Methylotrophic Bacteria: Biochemical Diversity and Genetics

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Summary. Bacteria that are able to use methane as a sole carbon and energy source also carry out a broad range of biotransformations, some of which have industrial and environmental significance. Genetic studies on methylotrophs, including the use of recombinant DNA techniques, show promise for the isolation and cloning of genes coding for specific functions.

Bacteria that can use, as the sole sources of carbon and energy, one-carbon compounds more reduced than carbon dioxide—methylotrophic bacteria—were first described in 1906 (*1*). Although methylotrophs are known to be ubiquitous and to contribute significantly to the global carbon cycle, it was not until 1970 that their widespread distribution, their taxonomic diversity, and their physiological properties were described (*2–5*).

Except for several species of yeasts and fungi that grow on methane or methanol, the microbes that can use one-carbon compounds as a carbon and energy source are prokaryotes (bacteria). Known methylotrophic bacteria are all Gram-negative, obligate aerobes, and exist in a variety of shapes (rods, vibrios, and cocci).

The term "methanotroph" designates those methylotrophic bacteria that can use methane for carbon and energy. Approximately 50 percent of the organic carbon degraded anaerobically is converted to methane, most of which is oxidized by methanotrophs to carbon dioxide before it reaches the atmosphere (*4*). In aquatic environments, these microbes are most numerous in regions where methane, produced from the anaerobic decomposition of organic matter, and oxygen from the atmosphere are present at concentrations optimal for their growth (*3*).

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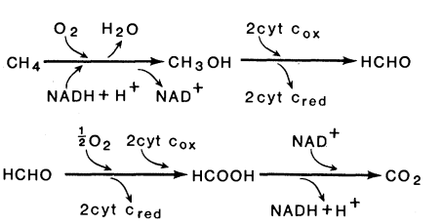
Physiology and Metabolism

Bacteria that grow on methane contain an abundance of intracytoplasmic membranes. The arrangement of these membranes together with biochemical pathways used for the assimilation of one-carbon units form the primary basis for their classification into two general groups (types I and II). They are further classified into subgroups on the basis of other criteria (*6*).

Methylotrophic bacteria contain enzymes that catalyze the following oxidative reactions



Carbon for the biosynthesis of cellular material is diverted from this pathway at the oxidation stage of formaldehyde. Two primary pathways (with variations) for the assimilation of formaldehyde have been described in bacteria and another in yeast (*6*). Type I methylotrophic bacteria use the efficient ribulose monophosphate pathway for formaldehyde assimilation. This pathway begins with the condensation of three molecules of formaldehyde with three molecules of ribulose monophosphate to yield three hexulose-6-phosphate molecules. These molecules are subsequently converted to intermediates that feed into biosynthetic pathways. The less efficient serine pathway found in type II methylotrophs begins with the condensation of two molecules of formaldehyde and two of glycine to yield two serine molecules. Rearrangement of the serine molecules in this pathway produces precursors for biosynthetic pathways. The bacteria for which the genetics of the one-carbon assimilation pathway are described possess the serine pathway and are all facultative type II methylotrophs. The use of methane by these bacteria requires at least ten enzymes (seven for the assimilation of formaldehyde, methanol dehydrogenase, a methane monooxygenase, and a cytochrome c_{552}) necessary to the metabolism of reduced one-carbon compounds (*3*).

Most methylotrophic bacteria are obligate methylotrophs, that is, they can utilize only compounds that do not contain carbon-carbon bonds as energy and carbon sources. The characterization of a facultative methylotroph that could grow on many multicarbon compounds and did not depend on the aforementioned pathways as an obligate means of existence has expanded the opportunities for studying the biochemistry and genetic control of one-carbon metabolism.

Scope of Biotransformations Catalyzed by Methylotrophs

Methane- and methanol-oxidizing organisms have been employed in the production of single-cell protein for use as an animal feed supplement (*4*). Their ability to oxidize a larger number of substrates that do not serve as carbon and energy sources has received much recent attention. The results of studies of the oxidative potentials of methylotrophs by Higgins, Dalton, Hou, and their co-workers (*7–17*) are summarized in Table 1. The range of biotransformations carried out by these organisms is obviously broad and includes reactions that yield products of potential commercial importance such as propylene oxide, a substrate for synthetic polymers. Other reactions, including the dehalogenation of organic compounds, have ecological significance.

The oxidation of most of these substrates has been attributed to a lack of specificity of the methane monooxygenase, the enzyme that catalyzes the conversion of methane to methanol, and to a lesser extent other enzymes which act in the further oxidation of methanol to carbon dioxide. A secondary alcohol dehydrogenase that catalyzes the oxidation of short chain secondary alcohols (products of the action of the methane monooxy-

genase on alkanes) to their corresponding methyl ketones is present in many methylotrophs (*13*). This enzyme has no discernible beneficial role in these bacteria, but its retention during evolution indicates a use for its activities in nature.

Mutagenesis in Methylotrophic Bacteria

In order to take full advantage of the abilities of methylotrophs to transform organic compounds to useful products, it will be necessary to develop techniques for their genetic manipulation. Attempts to isolate mutants in the methane oxidative and assimilatory pathways of obligate methane users have been frustrated by these organisms' requirements for a limited number of one-carbon sources of carbon and energy and by lack of success in isolating stable mutants with the use of chemical mutagens and ultraviolet light (*4*). Any mutation in the one-carbon catabolic or assimilatory pathways in obligate methylotrophs is lethal. An obligate methylotroph, *Methylosinus trichosporium* OB3b can be adapted from growth on methane to growth on methanol. When methanol-grown cells were examined, they were found to contain the first enzyme system in the oxidative pathway, methane monooxygenase, which is therefore assumed to be produced constitutively or induced by methanol in this organism. Thus it would be theoretically possible to obtain a nonlethal methane monooxygenase mutant that could be maintained on methanol.

Mutations outside of one-carbon functions have been difficult to isolate in obligate methylotrophs. Windass *et al.* (*18*) have, however, succeeded in isolating a temperature-sensitive glutamate synthase mutant of the obligate methylotroph *Methylophilus methylotrophus*.

Because facultative methylotrophs are capable of growing on both one-carbon and multicarbon compounds, nonlethal mutations can be induced in the enzymes for methane oxidation and the assimilation of formaldehyde. These mutants are unable to grow on methane or methanol but can grow on multicarbon substrates. The standard mutagenic techniques of ultraviolet light irradiation and chemical mutagenesis have successfully generated one-carbon metabolism mutants in *Methylobacterium organophilum* strain xx, a facultative methylotroph, and the facultative methanol- and methylamine-utilizing *Pseudomonas* strains AM1, MS, and *Pseudomonas aminovorans* (*6*, pp. 102–106). Subsequent analyses of the mutants have shown that several of the genes that code for proteins of the one-carbon oxidative and assimilatory pathway (except those coding for formate dehydrogenase and cytochrome c_{522}) are linked by transformation on the *M. organophilum* strain xx chromosome (*19*). Methanol dehydrogenase, the assimilatory enzymes, cytochrome c_{522}, and other catabolic enzymes were found to be induced by methanol in this organism (*20*). Curiously, mutants obtained were almost entirely confined to genes coding for enzymes that enable growth on methanol. Only one stable auxotroph was isolated in many attempts. A summary of mutants isolated is shown in Table 2.

As an alternative to the use of chemicals and ultraviolet light, transposons can be employed to generate mutations in bacterial genomes. Transposons are DNA sequences that code for resistance to an antibiotic and can insert with a general degree of randomness into the bacterial DNA (transposition). The gene into which the transposon is inserted is mutated because its continuity is interrupted by the transposon. Transposons are usually introduced into the cell on a plasmid, from which they can transpose into the cellular DNA. An effective transposon delivery system developed by Weiss *et al.* (*21*) consists of a conjugative plasmid derived from the plasmid RK2 that has a broad host range; RK2 and its derivatives are transferred to *Methylobacterium organophilum* strain xx at high frequencies (as described later). The transposon delivery vector (designated pUW964) contains the transposons Tn5 (kanamycin resistance) and Tn7 (trimethoprim and streptomycin resistance). In addition, it contains the replicative functions from the plasmid Col E1, which are only expressed in *Escherichia coli*. Thus the vector can transfer itself by conjugation into a broad range of recipient strains but cannot replicate. Under selective pressure of antibiotics the only recipient bacteria that can grow are those in which the transposons have inserted themselves into cellular DNA and are therefore replicated by the recipient cells. With the use of the pUW964 delivery system, kanamycin resistance has been transferred to *M. organophilum* strain xx at a frequency of 10^{-4} per recipient cell (*22*). Although

Table 1. Compounds oxidized by methylotrophs.

Compound	Product	Reference
Ethane	Ethanol	(*7, 8, 9, 10, 11*)
	Ethanal	(*8, 12*)
	Acetate	(*8*)
Propane	1-Propanol	(*8, 9, 10, 11, 12*)
	2-Propanol	(*7, 8, 9, 10, 11*)
	Propanal	(*7, 8*)
	Acetone	(*7, 13*)
Butane	1-Butanol	(*8, 9, 10, 11*)
	2-Butanol	(*7, 8, 9, 10, 11*)
	n-Butanal	(*7, 8*)
	2-Butanone	(*7, 13*)
Isobutane	Isobutanol	(*11*)
	tert-Butanol	(*11*)
Pentane	1-Pentanol	(*8, 11*)
	2-Pentanol	(*7, 8, 11*)
	n-Pentanal	(*8*)
	2-Pentanone	(*7, 13*)
Hexane	1-Hexanol	(*8*)
	2-Hexanol	(*7, 8, 11*)
	n-Hexanal	(*8*)
	2-Hexanone	(*7, 13*)
Heptane	1-Heptanol	(*8, 11*)
	2-Heptanol	(*8, 11*)
	n-Heptanal	(*8*)
Octane	1-Octanol	(*8, 11*)
	2-Octanol	(*8, 11*)
Hexadecane	1-Hexadecanol	(*14*)
Ethene	Epoxyethane	(*8, 10, 11, 12*)
Propene	1,2-Epoxypropane	(*8, 10, 11, 12, 14*)
1-Butene	1,2-Epoxybutane	(*8, 10, 11, 12*)
trans-2-Butene	*trans*-2,3-Epoxybutane	(*8, 11, 12*)
	trans-But-2-en-1-ol	(*8, 11, 12*)
	trans-But-2-en-1-al	(*8*)
cis-2-Butene	*cis*-2,3-Epoxybutane	(*8, 11, 12*)
	cis-But-2-en-1-ol	(*8, 10, 11*)
	cis-But-2-en-1-al	(*8*)
	Butanone	(*8*)
Butadiene	1,2-Epoxybutene	(*10, 11*)
Isoprene	1,2-Epoxyisoprene	(*11*)
2-Propanol	Acetone	(*12*)
2-Butanol	2-Butanone	(*10, 13*)

this transposition frequency is sufficient for detailed analysis, screening of hundreds of kanamycin-resistant transconjugants has not yielded any nutritional mutants.

Because auxotrophic mutants have been difficult to isolate, more novel methods for mapping chromosomal markers are needed. Holloway and coworkers (*23*) have used *Pseudomonas aeruginosa* genetics to aid in mapping the chromosome of *Methylophilus methylotrophus* AS1. Unlike methylotrophs, *P. aeruginosa* is sensitive to chemical and ultraviolet light mutagenesis, and many auxotrophic mutants have been obtained (*23*). Plasmids from the Inc P1 (broad host range) incompatibility group, such as R68.45, are capable of mobilizing chromosomal DNA during conjugal transfer. These fused molecules, containing plasmid and chromosomal DNA are termed "R-prime" plasmids and are discussed below. Holloway and coworkers have generated R-prime plasmids that contain *M. methylotrophus* AS1 DNA, using the R68.45 derivative plasmid, pMO172. Upon conjugative transfer to *P. aeruginosa* mutants, complementation of the recipients' mutations by the *M. methylotrophus* AS1 DNA was observed. Thus R-prime plasmids able to complement more than one mutation in *P. aeruginosa* contained more than one marker from *M. methylotrophus* AS1, and these markers are therefore linked on the *M. methylotrophus* AS1 chromosome. In this manner, Moore *et al.* (*24*) were able to construct several linkage groups within the chromosome of *M. methylotrophus*.

A Useful Obligate Methylotroph for Genetic Studies

One organism that shows promise in the isolation of nutritional mutations is *Methylomonas* sp. 761M (*25*). This obligate type I methane-oxidizer is unusual in that it possesses a complete tricarboxylic acid cycle. In particular it has a functional α-ketoglutarate dehydrogenase that is lacking in other type I organisms. While it has an obligate requirement for one-carbon compounds as energy sources, its growth is affected by other nutrients. Growth of several obligate methane-oxidizers is enhanced by the addition of nutrients to methane-grown cultures, but this has not been analyzed further (*6*, p. 8). In *Methylomonas* sp. 761M, the rate of growth is stimulated four- to fivefold and cell yields are increased sixfold when amino acids are present in the culture medium in addition to methane (Fig. 1). Asparagine, glutamine, lysine, and valine are the most stimulatory; threonine and tyrosine alone are inhibitory (*26*). Glucose, when added to minimal media plus methane, also stimulates growth. Growth has never been observed in the absence of methane or methanol as energy sources.

Methylomonas sp. 761M also incorporates acetate into cellular materials and uses several dicarboxylic acids as substrates for respiration (*26*). Uptake systems for these compounds, as well as for glucose and amino acids, must therefore be present. It should be possible to obtain mutants whose behavior in the presence of amino acids and glucose differs from that of the wild type of strain.

A very interesting and useful property of *Methylomonas* sp. 761M is its ability to grow on methanol in the absence of methane. Under these conditions the same effects of growth stimulation and inhibition by different nutrients are observed (*26*). *Methylosinus trichosporium* OB3b is another obligate methylotroph that can grow on either methane or methanol, and in this organism the methane monooxygenase is produced constitutively during growth on methanol (*4*). The function of methane monooxygenase is not known to be required for methanol utilization, and methane-oxidizing strains capable of growth on methanol provide a workable system for obtaining nonlethal mutants in this important enzyme.

Preliminary experiments have indicated that *Methylomonas* sp. 761M is more sensitive to the mutagenic effects of ultraviolet light than are other obligate methylotrophs. This finding, along with the response of the organism to nutrients and metabolites and its ability to grow on methanol, establishes *Methylomonas* sp. 761M as a promising candidate for genetic analysis.

Naturally Occurring Plasmids in Methylotrophs

Plasmid DNA has been identified in many different kinds of autotrophic bacteria (*27*) and methylotrophs. In *Alcaligenes eutrophus*, plasmid DNA has been shown to be involved in hydrogen oxida-

Table 1 (continued).

Compound	Product	Reference
2-Pentanol	2-Pentanone	(*10*)
2-Hexanol	2-Hexanone	(*10*)
Chloromethane	Formaldehyde	(*12, 15, 16*)
Dichloromethane	Carbon monoxide	(*15, 16*)
Trichloromethane	Carbon dioxide	(*15, 16*)
Bromomethane	Formaldehyde	(*11, 12, 15, 16*)
Fluoromethane	Formaldehyde	(*11*)
Dimethylether	Methanol	(*11, 12, 16*)
	Formaldehyde	(*11, 12, 16*)
Diethylether	Ethanol	(*12, 16*)
	Ethanal	(*12, 16*)
Methylformate	Formaldehyde	(*16*)
	Formate	(*16*)
Cyclopropane	Cyclopropanol	(*17*)
Methylcyclopropane	Cyclopropylmethanol	(*17*)
Cyclohexane	Cyclohexanol	(*11, 16*)
Benzene	Phenol	(*11, 14, 16*)
	Hydraquinone	(*16*)
Toluene	*p*-Cresol	(*11, 14, 16*)
	Benzyl alcohol	(*14, 16*)
	Benzoic acid	(*14*)
Ethylbenzene	*o*-Hydroxyethylbenzene	(*14*)
	p-Hydroxyethylbenzene	(*14, 17*)
	Phenylethanol	(*14, 17*)
Phenylacetic acid	Benzoic acid	(*14*)
Styrene	Styrene oxide	(*17*)
	p-Hydroxystyrene	(*17*)
α-Methylstyrene	*p*-Hydroxy-α-methylstyrene	(*17*)
Propylbenzene	*p*-Hydroxypropylbenzene	(*17*)
1-Phenylheptane	1-Hydroxy-1-phenylheptane	(*14*)
	1-Phenylheptane-7-al	(*14*)
	1-Oxo-1-phenylheptane	(*14*)
m-Cresol	*m*-Hydroxybenzaldehyde	(*14*)
	p-Hydroxybenzaldehyde	(*14*)
o-Cresol	5-Methyl-1,3-benzenediol	(*14*)
m-Chlorotoluene	Benzyl alcohol	(*14*)
	m-Methyl benzyl alcohol	(*14*)
	p-Methyl benzyl alcohol	(*14*)
Pyridine	Pyridine-*N*-oxide	(*16*)
Naphthalene	α-(1,6)-Naphthol	(*17*)
	β-Naphthol	(*17*)

tion (*28*). A plasmid in *Methylobacterium organophilum* strain xx is associated with growth on methane (*3*). Many methylotrophs also contain resident plasmids which are cryptic in that nothing is known of the functions they encode. The plasmids in obligate methylotrophs are large (greater than 100 kilobase pairs), and occasionally more than one plasmid is present in a strain (*29*).

Organism SB-1, a type II obligate methylotroph isolated in our laboratory, contains two resident plasmids of 130 and 210 kilobase pairs. The smaller of the plasmids, designated pR6-1, is being studied in detail. Restriction enzyme analysis has revealed that pR6-1 contains several sites for enzymes recognizing hexanucleotide sequences (Fig. 2). Clone banks of pR6-1 DNA have been constructed in different restriction sites of the plasmid cloning vector pBR322 (*30*) and maintained in *E. coli* HB101. The use of *E. coli* clones carrying the hybrid plasmids has enabled the isolation of the inserted fragments in yields four orders of magnitude higher than the plasmid yields from organism SB-1. The hybrid plasmids have been stably maintained in *E. coli* HB101 for 1 year by monthly transfers.

The nature of these plasmids with regard to conjugation is speculative since their cryptic nature does not allow selection for conjugal transfer. Other plasmids of similar size have generally been shown to encode conjugal transfer functions (*31*). The transfer of chromosomal markers can be facilitated by some conjugative plasmids through mobilization of these markers during exchange of plasmid DNA between mating pairs. In order to determine whether plasmids of methylotrophs can function in this manner, matings in broth cultures have been carried out between organism SB-1 and several *Pseudomonas aeruginosa* auxotrophic mutants. In one such mating with *P. aeruginosa* PAO 905 (a leucine auxotroph), complementation to leucine prototrophy was observed at a frequency of 2×10^{-4} Leu^+ (does not require leucine) transconjugants per recipient cell (*22*). Plasmid DNA obtained from these transconjugants is structurally different from pR6-1 and the resident plasmid in *P. aeruginosa* PAO 905 but shares sequence homology with each (*22*). The pR6-1 DNA present in the transconjugants and the organism SB-1 DNA that complements the leucine mutation have not yet been identified.

Table 2. Mutations in methylotrophic bacteria obtained through chemical or ultraviolet light mutagenesis.

Organism	Mutation	Reference
Methylophilus methylotrophus	Glutamate auxotroph; tryptophan auxotroph	(*18, 39*)
Methylobacterium organophilum xx	Glutamate auxotroph; one-carbon metabolism (eight different functions)	(*34, 19*)
Pseudomonas AM1	One-carbon metabolism (ten different functions); malyl coenzyme A hydrolase; acetyl coenzyme A synthetase; phosphoserine phosphatase; carotenoid synthesis	(*6*, pp. 102–106)
Pseudomonas aminovorans	One-carbon metabolism (five different regulatory mutants)	(*6*, pp. 102–106)
Pseudomonas MS	One-carbon metabolism	(*6*, pp. 102–106)

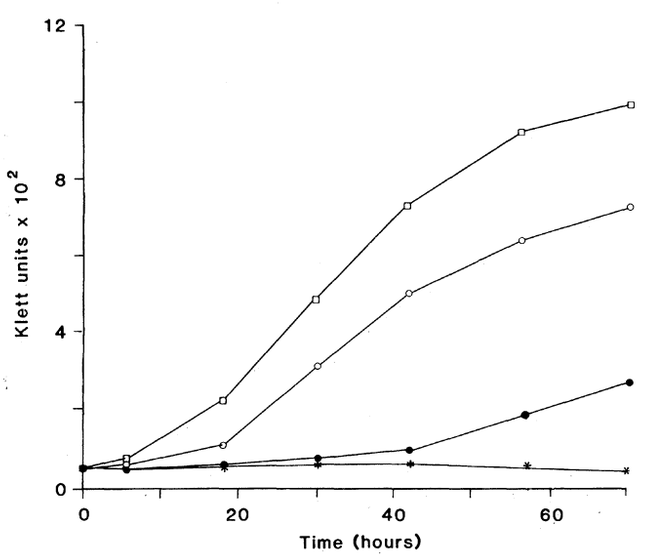


Fig. 1. Growth of *Methylomonas* sp. 761M in broth culture under different conditions: (*) minimal medium plus 0.1 percent peptone, 0.1 percent glucose, and no methane; (●) minimal medium plus methane: air (1:1); (○) minimal medium plus 0.1 percent glucose plus methane:air (1:1); and (□) minimal medium plus 0.1 percent peptone plus methane:air (1:1).

Strategies for Gene Cloning in Methylotrophs

We and others have been attempting to isolate DNA that codes for oxidative and assimilatory enzymes involved in growth on methane and methanol through recombinant DNA techniques. The biological activities of cloned gene products can be determined by several means. Expression of plasmid-borne foreign DNA in *E. coli* can be tested in minicells that synthesize proteins encoded on the plasmids (*32*). Alternatively, the foreign DNA fragments can be transcribed and translated in vitro with the use of an extract of *E. coli* (*33*). Both methods require pure protein standards for identification of the gene products. Methane monooxygenase from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b have been characterized; each contains three subunits, but the subunits differ substantially in size as well as in biochemical properties. (Purified monooxygenase from organism SB-1, *Methylomonas* sp. 761M, and *Methylobacterium organophilum* strain xx are not yet available.) These methods also assume that the heterologous cloned methylotroph DNA will be recognized by *E. coli* transcriptional and translational proteins. At present no methylotroph DNA has been thus expressed. A more straight forward method for assigning a biological function to the cloned DNA from methylotrophs is complementation of mutations in methane oxidative and assimilatory enzymes in *M. organophilum* strain xx.

Complementation of markers requires a method for the transfer of the DNA fragments to be assayed into the mutant cells. The majority of systems for genetic transfer have been established in *Bacillus subtilis*, *P. aeruginosa*, and *E. coli*, and these procedures have not been readily adaptable to methylotrophic bacteria. Transformation, the uptake of naked DNA by bacterial cells, has been demonstrated with *M. organophilum* strain xx (*34*). This occurs only with large amounts of linear DNA. Transformation with circular plasmid DNA (pRK290) has been achieved in our laboratory, but the transformation frequencies are too low to be suitable for "shotgun" cloning of genetic material. Transduction, or the transfer of bacterial genes between cells by bacterial viruses (bac-

teriophages), has not been observed in these bacteria, although lysogenic bacteriophages that infect methylotrophs have now been isolated (*35*).

The most successful method for the transfer of cloned DNA into methylotrophs has involved the use of conjugative or mobilizable cloning vectors. Conjugative plasmids can be transferred between bacterial cells by simple mating techniques. The transfer of mobilizable vectors requires the assistance of another mobilizing plasmid that codes for the gene products necessary for conjugal transfer of the cloning vector. Some promising mobilizable cloning vectors for use in methylotrophs are the plasmids pRK290 (and derivatives) (*36*), pTB70 (*18*), pKT230 (*37*), and RSF1010 (*38*). These plasmids have specific restriction sites for the insertion of DNA fragments and have been transferred to methylotrophic bacteria with Inc P1 plasmids as mobilizing agents. A summary of the use of these and other conjugative plasmids and mobilizable cloning vectors in methylotrophs is presented in Table 3. Several of these vectors contain regions that enable them to be packaged into the *E. coli* bacteriophage λ. These so-called cosmid vectors allow insertion of large segments of cloned DNA in vitro, and are useful for constructing gene banks of chromosomal and plasmid DNA. For propagation and maintenance of cloned DNA, cosmids can be transferred to *E. coli* after being packaged in infectious λ particles.

In vivo Cloning with R-Prime Plasmids

Plasmids of the P1 incompatibility group (Inc P1 plasmids) have been transferred between several Gram-negative organisms—for example, *Pseudomonas aeruginosa*, *E. coli*, *Erwinia chrysanthemi*, *Rhizobium meliloti*, and methylotrophs (*39*). The Inc P1 plasmids include RP1, RP4, RK2, and R68. These very similar conjugative plasmids are approximately 56 kilobase pairs in size, and all specify resistance to the antibiotics ampicillin, tetracycline, and kanamycin. A modified form of the plasmid R68, R68.45, carries a tandem duplication of a 2.1-kilobase-pair insertion sequence of R68 and has been shown to promote the transfer of chromosomal DNA between several Gram-negative bacteria.

An R-prime plasmid which contains R68.45 DNA and a chromosomal fragment of *E. coli* that codes for the genes required for tryptophan biosynthesis has been generated from R68.45 (*39*). Another R-prime form of R68.45 which carries the chromosomal trp A and trp B genes of *P. aeruginosa* has been constructed by Holloway (*39*). Moore *et al.* (*24*) have constructed R-prime plasmids in *Methylophilus methylotrophus* AS1 from an R68.45 derivative, and they have used these R-primes to map linkage groups of *M. methylotrophus* AS1 DNA relative to complementation of *P. aeruginosa* auxotrophs. Chromosomal DNA on R-prime plasmids can be transferred between Gram-negative organisms at relatively high frequencies because of the conjugative properties of R68.45.

We have used R68.45 in an effort to clone the genes of *Methylbacterium organophilum* strain xx that are involved in methanol oxidation. The R68.45 plasmid transfers from *P. aeruginosa* to *M. organophilum* strain xx and from *M. organophilum* into *E. coli* HB101 at frequencies above 10^{-2} transconjugant per recipient cell (Table 4).

Matings were achieved by mixing equal volumes of donor and recipient cultures (~ 10^9 cells per milliliter). The cells were collected onto membrane filters that were inverted and incubated overnight on nutrient agar plates at 30°C. Frequencies of transfer were determined by removing the filters, resuspending the cells in liquid media, and spreading dilutions onto media with and without kanamycin. These matings yielded higher transfer frequencies than did the other liquid or plate mating techniques attempted. Plasmid R68.45 can be isolated from *M. organophilum* strain xx transconjugants and can be transferred back

Table 3. Useful cloning vectors for methylotrophs. Abbreviations: Km, kanamycin; Tc, tetracycline; Ap, ampicillin; Su, sulfonilamide; and Sm, streptomycin.

Vector	Markers	Properties	Reference
R68.45	Km, Tc, Ap	Conjugative; broad host range; forms R-prime plasmids containing chromosomal DNA that are transferred at high frequency; transfers drug resistance at frequencies of 10^{-2} per donor in *Methylosinus trichosporium* OB3b and 1.4×10^{-2} per recipient in *Methylobacterium organophilum* xx	(*39, 42, 43*)
pM061	Km, Tc, Ap	Conjugative, with enhanced chromosomal mobilization, and host range and transfer frequencies similar to its parent plasmid R68.45; R-prime plasmids formed are more stable than those formed in R68.45	(*44*)
RSF1010	Su, Sm	Mobilizable; broad host range; transfers to *Methylophilus methylotrophus* AS1 and methanol-oxidizing pseudomonads; limited number of cloning sites	(*37, 38*)
pKT230, pKT231	Km, Sm	Mobilizable derivatives of RSF1010; containing cloning sites in resistance genes to allow selection for inserts	(*37*)
pTB70	Su, Sm, Km	Mobilizable; parent plasmid R300B is closely related to RSF1010; kanamycin resistance results from transposition of Tn5 into R300B; cloning sites in resistance genes; used to clone *E. coli* glutamate dehydrogenase gene into *M. methylotrophus* AS1	(*18*)
pRK290	Tc	Mobilizable; derived from plasmid RK2 (broad host range); contains two cloning sites that allow no selection for inserts; tranfers to *M. organophilum* xx with a frequency of 8×10^{-4} per recipient	(*36, 42*)
pLAFR1	Tc	Mobilizable cosmid derivative of pRK290	(*45*)
pVK100, pVK101, pVK102	Km, Tc	Mobilizable derivatives of pRK290 containing the kanamycin resistance gene of plasmid R6-5; pVK102 is a cosmid vector; cloning sites in resistance genes allow selection for inserts	(*46*)
pLA2901, pLA2905	Km, Tc	Mobilizable derivatives of pRK290 containing the kanamycin resistance gene of Tn5; several cloning sites allow selection for inserts	(*42*)
pLA2910, pLA2913, pLA2917	Km, Tc	Mobilizable cosmid derivatives of pLA2901	(*42*)

Table 4. Interspecies conjugal transfer of plasmid R68.45 and of an R-prime form containing *Methylobacterium organophilum* xx chromosomal DNA. Abbreviations: Km^R, kanamycin resistance; $MEOH^+$, ability to grow on methanol; and NA, data not available.

Donor strain	Recipient strain	Transfer frequency per recipient*	Marker	Plasmid recovery
Pseudomonas PAO 18 (R68.45)	*M. organophilum* xx	1.4×10^{-2}	Km^R	+
M. organophilum xx (R68.45)	*E. coli* HB101	3.2×10^{-1}	Km^R†	+
M. organophilum 17M (R68.45′)	*E. coli* HB101	8.6×10^{-2}	Km^R†	+
E. coli HB101 (R68.45′)	*M. organophilum* xx 17M‡ ($MEOH^+$)	1.9×10^{-2}	Km^R	NA
		1.9×10^{-2}	Km^R, $MEOH^+$	NA

*Some growth of cells occurs overnight on the filter. These frequencies are based on the assumption that the growth rate of the transconjugant does not differ from that of the total population on nonselective media. †The exceptionally high frequency noted in this experiment may result from transfer of R68.45 between *E. coli* cells after transfer from *M. organophilum* to the original *E. coli* recipients during the long mating period. ‡This is a mutant that lacks methanol dehydrogenase required for the oxidation of methanol to formaldehyde (*19*).

into *E. coli* HB101 by conjugation (Table 4).

Three-way filter matings have been performed with two different stable unlinked mutants, both unable to grow on methanol, and *Pseudomonas* PAO 18 carrying R68.45 as a mobilizer of chromosomal genes. Kanamycin-resistant transconjugants, able to grow on media containing methanol as the only carbon and energy source, were obtained in about one per 5×10^8 recipient cells. Mobilization of chromosomal DNA from one methanol mutant into another is required in order to obtain this result. It was anticipated that R-prime forms of R68.45 would be generated at low frequencies (*40*). The plasmids have been transferred from *M. organophilum* into *E. coli* HB101, and kanamycin-resistant transconjugants were selected. *Escherichia coli* HB101 lacks functional restriction, modification, and recombination systems, and this allows storage of a potential R-prime plasmid in an intermediate host. *Methylobacterim organophilum* strain xx may have a functional recombination system, and loss of cloned DNA from the R-prime plasmid could occur through homologous recombination if it were not transferred into *E. coli* HB101. When two of the *E. coli* HB101 intermediate plasmid hosts were mated separately with a methanol dehydrogenase mutant of *M. organophilum* strain xx (17M), kanamycin-resistant transconjugants that were able to grow on methanol as a sole carbon and energy source were obtained at high frequencies (Table 4). Most of the kanamycin-resistant transconjugants were converted to the methanol-plus phenotype (Table 4). These results indicate that the intermediate host contained an R-prime form of the plasmid R68.45 that was composed of the methanol dehydrogenase gene and R68.45 DNA. Because this R-prime proved too unstable for detailed analysis, another R-prime plasmid was constructed by the same approach, and with an R68.45 derivative (pMO61) as the chromosome mobilizing vector. Plasmid pMO61 is more stable in its chromosome mobilizing properties than R68.45 (*41*). $Methanol^+$ (does not require methanol) transconjugants derived from this mating should provide more detailed information on the region coding for enzymes of the one-carbon oxidative and assimilatory pathways and their regulatory elements.

A mobilizable plasmid pTB70, constructed in vitro, has been successfully employed for cloning the *E. coli* glutamate dehydrogenase gene into *Methylophilus methylotrophus*, a methylotroph used for single-cell protein production (*18*). A temperature-sensitive glutamate synthase mutant of *M. methylotrophus* was used as a host. The *E. coli* glutamate dehydrogenase gene (*gdh*) was inserted in vivo into a derivative of plasmid RP4, and RP4 prime plasmids containing this gene were selected by complementation with the use of an *E. coli* glutamate auxotroph. The glutamate dehydrogenase gene was then excised, inserted into pTB70, and transferred into the *M. methylotrophus* mutant with RP4 as a mobilizing agent. Transconjugants that grew without glutamate at the restrictive temperature were selected. The *E. coli* glutamate dehydrogenase gene was expressed in *M. methylotrophus*, and methanol-grown clones containing the *E. coli* gene for the more efficient of the two pathways for glutamate synthesis produced higher yields of single-cell protein than the parent bacterium grown on methanol.

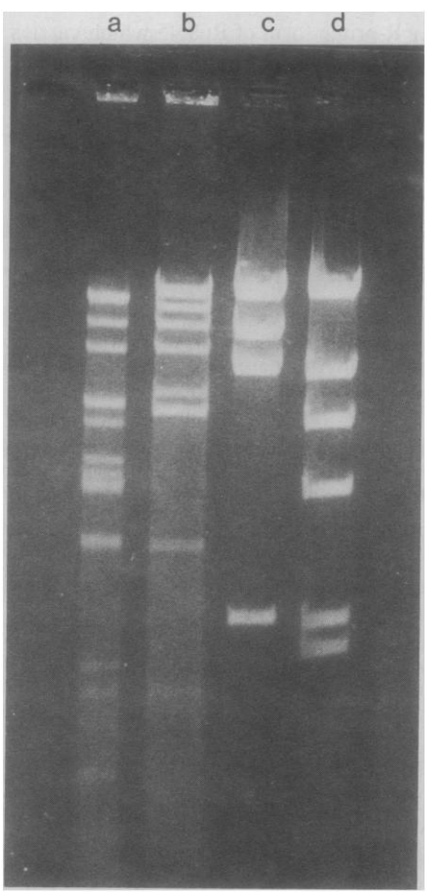


Fig. 2. Restriction digests of plasmid pR6-1 isolated from organism SB-1. Agarose gels (0.7 percent) are shown containing digests of the plasmid and bacteriophage λ DNA. Lanes: (a) pR6-1 (Bgl II), (b) pR6-1 (Hind III), (c) λ (Bgl II), and (d) λ (Hind III).

Concluding Remarks

The successful exploitation of methylotrophs as biocatalysts may require the application of modern genetic techniques for the optimization of the reactions we have described. It is encouraging that the genetics of *E. coli* and *P. aeruginosa* appear to be adaptable to these purposes.

Although the biochemistry of methylotrophs with regard to one-carbon metabolism has been studied in depth, uncharacterized biochemical and physical factors have impeded progress in obtaining nutritional mutants for genetic studies. The development of new mutagenic techniques, such as transposon mutagenesis, applicable to methylotrophs will aid in overcoming this barrier.

The examination of new methylotrophic isolates often proceeds no further than the standard tests used for

classification purposes. Additional studies could indicate strains with properties that provide useful genetic information. *Methylomonas* sp. 761M, with its responses to the presence of supplemented nutrients, now offers a system for studying the genetics of obligate methane-oxidizers.

Derivatives of broad host-range conjugative and mobilizable plasmids have been constructed and can serve as cloning vectors in methylotrophs. Encoding multiple drug resistances and possessing several cloning sites, these plasmids enable the construction of gene banks that can be maintained in *E. coli* and transferred to methylotrophs. The use of R-prime plasmids for the mobilization of chromosomal markers is an effective way to bypass in vitro recombinant DNA techniques. The application of such in vivo cloning methods to methylotrophs has provided a means for the identification of desired genes. A resident methylotroph plasmid has been implicated in chromosome mobilization, and this could have broader applications for gene transfer.

These developments establish the foundation for advancing the knowledge of methylotroph genetics and for applying this knowledge toward the beneficial use of this interesting group of bacteria.

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RESEARCH ARTICLE

Arctic Terrestrial Biota: Paleomagnetic Evidence of Age Disparity with Mid-Northern Latitudes During the Late Cretaceous and Early Tertiary

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During the past century abundant evidence has accumulated of a thriving biota at high Arctic paleolatitudes during the late Mesozoic and early Cenozoic eras. Arctic floras were first described by Heer in his seven-volume *Flora Fossilis Arctica*, published between 1868 and 1883 (*1*). Data on pollen and invertebrates in the Late Cretaceous and early Tertiary are more recent, the result of intensive geological exploration of Spitsbergen (*2*), Greenland (*3, 4*), and the Canadian Arctic Archipelago (*5, 6*). The predominantly terrestrial sequences characterizing the latest Cretaceous and early Tertiary have been dated mainly by comparing them with mid-northern latitude floras, although limited and at times tenuous association with marine or brackish-water beds containing datable invertebrates has been reported for North and West Greenland (*3, 7*), Spitsbergen (*8*), and west-central Ellesmere Island (*6*). The discovery of land vertebrate fossils of early to middle Eocene affinities in the upper part of the Eureka Sound Formation (*9*) at Bay Fiord on Ellesmere Island (locality 4, inset map in Fig. 1) provided an apparently reliable and independent source of relative age dates keyed to the land-mammal sequence of western North America (*10*).

Five areas on Axel Heiberg and Ellesmere islands (inset map in Fig. 1) have now yielded substantial megafloral and palynological collections (*11, 12*) as well as invertebrate (*10, 13*) remains and additional vertebrate material from the Bay

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