol as carbon source, and were maintained on agar slopes of the same medium. Where appropriate, the medium was supplemented with thiamin (5 μ g ml⁻¹) or adenine (20 μ g ml⁻¹). Strains of *Pseudomonas* AM1 carrying R68.45 were grown and maintained on media containing kanamycin.

All antibiotics were added as filter-sterilized aqueous solutions to the growth media at the following final concentrations: kanamycin, 500 μ g ml⁻¹ for *P. aeruginosa* and 200 μ g ml⁻¹ for *Pseudomonas* AM1; streptomycin, 200 μ g ml⁻¹; D-cycloserine, 250 μ g ml⁻¹; phosphonomycin, 300 μ g ml⁻¹.

Table 1. *Bacterial strains and plasmids*

Plasmid/Strains	Relevant properties	Reference
Plasmid R68.45	IncP Km ^R Tc ^R Cb ^R	Haas & Holloway (1976)
Donors		
<i>Pseudomonas aeruginosa</i> PAO8(R68.45)	Ilv ⁻ Met ⁻ Str ^R	Haas & Holloway (1976)
<i>Pseudomonas</i> AM1 PT1(R68.45)	Ade ⁻	This paper
<i>Pseudomonas</i> AM1 PT2(R68.45)	Thi ⁻	This paper
Recipients		
<i>Pseudomonas</i> AM1 PG1-1	Methanol dehydrogenase ⁻ Str ^R	This paper
<i>Pseudomonas</i> AM1 PG1-2	Methanol dehydrogenase ⁻ Pho ^R	This paper
<i>Pseudomonas</i> AM1 PG1-3	Methanol dehydrogenase ⁻ Cyc ^R	This paper
<i>Pseudomonas</i> AM1 PCT48-1	Serine pathway mutant Str ^R	Dunstan <i>et al.</i> (1972)

Isolation of mutants. Mutants of *Pseudomonas* AM1 unable to grow on C₁ compounds were isolated by the method of Heptinstall & Quayle (1970), using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as the mutagen. Auxotrophic mutants were isolated by the same method. Antibiotic-resistant strains were isolated either by selecting for spontaneous mutants or by treatment of exponential-phase cultures with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine followed by isolation of resistant clones. PT1 and PT2 were made auxotrophic before R68.45 was transferred to them.

Mating procedures. The antibiotic agar overlay method of Jeyaseelan & Guest (1979) was used to transfer plasmid R68.45 from *P. aeruginosa* to *Pseudomonas* AM1.

The patch mating method of Johnston *et al.* (1978) was used to screen clones for the ability to mobilize the chromosome, and was modified as follows to estimate frequencies of transfer of chromosomal markers. Late-exponential phase cultures of donor and recipient strains were mixed (1:1) at an approximate concentration of 10⁸ cells ml⁻¹ and mated on nutrient agar plates for 36 h. The growth was harvested from the surface of the plates and the cells were washed twice in sterile 0.9% (w/v) saline. A significant loss of bacteria occurred during the harvesting and washing stages. The bacteria were resuspended in 2 ml sterile 0.05 M-potassium phosphate buffer, pH 7.2, and 0.1 ml was plated in triplicate on to selective media to estimate the number of recombinants. The numbers of viable donor and recipient cells were determined on appropriate media. Sterile 0.9% (w/v) saline containing 0.01% (v/v) Nonidet P40 (BDH) (a nonionic detergent used to minimize clumping) was used as diluent. Recombinant clones were tested for unselected markers by streaking on to appropriate media; between 200 and 300 clones were tested from each mating.

RESULTS AND DISCUSSION

Plasmid R68.45 was transferred from *P. aeruginosa* to *Pseudomonas* AM1 at an average frequency of 10⁻⁶ transconjugants per recipient cell. Clones of *Pseudomonas* AM1(R68.45) were screened for their ability to transfer the genes coding for methanol utilization to recipients unable to grow on methanol. Of 1238 clones tested, 37 (3%) had chromosome mobilizing ability.

The frequency of transfer of the genes coding for methanol utilization was estimated using two auxotrophic strains, PT1 (Ade⁻) and PT2 (Thi⁻), as donors. Antibiotic-resistant strains of PG1, which lacks methanol dehydrogenase, and PCT48, which is probably blocked in the Icl⁻ part of the serine pathway (i.e. in the conversion of acetyl-CoA to glyoxylate, which does not involve isocitrate lyase; Dunstan *et al.*, 1972), were recipients. The frequency of transfer of the genes coding for methanol utilization was in the range 10⁻⁵–10⁻⁴ per donor cell.

Linkage of the genes coding for C₁ utilization and antibiotic resistance was investigated by testing recombinant clones for the unselected donor markers. When PG1 was used as recipient, cycloserine sensitivity was transferred with the methanol dehydrogenase gene in over 95% of the recombinants (Table 2), indicating that these markers are closely linked. The genes coding for methanol dehydrogenase and phosphonomycin sensitivity were transferred together in almost 90% of the recombinants but the methanol dehydrogenase gene and the streptomycin sensitivity marker did not show such close linkage, being cotransferred in only 65–72% of the recombinants. When PCT48 was the recipient, the wild-type allele of the serine pathway gene which was defective in this mutant was transferred with the streptomycin sensitivity marker more frequently, with about 80% of the recombinants containing both donor markers.

Table 2. *Cotransfer of chromosomal markers of Pseudomonas* AM1

Recipients which had received the donor markers which determined methanol utilization were selected initially. The selective medium contained no growth factors, thus preventing growth of the donor strains. Recombinant clones were tested for the unselected donor markers as described in Methods.

Donor	Recipient	Unselected marker	No. of recombinants expressing both donor markers	No. of recombinants tested	Percentage of recombinants expressing both donor markers
PT1	PG1-1	Str ^S	194	300	65
PT1	PG1-2	Pho ^S	240	270	89
PT1	PG1-3	Cyc ^S	291	300	97
PT2	PG1-1	Str ^S	177	246	72
PT2	PG1-2	Pho ^S	234	262	89
PT2	PG1-3	Cyc ^S	270	270	100
PT2	PCT48-1	Str ^S	241	290	83

Table 3. *Frequency of isolation of recombinants of Pseudomonas* AM1 on different selective media when PT2 was used as donor

No growth was detected when either the donors or the recipients were plated on to the selective media. Abbreviations: Str, streptomycin; Pho, phosphonomycin; Cyc, D-cycloserine; Thi, thiamin.

Donor	Recipient	Selective medium	No. of recombinants ml ⁻¹	No. of donors ml ⁻¹	Frequency of recombinant isolation per donor cell
PT2	PG1-1	Methanol + Str	360	1.7×10^6	2.2×10^{-4}
PT2	PG1-1	Methanol + Str + Thi	450	1.7×10^6	2.7×10^{-4}
PT2	PG1-2	Methanol + Pho	0	6.7×10^6	0
PT2	PG1-3	Methanol + Cyc	0	3.3×10^6	0
PT2	PCT48-1	Methanol + Str	375	5×10^6	7.5×10^{-5}
PT2	PCT48-1	Methanol + Str + Thi	378	5×10^6	7.5×10^{-5}

The frequency of recombinant isolation on different selective media in the crosses between PT2 and antibiotic-resistant derivatives of PG1 is shown in Table 3. Recombinants were detected when methanol utilization plus streptomycin resistance were used as selective markers, but not when resistance to either phosphonomycin or cycloserine were the counterselcted markers. This confirms the conclusion that the genes coding for phosphonomycin and cycloserine resistance are more closely linked to the gene coding for methanol dehydrogenase than is the streptomycin resistance gene. Approximately 2×10^{-4} recombinants were obtained per donor cell when selection was made on methanol plus streptomycin, whether or not thiamin was added to supplement the medium. Furthermore, all recombinants isolated on medium containing thiamin were able to grow in the absence of this vitamin. Similar results were obtained in crosses between PT2 and PCT48. This indicates that the gene determining thiamin auxotrophy in PT2 is not close to the genes coding for C₁ metabolism.

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