

Physiological and Genetic Aspects of the Utilisation
of Methylated Amines in M. methylotrophus .

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Submitted for the degree of Doctor of Philosophy, 1987.

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To my parents and Kevin:
for all their help.

ACKNOWLEDGEMENTS

Thanks to Bill for all his patience and help, particularly his patience, I'm sure he thought he was never going to be rid of me. Thanks also to everyone who has been in lab. 124 and then G2, especially Karen who managed to keep me sane. I'm grateful to Ian Charles for teaching me the sequencing, which never seemed to end, and to all the people who contributed their various methods to this work. I'm particularly grateful to my parents, who allowed me to keep eating for the last four years. Finally I'd like to thank Kevin for putting up with my intermittent bouts of rage and hysteria during the writing up period, I promise faithfully to visit you in the asylum every second Tuesday.

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ABBREVIATIONS

APS	ammonium persulphate
ATP	adenosine 5'- triphosphate
bp	base pair
CIP	cytidine 5'- triphosphate
DCPIP	dichlorophenol indophenol
DMA	dimethylamine
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetra-acetic acid
FMN	flavin mononucleotide
GTP	guanosine 5'- triphosphate
kd	kilo dalton
mdh	methanol dehydrogenase
MMA	methylamine
mmdh	methylamine dehydrogenase
NADP	nicotinamide adenine dinucleotide phosphate
OD	optical density
PEG	polyethylene glycol
PMS	phenazine methosulphate
RNA	ribonucleic acid
RNase	ribonuclease
SCP	single cell protein
SDS	sodium dodecyl sulphate

TEMED N,N,N',N' tetramethylethylenediamine
TMA trimethylamine
tmdh trimethylamine dehydrogenase
Iris tris (hydroxymethyl) aminomethane
TTP thymidine 5'- triphosphate
wt wild type

Additional Abbreviations.

DEP diethyl pyrocarbonate (1mM)
DMF dimethyl formamide
PQQ pyrrolio quinoline quinone
PVP poly vinyl pyrrolidone

CHAPTER 1

Introduction.

Methylotrophs are organisms which can synthesize all their cellular constituents from reduced carbon compounds containing one or more carbon atoms, but containing no carbon-carbon bonds. Obligate methylotrophs grow only on such compounds, whereas facultive methylotrophs are also able to utilise a variety of other organic multicarbon compounds. The utilisation of C_1 compounds in the environment is mostly confined to a small group of organisms which use methane, methanol, or methylated amines as their sole source of carbon and energy. Although the first methylotroph, Bacillus methanicus, was discovered some eighty years ago growing aerobically in methane (Anthony 1982), few other methylotrophs were discovered until the 1950's. At this stage biochemists discovered that many methylotrophic organisms assimilated C_1 units at levels more reduced than carbon dioxide by an assimilatory process then unknown. At the same time biotechnologists realised that methane and methanol offered cheap and readily available fermentation feedstocks, providing an additional stimulus for research and development. Since then, many new methylotrophic bacteria have been isolated, and interest in them has remained active in both the biochemical and biotechnological fields. Table 1.1 lists those substrates known to support the growth of methylotrophs. The bacteria

able to use these compounds are too numerous to mention, but include species of *Pseudomonads*, *Arthrobacter*, *Bacillus*, *Methylobacterium* and *Methylomonas*.

Compounds containing one carbon atom		Compounds containing more than one carbon atom	
Methane	CH ₄	Dimethyl ether	(CH ₃) ₂ O
Methanol	CH ₃ OH	Dimethylamine	(CH ₃) ₂ NH
Methylamine	CH ₃ NH ₂	Trimethylamine	(CH ₃) ₃ N
Formaldehyde	HCHO	Tetramethylammonium	(CH ₃) ₄ N ⁺
Formate	HCOOH	Trimethylamine N-oxide	(CH ₃) ₃ NO
Formamide	HCONH ₂	Trimethylsulphonium	(CH ₃) ₃ S
Carbon monoxide	CO		

Table 1.1 Substrates for methylotrophs.

There are four different pathways by which the aerobic methylotrophs assimilate carbon substrates, and each pathway has at least two potential variants; one route operates in yeasts, (Dijken et al. 1981), while the other three, the ribulose monophosphate, ribulose biphosphate, and the serine pathway operate in bacteria (Anthony 1982). The organism used in the studies in this thesis, Methylophilus methulotrophus, is a Gram-negative obligate methylotroph, depending on the presence of methanol or one of the methylated amines for its growth. Conversion of methanol to cell carbon in this organism

occurs through the oxidation of methanol to formaldehyde by methanol dehydrogenase, with the assimilation of the formaldehyde into cell carbon via the ribulose monophosphate pathway (Beardsmore and Quayle 1978), see Fig.1.1. Formaldehyde not assimilated in this manner is mainly oxidised to carbon dioxide via the dissimilatory ribulose monophosphate cycle with the formation of NAD(P)H.

Growth upon the methylated amines involves their ultimate oxidation to formaldehyde and ammonia. The term 'methylated amine' is used to denote amines containing only methyl groups without carbon-carbon bonds. In Methylophilus methylophilus the amine can serve as both a nitrogen and a carbon source, the ammonia being used for biosynthesis and the formaldehyde for both biosynthesis (assimilation), and oxidation to yield energy for growth (dissimilation).

The methylated amines are all oxidised to formaldehyde which can either be oxidised to provide metabolic energy, or assimilated into cell material. Each methyl group of the methylated amine is oxidised to one molecule of formaldehyde, either producing reducing equivalents or requiring NAD(P)H in the process.

The assimilatory pathways used for the bacterial growth upon methylated amines are the same as those used for the growth on other C₁ compounds, the ribulose monophosphate pathway. The dissimilation pathways involve conversion of trimethylamine to dimethylamine, and of dimethylamine to monomethylamine, (Fig.1.1). In M. methylophilus this involves the reactions (i) for trimethylamine to dimethylamine, (ii) for dimethylamine to monomethylamine, and (iii) of monomethylamine

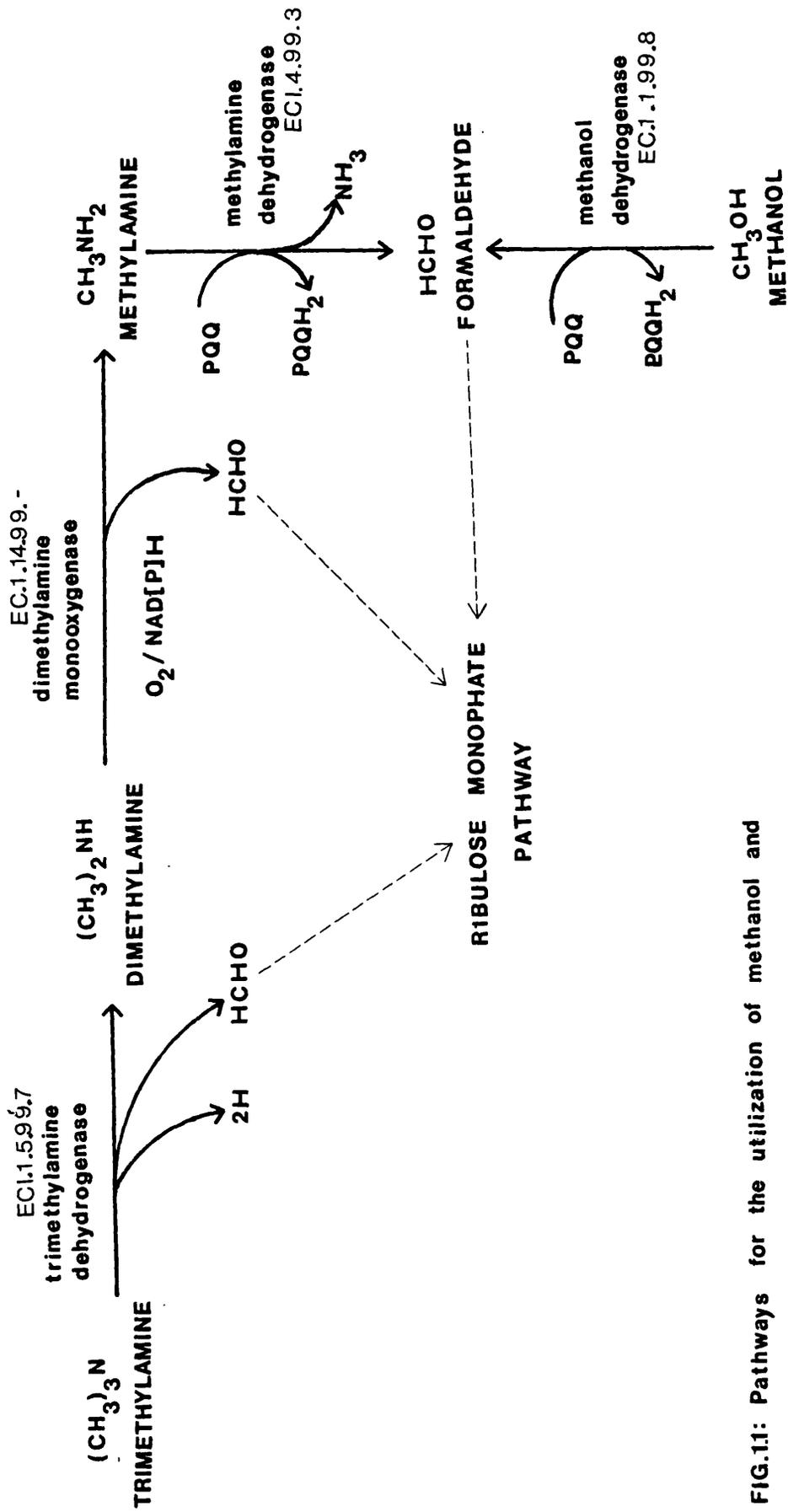
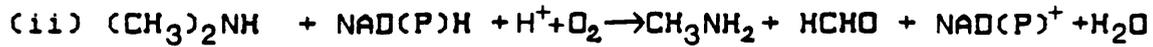
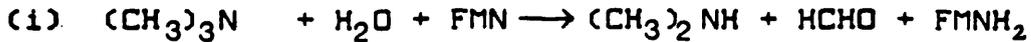


FIG.11: Pathways for the utilization of methanol and methylated amines.

to formaldehyde and ammonia;



where A is the acceptor

The enzymes involved in these reactions are respectively (i) trimethylamine dehydrogenase, (ii) dimethylamine monooxygenase (iii) monomethylamine dehydrogenase.

While the enzymes have been studied structurally, there is little evidence about the genetic control of their synthesis. Trimethylamine dehydrogenase has been shown to consist of two different sub-units, 1x 70,000, 1x 80,000 daltons, containing a covalently bound flavin 6-S-cysteinyl-FMN (Steenkamp et al. 1978), and a single tetrameric Fe-S centre (Hill et al. 1977). Although unstable once isolated, studies on dimethylamine monooxygenase suggest that it is an unusual carbon monoxide-sensitive haemoprotein (Eady et al. 1971), probably containing flavin as well as nonhaem iron. Methylamine dehydrogenase consists of two light subunits (13,000 daltons), and two heavy subunits (40,000 daltons, Matsumoto et al. 1980). There are, however, no significant quantities of iron in monomethylamine dehydrogenase, and no evidence for the presence of flavin (Eady and Large 1971).

1.1 Genetical Aspects.

Though the physiological and biochemical aspects of M. methylophilus have been extensively studied, little

effort was put into the genetics of the organism until Imperial Chemical Industries began to utilise it to produce Single Cell Protein (S.C.P). S.C.P. for animal nutrition, as well as possibly direct incorporation into human food, was a comparatively novel commodity which required much research and development involving genetic, technical and engineering aspects of production. The production of biomass as S.C.P. is of necessity a large-scale capital business and the cost of the carbon source is an important element in profitability calculations. Glucose, at two to three times the cost of methanol, is economically less attractive than methanol as a basic carbon source, and in recent years C_1 -utilisers have taken over the field of S.C.P. production.

ICI's first full-scale S.C.P. plant was successfully commissioned and operated in 1979. Fermentation involved M. methylotrophus to produce 'PRUTEEN', the registered trade mark for ICI's S.C.P. The product competed favourably in the animal food industry, and with competition such as soya meal, sold as a high energy/protein commodity. There was initially much scope for strain improvement, and one of the first tasks undertaken in this field by the ICI Corporate Laboratory was to eliminate the energetically sub-optimal, nitrogen-assimilation pathway involving glutamine/glutamate synthetase, and to introduce the gene for the energetically more favourable glutamic dehydrogenase, cloned from E. coli into a broad host-range plasmid vector (Windass et al. 1980). Although this manipulation was successful in improving carbon-conversion in the methylotroph, the improved strain was never used for commercial production.

The success in this endeavour led to further investigations into the opportunities offered by recombinant DNA technology to the field of S.C.P. science. One might wish to manipulate a S.C.P. organism in order to understand the strain better, to improve the strain itself, or to enable the organism to produce some medical or industrial product at a commercially viable rate. Recombinant DNA techniques have today made it relatively easy to clone genes from almost any organism and to attempt to express them in a foreign cellular environment. Despite the complexities of eukaryotic gene structure, it is proving possible to make products of mammalian genes in bacterial cells by cloning DNA copies (cDNA) of mammalian messenger RNAs, and expressing them within the bacterial cell (Old and Primrose 1980).

In order to use methylotrophs or other S.C.P. organisms as hosts for the expression of prokaryotic or eukaryotic coding sequences there needs to be much more effort towards understanding the genetics of these organisms, knowledge of which remains primitive. Suitable vectors, capable of accepting DNA sequences and of replicating in the host strain have been developed (Sharp 1984), and several sequences have been successfully expressed within the organism (Hennam *et al.* 1982). We know little as yet, however, of the features that make a powerful promoter or an efficient ribosome-binding site, in methylotrophs or other S.C.P. organisms, although knowledge of these properties is essential for the high-level expression of cloned cDNA sequences.

From the expressed eukaryotic cDNA sequences (Hennam *et al.* 1982), and from the efficiency with which the

E. coli genes are expressed within M. methylophilus, it is possible that the features necessary for the efficient expression of DNA within M. methylophilus may be similar to other prokaryotes, notably E. coli sp. and Pseudomonas sp., with which it shares a high G plus C content (56%).

Standard genetic techniques, produced initially for the enterobacteria have required some modification for application to the methylophilus. Genetic variants for conventional genetic analysis have, for example, proved difficult to isolate in the methylophilus. In general, auxotrophic mutants with readily identifiable growth-requirements have been the basis for bacterial genetic analysis, yet despite successful mutagenesis as judged by the frequency of mutation to antibiotic-resistance, the isolation of auxotrophs remains limited. The difficulty can probably be attributed to the dearth of permeases for exogenous compounds, making auxotrophy a lethal condition.

A significant tool in advancing the molecular genetics of M. methylophilus and many other bacterial species has been the highly mobile genetic elements, the insertion sequences and transposons. The existence of such elements has long been inferred from the analysis of controlling elements in maize (McClintock, 1956, Fincham and Sastry 1974), and it is today evident that such mobile sequences are common in both eukaryotic chromosomes and bacterial plasmids (Bukhari et al. 1977). Transposons are useful in bacterial genetics primarily because they are highly mutagenic, and the mutations they confer have a readily selectable physical and genetic marker, usually an antibiotic-resistance determinant, at the

site of mutation. Their readiness to integrate freely and randomly into the host chromosome is as useful as their ability to remain stable once integrated.

While the process of mutation and selection is useful in generating strains with desirable characteristics, the ability to transfer genes from one organism to another underlines the basis of controlled strain-construction. An ability to manipulate methylotroph genes in this fashion, to investigate their structure and function, or to produce useful gene-products, will be necessary if industrial companies are to exploit methylotrophs in the production of medically and commercially important substances. Transduction, at present, is not a viable proposition in the methylotroph as it has been impossible to isolate the bacteriophages which could be used for this purpose (Byrom, D., pers. comm. 1982, Holloway 1980). Indeed, although a transformation system has been described for a facultative methane-user, Methylobacterium organophilum (O'Connor and Hanson 1978), conjugation remains the only way of transferring genes between strains of Methylophilus. Most vectors developed to date have been specifically designed for the enterobacteria, in particular E. coli strains. For use in M. methylophilus plasmid vectors have been developed which incorporate the broad host-range characteristics from the IncQ plasmids, but which in addition contain the antibiotic-resistance genes from pBR322. The stability of the vectors within M. methylophilus is complete as long as a continual selection pressure is applied (Sharp 1982).

Mobilisation of these plasmid vectors, which cannot normally

promote their own transfer, is mediated by the presence of transfer agents such as the broad host range R plasmids. These mobilisation plasmids act in a similar manner to the F or "fertility factors" in that they code for the production of pili, and have been shown to enhance chromosome-mobilisation at a low frequency. The IncI based plasmids used for chromosome mobilisation in this thesis work can promote the transfer of these M. methylophilus vectors, but are not themselves stable within the recipient cell (Sharp, G., pers. comm. 1982)

1.2 Deciding on a Pathway to Study.

Using these techniques a number of mutants have already been obtained which show limited growth on methanol; these mutants have however proved unsuccessful in allowing the isolation via genetic replacement from a wild type gene bank, of any DNA sequences which relieve the mutant phenotypes (Burt 1982). The trimethylamine pathway, however, has been given little genetic attention, although it represents a prime target for transposon mutagenesis and subsequent genetic analysis. The pathway is non-essential to the organism, and thus the generation and isolation of mutants should be a simplified task. The pathway is not constitutive, and some regulatory control has been implied; a genetic analysis of this system might therefore throw light on the control of gene expression in the methylotroph. In addition, all three enzymes involved, trimethylamine dehydrogenase, dimethylamine monooxygenase and methylamine dehydrogenase are easily assayed (Jones et al., 1982).

1.3 Summary

In summary the purpose of the studies in this thesis is to find out more about the molecular genetics and physiology of M. methylophilus. Transposon mutagenesis is used to generate mutants, leading to a study of the effect of these mutations on the regulation of the trimethylamine-assimilation pathway, and subsequently to the isolation of a M. methylophilus gene. Isolation of the gene is of considerable interest, to allow an investigation into the molecular make-up of the gene, including its codon usage, and the sequences that govern its transcription and messenger RNA translation.

CHAPTER 2

Materials and Methods.

2.1 General Methods.

Precautions against bacterial and enzymic contamination.

All solutions and equipment which were not heat-labile were autoclaved at 15 p.s.i. for 15 minutes. Those solutions affected by heat were sterilised by passing through a membrane filter. Heat-labile equipment was sterilised by first rinsing in ethanol, and then twice in sterile water.

In the handling of DNA and RNA, gloves were always worn.

Siliconisation of Glassware.

Glass equipment for DNA or RNA use was siliconised to prevent adherence of DNA or RNA to glassware. This was achieved by coating with 2,4-dichlorodimethylsilane. The procedure was carried out in a fume cupboard.

2.2 Sources of Chemicals and Biochemicals.

Unless otherwise stated, all chemicals were obtained from:

Fisons Scientific Equipment, Loughborough, U.K.

All radiochemicals were supplied by: Amersham International, Amersham, U.K. Ribonuclease A, Deoxyribonuclease I, Pronase and Lysozyme were obtained from: Sigma Chemical Co. Ltd., Poole U.K.

Restriction endonucleases and DNA modifying enzymes (Klenow, DNA Polymerase-I etc.) were supplied by either Bethesda Research Laboratories, West Germany, or Pharmacia Laboratories (P-L) Biochemicals, Milton Keynes, U.K.. DNA Ligase, and Alkaline Phosphatase(calf thymus) was also supplied by P-L Biochemicals.

Nitrocellulose paper and filters were from: Schleicher and Schuell.

X-Ray film was Fugi RX 100.

Stained gels were photographed using a Polaroid Land camera and either type 55 or type 57 Polaroid film.

2.3 Preparation of Stock Solutions and Buffers.

Stock solutions and buffers mentioned in the following sections, but not detailed, were made as follows and autoclaved at 15 psi. for 15 minutes unless otherwise stated.

2.3.1 Buffers and solutions.

Set out below are buffers and solutions which are not specifically described in the text.

Coomassie Blue Stain/Destain.

Stain	0.1% Coomassie Blue	Destain	5% Methanol
	50% Methanol		10% Acetic acid
	10% Acetic Acid		

H-Iop (1 litre)

Bactotryptone	10.0g
NaCl	8.0g
Agar	8.0g

Luria Broth. (1 litre)

Bactotryptone	10.0 g
Yeast Extract	5.0 g
NaCl	5.0 g
MgSO ₄	2.5 g
Glucose	1.0 g
Add 15.0g agar for L-Agar	

M9 Salts x5. (1 litre)

Na ₂ HPO ₄ .H ₂ O	30.0 g
KH ₂ PO ₄	15.0 g
NaCl	2.5 g
NH ₄ Cl	5.0 g

after autoclaving, add;

MgSO ₄ (1 M)	5.0 ml
CaCl ₂ (0.1 M)	5.0 ml

M. methylophilus Salts x4

NaH ₂ PO ₄ .2H ₂ O	5.6 g
K ₂ HPO ₄	7.6 g

dissolve in 800 ml. H₂O, add;

$(\text{NH}_4)_2 \cdot \text{SO}_4$ 7.2 g

autoclave and add;

MgSO_4 0.8 g in 200 ml. sterile H₂O

adjust to pH 6.8 if necessary.

Nick Mix x10.

0.5 M Tris-HCl, pH 7.8

50 mM MgCl_2

0.1 M 2-Mercaptoethanol

Quench Mix x10.

2% SDS

50 mM EDTA

10 mM Tris-HCl, pH 7.5

Restriction Buffers.

Buffer	NaCl	Tris-HCl (pH 7.5)	MgCl_2	Dithiothreitol
Low	0	10 mM	10 mM	1 mM
Medium	50 mM	10 mM	10 mM	1 mM
High	100 mM	50 mM	10 mM	1 mM

SmaI 20 mM KCl, 10 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 ,
1 mM Dithiothreitol

Sample Buffer. (Acrylamide I)

(Formamide Dye Mix)

100 ml De-ionised Formamide

0.1 g Xylene Cyanol FF

0.1 g Bromophenol Blue

2 ml EDTA

Sample Buffer. (Acrylamide II)

10 mM 2-Mercaptoethanol

10% Sucrose

4% SDS

0.125 M Tris-HCl pH 6.8

Sample Buffer. (Agarose) x6

0.25% Bromophenol Blue

40% Sucrose

SSC

0.15M NaCl

0.015M Sodium Citrate, pH 7.0

T4 DNA Ligase Buffer x10.

1.0 M Tris-HCl, pH 7.5 66 μ l

0.2 M EDTA, pH 9.0 5 μ l

1.0 M MgCl₂ 10 μ l

1.0 M 2-Mercaptoethanol 10 μ l

0.1 M ATP pH 7.0 1 μ l

Tris-Acetate Buffer.

0.4 M Tris-HCl

0.2 M Sodium Acetate

0.1 M EDTA

adjust to pH 8.2 using Acetic Acid.

IE Buffer.

10 mM Tris-HCl, pH 7.5

1 mM EDTA

IM Buffer.

100 mM Tris-HCl, pH 7.5

50 mM MgCl₂

Trace Elements. (for *Methylophilus methylotrophus*) (1 litre)

CuSO ₄ .5H ₂ O	0.02 g
MnSO ₄ .4H ₂ O	0.01 g
ZnSO ₄ .7H ₂ O	0.01 g
CaCO ₃	1.80 g
1 M HCl	36.6 ml
FeCl ₃	0.97 g

make up in 800 ml, add H₂O to 1000 ml.

Iris-Glutamine Gel Buffer x10.

Iris-HCl	30 g
Glycine (NH ₄ ⁺ free)	144 g
SDS	10 g
H ₂ O	1 l

autoclaving not required.

Tryptone-Yeast Agar. (1 litre)

Bactotryptone	8.0 g
Yeast extract	5.0 g

NaCl 2.5 g

add 18 g agar for plates, 6 g agar for soft agar.

Tryptone-Yeast Broth (TY) x2.(1 litre)

Bactotryptone 16 g

Yeast Extract 10 g

NaCl 5 g

2.3.2 Stock Solutions.

DNase 1 Stored as a solution of 0.1 mg/ml in 0.02 M sodium acetate, (pH 5.2).

RNAse A RNAse free of DNase was prepared by dissolving RNAse at a concentration of 10 mg/ml in 10 mM Tris-HCl, pH 7.5 and 15 mM NaCl. The solution was heated to 100°C for 15 minutes and allowed to cool slowly to room temperature. Aliquots were stored at -20°C.

Lusozyme Stock solution of 50 mg/ml in water, aliquots stored at -20°C. Aliquots were discarded after one use.

Dideoxy Solutions

10 mM ddTTP	: dissolve 10 mg/1639 µl	TE
10 mM ddCTP	: dissolve 5 mg/862 µl	TE
10 mM ddGTP	: dissolve 5 mg/806 µl	TE
10 mM ddATP	: dissolve 5 mg/323 µl	TE

Deoxy Solutions

50 mM dTTP	: dissolve	25 mg/779	μl TE
50 mM dCTP	: dissolve	100 mg/3378	μl TE
50 mM dGTP	: dissolve	10 mg/316	μl TE
50 mM dATP	: dissolve	10 mg/339	μl TE

Ampicillin 10 mg/ml stock solution in water, stored at -20°C

Chloramphenicol 30 mg/ml stock solution in 100% ethanol,
stored at -20C.

Kanamycin 10 mg/ml stock solution in water, stored at -20°C.

Tetracycline 2 mg/ml stock solution in ethanol/H₂O (50:50),
stored at -20°C.

2.4 Maintenance and Propagation of Bacterial and Viral Strains.

E. coli strains (DH1, JM101, etc.) were maintained on L-Agar plates at 4°C for up to four weeks, when the strain would be subcultured.

M. methylophilus strains were kept on minimal/ methanol plates, at 4°C, and subcultured every two weeks.

All bacterial strains were also maintained as glycerol cultures at -20°C. These were prepared by mixing 1 ml fresh overnight with 1 ml 80% glycerol.

Phage stocks of M13mp19 were stored as a lysate at 4°C and -20°C in TM buffer.

2.5 DNA-related Techniques.

Isolation of High Molecular Weight Bacterial DNA.

For bulk preparations, 200 ml of an overnight cell culture was used. These were harvested and washed twice with an equal volume of ice-cold TEN buffer (0.01 M Tris-HCl, pH 7.9, 0.001M EDTA, 0.1 M NaCl), and finally resuspended in 25 ml of the same, 5ml of 10 mg/ml lysozyme solution was added and incubated at 37° C. After 20 minutes, 30 ml buffer TEN containing; 2% sarcosyl NLS7 and 0.02 mg/ml RNA'se A, was added and incubated at 42° C for 60 minutes. Pronase was then added to a final concentration of 1 mg/ml, incubated at 42° C for a further four hours. The resultant lysate was extracted three times with phenol equilibrated against 0.5 M Tris HCl, pH 8.0. The aqueous phase, containing the DNA, was then precipitated by the addition of ethanol and acetic acid (10:1). The precipitated DNA was resuspended in 20 ml TE, and dialysed against the same buffer for 24 hours, with three 2 litre changes. The DNA was then sized on a 0.2% agarose gel, using ligated plasmid markers (Maniatis et al 1982).

Preparation of Plasmid DNA.

Using a suitable selection (usually 0.1 mg/ml ampicillin), 250 ml of cells were grown in a 2 litre baffled flask shaking at 200 rpm at 37° C for at least 18 hours. The cells were resuspended gently in 8 mls of 25 mM Tris-HCl, pH 8.0, 10 mM

EDTA, 50 mM glucose. 2 mls of the above buffer containing 10 mg/ml lysozyme was added, and the lysate left on ice for 5 minutes, after which 20 mls of alkaline SDS (0.2 M NaOH, 1% SDS) was added and the mixture again left for 5 minutes at 0°C. The lysate was mixed with 15 ml of 3 M potassium acetate at 0°C, and the mixture poured into a 100 ml polycarbonate tube and spun at 8,000 rpm for 10 minutes. The run was stopped without the rotor brake, and the entire contents of the tube passed through a funnel containing a siliconised glass wool plug. Isopropanol, 50 ml, was added to precipitate the DNA, which was subsequently pelleted at 8,000 rpm for 10 minutes. The pellet was covered with 3 mls of ethanol, left 5 minutes, then removed and the pellet layered with ether for a further 5 minutes. The pellet was dried using a nitrogen stream for 15 minutes, and resuspended in 4 ml of TE buffer. Solid CsCl, 1.12 times the weight of the DNA solution was added together with 50 µl of ethidium bromide, 10 mg/ml: the solution was spun in a 10 x 10 titanium rotor head for 48 hours at 40,000 rpm and 20°C, in an MSE Prespin or Superspeed. When viewed under short wave ultraviolet light the spun tube normally showed only one band, which was the plasmid DNA; any chromosomal DNA banded higher. The plasmid band was removed and extracted with an equal volume of CsCl saturated isobutanol to remove the ethidium bromide. Dialysis was carried out against 4 changes of 2 litres TE buffer, using dialysis tubing pre-boiled in 10 mM EDTA.

Preparation of λ Phage DNA for HindIII markers.

A single plaque of bacteriophage λ CI⁻ was picked, suspended in 500 μ l of phage buffer and 100 μ l of this mixed with 100 μ l of plating cells (prepared from an overnight culture of HB101 resuspended in 10 ml of 10 mM MgSO₄). The phage were allowed to absorb at room temperature for 15 minutes, then diluted into 250 ml L-broth at 37°C, and aerated overnight at 37°C. When the culture had lysed (indicated by debris in the culture), 500 μ l of chloroform was added and the cell debris pelleted by centrifugation at 8,000 rpm for 10 minutes. The phage were precipitated by the addition of 4 g NaCl (4°C, 1 hour), and 20 g PEG 6000 (4°C, overnight). The phage were then pelleted at 4,000 rpm for 5 minutes, resuspended in 1 ml phage buffer and purified on a CsCl step gradient (Maniatis ~~et al~~ 1982). The phage band was removed with a hypodermic needle and syringe and dialysed for 2 hours in 2 l TE buffer. Any protein was digested with 1 mg/ml pronase at 37°C for 1 hour, and residual protein removed by phenol-extraction, ether-extraction and ethanol precipitation. The DNA was resuspended in TE buffer to a concentration of 200 μ g/ml, then digested by HindIII and stored at -20°C. When required, 0.2-0.5 μ g of DNA was loaded as a marker.

Preparation of M13mp19 DNA.

A 10 ml quantity of host cells (usually JM101) were grown to

logarithmic phase (OD_{600} 0.6), and infected with the appropriate phage. The infected cells were grown overnight to ensure a good titre of phage in the supernatant, which was used to infect 250 ml logarithmic phase cells the next day. The bulk infected cells were grown for 1-2 hours, when chloramphenicol was added to a final concentration of 200 μ g/ml. Amplification was allowed to continue for a further 2 hours. The cells were then spun down, and the pellet resuspended in 2 ml of a solution containing 15% (w/v) sucrose, 50 mM Tris-HCl, pH 8.0 50 mM EDTA, and treated with 250 μ l of 10 mg/ml lysozyme. The mixture was left on ice for 5 minutes and the cells lysed by the addition of 2 μ l of 2% Triton-X 100. After 10 minutes at 0 $^{\circ}$ C the lysate was cleared by centrifuging at 17,000 rpm for 45 minutes. To every 1 ml of cleared lysate, 1.22 g of CsCl were added and dissolved. Another centrifugation of 8,000 rpm for 10 minutes removed most of the protein which appeared as a surface leaf that could be removed with a spatula. For every 1 ml of liquid, 15 μ l of 10 mg/ml ethidium bromide and 170 μ l of H₂O was added. The solution was added to 5 ml self-seal Beckman tubes and centrifuged overnight in a Beckman Ti-50 rotor. Two bands were visible; the lower circular supercoiled plasmid DNA was harvested using a hypodermic syringe and needle. Ethidium bromide was removed by 2 to 3 extractions with CsCl-saturated isopropanol, and the CsCl removed by dialysis with 4 successive changes of TE. The DNA solution was further cleaned by phenol extraction followed by ether extraction and ethanol precipitation. The subsequent DNA was resuspended in 1 ml TE.

Minipreparation of Plasmid DNA

Two main methods were used to isolate plasmid DNA from small (up to 10 ml) cell cultures; both methods yielded 2-3 µg plasmid DNA. The boiling method (based on Holmes and Quigley) was used on M. methylophilus cultures while the Alkaline Lysis Method (based on Birnboim and Doly) was primarily used on E. coli strains.

(i) Boiling Method. (Holmes and Quigley 1981)

10 ml of growth medium containing an appropriate antibiotic was inoculated with a single colony, incubated overnight at 37 °C with vigorous agitation, and 1.5 ml of the grown culture was transferred to an Eppendorf tube, centrifuged for 30 seconds and the supernatant removed. The cell pellet was resuspended in 350 µl of;

8% Sucrose

0.5% Triton-X 100

50 mM EDTA, pH 8.0

10 mM Tris-HCl, pH 8.0

and lysed by the addition of 25 µl, 10 mg/ml lysozyme. The tube was placed in boiling water for 40 seconds to precipitate protein and chromosomal DNA, and then spun immediately for 10 minutes at full speed in a microfuge. The resultant chromosomal and protein pellet was removed with a toothpick. 40 µl of 2.5 M sodium acetate, and 800 µl of ethanol was added to the supernatant and the mixture stored at -70° C for 10

minutes. The DNA pellet was resuspended in 200 μ l of TE, and RNA removed by the addition of an equal volume of 5 M LiCl, leaving at 0°C for 5 minutes, then microfuging for 10 minutes. The supernatant was removed and re-precipitated with ethanol, and the DNA pellet then suspended in 20 μ l TE. The DNA was either digested with the appropriate restriction endonuclease or stored at -20°C.

(ii) Alkaline Lysis Method. (Modification of Birnboim and Doly 1979)

From an overnight inoculum 1.5 ml of the culture was microfuged for 30 seconds in an Eppendorf Centrifuge, and the supernatant removed by leaving the pellet as dry as possible. The pellet was resuspended in 100 μ l ice-cold solution of;

- 50 mM Glucose
- 10 mM EDTA
- 25 mM Tris Cl, pH 8.0

and, after 5 minutes, 200 μ l of 0.2 M NaOH, 1% SDS was added and the contents mixed by inverting the tube gently several times. Once clear, the lysate was stored at 0°C for five minutes, then 150 μ l of potassium acetate added (pH 4.8, 3 M with respect to potassium, 5 M with respect to acetate), the tube vortexed and stored on ice for five minutes. The contents were subsequently centrifuged in an Eppendorf Centrifuge at 4°C and ethanol precipitated. An equal volume of LiCl was added to the DNA (resuspended in 200 μ l TE) and left on ice for five minutes. The contents were centrifuged in an Eppendorf Centrifuge at full speed for five minutes, and the

supernatant then precipitated with two times the volume of ethanol. The RNA-free DNA pellet was finally resuspended in 20 μ l of TE.

Isolation of 10 kb Fragments via a Salt Gradient.

To isolate restriction fragments of approximately 10 kb long for cloning, it is necessary to partially digest the DNA with the restriction enzyme of choice, and then to separate the fragments on some kind of gradient. In this case a NaCl gradient was used.

The salt solutions required are 1.25 M NaCl and 5 M NaCl made up as follows;

<u>1.25 M</u>	<u>5 M</u>
1.25 M NaCl	5 M NaCl
10 mM Tris-HCl pH 7.5	10 mM Tris-HCl pH 7.5
1 mM EDTA	1 mM EDTA

8.5 ml of each solution was placed in the two wells of a gradient maker, and the peristaltic pump allowed to slowly draw off the combined liquid gradient.

After formation of the gradient, 90 μ l of restricted DNA was layered above the gradient, and spun at 16,000 rpm for 2 hours at 20°C. After centrifugation, a fraction collector was used to distribute fractions of the gradient into Eppendorf tubes. These DNA fractions were ethanol precipitated and resuspended in 40 μ l TE buffer. Two μ l of each third sample was run on a 0.7% agarose gel (Fig. 5.2), and the fractions 5-8 pooled to collect the 5-15 kb restriction fragments. These were ethanol

precipitated and resuspended in TE.

Preparation of Competent E. coli Cells

To facilitate the entry of plasmid DNA into E. coli, the cells were treated chemically with $MgCl_2$ and $CaCl_2$ to induce competence, 500 μ l of an overnight inoculum was sub-cultured into 25 ml of L-broth and growth continued at 37°C until the OD_{600} was 0.60. The cells were then harvested by centrifugation at 5,000 rpm for 10 minutes and resuspended in 20 ml of 0.1 M, $MgCl_2$ and left on ice for 10 minutes. The cells were reharvested by centrifugation, and suspended in 10 ml $CaCl_2, 0.1M$, 0°C. After 20 minutes on ice, the cells were recovered by centrifugation at 5,000 rpm for 6 minutes, resuspended in 1 ml $CaCl_2$ and stored at -20°C or used immediately.

As trace amounts of detergent present on the surfaces of laboratory glassware were found to cause drastic reductions in the efficiency of transformation, sterile plastic-ware was used whenever possible.

Transformation of Competent Cells.

The DNA to be used in the transformation (less than 1 μ g) was diluted to 50 μ l total volume by TE. This was added to 200 μ l of competent cells. The DNA-cell mixture was left at 0°C for one hour, given a 2-minute heat-shock at 42°C to facilitate DNA uptake, then returned to the ice. After 10

minutes 1 ml of L-broth was added and the cells incubated at 37°C for 30 minutes. The mixture was centrifuged at 5,000 rpm for 10 minutes, the cells were resuspended in 100 µl of L-broth and plated on to an L-agar plate containing a suitable antibiotic selection.

For all transformations, positive and negative controls were included; the positive controls containing 100 ng pBR322.

Transformation of JM101 with Recombinant M13 DNA.

Competent cells were prepared as above and the transformation carried out in the same manner. After the heat-shock 300 µl of exponentially growing cells were added to the transformed cells followed by 3 ml of K-top, 25 µl 100 mM IPTG and 125 µl 2% X-Gal (in DMF). The mixture was poured on to a minimal plate containing glucose and thiamine, and incubated overnight at 37°C.

Purification of DNA using G50 Sephadex.

In some of the processes used in this thesis it was necessary to obtain pure DNA, free from any contaminating salts or phosphates. To ensure complete removal of small inorganic molecules a mini-G₅₀Sephadex column was applied. The column was constructed for this purpose in a large blue Gilson tip, blocked at the tip with siliconised glass wool and supported in a 7ml Sterilin tube containing a small Eppendorf tube to

collect the sample. 400 ul of G₅₀ Sephadex suspension solution was centrifuged through the tip at 5,000 rpm for 10 minutes; this gave a column of 1.3 to 1.5 cm in height through which the DNA sample (in 100 ul TE) was passed via centrifugation as above.

Agarose Gel Electrophoresis.

DNA samples were analysed by electrophoresis in agarose gels containing ethidium bromide. The concentration of the gel depended on the size of fragments to be analysed; smaller fragments (0.5-4 kb) required 0.8-1.0% gel, while larger fragments and undigested DNA were usually run on a lower percentage gel. Very large fragments (>50 kb) required a 0.2% gel, which was layered on top of a 1% gel bed for support.

The samples to be analysed in each case were mixed with 1/3 volume of agarose loading sample buffer, and the samples run into the wells at 100 V, then turned down to the appropriate voltage (this could vary from 30 V overnight to 100 V for 2 hours). In addition to the regular 13x25 cm gel beds, smaller, 8x8 cm beds were used for quick analysis. These required a smaller voltage, and were usually run at 70-80 V for 1-2 hours..

After the gel had run, it was photographed or viewed using a short-wave ultraviolet transilluminator and a Polaroid camera.

Restriction enzyme digests

One unit of restriction enzyme will digest 1ug of DNA in one hour at 37°C, in the stated restriction buffers. Any deviations to this rule are stated in the work where necessary.

Preparative Agarose Gel Electrophoresis.

This was carried out in essentially the same way as analytical agarose gel electrophoresis, with the exception that low-melting point agarose (Bethesda Research Laboratories Inc.) was used. The gels were never of a lower concentration than 0.8%, and all gels were run at 4°C. The gel was viewed under weak short-wave ultraviolet light and the appropriate bands cut out with a scalpel. The agarose/DNA slices were added to an equal volume of TE buffer and placed at 70°C for 10 minutes. An equal volume of phenol was added, also at 70°C, and the mixture lightly shaken at 37°C for 10 minutes. The sample was then spun in a microfuge at room temperature for 10 minutes, the aqueous layer removed, phenol-extracted again, ether-extracted, and ethanol precipitated. The DNA was resuspended in 10 µl TE.

Southern Blot (Southern 1980)

DNA samples (1-5µg) were treated with the appropriate restriction enzyme before being run on a 0.7% agarose gel, 30 V overnight with EtBr. The gel was subsequently photographed under ultraviolet and the DNA denatured by treating the gel for 2 hours in 3 M NaCl, 1 M NaOH, 2 mM EDTA, with one change of solution after one hour. The gel was subsequently neutralised by washing for 2 hours in several changes of 3 M NaCl, 0.5 M Tris-HCl, pH 7.0; washing was continued until the pH of the gel had dropped to 7.0. The gel was finally washed

in 20xSSC for 20 minutes and placed on the transfer apparatus. A sheet of nitrocellulose paper was cut to the same size as the gel and soaked in 3x SSC. The nitrocellulose paper was placed on top of the gel and covered by 3 sheets of Whatman 3MM filter paper (also soaked in 3xSSC). Several sheets of dry 3MM paper were placed on top, followed by 6 inches of absorbant tissues and a 1 kg weight. The transfer was carried out at 4⁰C overnight, using 20x SSC as a transfer buffer. The DNA could be identified on the filter by staining with ethidium bromide and the filter cut into different probe regions if necessary. The filters were washed in 3xSSC for 15 minutes, and were baked at 80⁰C for 2 hours: they were then ready for hybridisation.

Analysis of DNA by Spot Hubridisations.

Spot hybridisations on filters provide a rapid and sensitive method for estimating the nucleotide sequence homologies between different DNA's. 1 mg/ml solutions of DNA's were required, 5 μ l of which were vacuum dried and redissolved in 5 μ l of 1 M NaCl, 0.1 M NaOH, 10 mM EDTA. The samples were heated at 100⁰ C for five minutes, then spotted onto a nitrocellulose filter. After leaving the filters at 21⁰ C for 30 minutes they were rinsed in 3xSSC for 4 hours.

Radioactive Labelling of DNA by Nick-translation

The nick-translations were carried out in a standard reaction mix, prepared as follows;

DNA (20 ug/ml)	5 μ l
10x Nick Mix	2.5 μ l
dATP, 50 μ M	2 μ l
dGTP, 50 μ M	2 μ l
dTTP, 50 μ M	2 μ l
H ₂ O	8.5 μ l
DNA'seI 8 ng/ml	1 μ l
<u>E.coli</u> DNA polymerase I	1.5 μ l (4 units)
³² P dCTP 10 μ C/ μ l	2 μ l (20 $^{\circ}$ C)

and incubated at 15 $^{\circ}$ C for one hour. The DNase was prepared immediately before use from a stock solution of 0.1 mg/ml. The reaction was stopped by the addition of 25 μ l of quench mix and the DNA recovered by phenol-extraction followed by ethanol precipitation using 100 μ g of denatured salmon sperm DNA as a carrier. After rinsing the pellet with 70% ethanol, a second ethanol precipitation was employed to ensure all unincorporated deoxy nucleoside triphosphates were removed. The DNA was finally resuspended in 500 μ l, 10 mM Tris-HCl, pH 7.5.

Hybridisation of DNA.

All solutions were de-gassed before use. Nitrocellulose filters to be hybridised were washed in 3xSSC at 65°C for 30 minutes, then in Denhardt's solution for 2-3 hours; (0.2% BSA, 0.2% Ficoll 400, in 3xSSC.). This was followed by 50-60 minutes in DDS (0.1% SDS, 50 µg/ml salmon sperm in 1x Denhardt's). Finally an overnight incubation of DDS plus probe completed the hybridisation. The filters were subsequently washed 5-6 times in DDS without probe, at 65°C for 10 minutes each wash; twice in 0.2xSSC, 50 µg/ml salmon sperm DNA, 1% SDS at 65°C for 10 minutes; and then in 3xSSC at room temperature for 2-3 minutes. The filters were then dried and autoradiographed overnight on Fugii X-ray film at -70°C.

Preparation of Template DNA from M13 Recombinants.

Template DNA's were prepared using fresh overnight cultures of JM101 and either a fresh plaque or a plaque resuspended in phage buffer and kept at -20°C. 50 µl of JM101 was added to 25 ml of L-broth, which was subsequently divided into smaller 1 ml aliquots. The plaques were picked using the small end of a disposable 1 ml glass pipette, and placed in the L-broth. The cultures were incubated, shaking, overnight, then spun in a microfuge for 3 minutes to pellet the cells. An 800 µl quantity of the supernatant was transferred into a fresh Eppendorf and 200 µl, 2.5 M NaCl, 10% PEG 600 added. The phage

were allowed to precipitate at room temperature for 30 minutes, then harvested by centrifuging for 5 minutes. All the supernatant was carefully removed. The pellet was resuspended in 100 μ l of 1.1 M NaAc, pH 7.0, with 50 μ l of Iris-saturated phenol and 50 μ l CHCl_3 /isoamylalcohol (50:1). After vortexing for 10 seconds, the mixture was stood at room temperature for 5 minutes, then microfuged for one minute. The aqueous layer was removed to a fresh tube and 60 μ l CHCl_3 : isoamylalcohol (50:1) added. The mixture was re-spun and the aqueous layer again removed to a fresh tube. 250 μ l of ethanol was added to precipitate the DNA, and left at -70°C for 10 minutes. After centrifugation for 10 minutes, the pellets were rinsed in 70% ethanol, resuspended in 20 μ l TE and stored at -20°C .

Construction of Systematic Deletion Sub-Clones by DNase I Treatment.

(1) DNase treatment and first digestion.

DNase I was prepared to a stock concentration of 10 mg/ml in sterile water. DNase I buffer, 10x concentration (0.2 M Tris-HCl, pH 7.5, 10 mM MnCl_2 , 1 mg/ml BSA; Hong 1982) was used within one hour of preparation. The replicative form (RF; double stranded) of M13 DNA, 15 μ g, was suspended in 140 μ l 1x DNase I buffer and aliquoted into five Eppendorf tubes. The DNase I was serially diluted to 0.5 ng/ μ l, and added to each tube. Digestion was allowed to continue for 1, 2, 3, 4, and 5 minutes incubated at room temperature. The reaction was stopped by the addition of an equal volume of Tris-HCl

saturated phenol, pH 7.6. After vortexing, the aqueous layer was removed and extracted twice with an equal volume of chloroform. 5 μ l of each digestion was removed and run on a 0.5% agarose gel to determine the optimal digestion products. In this case, samples 3, 4, and 5 were combined and ethanol precipitated (figure 6.5). The subsequent DNA pellet was resuspended in 200 μ l EcoRI buffer, and digested with EcoRI for two hours at 37°C, after which the reaction was stopped by heating at 68°C for 10 minutes. 200 μ l of PEG solution (13% polyethylene glycol 8000, 1.6 M NaCl) was added to the solution and the mixture placed on ice for 1.5 hours. After centrifuging in an Eppendorf microfuge at 4°C for 10 minutes, the DNA was resuspended in 100 μ l TE buffer, extracted with phenol, phenol plus chloroform (50:50), chloroform, and finally precipitated with ethanol.

(2) Klenow repair and second digestion

The DNA pellet was resuspended in 20 μ l H-buffer incorporating 5 units of Klenow fragment (DNA polymerase I, large fragment) and incubated at room temperature for 15 minutes, after which the reaction was stopped by ethanol precipitation. After resuspension in 20 μ l T-4 ligase buffer containing 10 units of T-4 ligase and 15% PEG, the DNA was ligated overnight at 4°C. The reaction was terminated by heating at 68°C for 15 minutes. A second restriction digest, to reduce the background original clones, was carried out by a second SmaI digestion, leaving 10 μ l of the sample undigested as a control. 5 μ l of the subsequent mixture was then used to transform 200 μ l of

competent JM101 E. coli cells, and plated as described above.

H-buffer

Iris 50 mM, pH 7.5
 MgSO₄ 100 mM
 dithiothreitol 1mM

DNA Sequencing.

Mixes were made from stock solutions previously described;

	<u>I-mix</u>	<u>C-mix</u>	<u>G-mix</u>	<u>A-mix</u>
0.5 mM dTTP	25	500	500	500
0.5 mM dCTP	500	25	500	500
0.5 mM dGTP	500	500	25	500
10 mM ddTTP	50			
10 mM ddCTP		8		
10 mM ddGTP			16	
10 mM ddATP				1
TE	1000	1000	1000	500

Primer Mix:

Universal Primer	2.5 ng/μl	1.0 μl
TM		1.0 μl
H ₂ O		3.0 μl

I screens

Template	1.25 μl
Primer Mix	1.25 μl

Anneal Mix

Template	5.0 μl
Primer Mix	5.0 μl

<u>Klenow/35-S dATP Mix</u>	35-S dATP (Amersham) 410 Curies/mMol	0.8 μ l (10mCi/ml)
	Klenow large fragment pol.	
	(5.2 u/ μ l)	0.73 μ l
	H ₂ O	7.6 μ l

The Primer Mix and template were annealed by placing in a 65°C water bath for 30 minutes, then allowed to return to room temperature slowly. To 2 μ l of the Primer/template mix, 2 μ l of the appropriate nucleotide mix was added together with 2 μ l of Klenow/dATP. After 20 minutes at room temperature 2 μ l of the 4dNTP chase mix was added, and the samples incubated for a further 20 minutes at room temperature then stored at -20° C until required.

Acrylamide Gel Preparation.

Gradient gels were prepared using 0.4 mm spacers. The glass plates were cleaned using first, detergent, then ethanol, followed by acetone. The smaller glass plate was always siliconised before use. The following solutions were made up, then filtered and de-gassed before use;

40% Acrylamide solution

38 g Acrylamide

2 g Bis-acrylamide

made up to 100 ml with H₂O, and stirred with amberlite for 10 minutes to de-ionise the solution.

0.5x TBE Acrylamide Urea Mixture 6%

4.3 g Urea

5 ml x10 TBE

15 ml 40% Acrylamide Solution

made up to 100 ml and stored in the dark at 4°C.

2.5x TBE Acrylamide Urea Mixture 6%

43 g Urea

25 ml x10 TBE

15 ml 40% Acrylamide Solution

5 g Sucrose

50 mg Bromophenol Blue

made up to 100 ml and stored in the dark at 4°C.

The glass plates to be used (clean and free from all grease) were assembled using 0.4 mm spacers. To 15 ml of the 2.5XTBE acrylamide solution 90 µl of 10% ammonium persulphate was added ; similarly a mixture was made from 60 ml of 0.5xTBE acrylamide solution with 360 µl of ammonium persulphate. A 15 µl quantity of TEMed was added to the 0.5x mix and 5 µl TEMED to the 2.5x mix. After mixing, 8 ml of the 0.5x mix was taken up in a 20ml pipette, followed by 12 ml of the 2.5x mix in the same pipette. Four air bubbles were introduced to form a rough gradient, and the mixture carefully poured down the side of the glass plates. The rest of the gel was poured using the 0.5x TBE acrylamide mix, ensuring that the gradient part of the gel was straight. The straight edge of a sharkstooth comb was clamped in position at the top of the gel, which was allowed to polymerise for at least one hour. Gels were pre-run

for at least 15 minutes prior to use to warm them.

2.6 RNA-Related Techniques.

Isolation of RNA from *M. methylophilus*.

50 ml of *M. methylophilus* cells grown overnight in methanol minimal media were diluted into four 2 litre baffled flasks, each containing 200 ml of minimal media supplemented with trimethylamine 0.2%, and methanol 0.01%, so that the OD_{600} was 0.1. The cultures were aerated at 37°C in a rotary shaker. At timed intervals, 100 ml of the media was removed and the cells spun down at 4°C. The cells were then resuspended in minimal media to an OD_{600} of 0.6. A 25 ml aliquot of the resuspended cells was rapidly diluted into 25 ml minimal media with 20 mM sodium azide at 4°C. The cells were swirled gently at 0°C then spun in a bench centrifuge at 4°C, 8,000 rpm for 10 minutes. The cells were resuspended in 0.4 ml, 20 mM sodium acetate, 1 mM EDTA, pH 5.6, at 0°C, and added to 0.8 ml of boiling 20 mM sodium acetate, 1 mM EDTA, 3% SDS in a large Eppendorf. When the lysate had become non-viscous (approximately 1 minute) the Eppendorf tube was transferred to a water bath at 60°C; 130 µl of 5 M NaCl (with 1mM DEP) was added to make a 0.5 M NaCl solution and the lysate split into two Eppendorfs. Both were extracted with an equal volume of hot phenol, and then with an equal volume of phenol

at room temperature. Two chloroform extractions removed any trace phenol, and an equal volume of iso-propanol precipitated the DNA and RNA. After 15 minutes at -70°C the samples were thawed and spun in an Eppendorf microfuge for 10 minutes. The RNA/DNA pellet was dried in a vacuum dessicator and resuspended in RNase free DNase-I buffer. Any DNA was subsequently digested to nucleotides by the addition of RNase free DNase-I for 5 minutes at 37°C . A further phenol extraction and ethanol precipitation removed any nucleotides and enzyme from the RNA preparation, which was then resuspended in 5 μl of TE, and stored at -70°C .

Dot-Blot Hybridisation of RNA.

A nitrocellulose filter, pre-soaked in sterile 20x SSC and air-dried, was used to spot on the RNA samples. The RNA's to be used were ethanol precipitated and resuspended using a 25 $\mu\text{g}/\mu\text{l}$ solution of E. coli tRNA in TE. 4 μl of the resuspended RNA, containing a maximum of 1 μg of mRNA (10 μg of total RNA), was spotted onto the filter and air dried. The nitrocellulose paper containing the RNA spots was baked for two hours at 80°C . Before hybridisation to the probe, the nitrocellulose filter needs to be pre-hybridised twice at 42°C for two hours in pre-hybridisation buffer:

50%	formamide
0.75 M	NaCl
75 mM	trisodium citrate

25 mM NaPO₄ pH 6.5
0.2% SDS
0.02% each of BSA, PVP, Ficoll.
1 mg/ml sonicated salmon sperm DNA

The filters were then incubated for a further two hours in pre-hybridisation buffer plus 10% dextran sulphate. The hybridisation was set up in pre-hybridisation buffer plus 10% dextran sulphate and the probe at 42°C overnight.

Six 20 minutes washes were performed after hybridisation to remove the background counts on the filter, with solution below;

50% formamide
0.75 M NaCl
75 mM sodium citrate
25 mM NaPO₄
0.2% SDS

Once the background counts had been reduced to an acceptable level (5-10 cpm) the filter was blotted dry and placed on Fuji X-ray film overnight.

2.7 Bacterial/Protein-related Techniques.

Membrane Mating.

As M. methylophilus has been shown to be refractory to the uptake of exogenous DNA, bacterial mating remains the only method of introducing exogenous DNA into the organism. Donor and recipient cells were grown overnight to a high density, then 500 μ l of each removed and mixed together. The mixture was drawn by vacuum through a Sartorius membrane filter, leaving the bacteria upon the filter. This was placed, bacteria uppermost, on a methanol plate and mating allowed to continue for at least 5 hours. The filter was then removed and its surface contents resuspended in 50 ml of methanol salt medium with an appropriate selection for the donor DNA. The cells were allowed to reach exponential growth, then plated on methanol-minimal salts plates with the appropriate selection for exconjugants.

Enrichment of Mutants in the Trimethylamine Pathway.

(Adapted from Byrom, 1982 pers. comm.)

M. methylophilus was membrane-mated with E. coli DS903 (In5) containing the In5 transposon on the plasmid pLG221, and resuspended in 50 ml minimal salts media supplemented with 0.2% trimethylamine or methylamine and 0.02% methanol. Growth of 4-6 hours was allowed before penicillin G was added to a concentration of 10 μ g/ml, and D-cycloserine to 25 μ g/ml.

After a further 6 hours of growth, the cells were harvested and resuspended in a small volume of H₂O. A further 50 ml of minimal salts media was added and the cells grown overnight in methanol kanamycin (10 µg/ml).

Isolation of Mutants.

Cells from the enrichment procedure were plated out on methanol minimal salt plates containing 10 µg/ml of kanamycin, to give 100-200 colonies per plate. Individual colonies were toothpicked onto both methanol and methylamine supplemented plates to identify mutants unable to utilise methylamine as a growth source.

Trimethylamine mutants were isolated in a similar manner, using trimethylamine instead of methylamine.

Measurement of Enzyme Activity in Whole Cells.

Wild type M. methylophilus cells were grown overnight in methanol-supplemented media, or media supplemented with one of the amines. Mutant cells (tmd 3, mad 1) were grown in a methanol/amine mixture. Two ml of the overnight cultures were transferred into 200 ml of similarly supplemented media and shaken vigorously at 40°C until the OD₆₀₀ was 0.63 (at 0.63, 1 ml of the culture is equal to 1 mg dry weight of cells). A 20 ml quantity of the cells were harvested and resuspended in 1 ml of glycyl-glycine buffer, pH 7.0.

The enzyme activities of the above cultures were measured using an oxygen electrode and the specific activities calculated from a chart readout. The oxygen electrode consisted of a 4 ml chamber kept at 40°C above the membrane electrode, a magnetic stirrer and an adjustable screw lid to keep an O₂-free environment. 3.85 ml of glycyl-glycine buffer at 40°C was placed in the chamber and the air expelled using the screw lid. When the initial chart reading was steady, 100 µl of cells was added to the chamber. When the reading became steady once again 50 µl of the desired substrate was added.

Substrate concentrations were as follows:

Methanol	10 mM
Trimethylamine	3 mM
Dimethylamine	3 mM
Methylamine	3 mM

Measurement of Enzyme Activity in Cell-free Extracts.

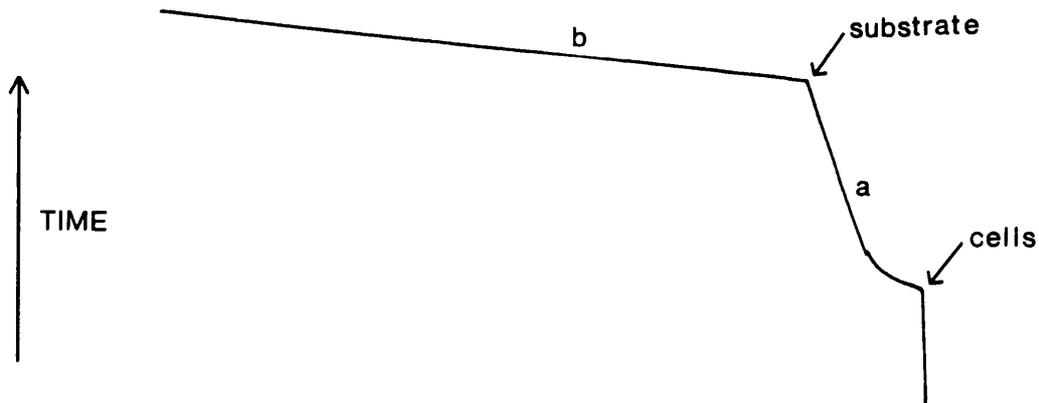
Cell-free extracts of M. methylotrophus, prepared as described below, were used to measure direct enzyme activity in cell environment. Oxygen uptake rates were measured at 40° C using substrate concentrations given above (Jones et al. 1982). Dehydrogenase activities were measured as the rate of reduction of dichlorophenolindophenol (DCPIP) at 40° C, pH 7.5 (trimethylamine and methylamine) or for methanol at pH 8.0, in the presence of phenazine methosulphate (0.43 mM),

ammonium chloride (15 mM), and potassium cyanide (1 mM) (Jones et al. 1982, Anthony and Zatman 1967, Eady et al. 1971).

Dimethylamine monooxygenase was assayed from the increase in the rate of NADH oxidation following the addition of dimethylamine (Eady et al. 1971).

Calculation of Results:

The measurement of enzyme activity is taken as the uptake of Oxygen as indicated by the slope of the chart readout, shown diagrammatically below:



The endogenous respiration of the cells (a), must be taken away from the oxygen uptake when the substrate is applied (b). Thus the true oxygen uptake equals (slope b) - (slope a), i.e. if the volume of the chamber is 4 ml, temperature 40° C, the solubility of oxygen at this temperature is 0.380 g-atom O/ml, and 100 µl of cells is equal to 2 mg, then the rate of oxygen uptake in ng-atoms O/min/mg dry weight;

$$= \frac{(a-b) \times 4.0 \times 0.380}{2.0 \times 90}$$

Preparation of Cell Free Extracts.

M. methylophilus grown to mid-logarithmic phase were harvested at 8,000 rpm, 15 minutes at 20°C. The pellets were washed twice in 0°C, 10 mM sodium phosphate buffer, pH 7.5. At this stage, the pellets would be stored at -20 °C until required. 1 ml of washed cell suspension (containing 150 mg wet weight of cells per ml) in 100 mM sodium phosphate buffer was sonicated at 20 kHz for 5 minutes in one minute bursts followed by a one minute cooling period at 0 °C. Whole cells and debris were removed by centrifuging the crude extract in an Eppendorf centrifuge for 1 minute. The extract was usually used immediately, but could be stored at 0 °C for up to 12 hours.

Preparation of Cytoplasmic and Periplasmic Fractions.

M. methylophilus cells were grown to an OD_{600} of 0.60. After harvesting by centrifugation at 8,000 rpm for 15 minutes, the cells were resuspended in 20 mM Tris-HCl at pH 7.5, 0.75 M Mannitol to a cell suspension of 5.0 mg/ml dry weight of cells. 100 μ l of a 10 mg/ml solution of lysozyme was added to the cells and the mixture gently agitated at 30 °C for 12 minutes. Ten 0.1 μ l aliquots of EDTA (100 mM) were then added at one minute intervals and the mixture incubated 5 minutes prior to the addition of 0.5 ml $MgCl_2$ (1 M), and 0.1 ml RNase/DNase (10 mg/ml each). After a further two minutes incubation, the mixture was rapidly cooled on ice and then

centrifuged at 15000xg for 15 minutes to yield a clear pink-orange supernatant (periplasm) and a pellet (sphaeroplasts). The sphaeroplasts were lysed by the addition of 0.5 ml of 20 mM Tris-HCl pH 7.5.

SDS-Polyacrylamide Gel Electrophoresis.

Acrylamide gels for the analysis of proteins were usually 12.5% and 2 mm thick. The recipe for a 12.5% SDS polyacrylamide gel is shown below;

	<u>Plug(ml)</u>	<u>Main gel(ml)</u>	<u>Stacker(ml)</u>
30:0.8 Acrylamide:Bis	12.50	18.75	1.25
3M Tris-HCl pH 8.8	3.75	5.62	1.25(1M Tris pH 6.8)
10% SDS	0.30	0.45	100 μ l
H ₂ O	13.28	19.92	6.84
TEMED	15 μ l	25 μ l	10 μ l
10% Ammonium Persulphate	150 μ l	250 μ l	70 μ l

The gel solution was de-gassed before the addition of the APS and TEMED. The acrylamide plug was poured into the gel apparatus first, allowed to set for 20 minutes and any excess water removed before the main gel mix was applied. To ensure an even surface to the main gel, a layer of water was carefully applied to the top of the unset gel mix. This was discarded before the stacker mix and gel comb were applied (1/2-1 hour after the main gel mix). The gel was run in

a Tris-glycine buffer for four hours at 120 volts or overnight at 30 volts.

RF Values and Calibration Curves.

The relative mobility (RF) of separate protein bands may be calculated by constructing a calibration curve for the calibration kit proteins. The Rf is defined as;

Distance the protein has migrated from origin

Distance from the origin to reference point.

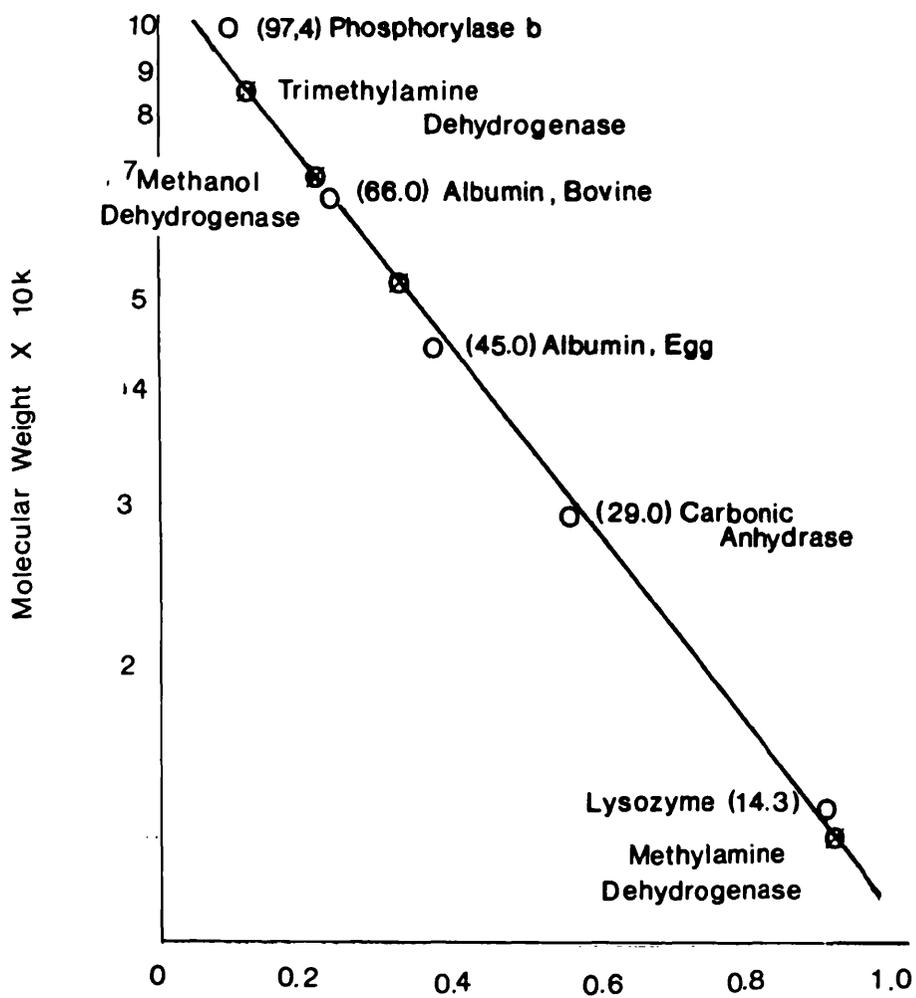
The dye-front was used as the reference point. The calibration curve (Fig. 2.1) was plotted using the Rf values of the calibration kit proteins versus the logarithms of their corresponding molecular weights. The molecular weights of the major protein bands from whole cell extracts were determined by calculating their Rf values and relating this to the molecular weight value on the calibration curve.

Coomassie Blue Staining of Proteins

Protein bands on acrylamide gels were detected by staining for one hour in Coomassie blue stain (page 13), and then overnight in destain. Gels were subsequently photographed wet or dried down to produce a record of the gel.

Figure 2.1 Calibration Curve for Polyacrylamide Gel Electrophoresis of Proteins.

The relative mobility of protein bands may be calculated from such a calibration curve. The curve was plotted using the Rf values of the calibration kit proteins versus the logarithms of their corresponding molecular weights. The molecular weights of protein bands of interest could then be calculated by using their Rf values. The relative positions of trimethylamine dehydrogenase and methanol dehydrogenase are shown. The molecular weights of the calibration proteins are shown in brackets, in K. daltons.



CHAPTER 3

Growth of Methulophilus methulotrophus on Methanol and the
Methylated Amines.

M. methulotrophus expresses or synthesises all the appropriate enzymes it requires for the metabolism of methanol and the methylated amines to formaldehyde. The enzyme methanol dehydrogenase converts the substrate methanol to formaldehyde; trimethylamine dehydrogenase converts trimethylamine to dimethylamine, the conversion of dimethylamine to methylamine is mediated by dimethylamine monooxygenase and the conversion of methylamine to formaldehyde is carried out via methylamine dehydrogenase (Fig. 1.1).

Methanol dehydrogenase is an NAD-independent alcohol dehydrogenase, described originally in Pseudomonas M27 (Anthony and Zatman, 1964 a and b). Although its main function is to catalyse methanol oxidation, typical methanol dehydrogenases oxidise a wide range of primary alcohols using phenazine methosulphate as an artificial electron acceptor and ammonia or methylamine as an activator. The enzyme usually consists of two identical subunits of approximately 60,000 daltons each. In M. methulotrophus the subunits are each 62,000 daltons (Ghosh and Quayle 1981). A comparison of specific activities of crude extracts and of pure enzyme shows that the methanol dehydrogenase usually constitutes between 5% and 15% of the

total soluble protein in the cell, after cell breakage, although it is actually bound to the cellular membrane in which the components of the electron transport chain are situated.

Trimethylamine dehydrogenase, first described in organism 4B6 by Colby and Zatman (1973), occurs in obligate methylotrophs and the more restricted facultative methylotrophs. It catalyses the anaerobic oxidative demethylation of trimethylamine, so producing dimethylamine and formaldehyde. Trimethylamine is its best substrate, with phenazine methosulphate as an artificial electron acceptor. The molecular weight of the enzyme is approximately 150,000 daltons in M.methylotrophus and organism W3A1, consisting of two non-identical sub-units of approximately 70,000 and 80,000 daltons (Steenkamp et al. 1978). Early experiments with W3A1 suggested that it might have two prosthetic groups; one of these has now been identified as an FeS core unit, probably present as a ferredoxin-type, cysteine-ligated cluster ($\text{Fe}_4\text{S}_4(\text{S-Cysteine})_4$), (Hill et al. 1977). Each molecule of enzyme contains one of these iron-sulphur centres. The second prosthetic group is 6-S-cysteinyl-FMN, the cysteinyl residue being part of the polypeptide chain in the complete dehydrogenase. The enzyme can be found on the cytoplasmic side of the membrane, donating its reducing equivalents to the respiratory chain at the level of ubiquinone/cytochrome b (Burton et al. 1982).

Dimethylamine monooxygenase was first described in Pseudomonas aminovorans (Eady and Large 1971), and has been shown to be the only enzyme oxidising dimethylamine to

methylamine during the aerobic growth of methylotrophs on methylated amines. Dimethylamine is the best substrate, although some other secondary amines are also substrates. The enzyme shows no activity with primary, tertiary, or quaternary amines. A preliminary study of the enzyme, at 10% purity, showed the presence of flavin, acid-labile iron, and non-acid-extractable iron. A further kinetic investigation by Brook and Large (1976), using 100-fold purified enzyme, characterised the most unusual feature of the monooxygenase, that being its extreme sensitivity to carbon monoxide. The enzyme, in some respects, shows a greater similarity to haemoglobin than to microsomal monooxygenases. Like trimethylamine dehydrogenase, dimethylamine monooxygenase is found on the cytoplasmic side of the membrane interacting with the electron chain in a similar manner to trimethylamine dehydrogenase.

Methylamine dehydrogenase catalyses the oxidation of methylamine to formaldehyde and ammonia. The enzyme was first described in Pseudomonas AM1, and investigated further by Eady and Large (1968, 1971) and by Matsumoto et al., 1980. The methylamine dehydrogenases from several different obligate methylotrophs have been isolated, and appear to be similar to each other. Methylamine dehydrogenase constitutes 3-5% of the soluble protein of methylamine-grown cells, its substrate specificity depending upon the source of the enzyme. Dehydrogenases from obligate methylotrophs oxidise a very limited range of primary amines, with methylamine as the best substrate, while the enzyme from Pseudomonas AM1 oxidises a wide range of primary aliphatic amines and diamines at rates

as high, and sometimes higher than, that observed with methylamine. The best primary electron acceptor is phenazine methosulphate, (PMS). The prosthetic group, which donates electrons to PMS, is almost certainly the same as that of methanol dehydrogenase, pyrolo-quinoline quinone, and similarly to methanol dehydrogenase, may also be found on the periplasmic side of the cytoplasmic membrane, probably interacting with cytochrome c.

3.1 Growth Curves and Growth Rates.

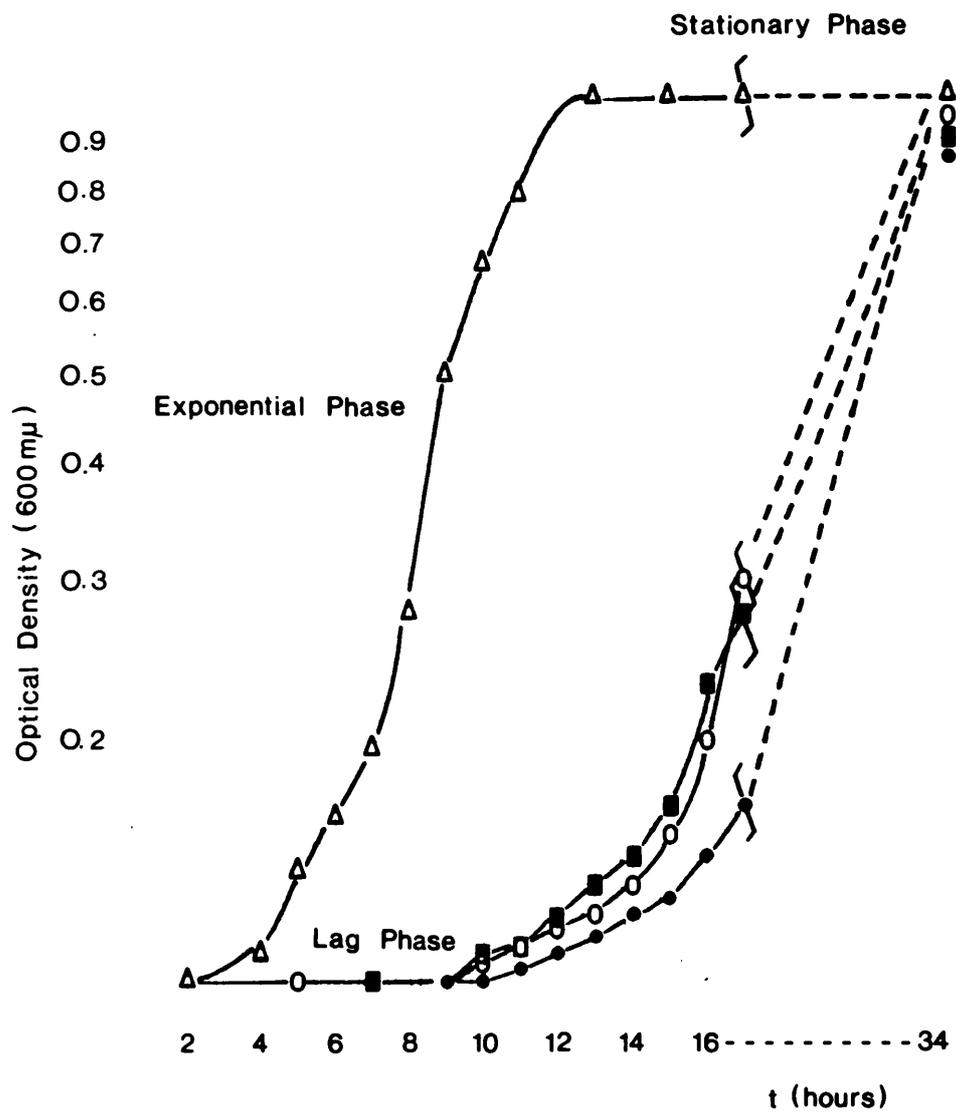
The response given by M. methylotrophus to the introduction of methanol or methylated amines to its surrounding environment may be studied by several different strategies. Initially, growth curves based on the increase in the optical density of the cultures are sufficient to distinguish between a greater or lesser capability to grow upon an individual compound; Figure 3.1 shows the comparative growth curves of M. methylotrophus using methanol and the series of methylated amines chosen for the project. Three principal phases may be defined within the growth curve; these are the lag phase, in which the growth rate is zero; the phase of exponential growth (logarithmic phase) in which the growth rate reaches a constant maximal value; and the stationary phase, in which the growth rate falls again to zero, when there is insufficient nutrient to support growth. The lag phase arises primarily from either the use of an inoculum taken from an old culture in which the population is in the stationary or death phase,

Figure 3.1 The Growth of M. methylophilus as measured by the Increase in Optical Density (OD_{600}).

The cells were initially sub-cultured from a methanol minimal salts medium.

- △ Growth on methanol.
- Growth on trimethylamine.
- Growth on dimethylamine.
- Growth on methylamine.

Original density for all cultures was $OD_{600} 0.02$.



or by transfer to a chemically different medium. The lag observed in the methanol culture is a reflection of the fact that, after the cessation of growth, cells often undergo changes in their chemical composition, and in particular a decline in the cellular population of ribosomes which must be replaced before a high rate of protein synthesis can occur. A similar lag period would be observed for the methylated amines, after subculture from a chemically similar medium. The lag period observed in Figure 3.1 however, is due to the change in media composition; that of methanol to a methylated amine culture. The lag observed in this case is due to the adjustment of the cells to their chemically new medium, that is the synthesising of the new components required to utilise the new medium (Section 3.3). The exponential phase of growth begins when the growth rate of the culture has reached a constant value. During this phase the cells are nearly all of constant size, and are all viable, thus cell mass and cell number increase in parallel. The comparative differences between the exponential phases of growth on methanol and the methylated amines are characterised by the differences in their growth rate constants, (k). As the growth rate varies during the different phases of growth, k is specifically defined as the 'exponential growth rate constant'; the number of doublings per unit time, usually expressed as the number of doublings per hour. The number of doublings per hour of such a growing population may be calculated by subtracting the logarithm (base 2) of the initial population (N_0) from the logarithm (base 2) of the final population (N_t), and dividing this figure by the time, t , elapsed in hours;

$$k = \frac{\log N_t - \log N_0}{t}$$

In addition it is often convenient to express the growth rate in terms of the time required for the population to double, or the 'mean doubling time'. This is the reciprocal of the exponential growth rate, $1/k$, (Table 3.1). In this manner we find that growth upon methanol, with a mean doubling time of 1.9 hours is much faster than growth upon trimethylamine, dimethylamine, or methylamine with the respective mean doubling times of 2.8 hours, 4.2 hours, and 2.9 hours. The numerical value for the growth rate constant is influenced by both genetic and environmental factors, although the maximal reproductive potential of any given microorganism in an optimal environment is an inherent property of the species, and will differ widely from species to species. Subject to this genetically determined upper limit, the actual growth rate of a microorganism is determined by its environment. Many factors affect the growth rate, including the nature and concentration of the nutrients, the temperature, pH, and ionic strength of the medium. Thus the growth rates given here are all subject to the experimental conditions specified.

Number Of Cells $\times 10^6$.				
Time/ Hours	Growth Substrate.			
	Methanol	Trimethylamine	Dimethylamine	Methylamine
5	1.20	0.81	0.59	0.93
6	2.31	1.47	1.01	1.59
7	3.60	2.31	1.47	2.27
8	4.93	2.88	2.03	2.80
9	6.01	3.51	2.41	3.53
10	7.26	4.29	2.88	3.27
Growth Rate Constant (k).	0.52	0.36	0.24	0.34
1/k	1.9	2.8	4.2	2.9

Table 3.1 Growth Rate Constants, Taken During the Exponential Phase of Growth (see Appendix 2).

3.2 Polyacrylamide Gel Electrophoresis of M. methylotrophus Cell Extracts.

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) of whole cell extracts was carried out as described in the methods section. After staining the gels were dried down or photographed wet to produce a permanent record of the gels. Figure 3.2 shows whole cell extracts of M.methylotrophus , when grown in a methanol-supplemented medium or an amine-supplemented medium. One set of extracts has been separated into cytoplasmic and periplasmic components to help identify the proteins of the methanol and trimethylamine pathways.

The most prominent band in the periplasmic fraction has an apparent size of 67,000 daltons, and although slightly higher than the documented size (62,000 daltons), this band is likely to be methanol dehydrogenase, which we would expect to be loosely bound to the cell membrane on the periplasmic side (Burton et al., 1982). This is strengthened by noting that the band is present, not only in methanol-grown cultures, but also in amine-grown cultures. When grown upon trimethylamine or a trimethylamine /methanol mixture, the cell extracts contain additional bands that arise from the induction of the enzymes of the amine pathway. Trimethylamine dehydrogenase is the most conspicuous, producing a prominent band above that for methanol dehydrogenase, which is present only when grown upon a methylated amine. Under PAGE the two sub-units of trimethylamine dehydrogenase are usually seen to migrate together at about 75,000 daltons , but here they were found to

Figure 3.2 Periplasmic, Cytoplasmic, and Total Cell Extracts
of M. methylophilus grown on a Methanol or
Trimethylamine supplemented medium.

The relative molecular weights and positions of
trimethylamine dehydrogenase (tmdh), and methanol
dehydrogenase (mdh), are shown.

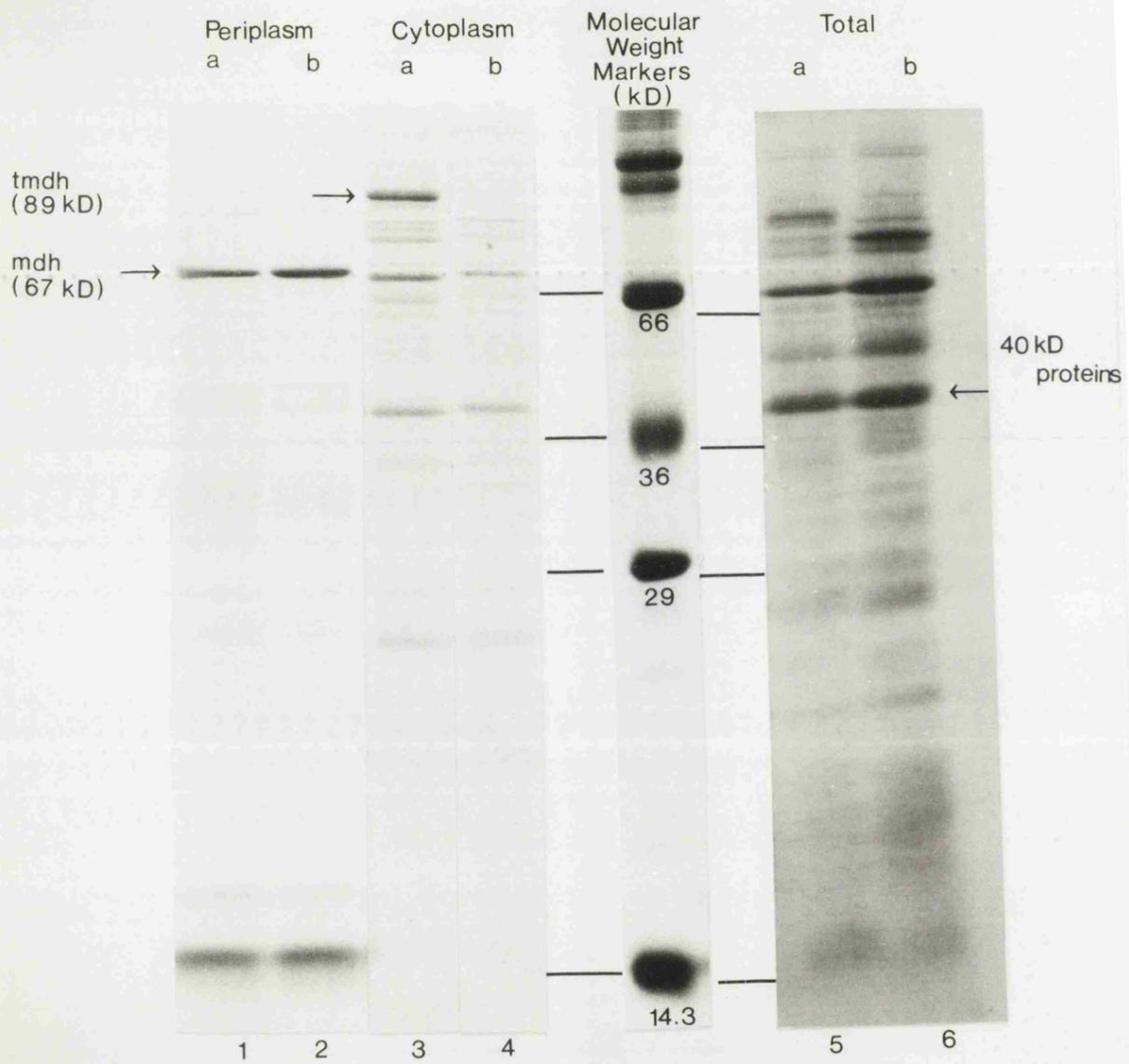
Tracks (a) represent trimethylamine grown cultures

Tracks (b) represent methanol grown cultures.

Tracks 1-2 show the periplasmic compartment.

3-4 show the cytoplasmic compartment.

5-6 show whole cell extracts.



run consistently at a mobility suggesting a size of about 89,000 daltons . Although this is high for the quoted molecular weights, the band is present only in cytoplasmic preparations which would be expected to be induced for trimethylamine dehydrogenase, and in addition all mutants obtained which have lost the ability to utilise trimethylamine have simultaneously lost this 89,000 dalton band (Chapter 4).

There is no current literature relating to the size of dimethylamine monooxygenase, probably due to the instability of the enzyme outside its cellular environment. Thus no bands could be positively linked with the enzyme in the cellular preparations.

There is also some difficulty in identifying the sub-units of methylamine dehydrogenase; the 40,000 daltons sub-units are obscured by the presence of similarly sized cytochromes and the Fp¹ protein (Burton et al. 1982). There is a band at the 13,500 daltons mark which may possibly be the smaller sub-unit. The band seems present only in the amine grown culture, which strengthens the possibility that it is the 13,000 daltons sub-unit of methylamine dehydrogenase.

3.3 Constitutive and Inducible Expression.

It was stated in the introduction that it is reasonable and economic that microorganisms do not synthesise, all the time, all the enzymes they are able to make, but only those that are necessary for their metabolism under the current physiological conditions. The phenomenon that certain enzymes are formed

only in the presence of certain specific substrates is termed induction, while the continual production of an enzyme despite changes of substrate is the constitutive expression of that enzyme. Although the growth curves represented in Figure 3.1 show that a finite time is necessary before the utilisation of the methylated amines may be accomplished after sub-culture of M. methylophilus cells from a methanol medium, little more information can be gleaned. A more sensitive assay, analysing the levels of the enzymes present in the cell during growth on the various substrates is required. Such information can be obtained by measuring the oxygen-uptake of the cells when they are presented with different utilisable substrates (Methods, Section 2.8). By measuring the comparative oxygen-uptake of the cells trimethylamine dehydrogenase and methylamine dehydrogenase can be shown to be inducible enzymes (Table 3.3). while methanol dehydrogenase, present in both methanol and amine cultures, is largely constitutive. In this thesis the induction of dimethylamine monooxygenase was of a much lower level than has been reported in other literature (unpublished results, see appendix 1), and thus further enzyme assays on the monooxygenase are not included in the work.

In the absence of a methylated amine, the levels of the amine dehydrogenases in M. methylophilus are barely detectable; the low activity detectable for trimethylamine dehydrogenase is possibly a basal amount of enzyme required for the induction process in a similar manner to that of the beta-galactosidase gene system in E. coli (see Discussion; Chapter 7). The hundred-fold induction of trimethylamine dehydrogenase seen upon growth on trimethylamine (Table 3.3) might either be caused by activation of an inactive precursor

Grown On.	Substrate.									
	MeOH					IMA				
	1	2	3	4	AM	1	2	3	4	AM
MeOH	1086	988	997	934	1001	4.2	4.0	2.1	2.2	3.1
MeOH/ IMA	995	721	731	682	732	390	308	343	304	336
IMA	812	735	703	712	740	405	415	372	318	377
Grown On.	substrate MMA									
	1	2	3	4	AM					
MeOH	0.0	2.1	0.0	1.4	0.8					
MeOH/ IMA	351	335	315	308	327					
IMA	365	382	342	321	352					

Table 3.2 Enzymic Activities of Whole Cells of M. methylotrophus Grown on Methanol and the Methylated Amines;

The respiration rates are measured as ng atom O/min/mg dry weight. The four columns represent individual preparations of cells (Methods 2.7). AM=Arithmetic Mean.

form of the enzyme already present in the cell, or by de novo synthesis of enzyme molecules. Examination of SDS polyacrylamide gels, however, shows a total absence of the trimethylamine dehydrogenase band at the 89,000 molecular weight mark after growth on methanol, but a strong band appearing when trimethylamine or one of the other amines was used as a substrate (Fig. 3.2). As no larger band (above 90,000 daltons) can be seen to disappear upon the addition of trimethylamine to the culture, it is unlikely that it is the cleavage of larger, precursor molecules that results in activation of the enzyme. It thus seems probable that induction is due to the de novo synthesis of the enzyme from the gene system.

Although it is difficult to detect the sub-units of either methylamine dehydrogenase or dimethylamine monooxygenase upon these SDS-polyacrylamide gels, it is probable that their induction is also due to de novo synthesis of the enzyme molecules.

The induction of the expression of any gene, or gene system, is usually triggered by the substrate for the enzymes, or by molecules which are closely related to the substrates. It may not however be the actual substrate that interacts with the gene system to enable induction to occur; in the induction of the E. coli beta-galactosidase operon the inducer is allolactose, formed when lactose enters the cell by the action of a basal level of beta-galactosidase. In a similar manner the basal level of

trimethylamine dehydrogenase may act upon trimethylamine entering the cell to form the true inducer of the trimethylamine gene. In the case of the trimethylamine pathway however, it seems that there may be several inducers for the system. Enzyme assays and SDS-polyacrylamide gel electrophoresis show that growth upon both methylamine and dimethylamine induces not only methylamine dehydrogenase, but dimethylamine monooxygenase and trimethylamine dehydrogenase as well (Table 3.3, Figure 3.3). Thus methylamine and dimethylamine, or some common product of these, must act as the inducer for both the other enzymes in the pathway. It may be that each substrate does act as an inducer for all three enzymes, either by the enzymes being in a correlated unit on the genome and thus switched on coordinately by the spatially similar substrates, or by separately inducing each gene. In both cases the control circuits for the genes must be flexible enough to cope with the structural differences in the compounds, and in the latter case the basal level of trimethylamine dehydrogenase would form a small amount of dimethylamine from the trimethylamine entering the cell, and similarly a basal level of dimethylamine monooxygenase and then methylamine dehydrogenase would activate the rest of the pathway. Although the production of trimethylamine dehydrogenase and dimethylamine monooxygenase during growth on methylamine is wasteful to the cell, it is less wasteful than, say, the constitutive production of methanol dehydrogenase.

There is a small amount of regulation of methanol dehydrogenase: Table 3.2 shows a slight increase in methanol dehydrogenase activity upon growth on methanol, compared to

Grown on	substrate MeOH					substrate IMA				
	1	2	3	4	AM	1	2	3	4	AM
MeOH	26.2	49.7	42.0	36.4	38.5	0.0	0.0	0.0	0.0	0.0
IMA	18.4	40.6	39.7	29.1	31.9	18.1	20.9	31.4	21.3	22.9
DMA	16.8	44.4	38.6	32.4	33.0	12.6	21.0	26.5	18.1	19.5
MMA	16.3	41.2	39.9	29.7	32.3	17.1	19.8	27.1	22.4	21.6

Grown on	substrate MMA				
	1	2	3	4	AM
MeOH	0.0	0.0	0.0	0.0	0.0
IMA	15.2	28.8	37.4	25.5	26.7
DMA	17.8	32.4	31.1	28.3	27.4
MMA	18.3	31.1	28.6	24.0	25.5

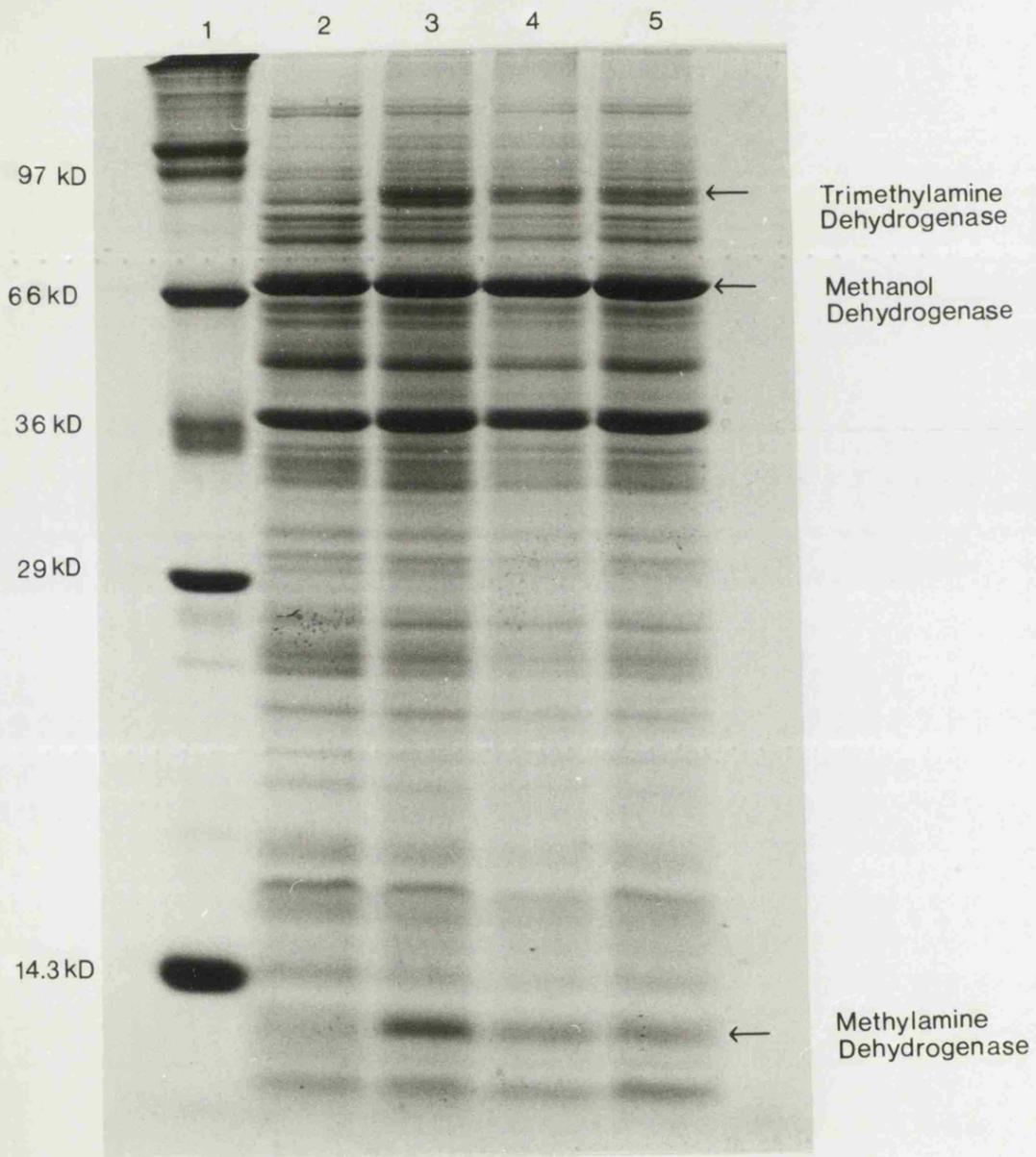
Table 3.3 Enzyme Activity of the Amine Dehydrogenases in M. methylotrophus

enzyme activity of the amine dehydrogenases measured as the rate of reduction of dichlorophenol indophenol (DCPIP)/minute/mg dry weight of cells. Columns 1-4 represent individual whole cell extract preparations, AM=Arithmetic Mean.

Figure 3.3 Whole Cell Extracts of M. methylophilus grown on Methanol or one of the Methylated amines.

Whole cell extracts were prepared from log phase cultures by the protocol previously described in Methods 2.7.

- (1) Molecular weight markers
- (2) Methanol supplemented culture.
- (3) Trimethylamine supplemented culture.
- (4) Dimethylamine supplemented culture.
- (5) Methylamine supplemented culture.



Grown on	MeOH					substrate				
	1	2	3	4	AM	1	2	3	4	AM
MeOH	26.2	49.7	42.0	36.4	38.5	0.0	0.0	0.0	0.0	0.0
IMA	18.4	40.6	39.7	29.1	31.9	18.1	20.9	31.4	21.3	22.9
DMA	16.8	44.4	38.6	32.4	33.0	12.6	21.0	26.5	18.1	19.5
MMA	16.3	41.2	39.9	29.7	32.3	17.1	19.8	27.1	22.4	21.6

Grown on	substrate MMA				
	1	2	3	4	AM
MeOH	0.0	0.0	0.0	0.0	0.0
IMA	15.2	28.8	37.4	25.5	26.7
DMA	17.8	32.4	31.1	28.3	27.4
MMA	18.3	31.1	28.6	24.0	25.5

Table 3.4 Enzyme Activity of the Amine Dehydrogenases in M. methylotrophus

enzyme activity of the amine dehydrogenases measured as the rate of reduction of dichlorophenol indophenol (DCPIP)/minute/mg dry weight of cells. Columns 1-4 represent individual whole cell extract preparations, AM=Arithmetic Mean.

growth on the methylated amines. This increase is a 20% induction of the enzyme allowing even greater efficiency in the utilisation of methanol in the media.

3.4 Diauxic Growth.

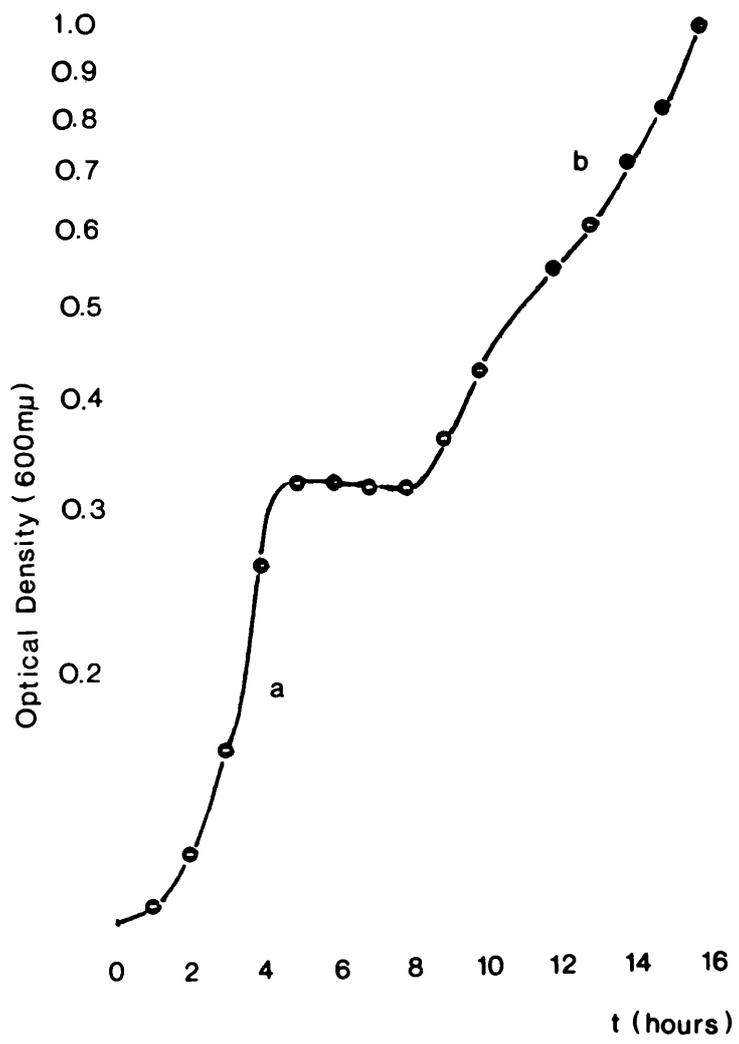
When grown upon a mixed substrate medium, such as methanol and a methylated amine, M. methylophilus exhibits diauxic growth (Figure 3.4). This is the result of initial growth upon methanol (four hours), followed by a transient lag, and then the expected growth upon the methylated amine. Many of the enzymes of catabolic pathways are subject to such catabolite repression (De Crombrugghe and Pastan 1978). If the cell is provided with a rapidly metabolizable energy source, the resulting increase in the intracellular concentration of ATP leads to the repression of the enzymes that degrade less rapidly metabolised energy sources. The increase in intracellular ATP leads to a decrease in available 3'-5' cyclic AMP in the cell, which is usually required for the transcription of the less rapidly metabolised energy source.

3.5 Summary.

The enzyme studies and polyacrylamide gel electrophoresis results with M. methylophilus grown on methanol or the methylated amines show that whereas methanol dehydrogenase is a constitutive enzyme, the enzymes responsible for the

Figure 3.4 Diauxic Growth pattern Observed when M. methylophilus is grown on a Mixed Substrate medium of Methanol and a Methylated Amine.

M. methylophilus grown on a mixed substrate medium of (limiting) methanol and methylamine. Growth at a rate comparable to methanol-grown cells occurs at stage (a), which changes to a rate comparable to methylamine-grown cells at stage (b). Mixed substrate cultures of methanol and trimethylamine show a similar pattern of growth (unpublished results).



assimilation of trimethylamine, dimethylamine and methylamine are inducible. These enzymes, trimethylamine dehydrogenase, dimethylamine oxygenase, and methylamine dehydrogenase are always induced together, and require the presence of at least one of the amines for induction. In addition, when grown upon a mixed substrate medium, such as methanol and a methylated amine, M. methylophilus exhibits a diauxic growth pattern similar to that sometimes observed in other organisms.

CHAPTER 4

Generation of Transposon Induced Mutations
and their Characterisation.

4.1 Transposons.

A number of reports have been published which describe the translocation, from one replicon to another, of small genetic elements termed insertion sequences (Bukhari et al. (Eds.)1977). Transposons are similarly specialised DNA segments which insert into many sites in bacterial genomes. They were originally distinguished from insertion sequences because transposons carry detectable genes, often conferring antibiotic resistance. Those which do encode antibiotic resistances provide powerful tools for microbial genetics, because their insertion into new sites in DNA generates mutations in which the mutant allele is linked to the selectable resistance trait (Kleckner et al. 1977). In addition to the central phenomenon of transposition, that is the appearance of a defined length of DNA (the transposable element) in the midst of sequences where it had not previously been detected, transposable elements typically display a variety of other properties: they can fuse unrelated DNA molecules and can mediate the formation of inversions and deletions nearby; they can be excised; and can contain transcriptional start and stop signals.

The drug resistance elements have in common their ability to insert into a large number of sites. The elements differ in the degree of specificity which they display; some appear to insert randomly, while others have distinct preferences for particular sites. In order to use the translocatable elements in bacterial genetics, one needs a vehicle such as a plasmid or phage to carry the transposon into the cell, and a means of selecting against the maintenance of the intact vehicle in the recipient bacterium. If this happens, then the only way for the recipient bacteria to become stably drug-resistant is for the transposon to become integrated into the bacterial genome. If this integration happens to be within a gene in the bacterial chromosome then that gene will become mutated, and probably inactivated. Such random insertional inactivations were ideal to produce random mutations in M. methylotrophus and in this case the transposable element In5 was used.

In5 is a 5400-base-pair-long transposon encoding the resistances to certain aminoglycoside antibiotics such as neomycin and kanamycin (Berg et al. 1978), (Figure 4.1). The element consists of a central unique sequence region containing the resistance gene flanked by inverted 1500-base-pairs long repeats (Berg et al. 1975). In5 transposons are strongly polar, because normal transcription in the operon distal to the transposon insertion site is prevented by termination signals present in the transposon. There are, however, low levels of expression from distal genes in approximately one-third of mutants generated by In5 insertion, due to a promoter associated with this transposon (Berg et al. 1980). In addition, In5 transposes at a high

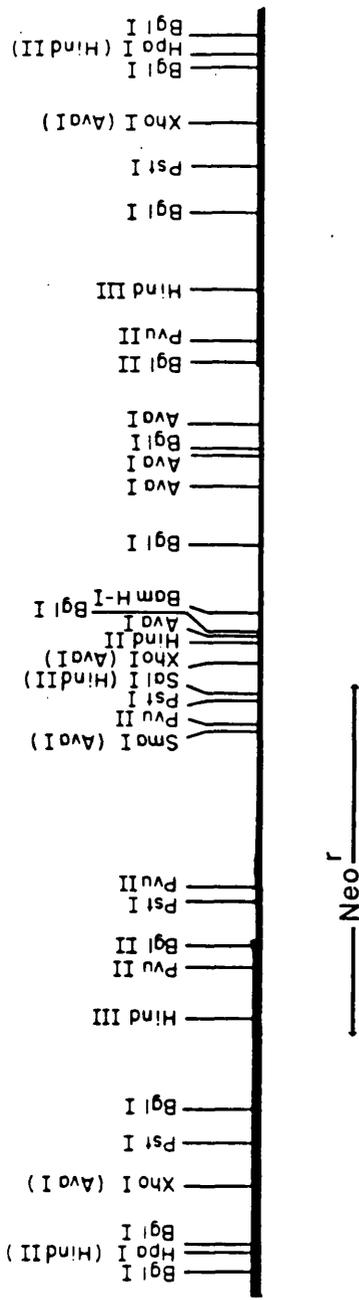


Figure 4.1: Map of Transposable Element InS.

Showing restriction enzyme cleavage sites, and position of Neomycin (Kanamycin) resistance gene (Neo^r). Inverted repeats are indicated by heavy lines.

(Diagram: Jorgensen *et al.* 1979.)

frequency, inserting into many sites in the genome, which makes it a useful mutagen for this study.

4.2 InS Mutation of M.methylophilus .

The simplest approach to transposon mutagenesis in this case was to use a transposon donor plasmid which could not be maintained in the target cell. For this reason the plasmid pLG221::InS was used, a InS donor based on the prototype IncIa plasmid ColIb (Boulnois et al. 1985). The plasmid has the capacity to promote conjugation between E. coli and a variety of Gram-negative organisms including M. methylophilus . Following conjugation, the plasmid is not inherited by M. methylophilus but, by selecting for the kanamycin resistance marker, transconjugants carrying the transposon can be isolated.

Mating between the donor E. coli cell, DS903, containing pLG221 and M. methylophilus took place on a membrane filter as described in Methods (Section 2.8). Conjugation was allowed to take place for 24 hours at 37°C in a minimal salt plate supplemented with methanol (0.1%). The resulting kanamycin-resistant derivatives were selected in 50 ml of methanol minimal salts media supplemented with kanamycin. Drug resistant derivatives of M. methylophilus appeared with a frequency of about 2×10^{-6} per donor cell (Table 4.1).

No. of Cells Plated.	No. of Drug Resistant Colonies.			
	1	2	3	4
1×10^5	0	0	1	0
1×10^6	2	1	3	3
1×10^7	23	15	28	21
1×10^8	219	186	272	215
MEAN FREQUENCY OF TRANSPOSITION = 2.2×10^{-6}				

Table 4.1 Frequencies of Transposition.

The frequency of transposition may be measured by the appearance of drug resistant elements in the population subjected to the transposable element. In this case, transposition of In5 carried on the donor plasmid pLG221 results in a transposition frequency of 2×10^{-6} when mated with M. methylotrophus.

4.3 Theory of Enrichment.

For selection of the mutants in the trimethylamine pathway, the cells were subjected to the enrichment and isolation procedure described in Methods (Section 2.7). The method uses antibiotics to eliminate growing cells in the media, thus actively selecting for those cells in the non-growth phase. In this case, growth of the whole population of transposon mutagenised cells in an amine medium results in the growth of the majority of the population: the rest of the population containing mutations vital for utilisation of the methylated amine are unable to grow and thus remain dormant in the growing population. The subsequent addition of penicillin-G breaks down the cell walls of actively growing cells by preventing terminal transpeptidation in the growing murein layer (Stanier et al. 1976). As a consequence, growth in the presence of penicillin leads to the formation of new murein chains which are not cross-linked and lack tensile strength. Cell walls containing such weak points are susceptible to changes in the osmotic pressure of the medium, and thus suspension in distilled water should eliminate these cells (Byrom, D. pers comm.). Additional treatment with cycloserine-D also acts on the growth of the cell walls, inhibiting the conversion of L-alanine to D-alanine, again preventing cross-linking by the absence of D-alanine in the murein layer. The cells unaffected by either of these treatments will be those unable to grow in the present medium and, thus enriched, they may be changed to a medium which they can utilise for subsequent growth. The success of the

antibiotic enrichment procedure, determined by the percentage of cells recovered containing mutations in one of the genes encoding the three enzymes of the trimethylamine pathway, was not as great as was first anticipated. Similar mutagenesis of M. methylophilus by In7, and enrichment for mutants affected in the enzyme methanol dehydrogenase, produced some 20-30% methanol dehydrogenase mutants (Sharpe 1984). In this case the most effective enrichment was for trimethylamine dehydrogenase mutants, where 20% of the mutants isolated in one enrichment procedure were trimethylamine dehydrogenase-defective cells. A far lower rate of enrichment and isolation was found for mutants affected in the other two enzymes of the pathway, dimethylamine oxygenase and monomethylamine dehydrogenase. The reason for this is unclear; it may be that the antibiotic enrichment procedure was not properly optimised for the organism or that the In5 mutagenesis was not as random and general in the methylophilus DNA as was hoped. The second of these explanations is probably the more feasible. Although several transposable elements have the ability to integrate at many positions in a DNA molecule, distinct insertion specificities have now been noted for different elements, and insertional hotspots have been described (Ely and Croft 1982, Lichtenstein and Brenner 1981). In5 appears to be non-specific with respect to its insertion sites in E. coli (Kleckner et al. 1977, Berg D. and Berg C. 1983), but some evidence that In5 has insertional preferences has been accumulated (Singer and Finnerty 1984), although the specificity of insertion is still unknown. Thus it is possible that a lower mutation rate for certain genes may be due to the

lack of integration sequences in the target DNA.

The rate of reversion of a population of cells mutated with In5 can give us some idea of the stability of a mutation. Reversion is associated with the loss of the inserted element and is assumed to be precise in most cases, thus restoring the gene function. In general, although transposons integrate freely into the DNA, they are fairly stable once they are integrated.

For most of the transposable elements, revertants may be detected at rates varying between 10^{-9} and 10^{-6} in the population of cells. For In5, reversion is independent of transposition (Berg and Egner 1980) and when excised the fate of the transposable element is not known, but it rarely integrates elsewhere. The reversion events for the trimethylamine and monomethylamine dehydrogenase mutants (Table 4.2) shows excision frequencies similar to those recorded by Berg and Egner 1980. However, excision did not concomitantly result in loss of the kanamycin-resistance, as would be expected from a single insertion event, and so it is possible that multiple insertion events might be occurring. To confirm this, a Southern transfer analysis was performed on EcoRI-restricted DNA from methylamine and trimethylamine dehydrogenase mutants, using ^{32}P -labelled In5 DNA as a probe, to estimate the number of transpositions that had occurred in the mutant cells. The results (Figure 4.2) suggest multiple transpositions in many cases. In5 specifies a diffusible negative regulator which represses the activity of In5 elements and reduces the activity of any additional In5 elements which are newly introduced. The net effect of this regulation is that

		methylamine Dehydrogenase mutant <u>mad</u> 1		trimethylamine dehydrogenase mutant <u>tmd</u> 3		
No. Cells Plated.	No. of Colonies Able to Grow Upon Methylamine.	Km		No. of Colonies Able to Grow Upon Trimethylamine.	Km	
		+	-		+	-
10^4	0	-	-	0	-	-
10^5	0	-	-	0	-	-
10^6	2	2	0	3	2	1
10^7	21	17	4	23	14	8
10^8	223	162	61	219	151	68

Table 4.2 Reversion of Mutants to the Wild Phenotype.

The reversion of methylamine dehydrogenase and trimethylamine dehydrogenase mutant cells to their wild phenotypes when selected for growth upon the original substrate.

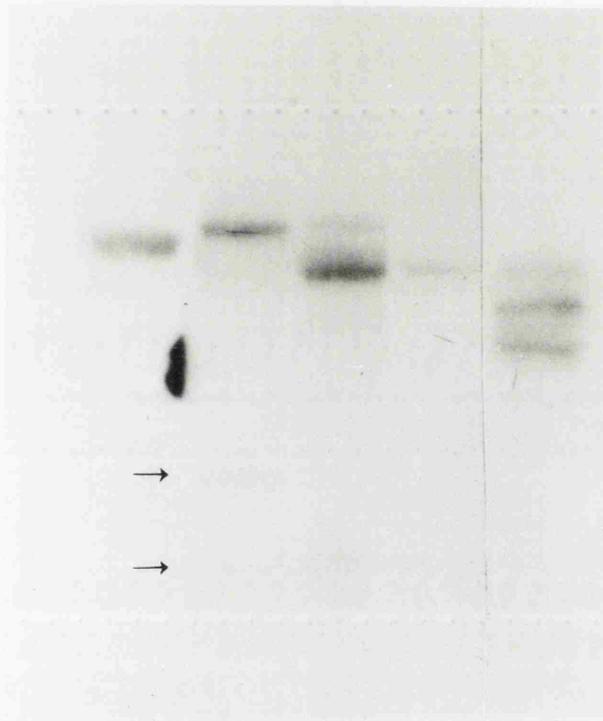
Figure 4.2 Evidence for Multiple Transpositions of Tn5 in
M. methylophilus .

EcoRI digested DNAs of

- (1) Mutant strain mad 1, deficient in the ability to utilise methylamine.
- (2) Mutant strain tmd 3, deficient in the ability to utilise trimethylamine.
- (3) Unknown mutation. }
- (4) Unknown mutation. } from conjugation
- (5) Unknown mutation. } with ::Tn5

probed with 32-P labeled Tn5.

1 2 3 4 5



transposition of the element is more frequent when it first enters the new host cell than when it has become firmly established. Thus nearly all these transposition events are likely to have occurred at the same time following the introduction of the transposable element to the new cell.

This possibly means that each mutant cell may contain more than one affected gene within it, but it is unlikely that these additional mutations will affect one another.

4.4 Preliminary Characterisation of Mutant Cells.

Whereas growth of wild type M. methylotrophus upon a methylated amine will induce the enzymes required for the assimilation of that amine, the mutants obtained from the transposon mutagenesis procedure described previously, are unable to assimilate their specific amine growth compounds.

The sixteen mutants unable to utilise trimethylamine as a sole carbon source, and the two mutants unable to use methylamine as a carbon source, were tested for simple growth requirements. All sixteen trimethylamine mutants were unable to use trimethylamine as a sole carbon source, but were able to use methylamine at a much reduced rate on solid agar. Liquid media, however supported growth on methanol and on methylamine at a reduced rate. The two methylamine negative mutants were able to utilise trimethylamine and dimethylamine as a sole carbon source but were unable to utilise methylamine. For further studies a

single mutant was isolated from each group to represent that group: Trimethylamine negative isolate tmd 3 and methylamine negative isolate mad 1 were chosen.

Growth curves show that both mutants are able to metabolise methanol normally. In addition, tmd 3 was unable to utilise any of the amines at a rate comparable to the wild type cell, and mad 1 was unable to use methylamine (Figures 4.3 and 4.4).

Polyacrylamide gel electrophoresis of cell extracts of both mutants cultured in a trimethylamine/methanol minimal salts medium showed tmd 3 to be lacking trimethylamine dehydrogenase (Figures 4.5) with no additional polypeptides identifiable. Methylamine dehydrogenase negative isolate mad 1 is however able to utilise trimethylamine, and the prominent 89,000 dalton protein band is identifiable. Because of the difficulties associated with identifying the methylamine dehydrogenase sub-units, their absence cannot easily be proven.

These two mutants were further characterised by assaying the individual enzymes involved and comparing the mutant and wild-type strains. There is the possibility that the mutations were not interruptions in the genes coding for the enzymes of the trimethylamine pathway, but were mutations of the gene(s) encoding the permease which would be required by the cell for the uptake of the methylated amines. The entry of many nutrients into a bacterial cell is often mediated by specific permeases which actively facilitate uptake of the compound into the cell. The growth behaviour of permease-less mutants could be similar to the behaviour of both trimethylamine and methylamine dehydrogenase mutants, as entry of the carbon source into the cell would then be dependent upon passive diffusion. Even at one

Figure 4.3 Growth of the Mutant Strain tmd 3, Deficient in the Ability to Utilise Trimethylamine as a Sole Carbon Source.

Growth of tmd 3 on

- △ Methanol
- Trimethylamine
- Dimethylamine
- Methylamine

Original density, OD_{600} 0.02.

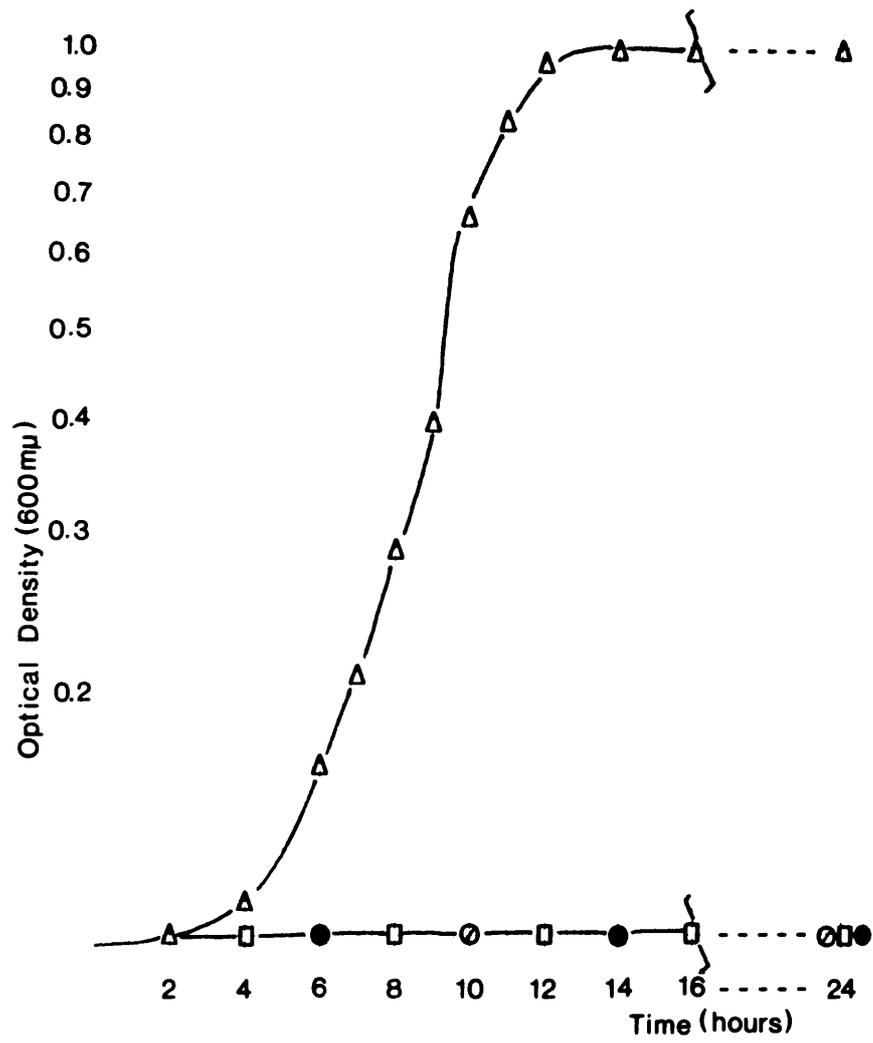


Figure 4.4 Growth of the Mutant Strain mad 1, Deficient in the Ability to Utilise Methylamine as a Sole Carbon Source.

Growth of mad 1 on

- Δ Methanol
- Trimethylamine
- ∅ Dimethylamine
- Methylamine

Original density, OD₆₀₀ 0.02.

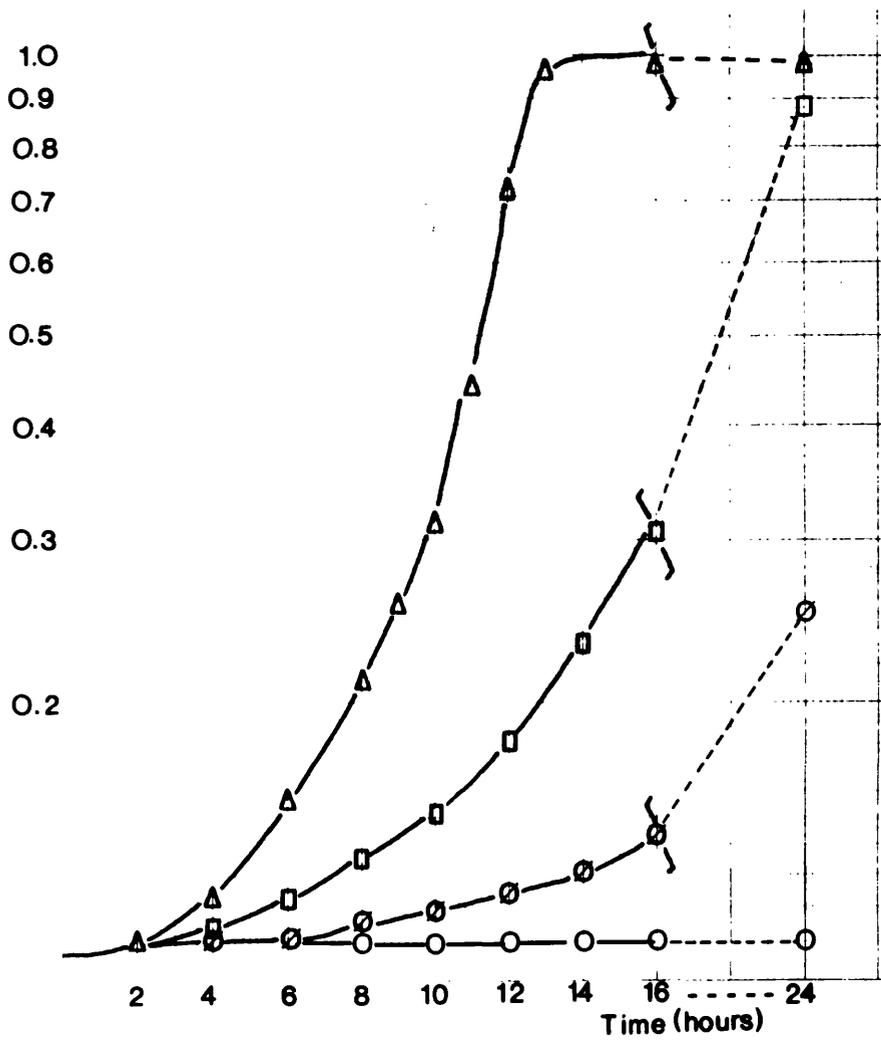


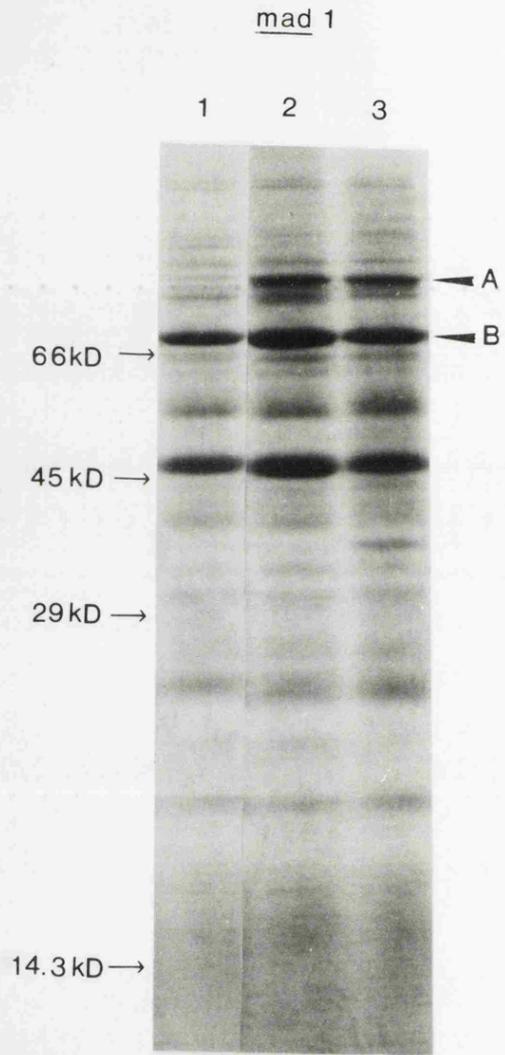
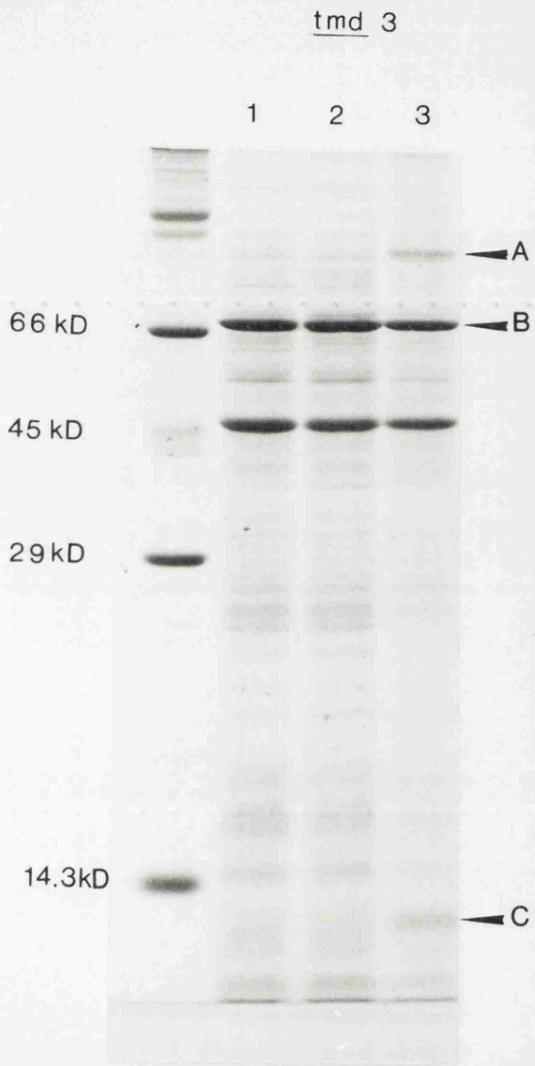
Figure 4.5 Polyacrylamide Gel Electrophoresis of Whole Cell Extracts of the Mutants tmd 3 and mad 1 compared to the Wild Type Cell Grown in Trimethylamine/ Methanol Supplemented Medium.

- (1) Methanol supplemented mutant cell
- (2) Trimethylamine/ methanol supplemented mutant cell
- (3) Trimethylamine/ methanol supplemented wild type M. methylotrophus .

A tmdh

B mdh

C mmdh



thousand times the normal concentration of the nutrient, this may not diffuse into the cell at a sufficient rate to allow the cells to grow comparably to their wild type counterpart. To eliminate the possibility that the mutational effects are due to permease deficiencies, it is necessary to directly register enzyme activity within the cell. Thus enzyme assays were carried out on broken cell extracts (Methods section 2.7) to allow direct access of the substrate to the enzymes involved. The trimethylamine and dimethylamine dehydrogenase activities were measured as the rate of reduction of 2,6-dichlorophenol-indophenol (DCPIP) at 40°C, with the respiratory electron transport chain terminated by the addition of KCN, and phenazine methosulphate (PMS) utilized as an artificial electron acceptor (Methods, Section 2.7). The results, tabulated in Table 4.3, are also represented as a percentage activity of the wild type M. methylotrophus cells, Table 4.4.

4.5 Statistical Analysis of Data.

The initial figures represented in Table 4.3 (columns 1-5) show the readings obtained from individual preparations of cell lysates, the sixth column giving the arithmetic mean. Although the arithmetic mean does give some indication whether mutant enzymic activities are similar or dissimilar with respect to the wild type enzyme, it will not necessarily give an unbiased view with so small a sample number. To eliminate the possibility of spurious results increasing or decreasing the arithmetic mean to give a false impression, standard deviation must also be taken

METHANOL						
CELL	1	2	3	4	5	AM
Wt	31.4	18.2	44.5	28.8	35.1	31.6
<u>tmd 3</u>	30.3	19.2	18.3	26.5	32.0	25.3
<u>mad 1</u>	34.0	13.9	20.9	28.1	34.1	26.2
TRIMETHYLAMINE						
Wt	10.4	6.1	13.9	11.4	12.3	10.8
<u>tmd 3</u>	0.0	0.0	0.0	0.0	0.0	0.0
<u>mad 1</u>	10.4	4.6	10.7	8.7	11.6	8.9
METHYLAMINE						
Wt	18.3	7.8	15.7	39.3	21.8	20.9
<u>tmd 3</u>	0.0	0.0	5.2	0.0	0.0	1.0
<u>mad 1</u>	0.3	0.0	0.0	0.2	0.0	0.1

Table 4.3 Enzyme Studies on Whole Cell Extracts.

Enzyme assays carried out on whole cell extracts of M. methylotrophus and of mutants cultured in methylated amine, or methanol salts media. Dehydrogenase activities are represented as the rate of reduction of dichlorophenol indophenol (DCPIP)/minute/mg dry weight of cells. Columns 1-5 represent individual preparations of whole cell extracts. AM = Arithmetic mean.

Cell	Substrate		
	Methanol	Trimethylamine	Methylamine
wt	100.0	100.0	100.0
<u>tmd 3</u>	80.0	0.0	5.4
<u>mad 1</u>	82.9	82.4	0.5

Table 4.4 Percentage Activity of Wild Type and Mutant Cells
(from Table 4.3).

into account. Because the sample size is so small we are unable to use the standard deviation as a measure of error. A sample size of five readings is too small to yield a good estimate of the population standard deviation. Thus, to avoid this error, a new variable must be introduced, the student or t distribution, given by:

$$t = \frac{\bar{x} - u}{s} \cdot \sqrt{n} \quad (1)$$

where \bar{x} is the sample mean,

s is the sample standard deviation,

n is the sample number

u is the population mean.

The t variable resembles the normal distribution and depends only on the value of n , providing that the variable x possesses a normal distribution. For a sample number such as five, this result is a flatter distribution curve to account for such small sample inaccuracies.

For a comparison between two means, equation (1) may be modified to include both samples being investigated. In this case, t becomes:

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{s (1/n_1 + 1/n_2)}} \quad (2)$$

and

$$s = \frac{(n_1 - 1) \cdot s_1^2 + (n_2 - 1) \cdot s_2^2}{n_1 + n_2 - 2} \quad (3)$$

Note that s becomes s^2 to account for the two different sample deviations.

It only remains to apply the number of degrees of freedom that apply to the sample size. The number of degrees of freedom is designated as the sample number - 1, as you automatically lose one degree of freedom for the calculation. Thus for two samples the number of degrees of freedom equals: $n + n - 2$, or in this case, $5 + 5 - 2 = 8$. The larger the sample size, the larger the number of degrees of freedom, which reflects on the greater accuracy expected with larger sample numbers. Applying eight degrees of freedom to a t distribution with a sample number of five gives, ± 2.31 . This means that if the t distribution for two means falls within this acceptance range, we may be 95% certain that the two sample means are the same. In a similar manner, if t lies outside this range, then we can be 95% certain they are statistically different.

4.6 Comparison of Mutant Enzyme Activities.

Initially, before any comparison of the enzyme activities can be undertaken, it is essential to determine whether both mutant and wild type cells are in a comparable physiological condition. Discrepancies in the physiological state between the two

cultures could result in altered enzyme activities which could lead to spurious results later on. It has been noted (unpublished observations) that cells considered to be in a poor physiological state give abnormally low enzyme activities for methanol dehydrogenase, and in particular cells which have been in contact with detergents rapidly lose their methanol and methylamine dehydrogenase activities, both enzymes being loosely bound to the periplasmic side of the cell membrane and thus sensitive to treatments which may disrupt this or the cell wall. Some indication of the physiological condition of the cell can thus be interpreted from the level of enzyme activity obtained for methanol dehydrogenase. If we use the methanol dehydrogenase activity of the wild type cell as a reference enzyme activity of a cell in normal physiological condition, then, by using the t-test distribution to see if the methanol dehydrogenase of the mutant cells is significantly different from the wild type cell activity, we should get some indication of the mutant's physiological state (assuming that the mutations in the trimethylamine dehydrogenase gene(s), and methylamine dehydrogenase gene(s), do not adversely affect the utilisation of methanol in any way). The third column on Table 4.6 shows the result of the test between the means of the wild type and the two mutant cells for methanol dehydrogenase; statistically both mutants, tmd 3 and mad 1, show enzyme activities comparable to the wild type M. methylotrophus, as far as a small sample size may allow.

The measurement of the enzyme activities of the amine dehydrogenases of both mutant and wild type cells, shown in Table 4.3, are converted to the more

Substrate									
Methanol			Trimethylamine			Methylamine			
Cell	1 AM	2 SD	3 t	1 AM	2 SD	3 t	1 AM	2 SD	3 t
wt	31.6	9.5	0.00	10.8	2.9	0.0	20.9	11.6	0.0
<u>tmd</u> 3	25.3	6.3	0.51	0.0	0.0	8.3	1.1	2.5	3.7
<u>mad</u> 1	26.2	8.7	0.43	8.9	2.5	1.1	0.1	0.1	4.0

Table 4.5 Statistical Analysis of data in Table 4.3.

Column 3 shows the t-test between the wild type and respective mutant means.

AM = Arithmetic Mean.

SD = Standard Deviation.

t = t-test between two means.

If t falls between +/- 2.31 then the means are statistically the same (95%).

significant test between two means in Table 4.5, using a comparison between the wild type cells and each mutant cell.

Assuming that the average batch of cells are in a similar physiological condition, as indicated by the enzymic activities of their methanol dehydrogenases, then the resulting differences in the activity of the amine dehydrogenases can be assumed to be real differences and not a result of poor cellular growth. Both mutants are shown to be strongly affected in their ability to utilize trimethylamine and methylamine respectively. Because the enzyme activities have been measured in whole cell extracts as opposed to intact M. methylophilus cells, the evidence supports the probability that the mutation in mad 1 is located actually within the gene(s) coding for the dehydrogenase, or within the DNA sequences controlling the structural gene. The inability of the trimethylamine dehydrogenase mutant tmd 3 to utilise trimethylamine or methylamine at a rate comparable to the wild type cell makes this case a little less simple than that for the methylamine mutant. It may be that the mutation is genuine, but polar, so that insertion of the transposable element has occurred early within the structural gene or the regulatory regions coding for trimethylamine dehydrogenase and that those gene(s) immediately distal to the mutated dehydrogenase might be similarly affected by a polar effect from the transposon. There is evidence that In5 can exert a polar effect on genes distal to the site of insertion, and that this effect is independent of the orientation of the transposable element (Berg, Weissand and Crossland 1980). The alternative explanation involves a mutation in the gene(s) encoding the permease required for the uptake of trimethylamine, preventing entry of the amine(s) into the cell,

and consequently preventing induction of the pathway. This explanation is only feasible if we assume that the reduced growth observed with tmd 3 upon methylamine is due to the limited uptake, possibly because passive diffusion of methylamine into the cell is insufficient to induce the trimethylamine dehydrogenase gene system. This involves methylamine being a more efficient inducer for the methylamine dehydrogenase gene(s) than for the trimethylamine dehydrogenase gene(s), which is feasible, although perhaps unlikely.

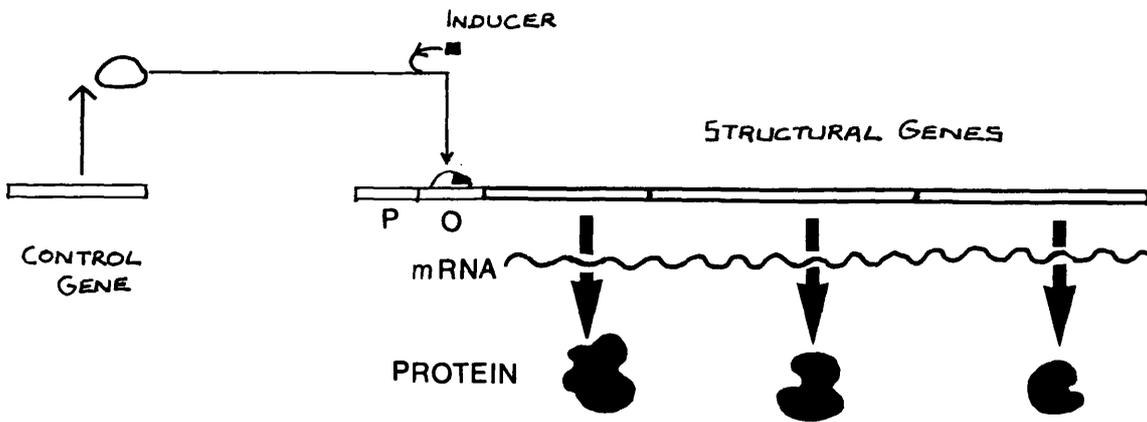
4.7 Implications for Structural Positioning and Genetic Control.

In some bacteria, the genes governing the synthesis of the various enzymes in a metabolic pathway are often closely linked to form a cluster on the genome, and are usually induced or repressed together. The first system of this type to be defined was the lactose operon of E. coli (Jacob and Monod 1961), and genetic mapping analysis has shown that the three genes required for lactose utilisation are adjacent to one another and preceded by a control region specifying the sites of action of regulatory proteins. Since then, many enzymes involved in both anabolic and catabolic pathways in bacteria have been found to be contiguous on the bacterial chromosome, and often regulated as an operon. Many more have been found to have no structural links on the genome with their enzymic neighbours in a metabolic pathway. From the data presented in both this chapter and chapter 3, it is difficult to categorise the genes of the trimethylamine pathway into either section. If the genes of the pathway act as

a unit of function and regulation, then a mutational event within either the regulatory regions or a structural gene could inactivate those genes distal to the disruption (figure 4.6). Thus a transposon insertion in the trimethylamine dehydrogenase gene could prevent synthesis of methylamine dehydrogenase if the gene was distal to the site of transposon insertion. However, in the absence of a suitable system for fine-structure genetic analysis, resolution of this problem will require physical location of the relevant genes by molecular cloning and 'chromosome walking'. The alternative possibility, that the related genes are unlinked but are governed by common regulatory mechanisms, must also be considered. The apparent inactivation of more than one gene by a single mutational event could be a consequence of the inactivation of a gene encoding a positive controlling protein, Figure 4.7.

4.8 Summary

The transposable element In5 was used to generate mutations in the chromosome of M. methylotrophus. Mutants unable to utilise trimethylamine or methylamine as a sole carbon source were isolated via an antibiotic enrichment procedure. One mutant from each class was isolated for further characterisation. Subsequent enzyme assays showed tmd 3 deficient in trimethylamine dehydrogenase, and methylamine dehydrogenase, while mad 1 seemed to lack only methylamine dehydrogenase.



Tn5 Insertion:

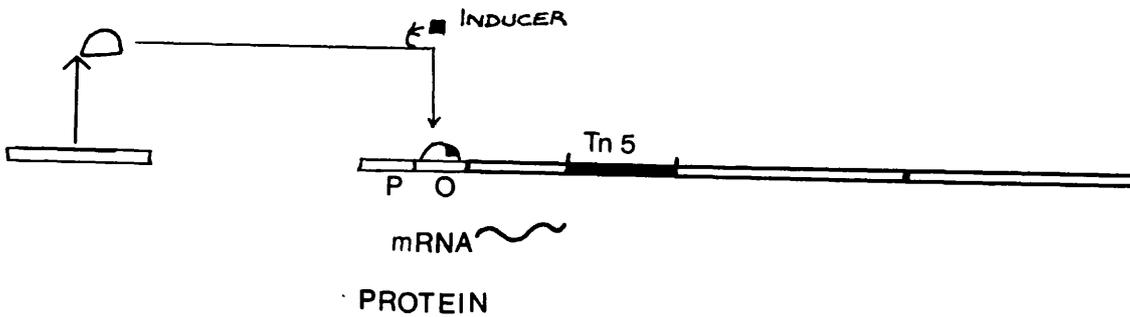
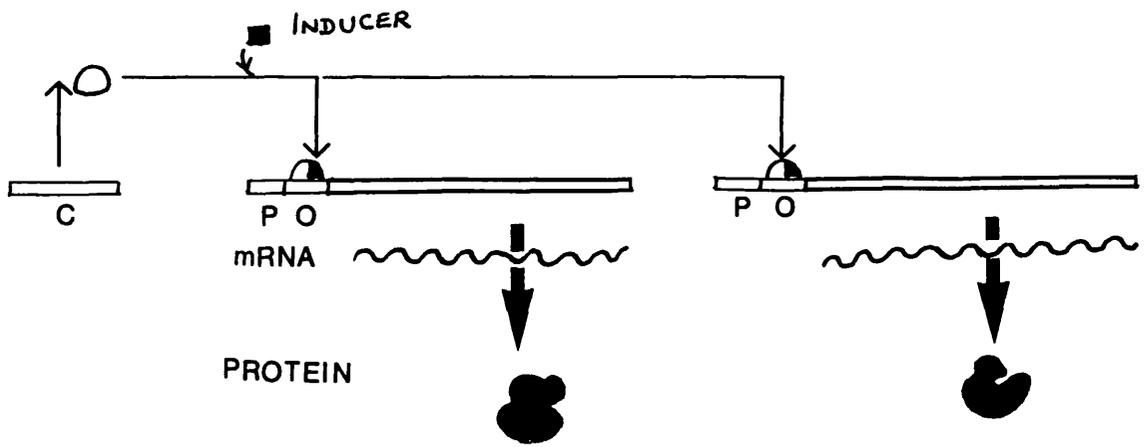


Figure 4.6 Effect of Transposon Insertion (i).

The genes are grouped together in an operon, acting together as a unit of function and regulation. The effect of transposon insertion results in the inactivation of the gene involved, together with a block of the transcription of genes distal to this insertion. O=operator, P=promoter.



Tn5 Insertion

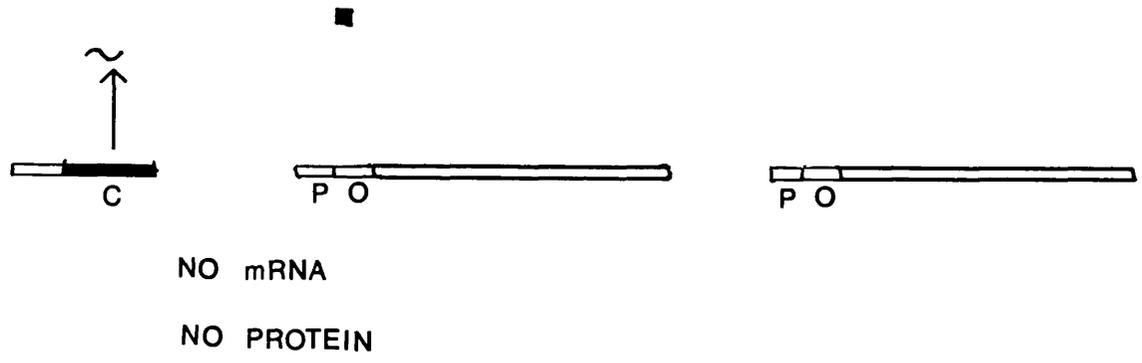


Figure 4.7 Effect of Transposon Insertion (ii).

The genes are not necessarily linked but may have a common control mechanism, involving the regulatory gene C and the target sites. O=operator, P=promoter.

These mutants, having been characterised as mutants in the trimethylamine pathway, could now be used to isolate the wild type genes from a M. methylophilus gene bank via the replacement of enzyme activity.

CHAPTER 5

Isolation of the Methylamine Gene(s).

Any DNA cloning experiment has four essential sections; a method for generating the genomic fragments, a method for ligating the fragment into the appropriate vector, a means of introducing the recombinant molecule into the required cell, and a method of selecting the clones that have acquired the desired recombinant. The diversity of methods that might be employed in the construction of a genomic library are wide (Primrose and Old 1980), but the choice of strategy is usually influenced by the simplest route which could achieve the desired recombinant library. This work involved the use of restriction enzymes to insert methylotroph DNA into a plasmid vector containing an antibiotic marker. Identification of the desired recombinants was through replacement of gene activity of the mutant phenotype.

5.1 The Probability of Obtaining a Given Gene.

The exact probability of having any one DNA sequence represented in a genomic library may be calculated from the formula;

$$N = \frac{\ln(1 - P)}{\ln(1 - f)}$$

where P is the desired probability of having any one DNA sequence represented, f is the fractional proportion of the genome in a single recombinant, and N is the necessary number of recombinants required (Clark and Carbon 1976).

The aim of constructing a genomic library is to isolate the desired sequence of DNA by screening the least number of recombinant clones. For this aim a vector able to handle large inserts of genomic DNA has the advantage of being able to reduce the number of recombinants that must be screened. For example, to achieve a 99% probability ($P=0.99$) of having sequence (x) represented in a library of a bacterial genome (3×10^6 base pairs), the number of recombinants to be screened (N), when insert size is 10kb

$$\frac{\ln(1 - 0.99)}{\ln(1 - 1.0 \times 10^4 / 3.0 \times 10^6)}$$

= 1379 recombinants.

This figure is acceptable in the screening of a genomic library, whereas a genomic library containing an average insert size of 3kb would require 4.6×10^3 clones to be screened to obtain the same probability of obtaining gene (x).

5.2 Construction of a Genomic Library.

5.2.1 Selection of an Appropriate Vector.

Because of the lack of receptor proteins for phage on the surface of M. methylophilus, it is necessary to use plasmid-based vectors to introduce exogenous DNA into the host cell. Many of the cloning vectors developed to date have been plasmids or phages specific for E. coli, and are not necessarily useful for generating a high copy number of the desired insert in M. methylophilus. Many wide-host-range cloning vectors have also been developed (Meyer et al. 1977), but these vectors tend to be large and to have a low copy number. For ease of manipulation and for a high gene dosage, small, high copy number, broad host range plasmids are required for M. methylophilus. The Inc Q group of plasmids meet these criteria, and although they themselves are non-conjugative, they may be effectively mobilised by conjugative plasmids of other groups (Grinter and Barth 1976). In this work, the composite plasmid pGSS33, containing several restriction sites from pBR322 while retaining the broad-host-range characteristics of the Inc Q group required for M. methylophilus (G.Sharp 1984), was used (figure 5.1). The plasmid contains a number of unique restriction sites available for cloning (Table 5.1).

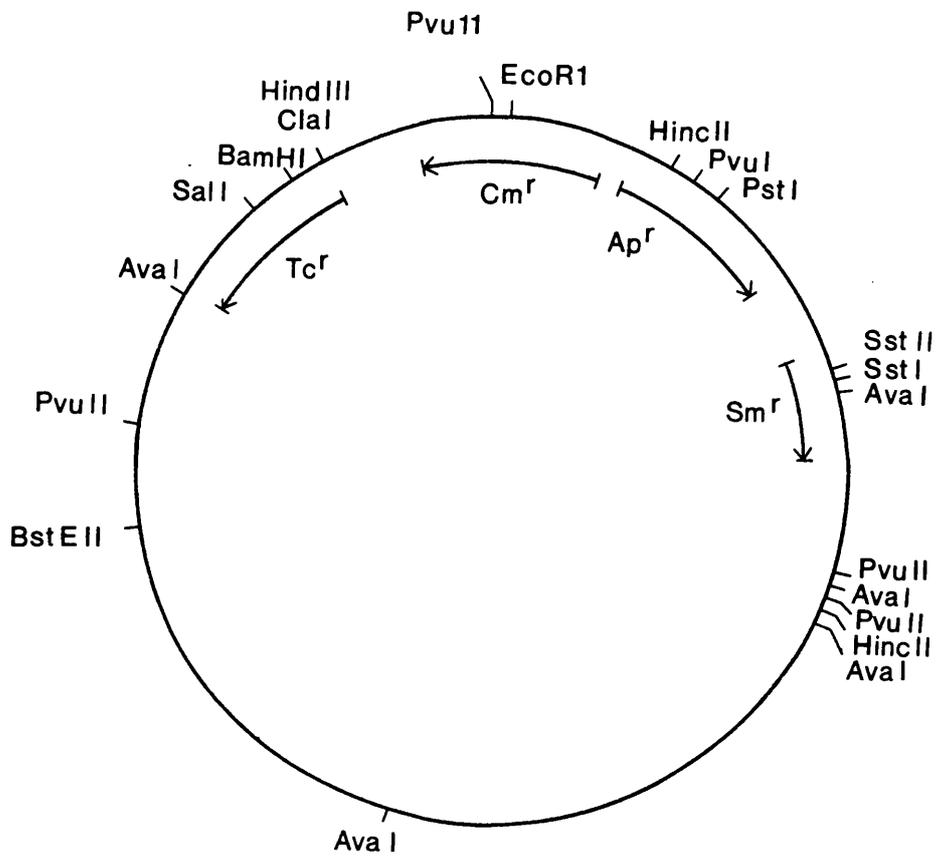


Figure 5.1 The Plasmid Vector pGSS33, 13.4 kb in length.

ENZYME	SITE	GENE
<u>SalI</u>	G ↓ I C G A C	Tc
<u>XhoI</u>	C ↓ T C G A G	Tc
<u>BamHI</u>	G ↓ G A T C C	Tc
<u>Sau 3a</u>	↓ G A T C	Tc
<u>BglII</u>	A ↓ G A T C T	Tc
<u>HindIII</u>	A ↓ A G C T T	Tc
<u>ClaI</u>	A T ↓ C G A T	Tc
<u>EcoRI</u>	G ↓ A A T T C	Cm
<u>EcoRI</u>	↓ A A T T	Cm
<u>PvuI</u>	C G A T ↓ C G	Ap
<u>PstI</u>	C T G C A ↓ G	Ap
<u>SstII</u>	C C G C ↓ G G	Sm
<u>SstI</u>	A G C T ↓ C	Sm
<u>HgiAI</u>	C T G G A ↓ C A T	Sm

Table 5.1 Insertional Inactivation of Antibiotic Resistance Genes Caused by Restriction of PGSS 33 by Various Endonucleases.

5.2.2 Generation of 10Kb Fragments from M. methylophilus DNA.

The DNA of M. methylophilus cells, grown in 0.1% methanol-salts medium, was extracted and purified according to Methods section 2.6. 4 mg of M. methylophilus DNA was obtained, of some 200 kb in average length from a 200 ml suspension of stationary cells ($OD_{600}=0.9$)

Libraries of prokaryotic or eukaryotic DNA may be prepared by two principal methods. The first approach involves digestion of the genomic DNA to completion with a restriction endonuclease and insertion of the resulting fragments into an appropriate vector. This approach has two main drawbacks; firstly, if the sequence of interest contains recognition sites for the particular restriction enzyme chosen, it will be cloned in two or more pieces. There is also a possibility that the sequence may not be cloned at all if it is contained within a fragment larger than the vector is able to accommodate. Secondly, the average size of fragments generated by many restriction enzymes is fairly small (3-4 kb), and thus an entire library will contain a very large number of recombinant clones which makes screening a laborious task. According to the formula given by Clark and Carbon, it is practical to aim for inserts of 10 kb in length to reduce the amount of screening required. In addition, if these fragments are generated by partial restriction of the bacterial DNA, this ensures that there is no systematic exclusion of sequences from the cloned library merely because of an unfortunate distribution of restriction sites.

After a variety of conditions had been tested, two units of EcoRI / μg of DNA 120 min was shown to generate fragments in the 5-15 kb range. A 250 μg quantity of M. methylophilus DNA was similarly digested to give this range of fragments, and 90 μg of the resultant digest was loaded onto a sodium chloride gradient (Methods, section 2.5), and spun at room temperature (20°C) for 20 hours at 16,000 rpm. After centrifugation, the fractions were collected from the gradient, purified, and 2 μl samples run on a 0.7% agarose gel (figure 5.2). Fraction 14-16, representing the 8-12 kb fragments, were pooled, and used to represent the M. methylophilus fragments (a) in the subsequent ligations.

A further 90 μg of the initial digest was loaded and run on a low melting-point agarose gel, and a block of fragments 8-12 kb in size was removed for phenol extraction, (Methods, Section 2.5) and ligated to pGSS33 (ligation b).

Finally, the partial digestion was used without any further purification of the DNA fragments (ligation c).

5.2.3 Ligation of Vector and Genomic DNA.

In any one ligation reaction involving restricted vector and genomic DNA, the recombination frequency may vary from 0-99%, depending on the conditions of ligation. The proportions of recombinants in dilute solutions may be low due to the circularisation of the linear fragments, which is relatively favoured due to the reduced frequency of intermolecular reactions. Increasing the concentration of genomic DNA molecules provides greater scope for reaction between vector

Figure 5.2 Fractions from the salt-gradient.

- (1) Hind III digest of λ DNA [1 μ g]
- (2) Fraction 2
- (3) ,, 4
- (4) ,, 6
- (5) ,, 8
- (6) ,, 10
- (7) ,, 12
- (8) ,, 14
- (9) ,, 16
- (10) ,, 18
- (11) ,, 20

1 2 3 4 5 6 7 8 9 10 11

23.1kb

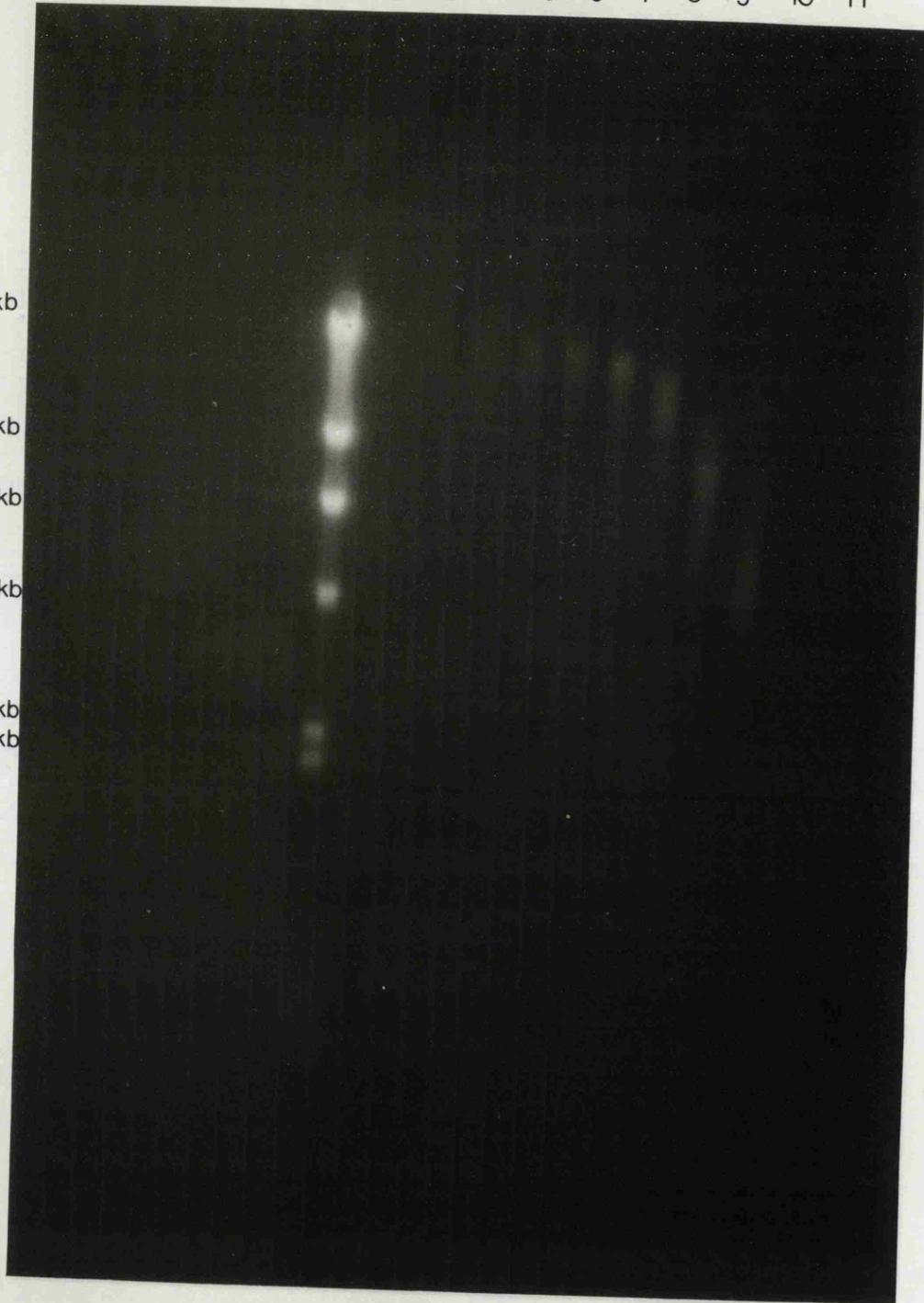
9.4kb

6.5kb

4.3kb

2.3kb

2.0kb



and genomic fragments, while treatment of the linearised plasmid with alkaline phosphatase, to remove 5' terminal phosphate groups, prevents both re-circularisation and plasmid dimer formation. In this state circularisation of vector DNA can occur only by insertion of non-phosphatase treated foreign DNA, which provides one 5' terminal phosphate at each join. One nick at each join remains unligated until after transformation, when the host cellular repair mechanisms reconstitute the intact duplex. Careful preparation of the vector DNA was found to be crucial for a high proportion of recombinants; pGSS33 previously digested with EcoR1 and subsequently cleaned by the phenol/ether/ethanol treatment was found to be far more susceptible to the phosphatase than when digestion and removal of the phosphate groups were carried out simultaneously. In addition the amount of phosphatase added was found to be critical to ensure an uninhibited ligation reaction later on, as an excess of the enzyme is difficult to remove at the final ligation stage. Thus two small aliquots of alkaline phosphatase (0.5u each) were added at half-hour intervals to the cleaned, restricted pGSS33 (5 µg in a total volume of 20 µl), at 37^oC to obtain maximum results. Solutions of plasmid DNA phosphatase treated in this manner contain large numbers of free phosphate groups which act as a strong inhibitor of T4 ligase: for a good ligation complete removal of these groups is essential and so a G-50 column was employed which selectively removes small molecules from the sample. Ligation was carried out between 2 µg of phosphatase-treated pGSS33 DNA and 10 µg of one of the different methylotrophus genomic fragments (a, b, or c) mentioned in

Section 5.2.2., in a total volume of 130 μ l using 2 units of 14-coded DNA ligase at 10°C for 16 hours.

Transfer of the ligated recombinant plasmids into M.methylotrophus was via the host E.coli strain DH1.R64(ddd11). 0.1 μ g of each of the recombinant plasmid mixes were used to transform 100 μ l aliquots of competent E.coli cells. The frequency of transformation (the number of cells transformed by 1 μ g of recombinant DNA), and the percentage of these cells that carried recombinant plasmids have been determined for each ligation and are shown in Table 5.2. The selection media contained ampicillin to allow growth of the transformed cells only.

5.3 Analysis of the Recombinant Clones.

The analysis of recombinants in the E. coli host strain DH1.R64(ddd11) is greatly complicated by the presence of the mobility plasmid R64(ddd11) which produces a large number of fragments in the 5-15 kb range when digested with EcoR1. To avoid this, recombinant clones randomly chosen from the total populations were individually transferred to M. methylotrophus where the mobility plasmid is unstable. These recombinant M. methylotrophus clones were grown up overnight at 37°C in methanol/minimal media containing ampicillin to a concentration of 100 μ g/ml, and analysed by the mini-preparation boiling method of Holmes and Quigley 1981, methods 2.6. The plasmid DNA subsequently obtained was restricted with 2 units of EcoR1 for two hours at 37°C, and the fragments separated by agarose gel electrophoresis

	LIGATION		
	(a)	(b)	(c)
Frequency of transformation (Transformants/ μg DNA)	1×10^2	2×10^2	1×10^5
Percentage recombinants	84	76	87
Average size of inserts (kb)	2.89	3.88	5.90
Total number of recombinants obtained	564	397	> 3000
Probability (P) of obtaining a given gene (see Appendix 3)	0.420	0.401	0.997

Table 5.2. Comparison of the Three Different Ligations

Using Fragments Obtained by

(a) Salt Gradient,

(b) Agarose Fragments

(c) Total Partial Genomic Digest.

(Figures 5.3, 5.4 and 5.5).

The average size of the recombinants from the three ligations using (a) fragments isolated from the salt gradient, (b) fragments cut out of the agarose gel, (c) total partial digest of genomic DNA, were calculated from Figures 5.3, 5.4 and 5.5 (see Table 5.2).

Preparing a library of genomic DNA inserts to provide adequately sized inserts for the subsequent recombinant clones is usually enhanced by preferentially selecting a 10Kb sized fragments for ligation to the vector DNA. However, when the partial digestion of M. methylophilus DNA by EcoRI, yielding fragments between 4-20 kb in size, was subsequently separated on the salt gradient to select for those 8-12 kb in size, it seems that the procedures required to clean the DNA for ligation reduced the efficiency of ligation, thus reducing the number of recombinants obtained (Table 5.2). In addition the average size of the inserts was not as large as was previously anticipated, making the procedure less successful than using the total partial digestion products (c), where a much larger number of recombinants were obtained containing a surprisingly high average insert size of 5.6 kb. The number of recombinants and average insert size of the clones containing inserts isolated by the agarose gel electrophoresis method showed a similar pattern to the salt-gradient isolates, probably for similar reasons, since both these gave a lower probability of obtaining a given gene than the recombinants containing the total genomic partial digest. This was subsequently used to screen the isolated mutants for replacement.

Figure 5.3 Selection of recombinants obtained from the salt-gradient fractions.

- (1) PGSS33 EcoRI .
- (2) EcoRI digest of DNA.
- (3) Recombinant 2.7Kb insert.
- (4) ,, NO ,,
- (5) ,, 6.6Kb ,,
- (6) ,, 1.0Kb ,,
- (7) ,, 3.6Kb ,,
- (8) ,, 3.45Kb ,,
- (9) Hind III digest of λ DNA.

1 2 3 4 5 6 7 8 9

21.4 kb

23.1kb

7.4 kb

9.4 kb

5.8 kb

6.5 kb

5.6 kb

4.8 kb

4.3 kb

3.5 kb

2.3 kb

2.0 kb

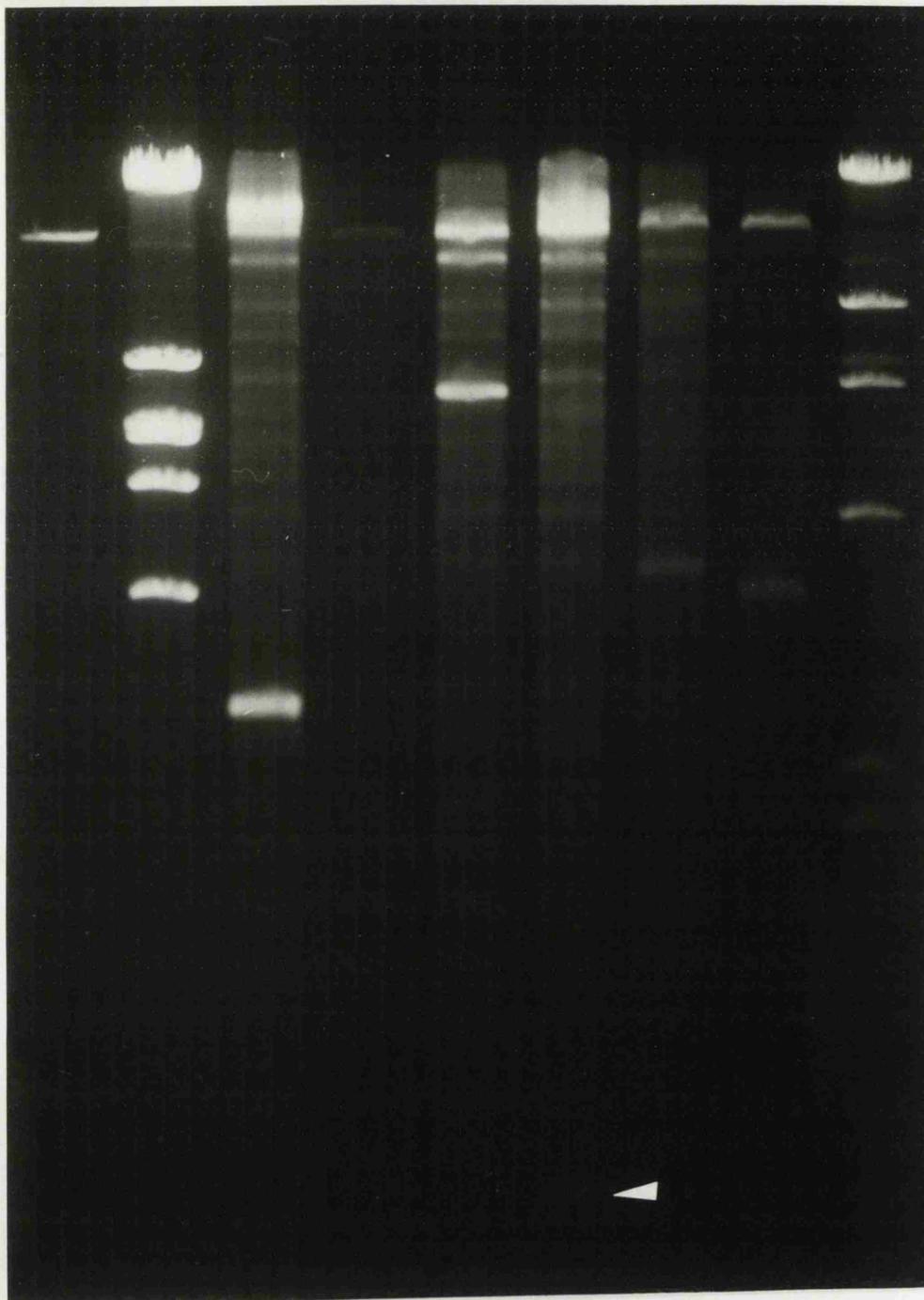


Figure 5.4 Recombinant Clones Obtained from the Agarose Fractions.

The recombinants have been restricted with EcoR1 .

- (1) pGSS33 undigested.
- (2) pGSS33 digested with EcoR1 (linearised).
- (3) Recombinant containing a 2.3 kb insert.
- (4) Recombinant containing a 1.8 kb insert.
- (5) Recombinant containing a 1.6 and 2.3 kb inserts.
- (6) Recombinant partially digested.
- (7) Recombinant containing no visible insert.
- (8) Recombinant containing no visible insert.
- (9) Recombinant containing no visible insert.
- (10) λ HindIII marker.

1 2 3 4 5 6 7 8 9 10

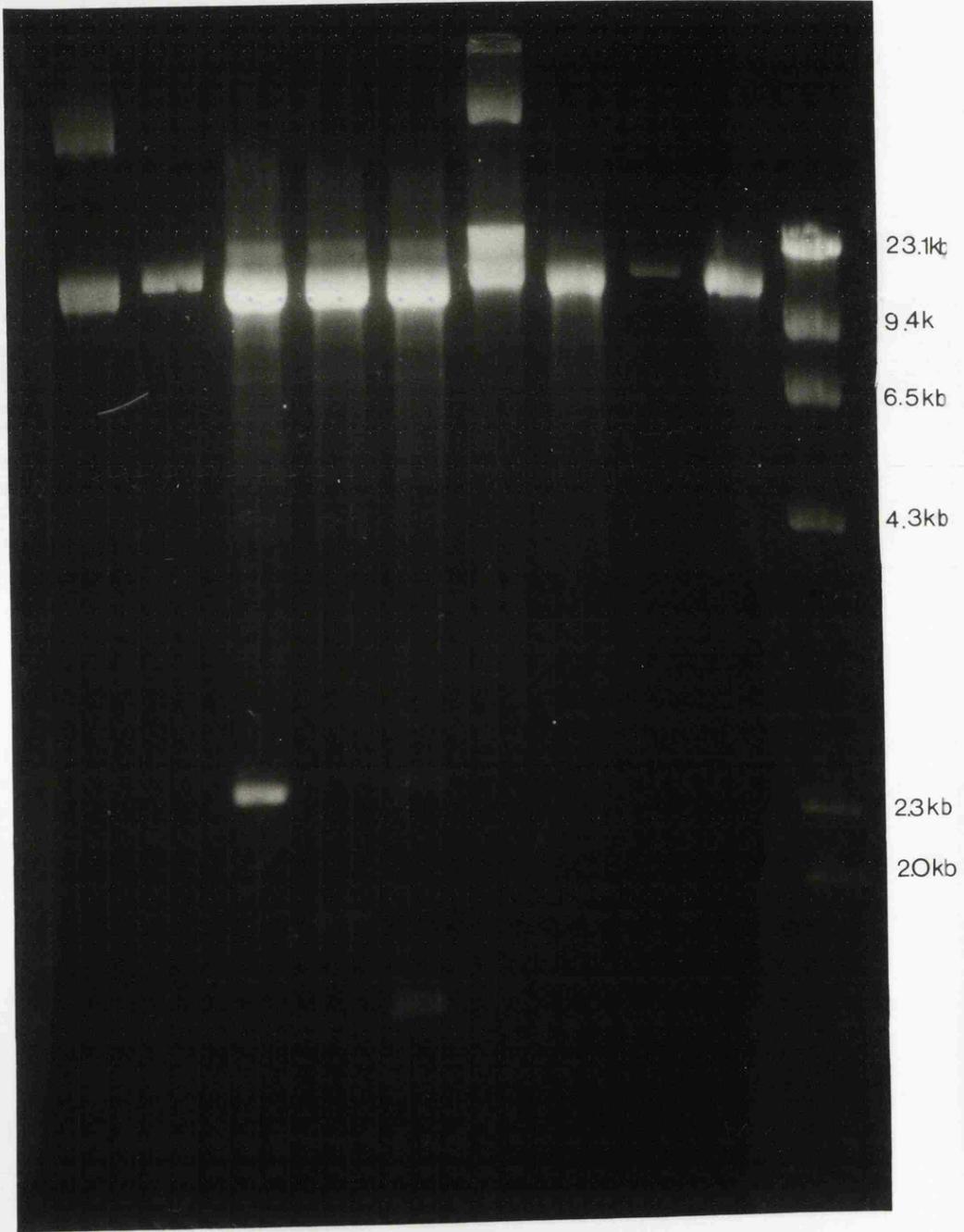


Figure 5.5 Recombinant Clones Obtained From the Total Partial Digest.

The clones recombinants have been digested with EcoR1 .

- (1) Recombinant containing a 1.9 kb insert.
- (2) Recombinant containing a 1.1 kb insert.
- (3) Recombinant containing a 4.3 kb insert.
- (4) Recombinant containing a 10 kb insert.
- (5) Recombinant containing a 10 kb insert.
- (6) Recombinant containing a 2.5 kb insert.
- (7) Recombinant containing a 4.3 kb insert.
- (8) Recombinant containing a 3.5 kb insert.
- (9) Recombinant containing a 5.8 and 11 kb inserts.
- (10) Recombinant containing a 6.0 kb insert.
- (11) Recombinant containing a 6.0 kb insert.
- (12) Recombinant containing a 10 kb insert.
- (13) Recombinant containing a 2.5 kb insert.

1 2 3 4 5 6 7 8 9 10 11 12 13

23.1kb

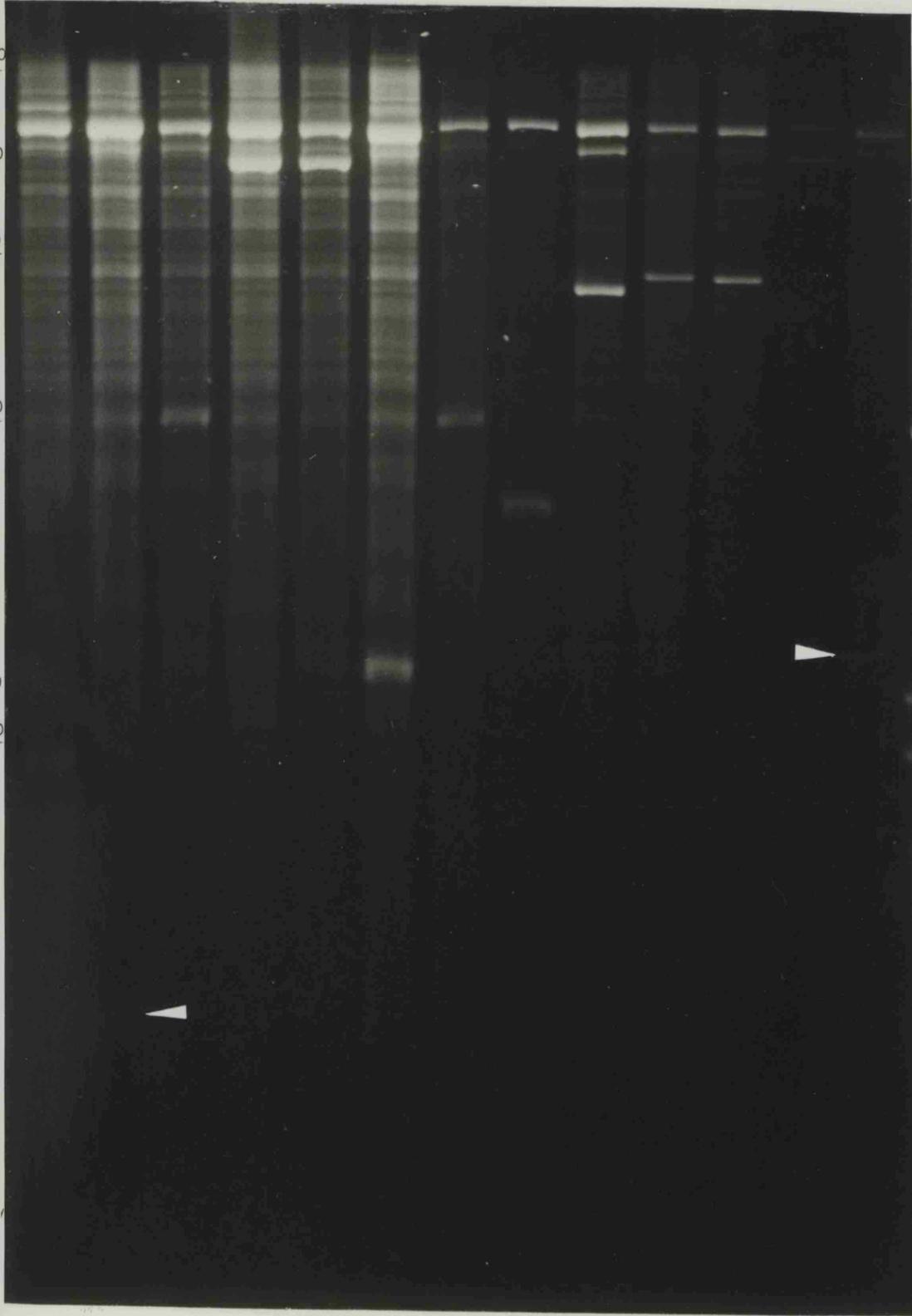
94kb

65kb

4.3kb

2.3kb

2.0kb



5.4 Screening the Recombinant Clones.

To screen for gene replacement of the mutants tmd 3 and mad 1, the recombinant plasmids had first to be transferred into M. methylotrophus. Transfer of pGSS33 into M. methylotrophus was not always as efficient as might be predicted, and transfer was found to depend upon the physiological condition of both the recipient and donor cells; maximum transfer being obtained when both cell cultures had reached logarithmic growth in liquid culture (Luria broth or methanol/minimal salts). To obtain maximum results from patch-plated recombinants, the donor DH1.R64(drd11) cells on Luria ampicillin (100 µg/ml) plates were used before 36 hours old, while the recipient methylotroph was cultured to mid-logarithmic phase before 100 µl was plated on the recipient plate. Patch-plating carried out in this manner gave clearly defined patches of cells on control methanol ampicillin (100 µg/ml) plates.

Thus cultures of mutant cells were grown to mid-logarithmic phase, and plated on trimethylamine ampicillin (100 µg/ml) for tmd 3, and on methylamine ampicillin (100 µg/ml) for mad 1. Three thousand recombinant clones were screened from the available gene bank, without giving any suppression of the tmd 3 mutation, but two putative-positive patches were obtained from the mad 1 testing.

5.5 Positive Identification of the Putative Mad⁺ Clones.

Isolation of recombinant pGSS33 DNA from the putative positive clones, and the introduction of that DNA back into the mutant mad 1 cells (via DH1.R64(drd11)), resulted in the loss of their mutant phenotype. This positively identifies the two recombinant plasmids as the agents able to restore to mad 1 the ability to utilise methylamine (Figure 5.6 and Table 5.3). These two plasmids, when restricted with EcoR1, were both found to contain an 11 kb insert and further restriction analysis with BamH1 gave four fragments (0.9 kb, 2.3 kb, 3.1 kb and 4.6 kb) in each case, identifying them as the same isolate.

To confirm that this insert contained the gene or genes that would complement the transposon-induced mutation in mad 1, a Southern blot was carried out on DNAs from both the wild-type and mutant mad 1 strains using the 11 kb insert as a probe (Methods, Section 2.5). Because the mutation in mad 1 is due to the insertion of In5, a 5.4 kb element, digestion of the genomic DNAs with EcoR1 (with no sites in In5), produces a larger genomic fragment from the mad 1 DNA (by 5.4 kb) than from that of the wild type strain. The respective genomic DNA fragments are highlighted by the 11 kb probe (Figure 5.7).

GENOTYPE	SUBSTRATE		
	MeOH	IMA	MMA
Wild type	32.4	11.2	39.5
<u>mad</u> 1	30.6	6.7	0.3
<u>Mad</u> +	32.7	12.0	0.0
<u>mad</u> B	31.9	11.8	7.1

Table 5.3. Enzymic Comparison of Wild Type, Mutant and Complemented (Mad +) Cells from Whole Cell Extracts.

Dehydrogenase enzyme activities were measured as the rate of reduction of DCPIP/minute/mg dry weight of cells in the presence of PMS, KCN, NH₄Cl (Methods, Section 2.7).

All cells were cultured in a trimethylamine minimal salts media to OD₆₀₀ of 0.65. spun down and resuspended to 1 mg/ml dry weight of cells before sonication.

The figures represent the arithmetic mean of five individual readings, (standard deviation less than 4.7).

Figure 5.6. Growth of wild type mad 1 and Mad⁺ clones, as measured by optical density OD_{600} in methylamine minimal media.

Original OD_{600} = 0.01, sub-cultured from an overnight trimethylamine-minimal salts media (containing Km^{10} for mad 1, Mad + isolates).

- ▲ Wild type
- ∅ Mad +
- mad 1

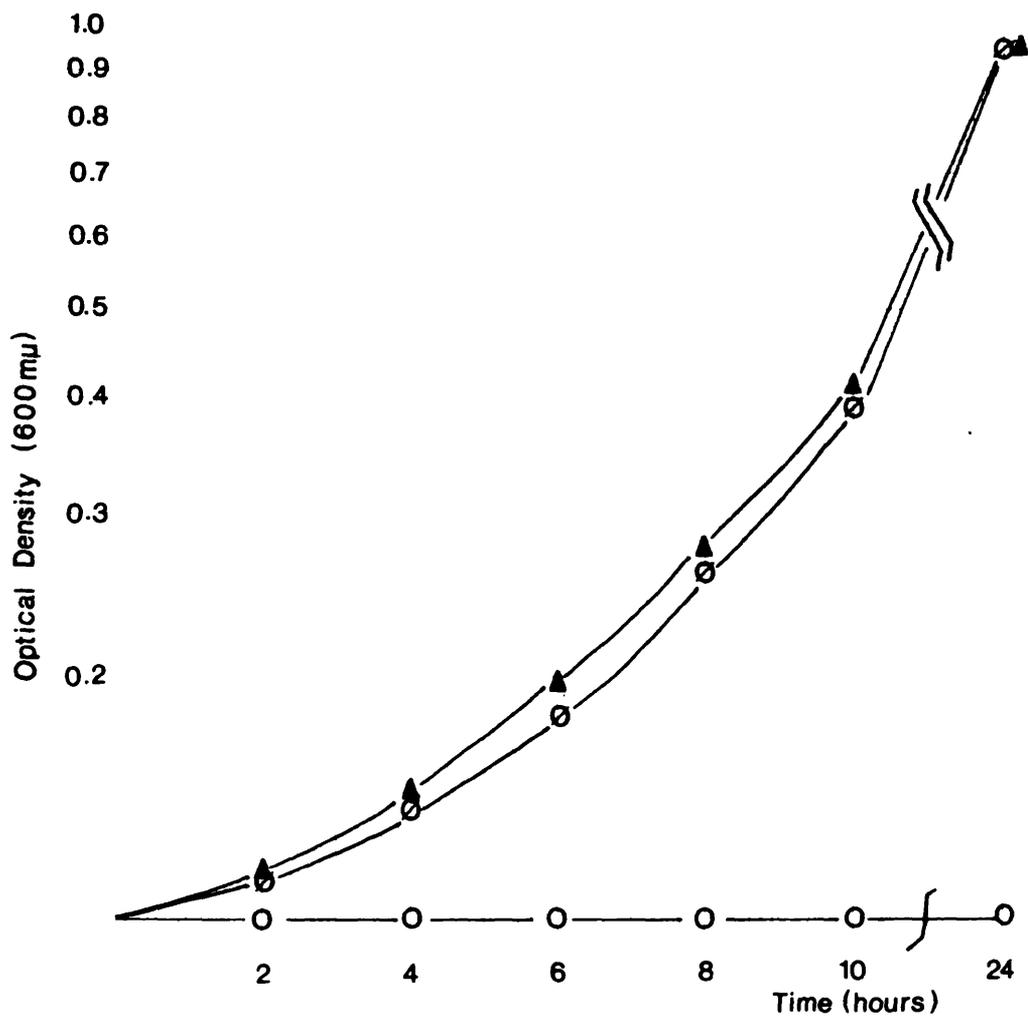


Figure 5.7 Southern blot of EcoRI digested mad 1 and M. methylophilus genomic DNA.

(1) Ethidium bromide stained DNA.

(2) DNA transferred and probed with ³²p-labelled DNA containing 11 kb DNA fragment.

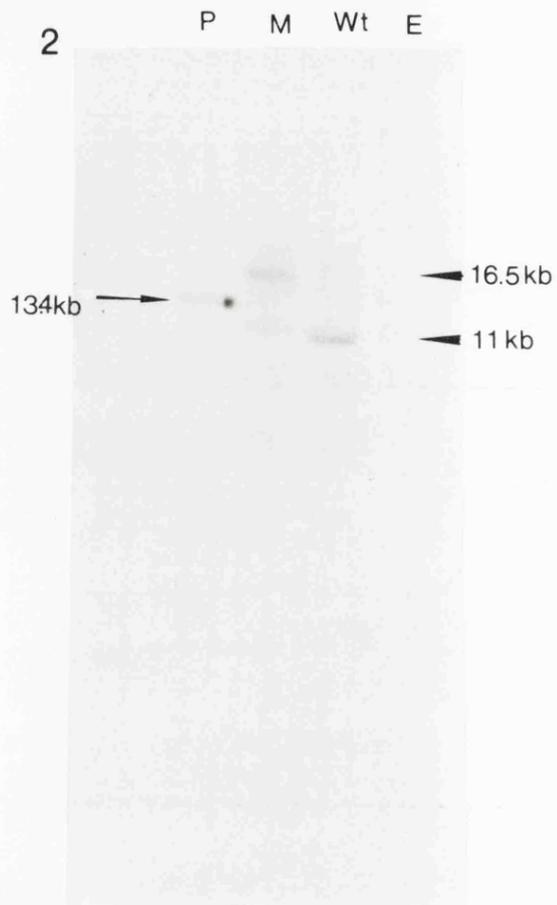
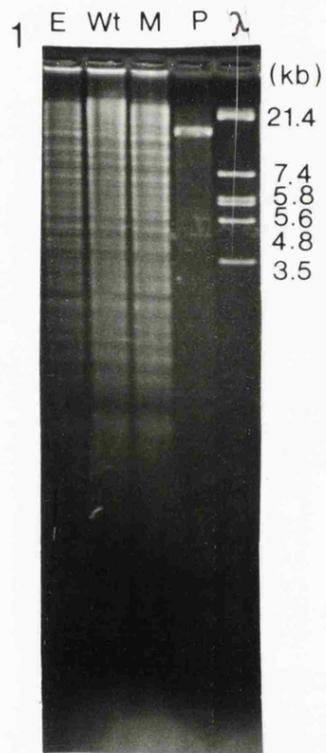
(E) E. coli genomic DNA.

(W) M. methylophilus genomic DNA.

(M) mad 1 genomic DNA.

(P) PGSS33 (linear).

(λ) λphage EcoRI DNA



5.6 Restriction Analysis of the 11 kb Mad⁺ Isolate, and sub-cloning to 2.3 kb.

To facilitate the subsequent sequencing of the gene that has conferred upon the mad 1 mutant the ability to utilise methylamine, the 11 kb fragment of M.methylophilus DNA which reversed the phenotype of the mad 1 mutant, was digested with BamHI. This digestion produced four fragments from the fragment of 0.9 kb, 2.3 kb, 3.0 kb, and 4.7 kb in length. Assuming that there is not an unfortunate distribution of restriction sites within the gene, one of these sub-fragments might now contain the wild-type methylamine dehydrogenase gene(s) (Mad⁺) that has allowed complementation of the transposon-induced mutation in mad 1.

To ascertain which of the fragments contain the Mad⁺ gene(s), these fragments and their mutated DNA counterparts in totally digested EcoRI/BamHI genomic mad 1 were probed with the original 11 kb fragment, labelled with ³²P. Because the transposon In5 contains no EcoRI digestion sites and only one BamHI site (near the centre of the transposon, Figure 4.1), the DNA containing the mutant gene coding for methylamine utilisation will be restricted into two fragments, cut in the transposon insertion. The effect of this, highlighted by the probe, will be to split one of the BamHI bands of the wild type into two bands. This confirms not only that the Mad⁺ gene(s) is contained within this fragment but that the mutation is truly due to transposon insertion. Figure 5.8 shows the result of the Southern blot, using EcoRI/BamHI digested mad 1 genomic DNA and BamHI

Figure 5.8. Southern blot of EcoRI/BamHI digested mad 1.
and M. methylophilus genomic DNA.

(1) Ethidium bromide stained DNA.

(2) Dna transferred to nitrocellulose and probed
with 32p-labelled 11 kb EcoRI fragment.

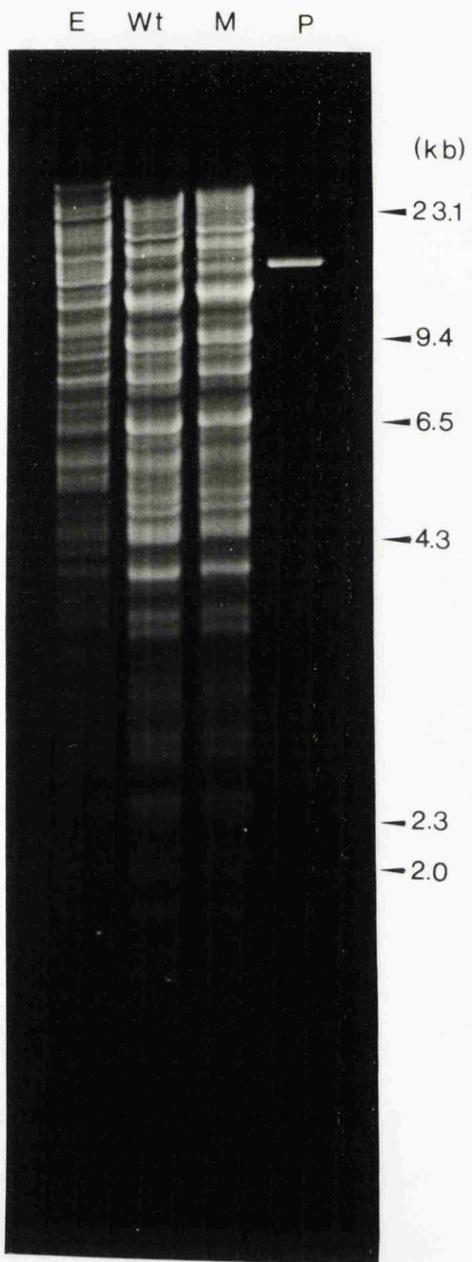
(E) EcoRI digested Ecoli genomic DNA.

(W) M.methylophilus genomic DNA.

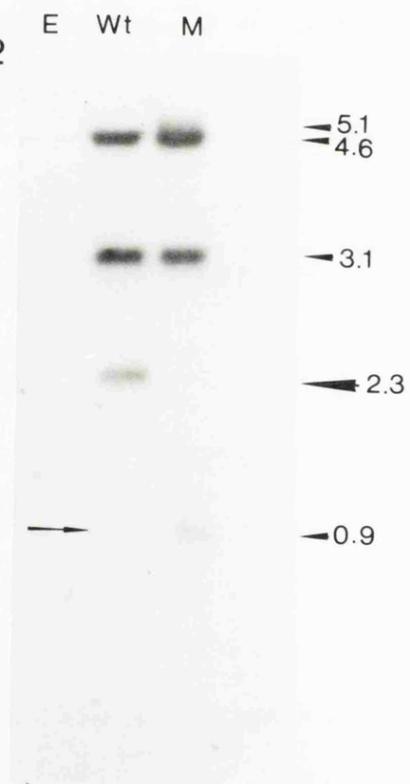
(M) mad 1 genomic DNA.

(P) linear pGSS 33 .

1



2



digested fragment DNA (track 2) as the control. The transposon insertion seems to have occurred in the 2.3 kb sub-fragment, as the corresponding fragment in the mutant genomic digest has been split into a 5.1 kb band and a 2.5 kb band, which is not visible to the eye, possibly because the transposon insertion seems to have occurred within a few hundred base pairs of one end of the 2.3 kb fragment, where the stringent washing procedures used might remove the probe. From this we can deduce that the gene which is affected by the transposon insertion is also within a few hundred base pairs of the 2.3 kb fragment.

5.7 Production of mRNA for the Methylamine Dehydrogenase

Gene(s)

Isolation of the gene(s) coding for the restoration to mad 1 the ability to utilise methylamine, allows us to use this DNA sequence as a probe to investigate the production of its corresponding messenger RNA (mRNA). It also enables some investigation into expression of the isolated gene, to determine whether control of this gene is at the transcriptional or translational level. Isolation of RNA from M. methylophilus was accomplished via a method adapted from Corbin et al. (1982), Methods section 2.6. Total RNA was isolated over an 18 hour time-course from an original culture of methanol grown M. methylophilus subcultured into a methylamine minimal salts medium (Figure 5.9). During the time-course, ten samples of the culture were removed and the

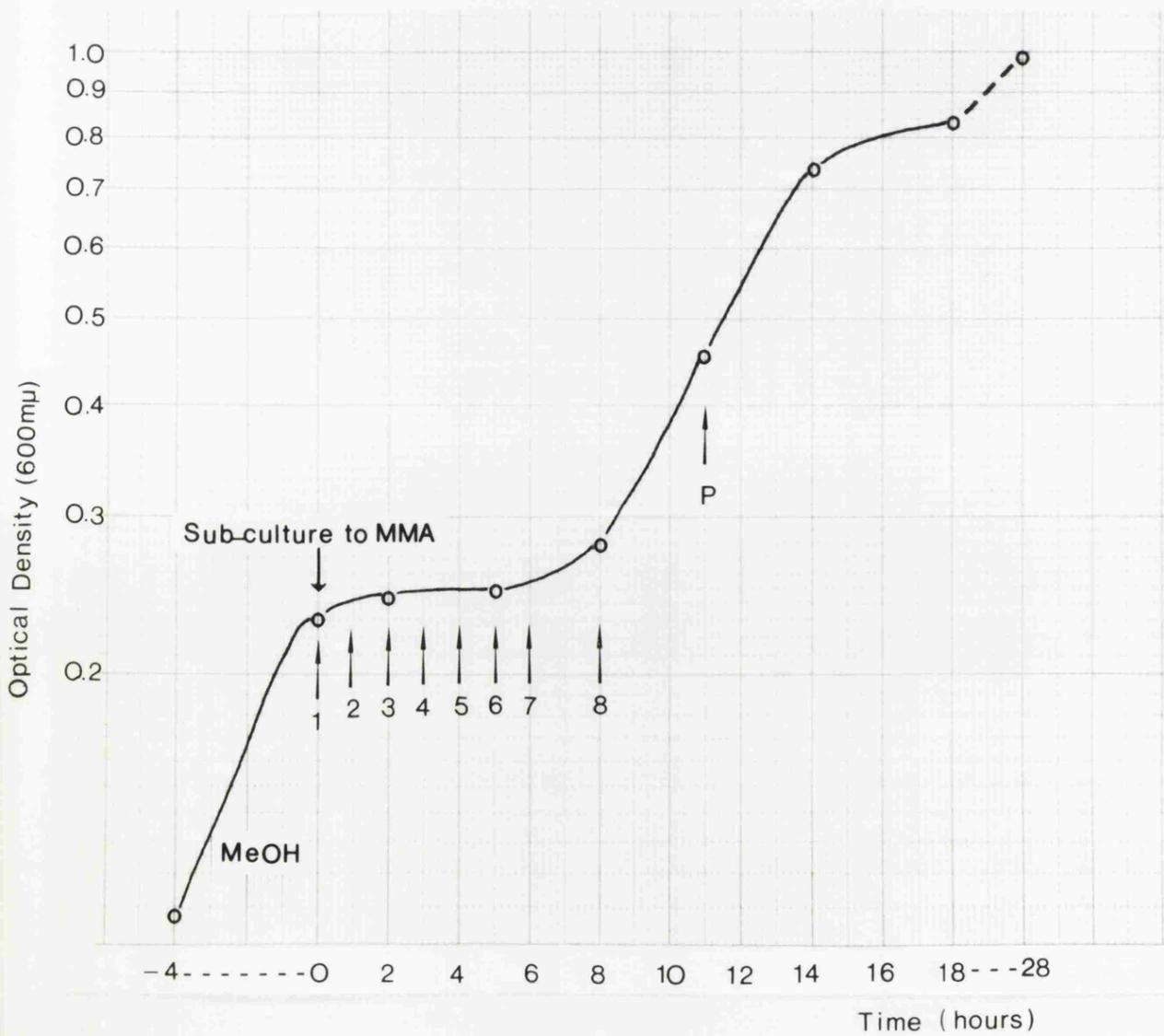
Figure 5.9 Time-Course for the Production of mRNA Related to the Isolated 11 kb Fragment.

1. Shows the appearance of mRNA relating to the 11 kb ³²-P labelled probe upon the introduction of methylamine into the medium.
2. The growth of M. methylophilus cells from a methanol inoculum subcultured into a methylamine supplemented culture. The arrows represent the samples removed for the isolation of RNA and hybridisation with the 11 kb probe.

1

N 1 2 3 4 5 6 7 8 P

2



total RNA immediately extracted from them.

Hybridisation of the DNA probe to the total RNA samples was carried out as described for the Dot Blot hybridisation of RNA to DNA in Methods section 2.6. The results, figure 5.9., indicate that the production of mRNA for these gene(s) is inducible, production of the mRNA occurring upon the introduction of methylamine to the medium.

CHAPTER 6

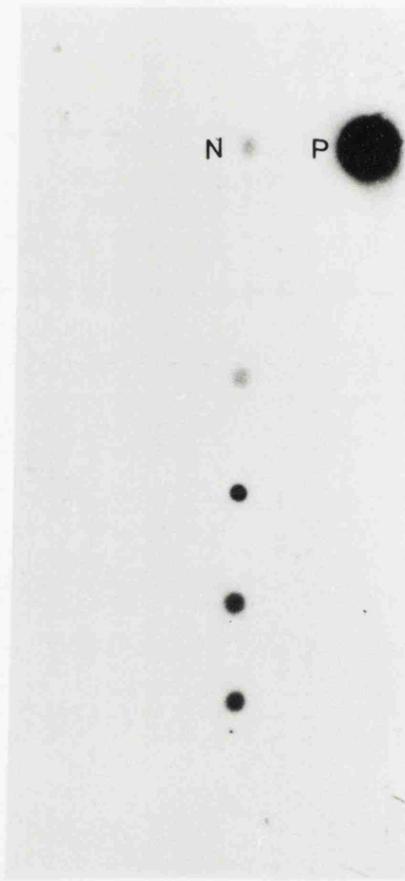
Sequencing the 2.3 kb Fragment

To enable further analysis of the 2.3 kb isolate at its most basic level, it was necessary to determine the sequence of nucleotide bases, thus identifying any regions of possible interest, such as open reading frames and the associated regulatory regions of any possible encoded proteins. To facilitate sequencing the 2.3 kb fragment was cloned into the BamHI site of the M13 vector, M13mp19. Recombinants were identified as white plaques deficient in B-galactosidase, opposed to the blue plaques of the original vector. To ensure the recombinants carried the correct insert, several white plaques were isolated, and the DNA prepared from each hybridised to the original 2.3 kb fragment (Figure 6.1). Those clones which gave a positive result were I-tracked to isolate the fragment inserted in both orientations.

Two sequencing strategies were followed, both detailed below, and their contribution to the total sequence data summarised in Figure 6.2. A sequencing gel is shown in Figure 6.3.

Figure 6.1 Hybridisation of M13mp19 Recombinants Containing the 2.3 kb Fragment to the Original 11 kb 32-P Labeled probe.

N	Negative control
P	Positive control
1 - 4	Samples



N

P

1

2

3

4

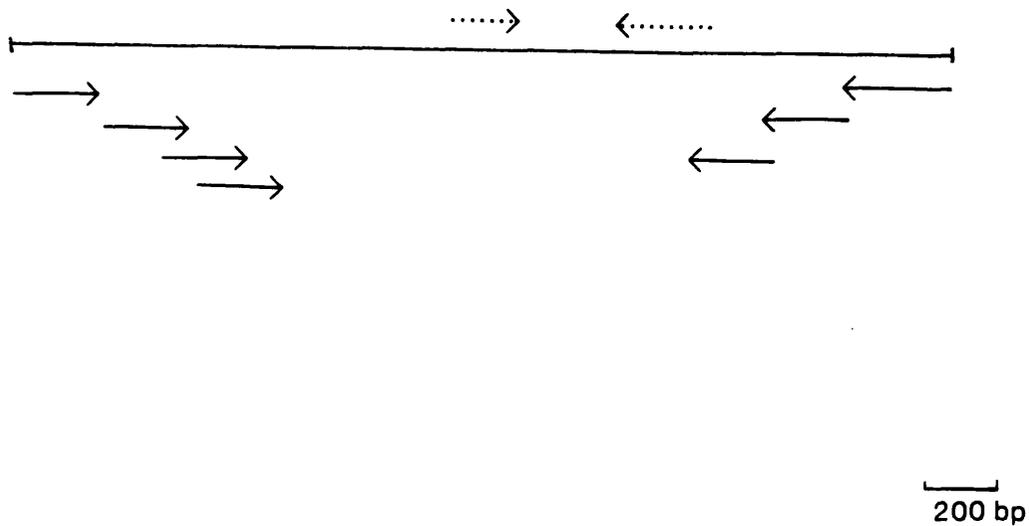
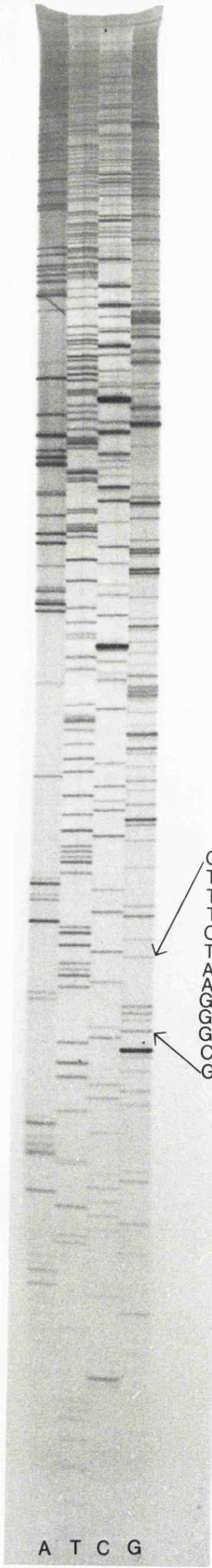


Figure 6.2 Summary of the Sequencing Strategies.

Arrows dictate the direction of sequencing and the length of individual sequencing runs. Unbroken arrows represent the oligonucleotide primer sequencing strategy, while the broken arrows represent the DNase I strategy.

Figure 6.3 A Sequencing Run; A Portion of the Gel has been Deciphered.



G
T
T
T
C
T
A
A
G
G
G
C
C
G

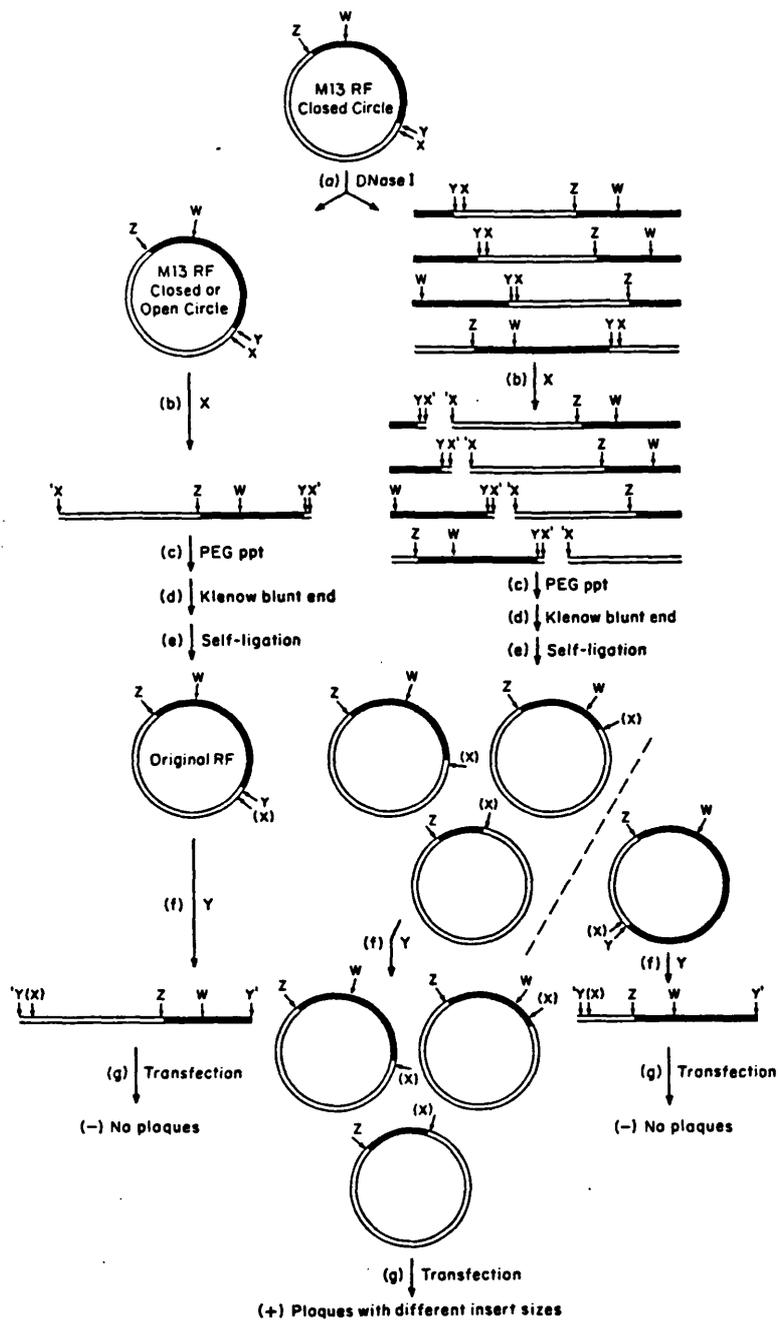
A T C G

6.1.1 A DNA sequencing strategy based on DNase I digestion, an improved DNA sequencing strategy by Lin, Lei and Wilcox 1985.

Improvements to Sanger's original dideoxy chain-termination method of DNA sequencing (1977) have been reported (Frischauf et al. 1980; Anderson 1981; Poncz et al. 1982; Hong 1982; Guo and Wu 1982; Deininger 1983; Henikoff 1984; Laughton and Scott 1984) using the single-stranded bacteriophage M13 as in Messing et al. (1981). The major problem in all of these methods, and in this thesis, due to the restricted number of endonuclease cleavage sites, is the generation of sub-clones: random sub-cloning of restriction fragments must overcome the preference for cloning certain DNA fragments (Anderson 1981) while the "shotgun approach" (Anderson 1981) has difficulties in finishing small gaps in the otherwise contiguous sequence.

The procedure used in this work is a modification of the strategy put forward by Lin, Lei and Wilcox (1985), originally based on Hong (1982): it is summarised in Figure 6.4. The details of the method are given in Methods, section 2.5. The appropriate amount of DNase I to use per μg of DNA, determined in the Methods section, was based on the sample showing least smear on the gel, yet still containing a fair proportion of linearised DNA, samples 3, 4 and 5 in Figure 6.5. Those samples with a high smear content were ignored to reduce the possibility of double digestions within the insert region, which could later confuse interpretation of the DNA sequence.

The purpose of the first restriction digest is to release part of the insert to generate the desired deletions, as shown in Figure 6.4, and to linearise any DNA not linearised in the



Outline of the sequencing strategy. W, X, Y, and Z represent different restriction endonuclease recognition sites. Solid lines indicate the insert DNA. Open lines indicate the M13 DNA. The priming site resides next to the X site.

Figure 6.4 Sequencing Strategy of Lin, Lei, and Wilcox (1985)

Figure 6.5 DNase I Treatment of M13mp19 recombinant RF DNA.

Panels (A) and (B) represent the two recombinants with the insert in opposite orientations.

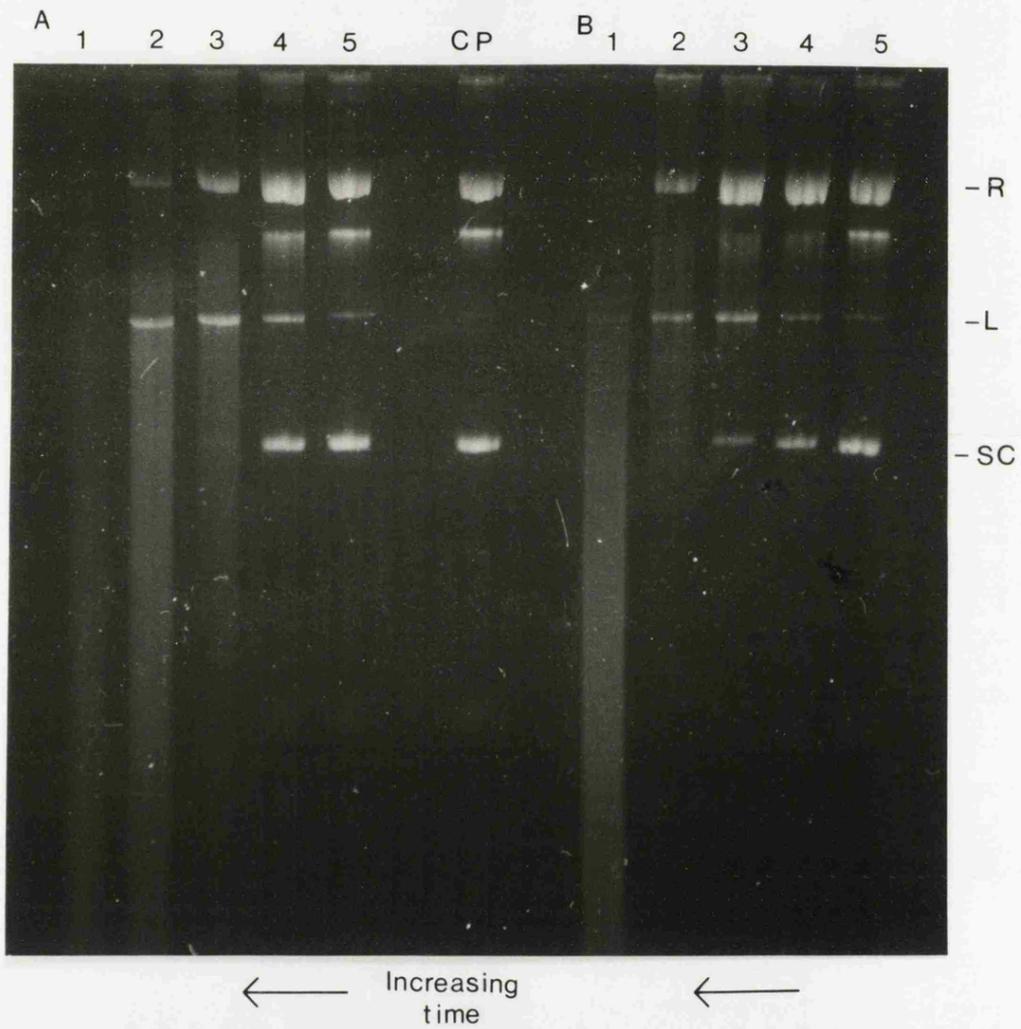
Track (1)- (5) DNA treated with decreasing time the same concentration of DNase I, Methods 2.5.

CP = Closed replicative form.

SC = Super coiled DNA

L = Linear DNA

R = Relaxed form of DNA



DNase I treatment. The addition of PEG precipitates the larger fragments of DNA, thus removing smaller fragments from the subsequent ligation stage. This stage is facilitated by the addition of Klenow polymerase to produce blunt ends for self-ligation. Lin, Lei and Wilcox (1985) report that after this ligation most of the circular DNA's were the original clone with the first restriction site altered; background reduction relies on the second restriction endonuclease cleavage. Elimination of the original clones thus relies on the sub-clones not containing the second restriction site if they have been cut within the insert by DNase I. However, although Lin, Lei and Wilcox observed a twenty-five fold decrease in the number of plaques when the ligated DNA was treated with a second restriction digestion, this work observed only a ten fold decrease in the number of plaques. In addition, subsequent analysis of the sub-clones' DNA by T-tracking showed only three out of thirty-five sub-clones tested to contain deletions of any size, in contrast to the wide range of deletions demonstrated by Lin, Lei and Wilcox (1985).

6.1.2 Using specific probes to "walk" along the DNA sequence.

A more reliable, but time-consuming method of sequencing involves the use of oligonucleotide primers to initiate extension of the Klenow reaction, Methods section 2.5.

The initial sequence data were obtained by using Universal Primer, but subsequent sequence data were obtained by using 17

bp long oligonucleotides as 'internal primers to overlap and extend the previous sequence data. Oligonucleotides were kindly supplied to order by John Kyte, University of Leicester, Biochemistry department.

6.2 Analysis of the Sequencing Data using the Wisconsin Package, University of Wisconsin.

The sequencing data is shown in Table 6.1, showing data from one end of the fragment and Table 6.2, giving data from the opposite end of the fragment. The following computer programs were used to analyse these data.

SEQED

SEQED uses the screen of the computer terminal as a window into the sequencing data. The sequencing data, once entered, can be edited.

FRAMES

Frames identifies all start and stop codons, and will print out a diagram of the open reading frames between these codons. Frames will ignore any start codons within an open reading frame, and any stop codons outside an open reading frame.

10	30	50
CGGCTTCAGAGCGAGCTGTGCGTGCATGCTGCAGATTTTACTGCTGATAATTGCCAGTCT		
70	90	110
GTGTAGCTGACGCAGCAATTAACAGCACAATACCCGGCAGATAACACATATCCGGCAGCT		
130	150	170
TACTTCGGCAGCTTTGTTTCAGGAAACGTTTCGGCACACGCGTACCAGCGCGCGGAGCGGC		
190	210	230
TGCATATGACCCAACGCAATGCAATAGCATACTGCAGCACCAAGCAGCACGTCGAGCGCG		
250	270	290
TTACGCGATCGTAGTGACGAGCAGTTGCGTACGCGCACACATATTCGCATCTCTTTTTTA		
310	330	350
CTTTTACCTCCCTACATCAGTTATACATTTCTGAGCAGATCGGTGGCAATCATCGAGTTG		
370	390	410
GAATCGGCAATGCTGTTGTTCTGGCXTTTCGGGCAGCGCAGXTTCTTCAGTGAGCTACCG		
430	450	470
GAACTGTTGTTCCCTGTGCTTCATCTTTAAACTGCAACGCACATCGATGGTCGCCAGCGCA		
490	510	530
CTGACCGGAXATCATCAGCXTCAGCTACGCCCAATAAAAACCATCATTGTGCGGCCTCCT		
550	570	590
TACTGTACTTTCTCCACAGCGCACTTGATCTCTTCTTCCCGACAGCGTAGCTTCAGCAT		
610	630	650
CGCCGCTGATACGATGAGTCCGTGTACCGTCATAGACGCATCTGCGCGCAATTGACACAG		
670	690	
AGCATCGCTAATAGCATGCTGCATACAGTA		

Table 6.1.
Sequencing Data. mm 1

CODONPREFERENCE

Codonpreference is a gene-finding tool for comparing the codon usage in each reading frame of a DNA sequence to a model pattern of codon usage.

The program plots a statistic for each reading frame that shows the similarity of the codons in a window of that reading frame to a previously calculated codon usage table. This codon usage table is generated by the program 'Codonfrequency'.

Codonpreference uses the method of Gribskov et al., (1984), to examine the codon usage in a DNA sequence. Codonpreference is useful for locating any protein-coding regions, determining their reading frames, and for estimating the maximum level of expression of a gene. Codonpreference is also useful in locating DNA sequencing errors, where 'frame shift' errors often disturb the pattern of codon choices; thus the correct reading frame may fall at the same time as one of the incorrect frames ascends.

The open reading frames are shown as boxes beneath the plot for their respective reading frames. Start codons are shown as short lines that extend above the height of the box, while stop codons extend below the bottom of the box. Rare codon choices in the sequence are marked below the open reading frame plot; a threshold is set by the operator below which a codon is considered to be rare.

CODONFREQUENCY

Codonfrequency tabulates codon frequencies from sequences and from existing codon usage files.

The program sums the codons used in as many gene regions as required within a sequence and writes a file with the sum of all the observations of each of the possible 64 codons. In addition Codonfrequency will normalise the codon observations to a frequency per thousand and to a fraction for each codon within its synonymous family. The existing codon usage files may be written by Codonfrequency or generated by hand. To write a codon usage table the 'Edit' mode was used.

TESTCODE

Testcode tries to identify protein coding regions in the DNA by plotting a measure of the non-randomness of the composition at every third base.

The program can help identify genes when there is no specific knowledge of the codon choices for the DNA being examined. Testcode plots a measure of the 'period three constraint' of each region of the DNA using a statistic developed by Flickett, J. (1982). The plot is divided into three regions for which the statistic makes predictions: the top region is supposed to predict coding regions to a 95% level of confidence, the bottom region is supposed to predict non-coding regions to a 95% level of confidence, while the

middle region is the 'window of vulnerability' for the method, where the statistic can make no significant prediction. Above the statistic plot there are markings that identify the start and stop codons for each reading frame of the sequence; starts are identified by short vertical lines, and stops by small diamonds.

TERMINATOR

Terminator searches for prokaryotic factor independent RNA polymerase terminators according to the method of Brendel and Trifonov (1984a).

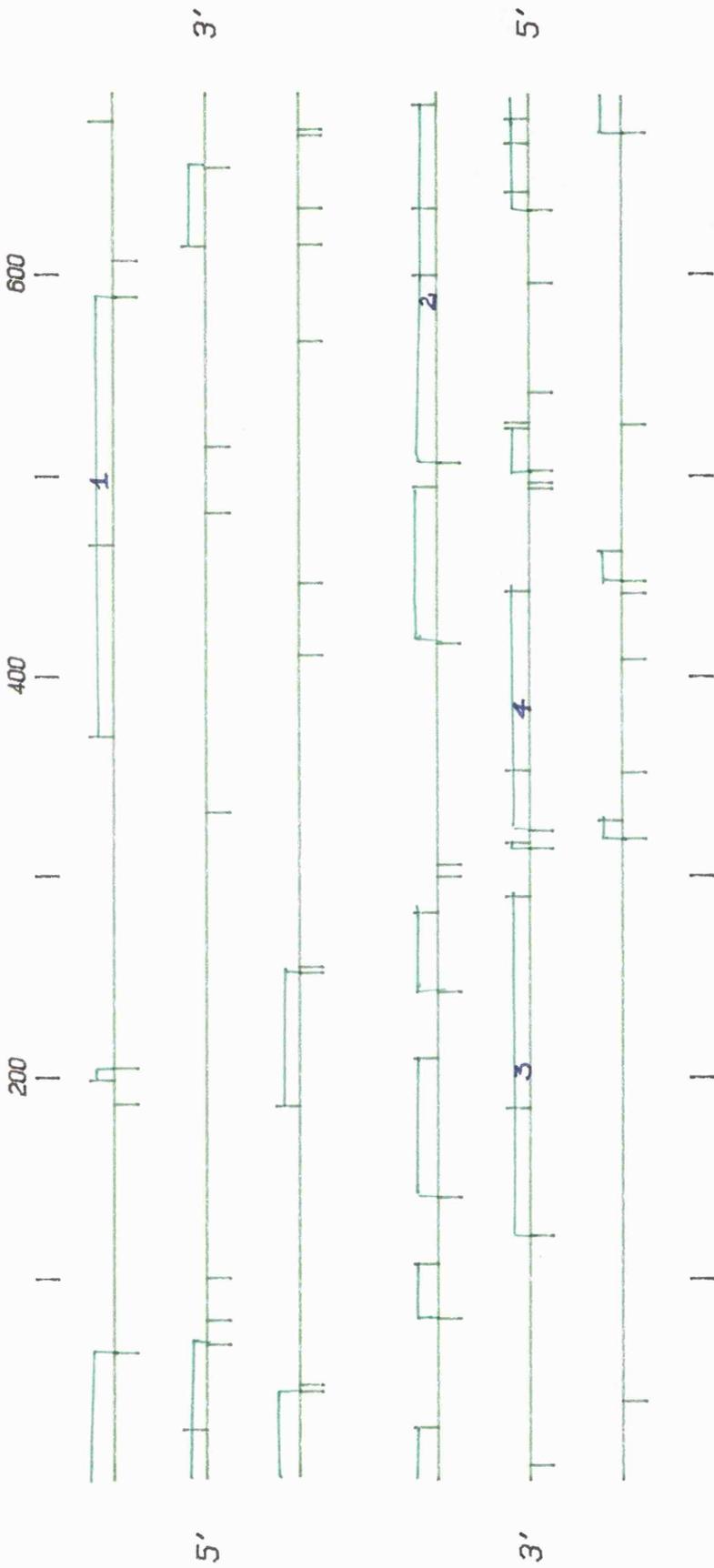
The program uses a table of the dinucleotide frequencies for each position from a set of known terminators to find places in the new sequence where terminator-like sequences occur. The method used by Terminator is described in detail in two papers; Brendel and Trifonov, 1984a , and Brendel and Trifonov 1984b.

TRANSLATE

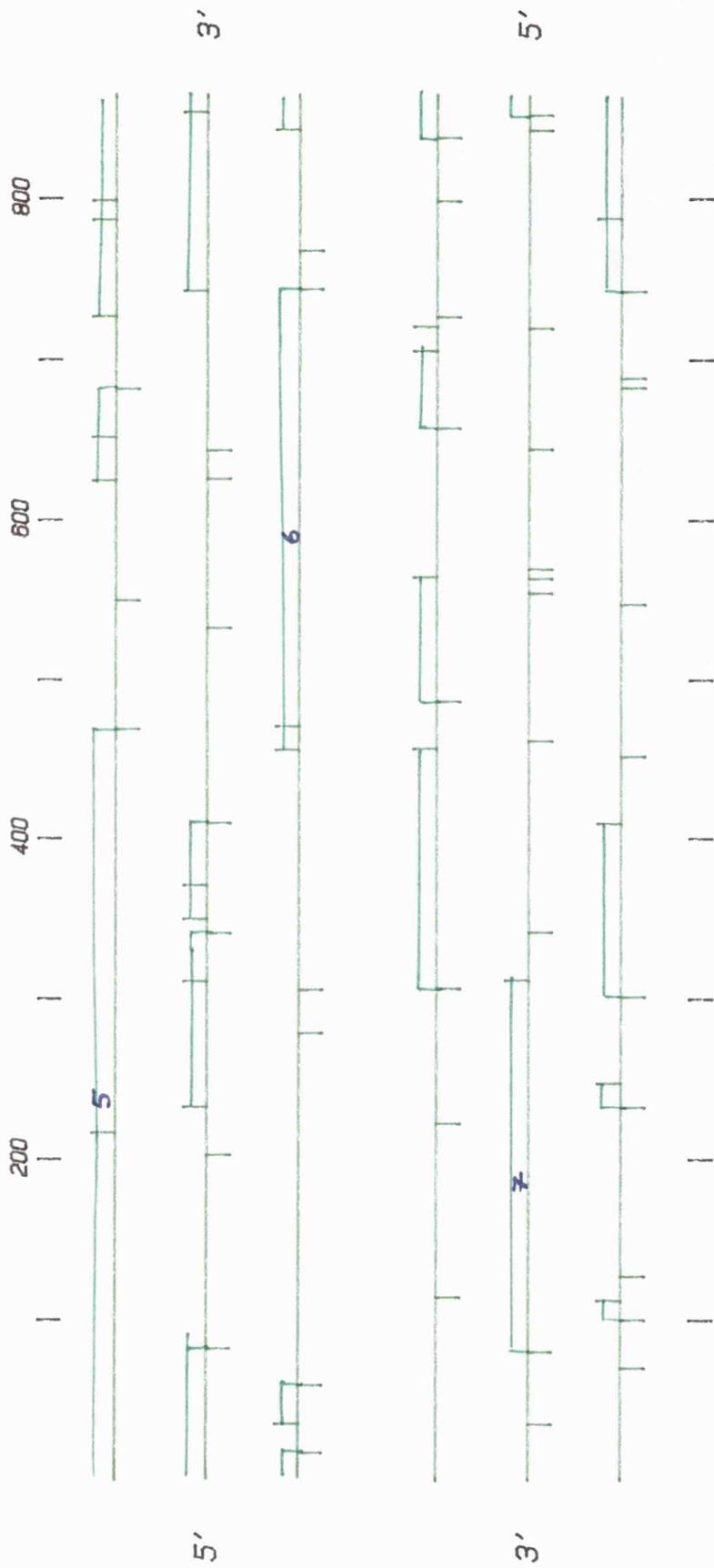
Translate identifies and translates the codons representing the amino-acids, start and stop codons.

6.3 Computer Analysis of the Sequencing Data; Results.

When deciding which of the sub-clones contained the wild-type region of the transposon insertion of mad 1, we also deduced that the mutation point in mad 1 was within ~300 bp of one end on the 2.3kb sub-clone. The sequencing data must thus contain the wild-type region of the transposon insertion position in mad 1. The 'Frames' program reveals some of the possible open reading frames, for each set of data, which occurs between consecutive start and stop codons (Figures 6.6 and 6.7). All other start and stop codons have independently been added (in black). A number of open reading frames are depicted, and any above 100 bp in length have been numbered 1-12. The most interesting frame, ORF-5, is the longest frame, which is over 475 bp in length. This could code for a protein over 16,000 molecular weight, and could be the large sub-unit of methylamine dehydrogenase (Molecular Weight 40,000). Other frames, which could possibly code for the methylamine dehydrogenase small sub-unit (Molecular Weight 13,000), possibly include frame ORF-6, and those frames which are incomplete in the sequencing data, and thus of unknown size. However, because the program Frames is using sequencing data which has been obtained from one direction only, a single unfortunate sequencing error can drastically change the length and position of a frame. To overcome this the program 'Testcode' was used, which uses a statistic analysis dependent not on a single codon, but a 'period three constraint'. In this case a set of statistics which fall above the 95%



FRAMES of: mm1q.dat Ck: 2553, 1 to: 690 10-NOV-1986 09:14

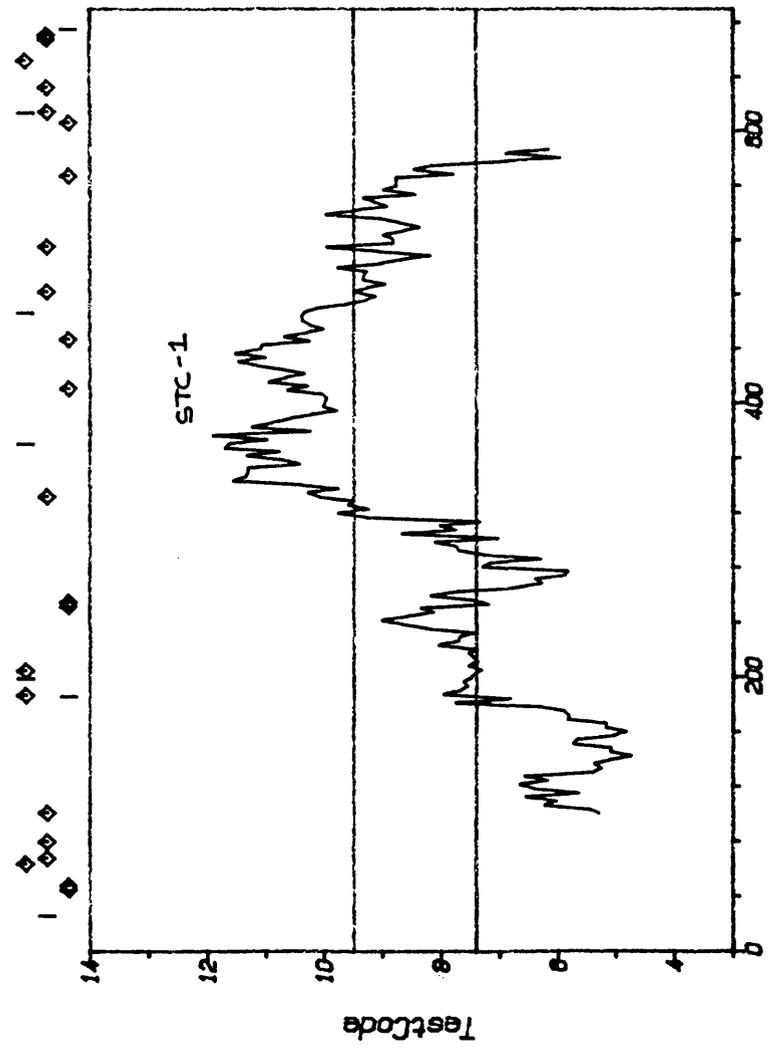


FRAMES of: mm2.dat Ck: 3581, 1 to: 865 10-NOV-1986 09:16

confidence limit can be considered an open reading frame. Figures 6.8 to 6.11 show the results of Testcode on the two sets of sequencing data. Several possible open reading frames are indicated in both orientations, and some of these can be matched to open reading frames in the Frames program. Frame ORF-6 in Figure 6.7 seems to be a good candidate for the set of statistics SIC-3 in Figure 6.10, while SIC-1 may relate to ORF-1 in Figure 6.6. Other statistic values above the 95% confidence limit which may indicate encoded proteins are SIC-2, SIC-4, and possibly SIC-5. These do not however match to any clearly defined frames in Figures 6.6 and 6.7. SIC-2 in particular shows a high confidence level, and this may well be an open reading frame which runs beyond the 5' sequencing data available. It is also interesting to note that the largest open reading frame, ORF-5, in Figure 6.7, does not represent a likely coding region in Testcode, it does in fact enter the 95% non-coding set of statistics.

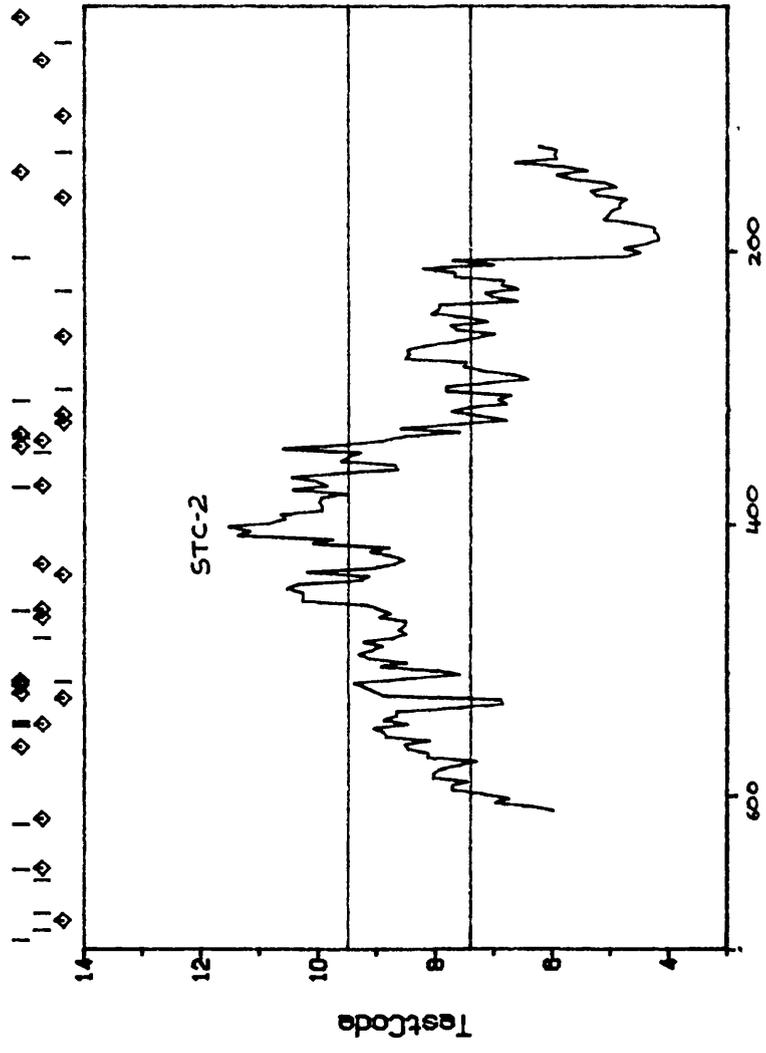
Although Test-code is a 'gene-finder' that plots the measure of non-randomness of the codon choices along a DNA molecule, the program does not provide information on the strand, reading frame, or level of expression of a gene. For this purpose the program 'Codonpreference' was used. Although codon preference tables from E. coli highly expressed genes (Wisconsin Package Data Bank), Figures 6.12-15, and an E. coli gene of low expression (the Irr R gene, Wisconsin Data Bank), Figures 6.16-20, were used, there were no obvious regions in the sequencing data which seemed to correspond to these codon usage tables. A similar result was obtained with

TESTCODE of: mm1q.dat cks 1298, 1 to: 689
Window: 200 bp 7-NOV-1986 11:03



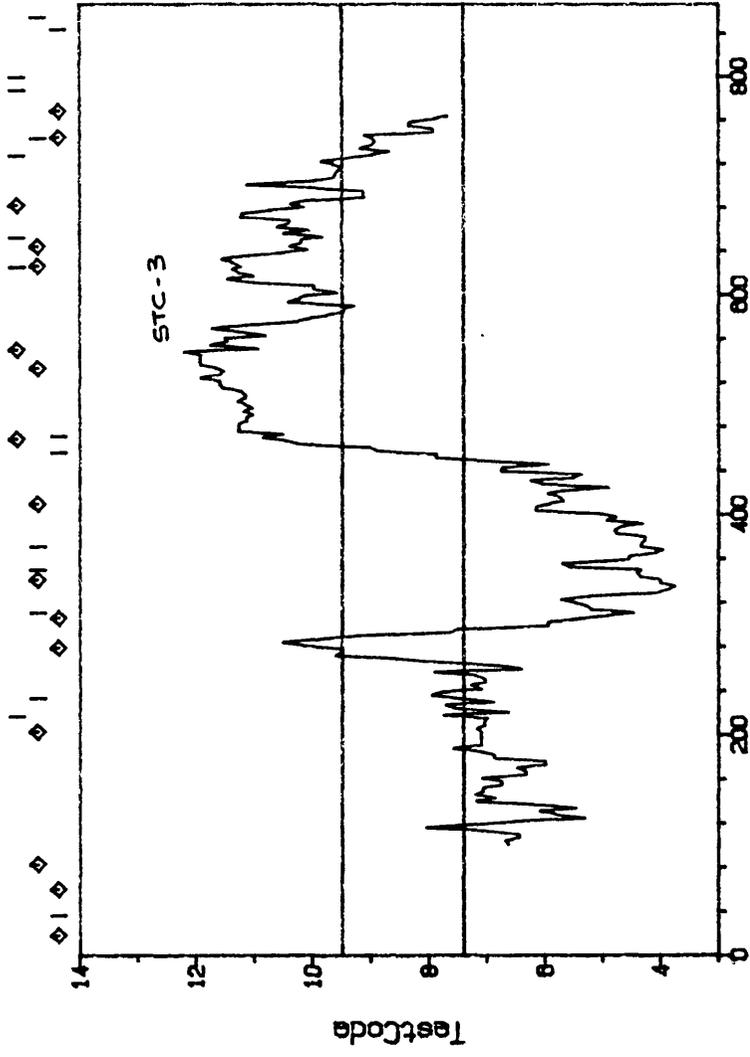
F6.8

TESTCODE of: mm1q.dat cks 1298, 1 to: 689
Window: 200 bp 7-NOV-1986 10:36



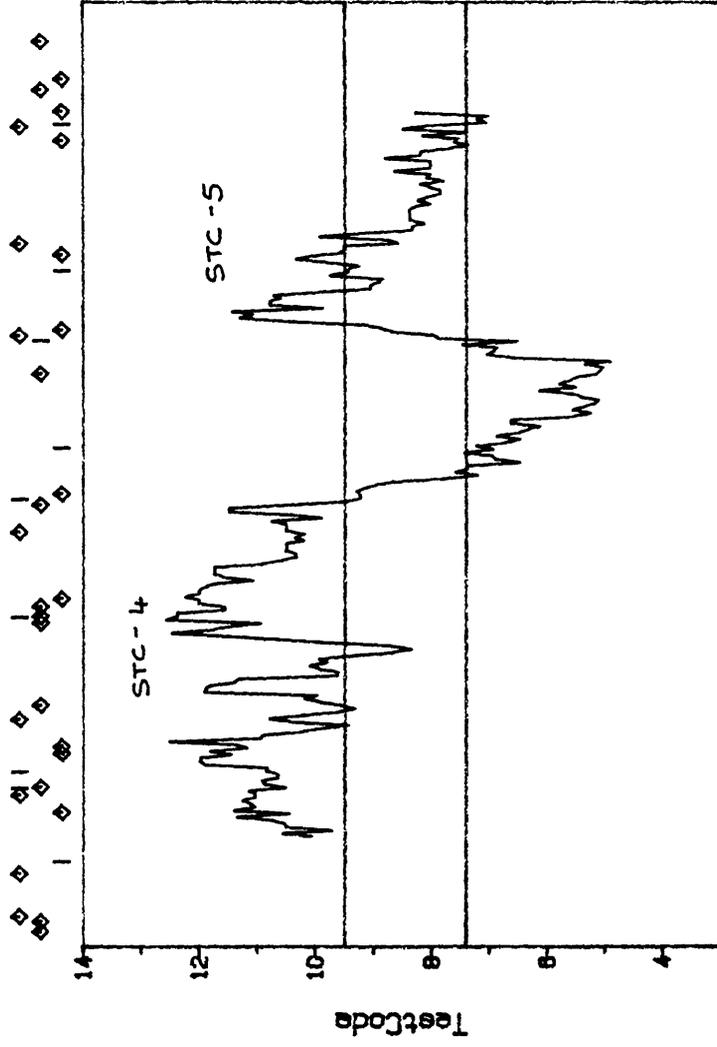
F 6.9

TESTCODE of: mm2.dat cks 3581, 1 to: 865
Window: 200 bp 7-NOV-1986 11:15



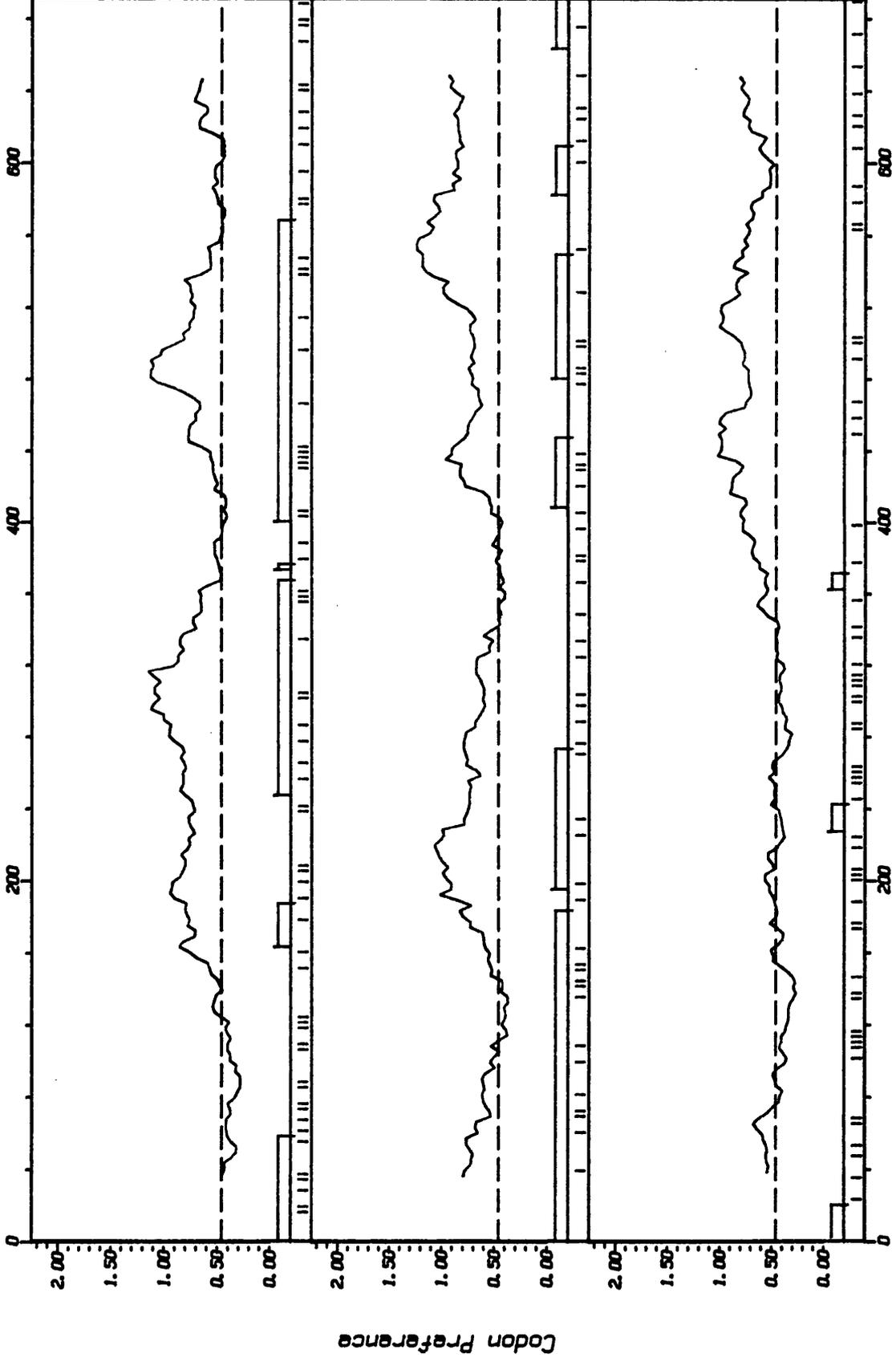
F6.10

TESTCODE of: mm2.dat cks 3581, 1 to 865
Windows 200 bp 7-NOV-1986 11:25

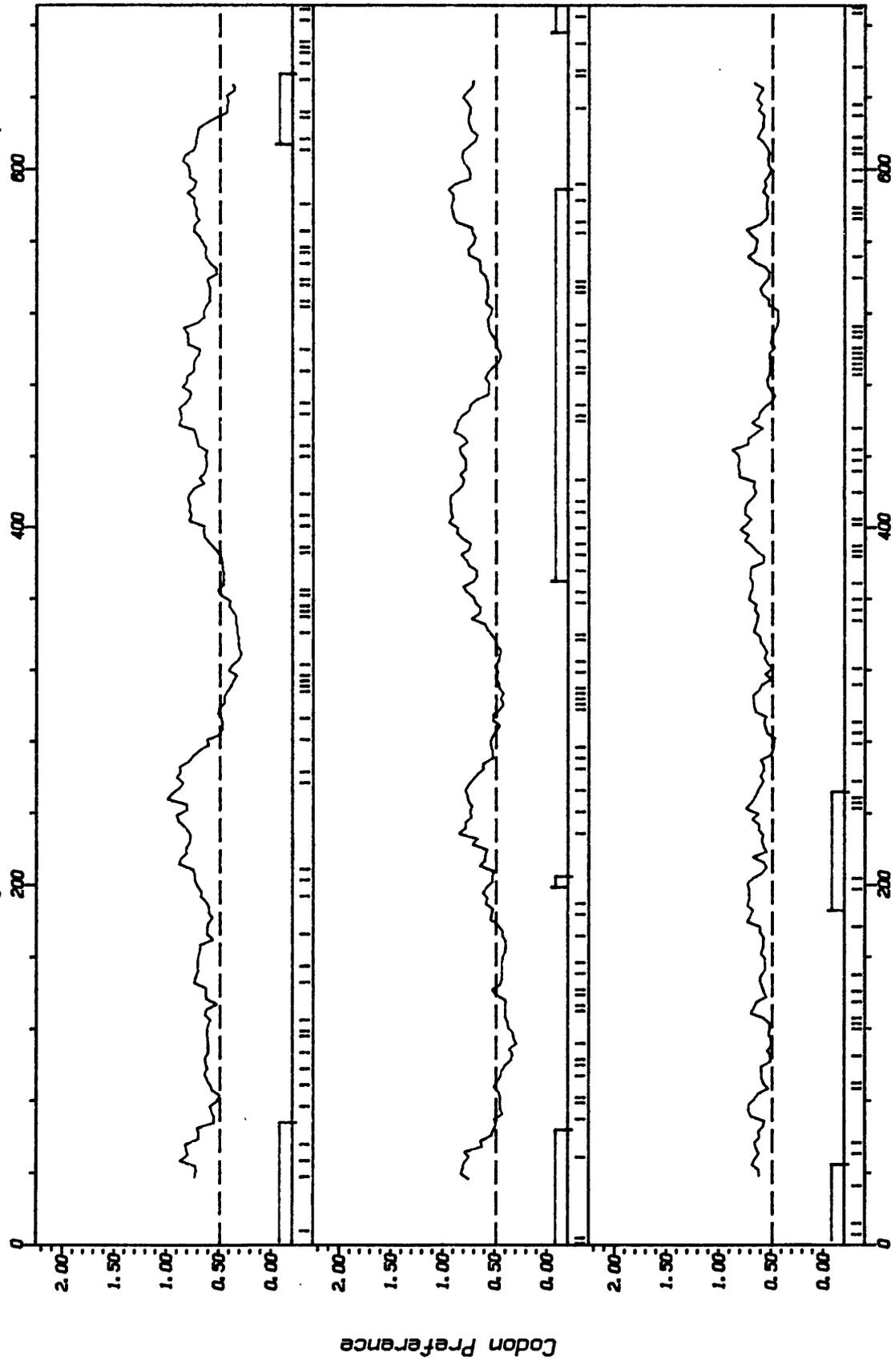


F6.11

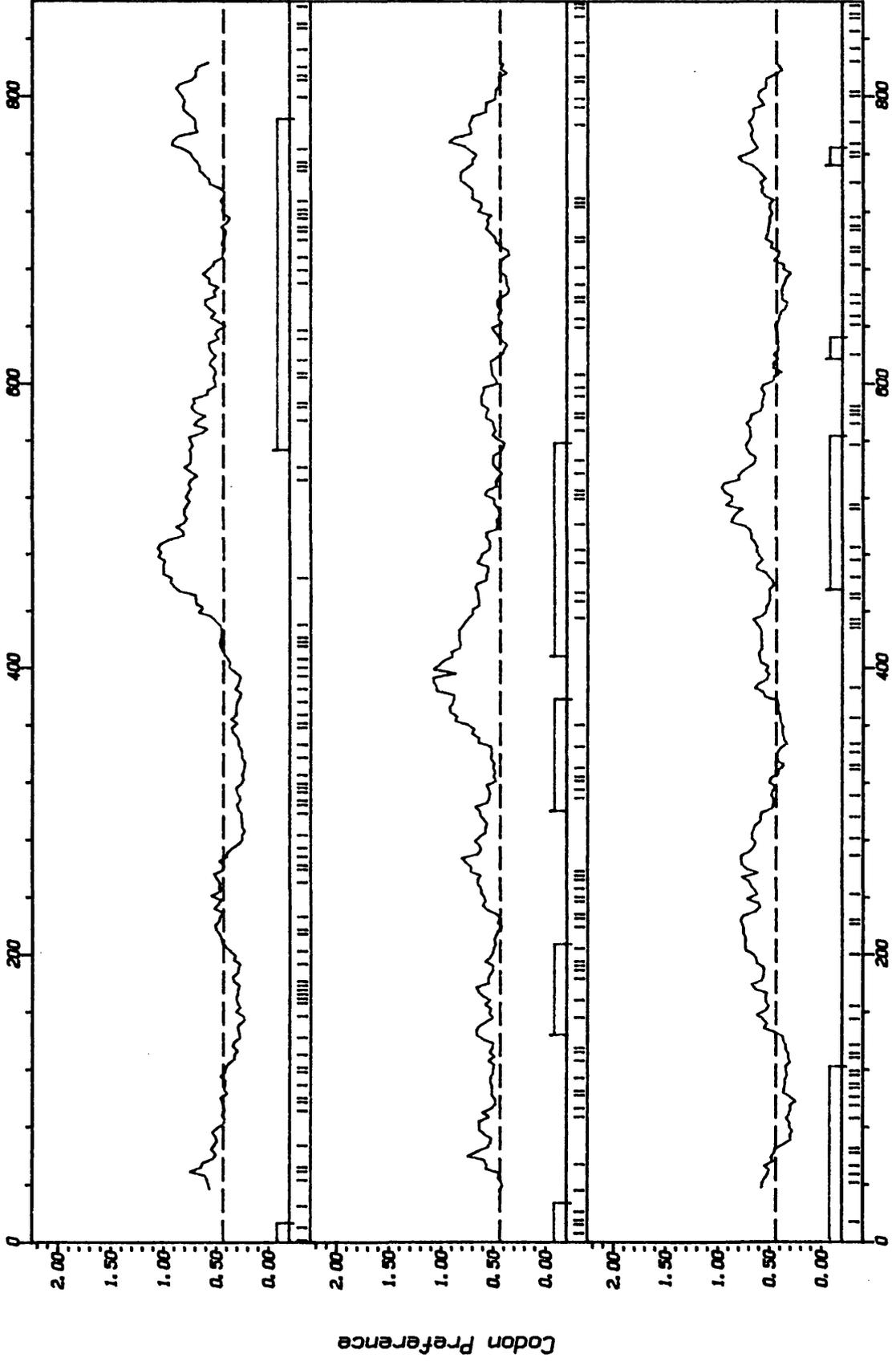
CODONPREFERENCE of: mm1q.dat Ck: 2553, 1 to 690 10-NOV-1986 12:00
Table: GenDocData:EcoHigh.cod FileCk: 5019 Window: 25 Threshold: 0.10 Density: 22.6



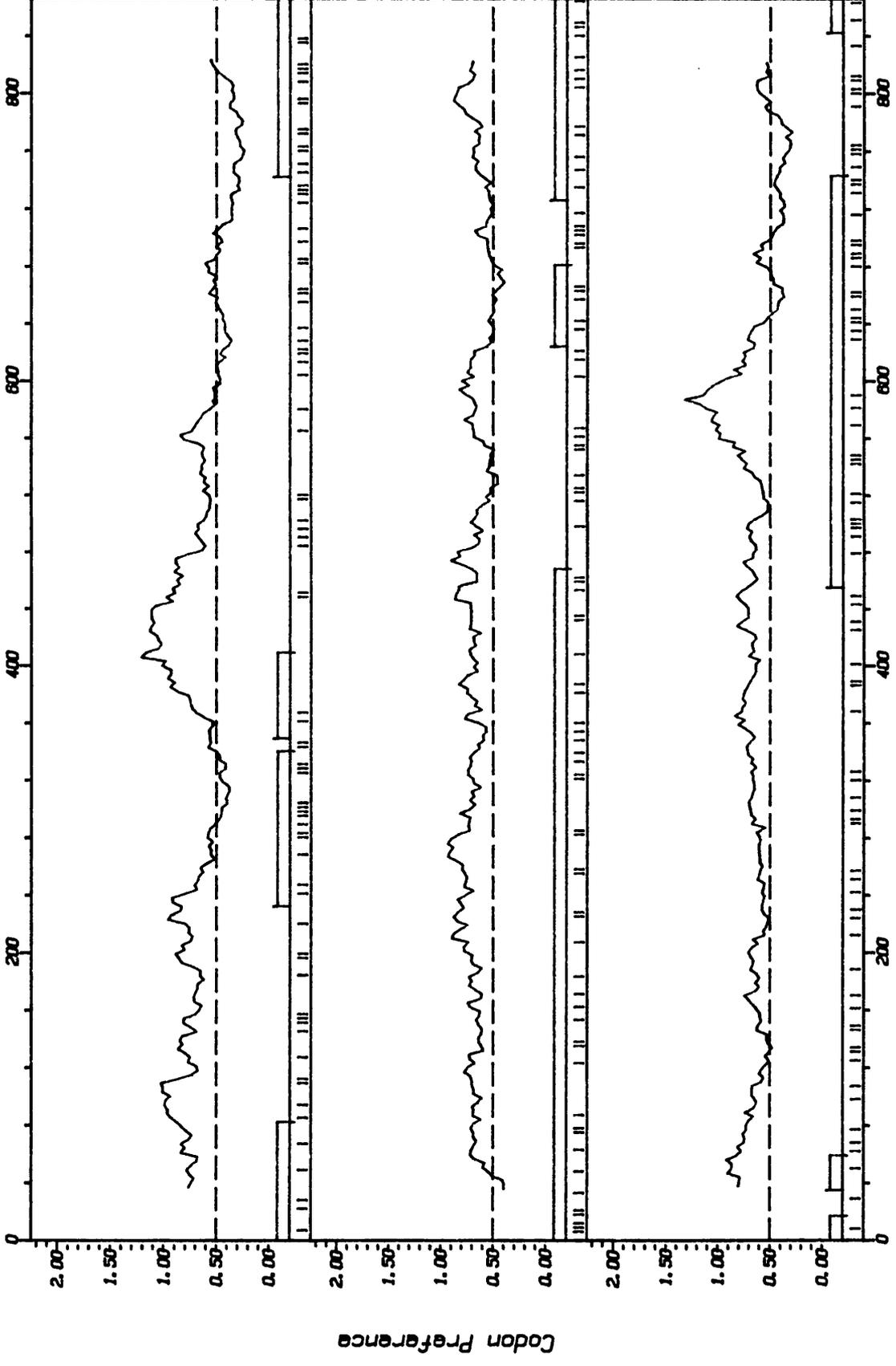
CODONPREFERENCE of: mm1q.dat Ck: 2553, 1 to 690 10-NOV-1986 11:54
Table: GenDocData: EcoHigh.cod FileCk: 5019 Window: 25 Threshold: 0.10 Density: 22.6



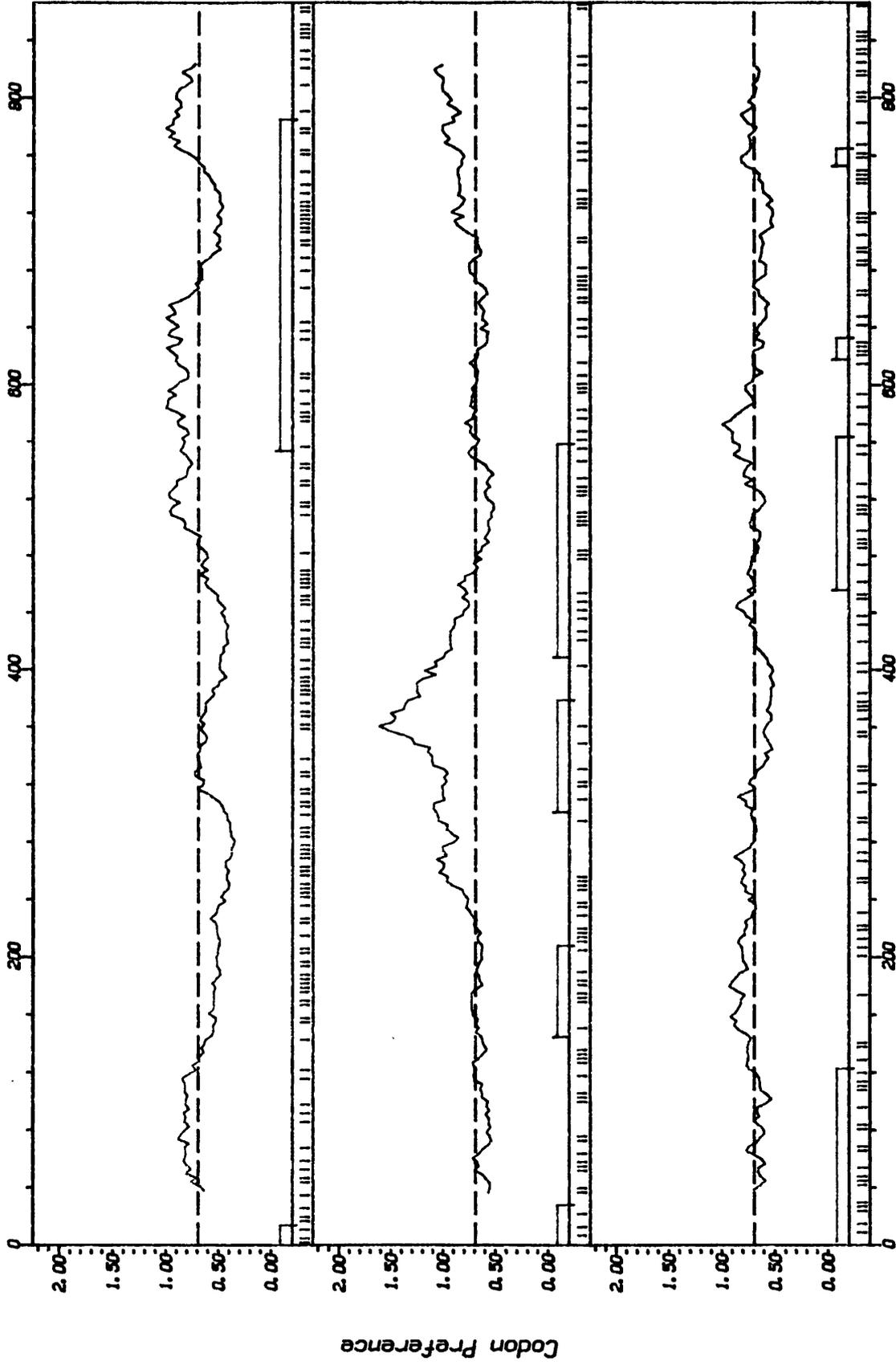
CODONPREFERENCE of: mm2.dat Ck: 3581, 1 to 865 10-NOV-1986 12:32
Table: GenDocData:EcoHigh.cod FileCk: 5019 Window: 25 Threshold: 0.10 Density: 28.4



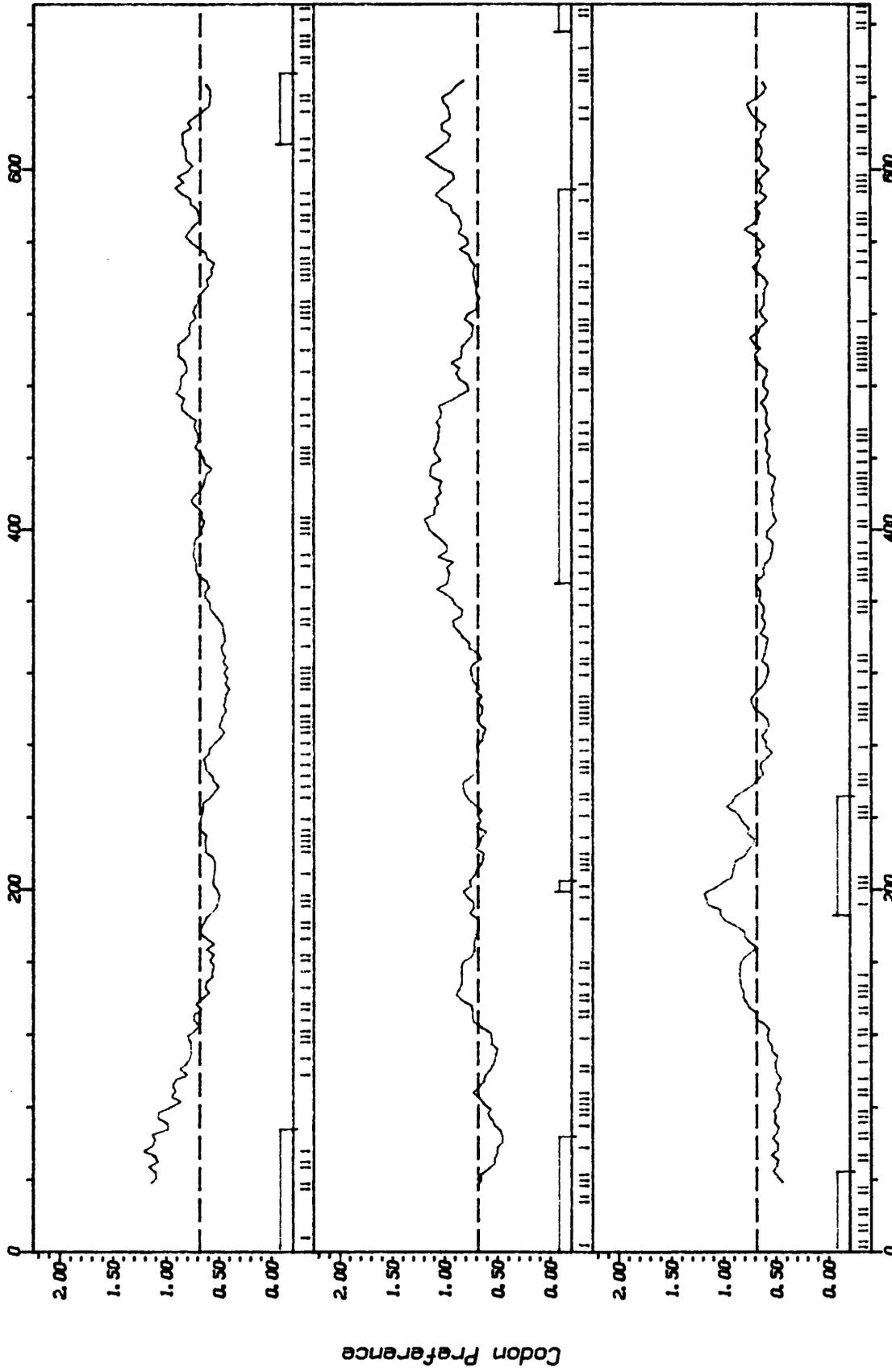
CODONPREFERENCE of: mm2.dat Ck: 3581, 1 to 865 10-NOV-1986 12:27
Table: GenDocData:EcoHigh.cod FileCk: 5019 Window: 25 Threshold: 0.10 Density: 28.4



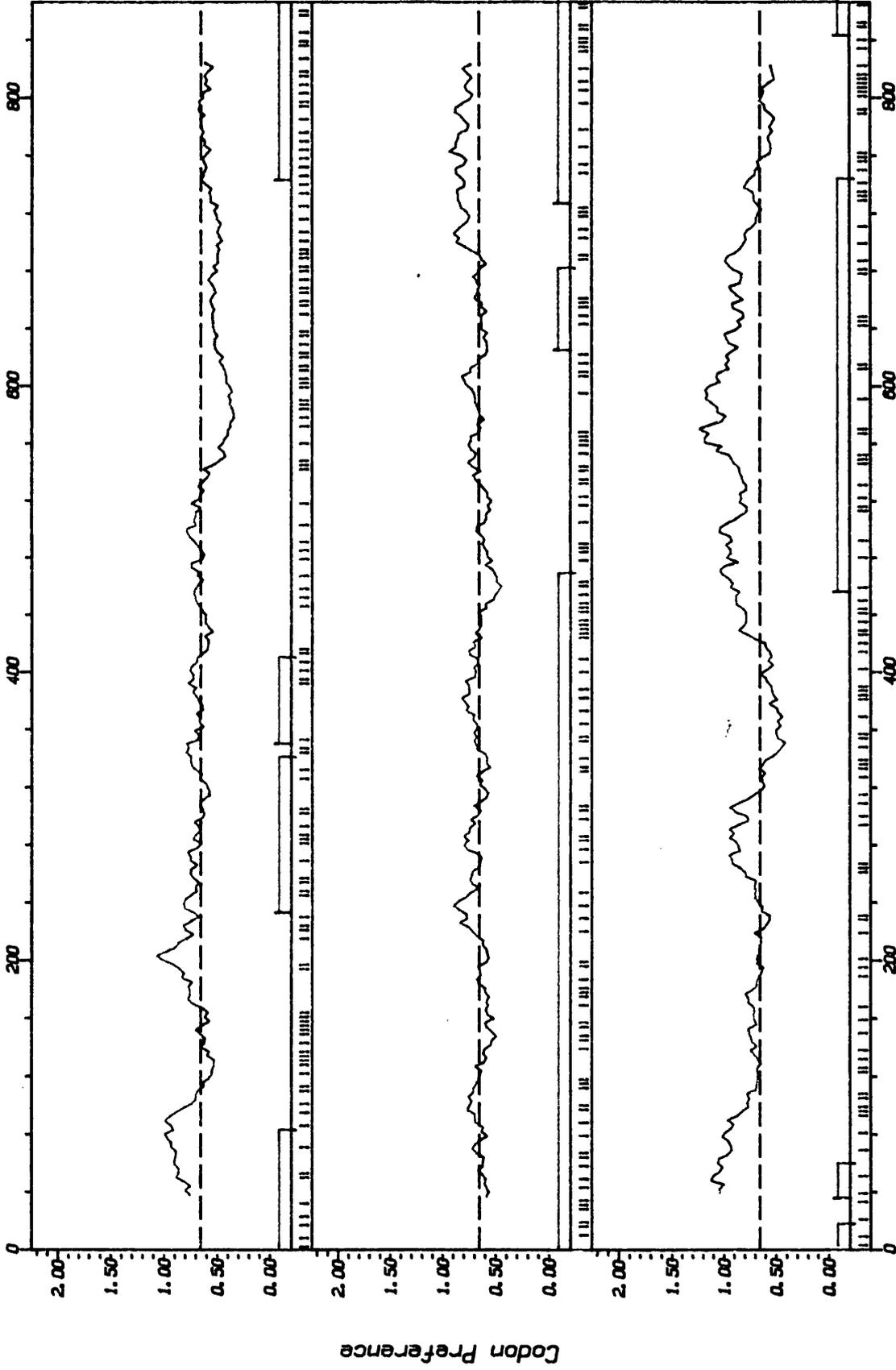
CODONREFERENCE of: mm2.dat Ck: 3581, 1 to 865 10-NOV-1986 13:01
Table: LOWCO cod; 4 FileCk: 2168 Window: 25 Threshold: 0.10 Density: 28.4



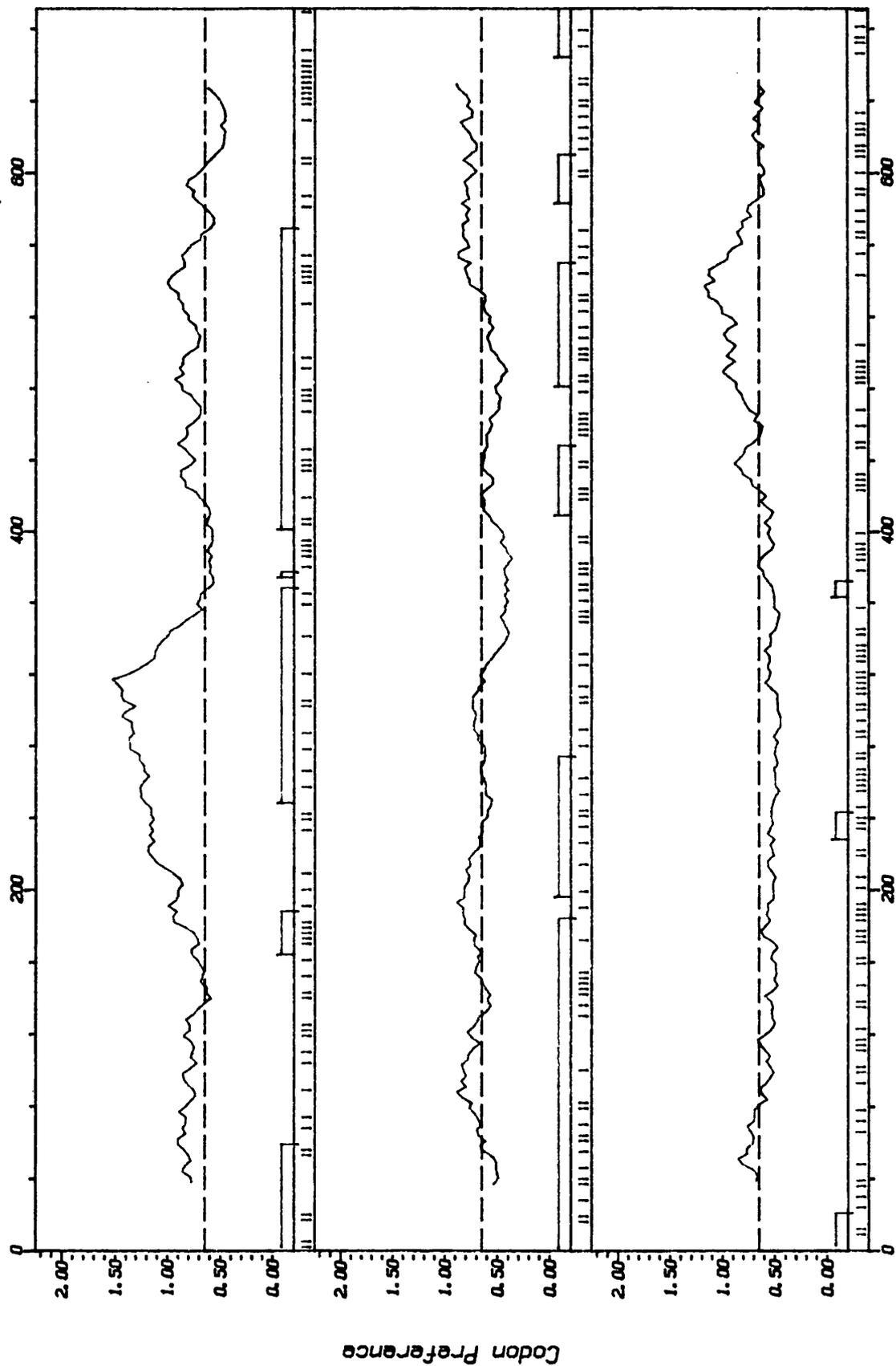
CODONPREFERENCE of: mm1q.dat Ck: 2553, 1 to 690 10-NOV-1986 12.43
Table: LOWCO cod: 4 FileCk: 2168 Window: 25 Threshold: 0.10 Density: 22.6



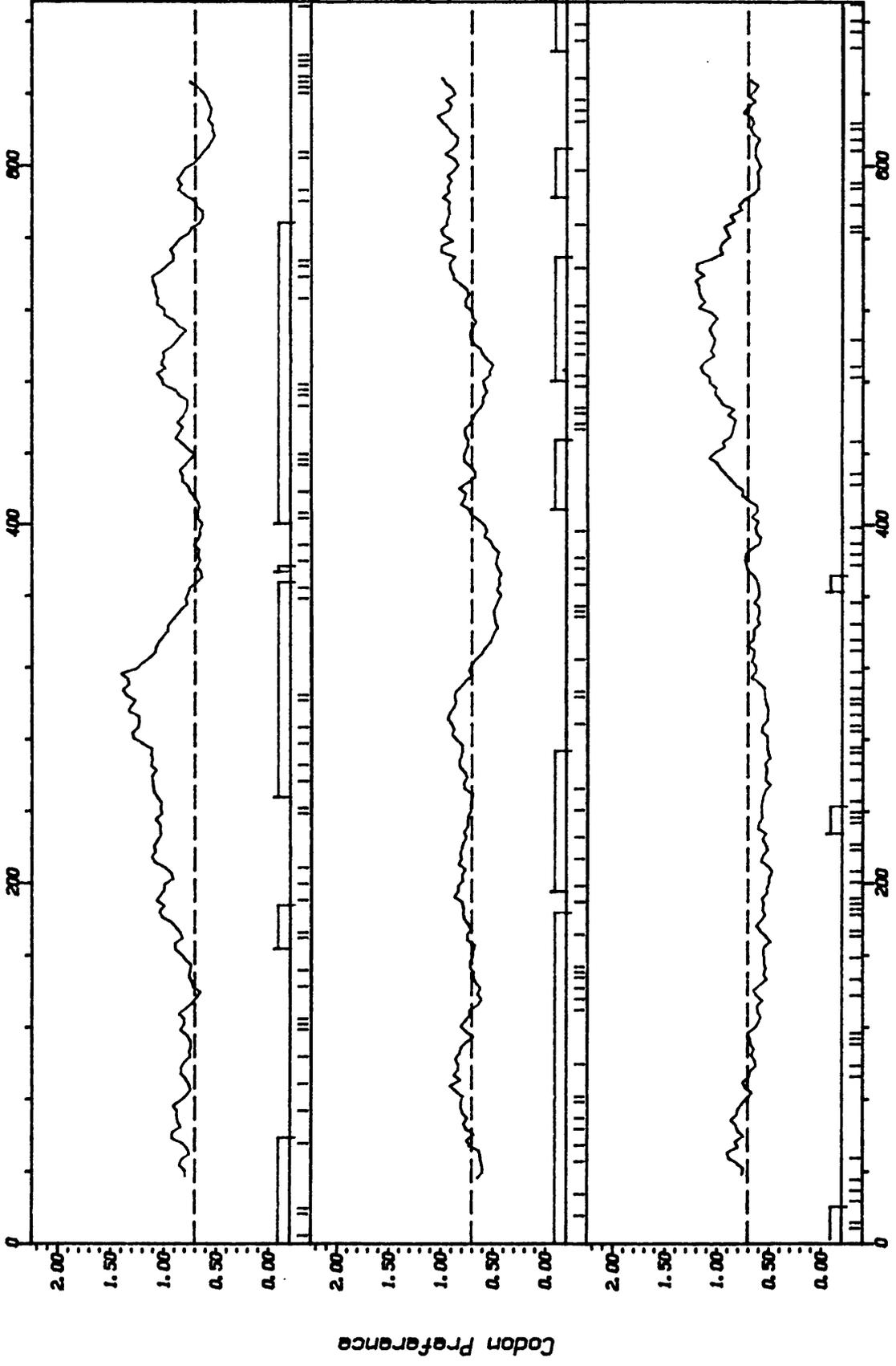
CODONPREFERENCE of: mm2.dat Ck: 3581, 1 to 865 10-NOV-1986 12:55
Table: lowco cod: 4 FileCk: 2168 Window: 25 Threshold: 0.10 Density: 28.4



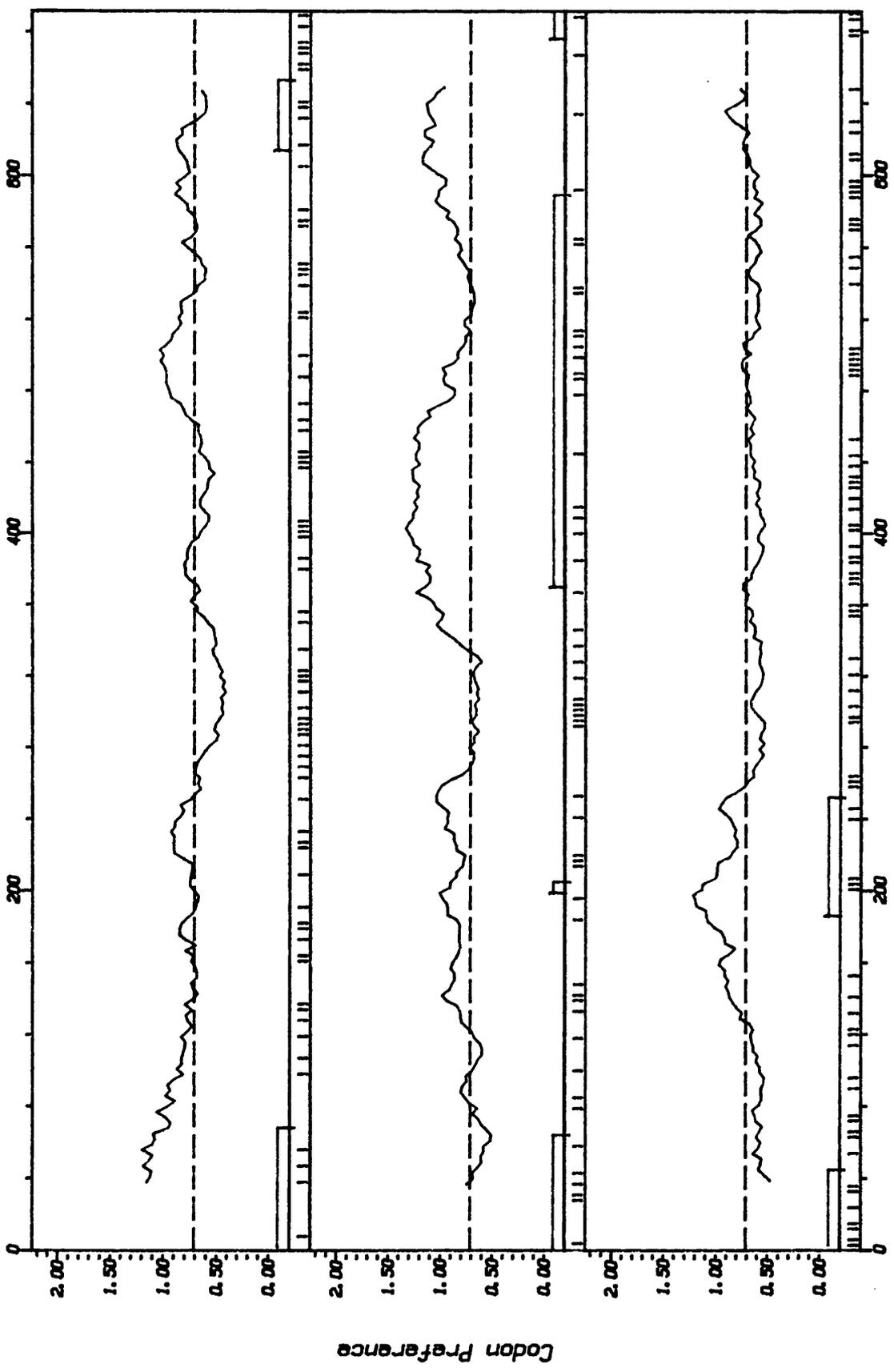
CODONPREFERENCE of: mm1q.dat Ck: 2553, 1 to 690 10-NOV-1986 12:49
Table: lowco cod:4 FileCk: 2168 Window: 25 Threshold: 0.10 Density: 22.6



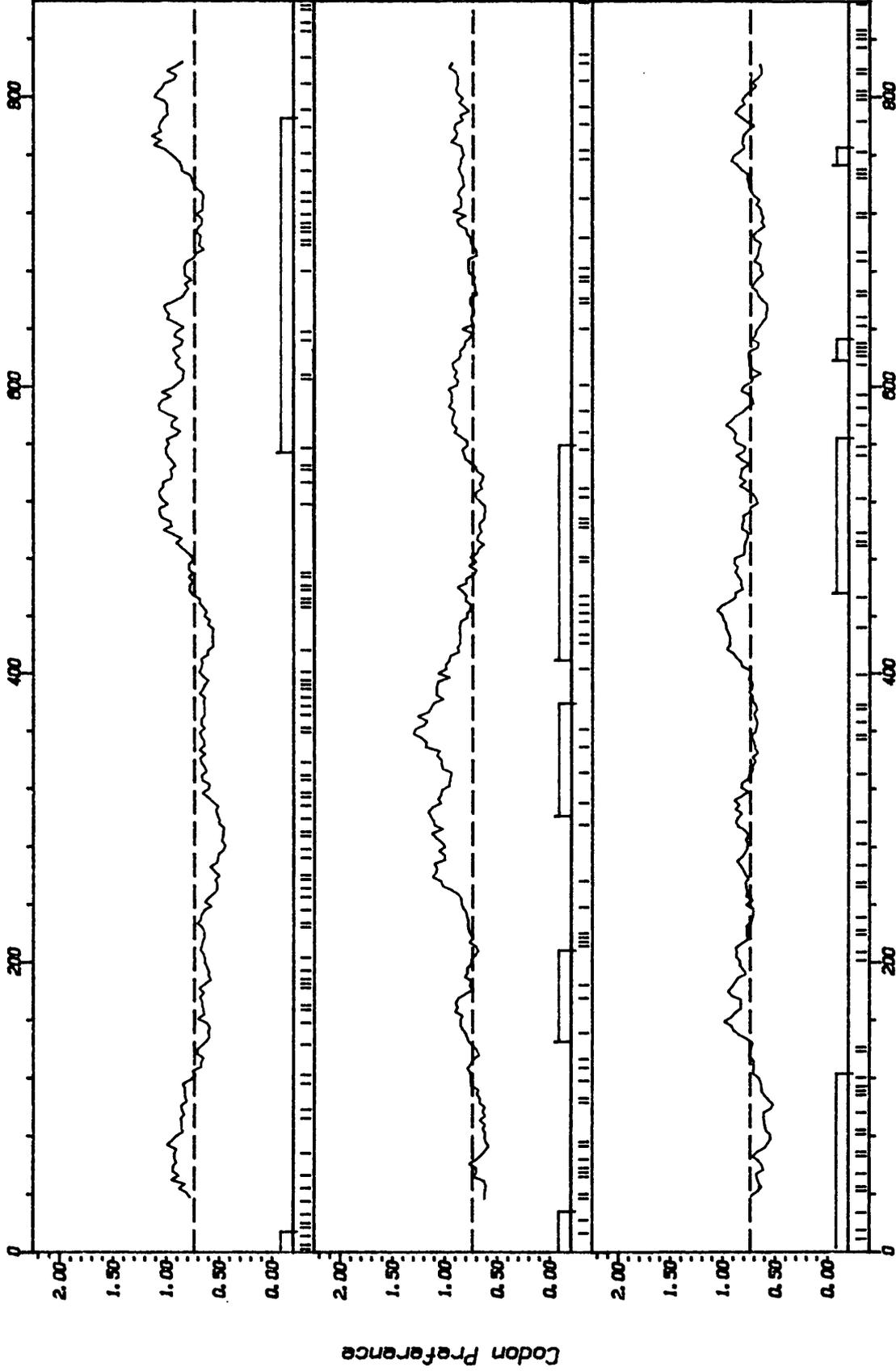
CODONPREFERENCE of: mm1q.dat Cks 2553, 1 to 690 10-NOV-1986 16:54
Table: psumet.cod FileCk: 2598 Window: 25 Threshold: 0.10 Density: 22.6



CODONPREFERENCE of: mm1q.dat Cks 2553, 1 to 690 10-NOV-1986 16:49
Table: psumet.cod FileCk: 2598 Window: 25 Threshold: 0.10 Density: 22.6

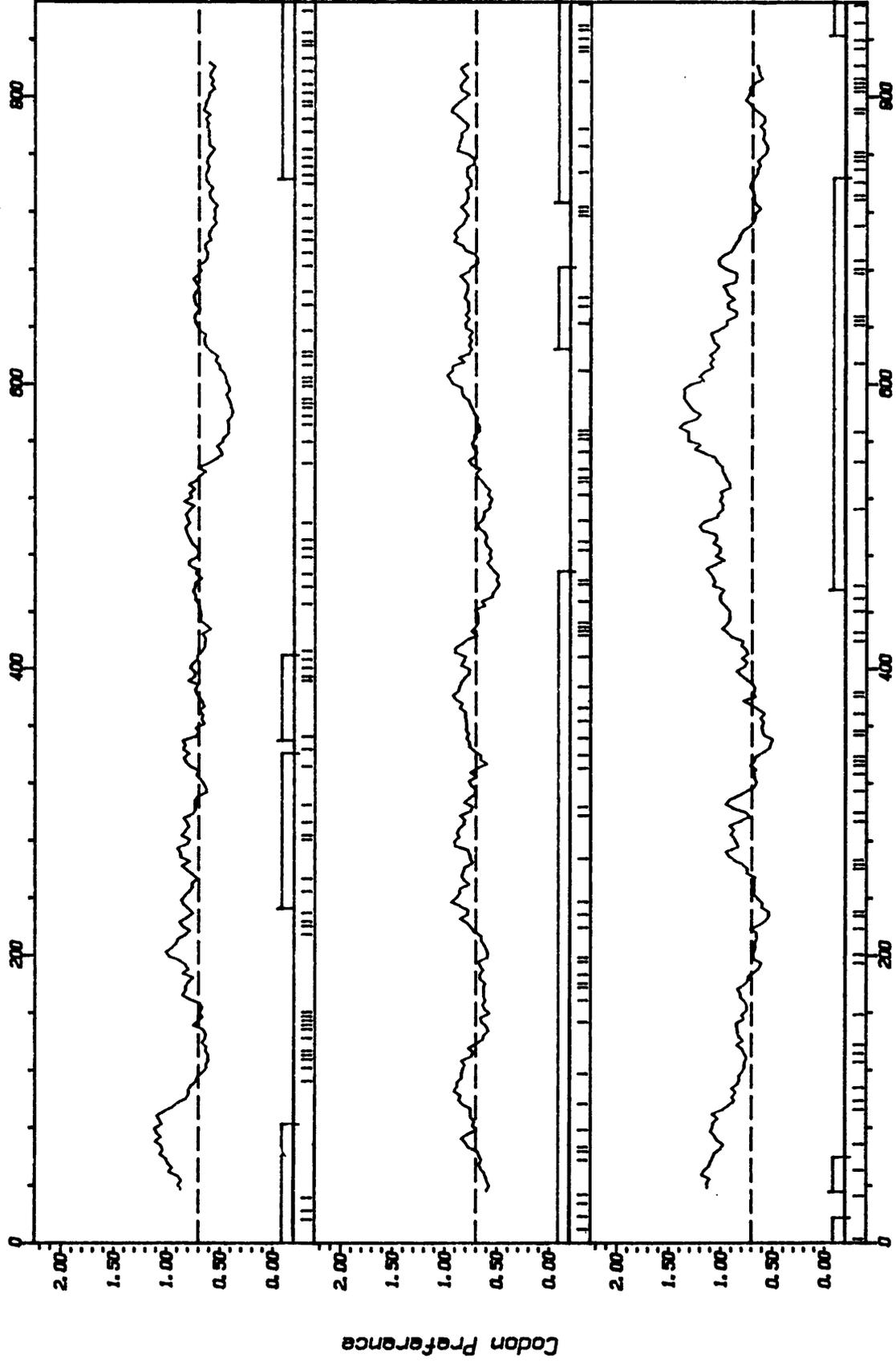


CODONPREFERENCE of, mm2.dat Ck, 3581, 1 to 865 10-NOV-1986 17:04
Table: psumet.cod FileCk, 2598 Window, 25 Threshold, 0.10 Density: 28.4

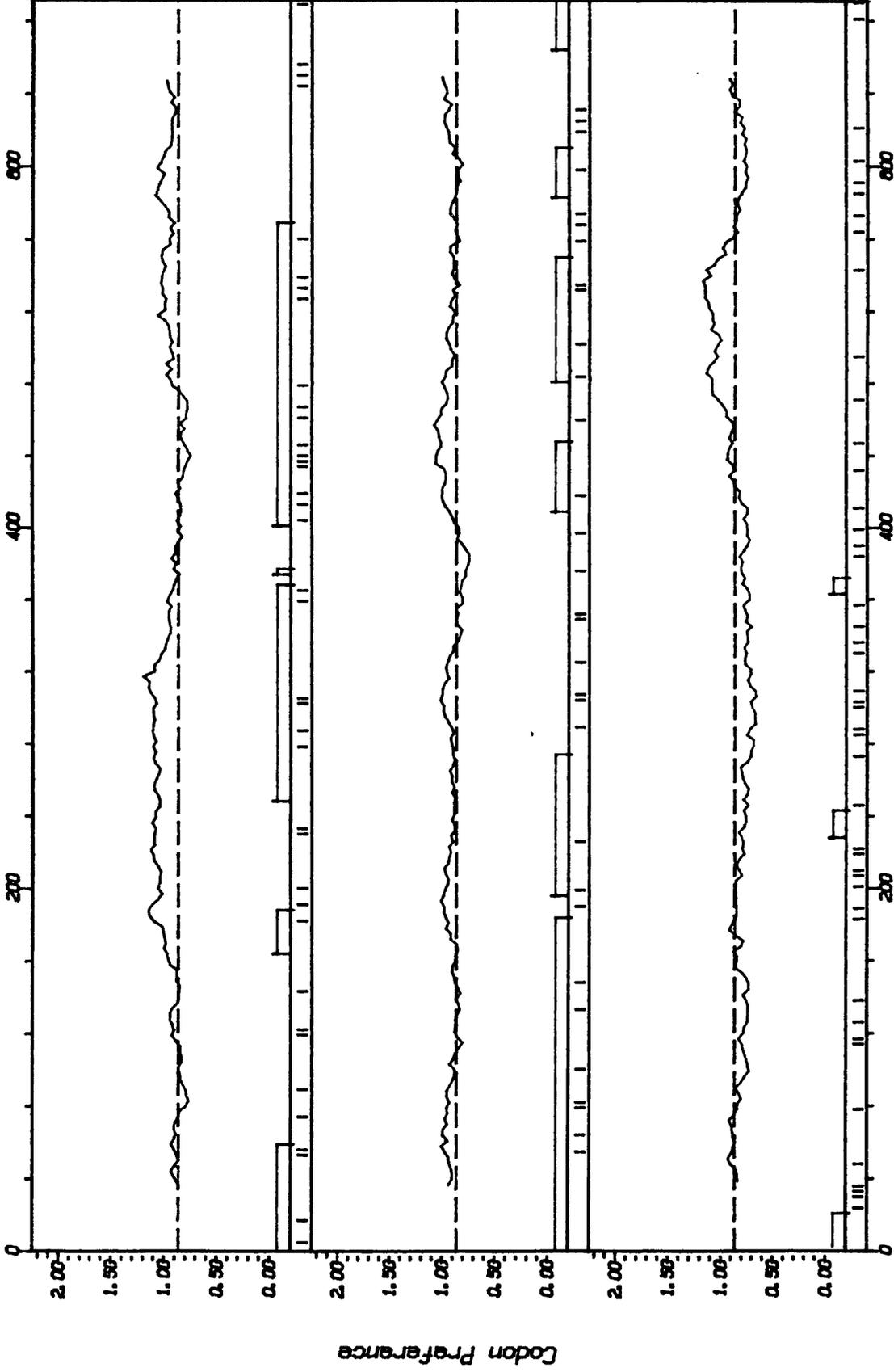


F 6 22

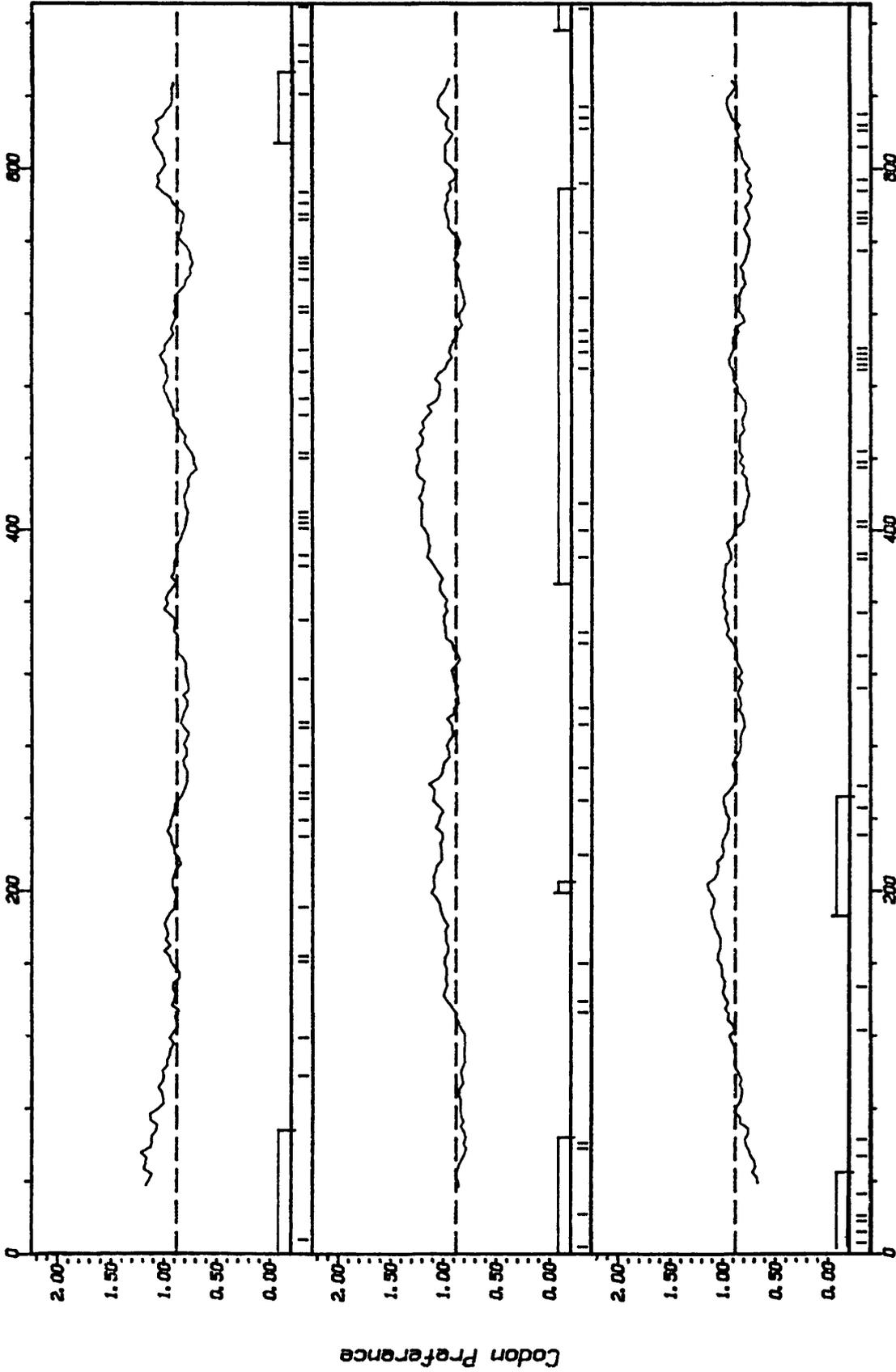
CODONPREFERENCE of: mm2.dat Ck: 3581, 1 to 865 10-NOV-1986 16:59
Table: psumet.cod FileCk: 2598 Window: 25 Threshold: 0.10 Density: 28.4



CODONPREFERENCE of: mm1q.dat Ck: 2553, 1 to 690 10-NOV-1986 17:15
Table: psuxyl.cod FileCk: 2767 Window: 25 Threshold: 0.10 Density: 22.6

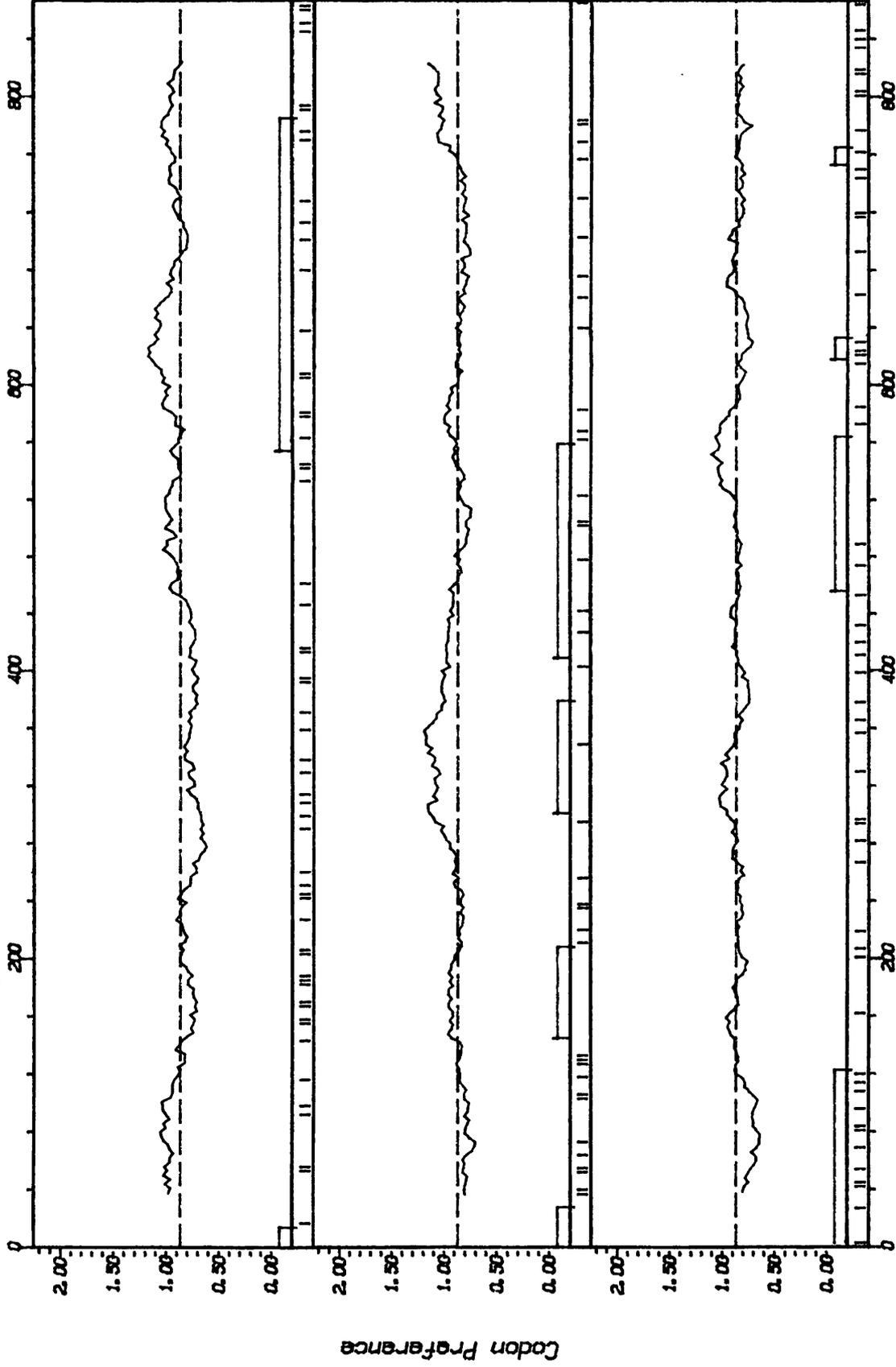


CODONPREFERENCE of: mm1g.dat Cks 2553, 1 to 690 10-NOV-1986 17.10
Tabler psuxyl.cod FileCk: 2767 Window: 25 Threshold: 0.10 Density: 22.6



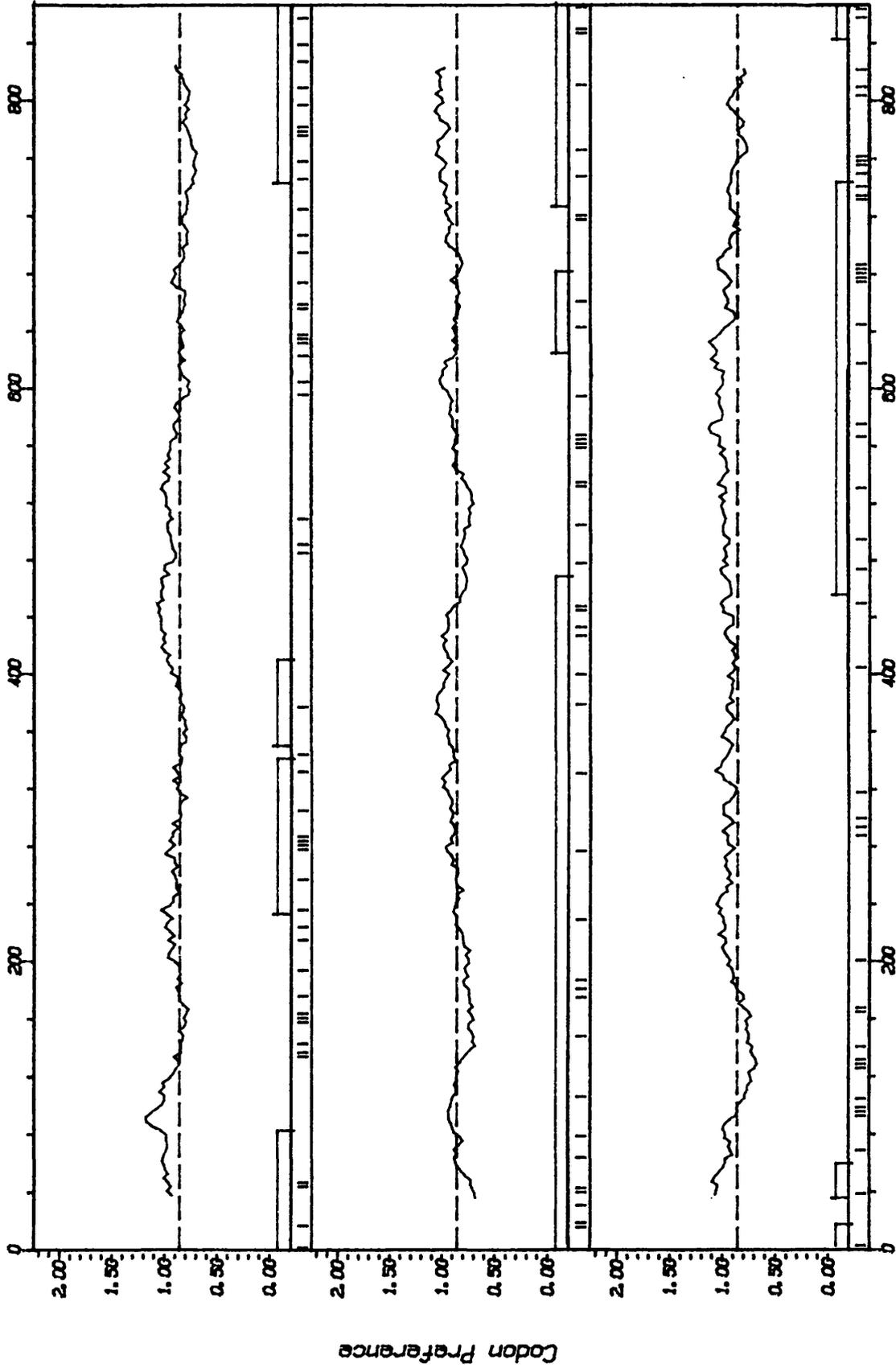
F 6 25

CODONPREFERENCE of: mm2.dat Cks 3581, 1 to 865 10-NOV-1986 17.23
Tablet psuxyl.cod FileCks 2767 Window: 25 Threshold: 0.10 Density: 28.4



F 6 26

CODONPREFERENCE of: mm2.dat Ck: 3581, 1 to 865 10-NOV-1986 17.19
Tablet psuxyl.cod FileCk: 2767 Window: 25 Threshold: 0.10 Density: 28.4



F 6 27

codon usage tables obtained from high and low level genes from Pseudomonads (the metapyrocatechase gene, Nikai et al. 1983, and the xyls gene of the IOL plasmid, Spooner et al. 1986). The only conclusions that may be drawn from this is that the codon usage table of Methylophilus methylophilus is probably dissimilar from these genes. Using the most probable protein encoding region, frame ORF-6, SIC-3, as a model for a M. methylophilus gene, a rough guide to codon usage in this organism can be obtained. Table 6.3 represents this codon usage together with the codon frequencies for the E. coli and Pseudomonad genes mentioned above.

Further analysis of the sequencing data was obtained with the program 'Search', which was used to find promotor-like TATAA sequences, and -35 binding sites (TTGACA sequences). Of particular interest is a TATAAC sequence at 320 bp on strand B of the mm1 data (shown as the complement strand in table 6.1), which lies 30 bp upstream of two ATG's, and a long run of A's. In addition, this possible promotor sequence lies a further 25 bp downstream from a possible -35 binding site (TTGCAA). However, although the program Frames identifies an open reading frame directly in front of these features, Iestcode does not recognise the region as a possible protein coding region, and if these are real regulatory regions they may belong to a gene downstream of the sequenced data.

'Terminator' was used to search for prokaryotic factor-independent termination sequences. Two termination sites were identified, at 140 bp 5' to 3', and 520 bp in the opposite

Codon	Metapyrocatechase	<i>M. METHYLO-TROPHUS</i> frequency/1000	<i>E. coli</i> mixture ^a	Codon	Metapyrocatechase	<i>M. METHYLO-TROPHUS</i> frequency/1000	<i>E. coli</i> mixture ^a
Arg				Val			
CGU	13	21	19	GUU	7	20	23
CGC	33	31	17	GUC	13	41	13
CGA	7	10	4	GUA	7	10	13
CGG	10	21	2	GUG	42	64	28
AGA	0	0	2	Lys			
AGG	0	0	0	AAA	13	10	40
Leu				AAG	33	10	9
CUU	3	0	8	Asn			
CUC	26	20	8	AAU	10	30	13
CUA	7	20	4	AAC	20	0	25
CUG	59	62	70	Gln			
UUA	0	10	10	CAA	3	0	15
UUG	16	4	9	CAG	23	10	31
Ser				His			
UCU	3	10	19	CAU	16		7
UCC	16	10	13	CAC	33		7
UCA	0	10	7	Glu			
UCG	3	30	10	GAA	39	30	32
AGU	7	0	5	GAG	29	10	20
AGC	3	10	13	Asp			
Thr				GAU	26	10	25
ACU	7	0	10	GAC	62	21	18
ACC	42	10	23	Tyr			
ACA	3	0	2	UAU	23	10	11
ACG	3	10	13	UAC	16	20	14
Pro				Cys			
CCU	0	10	4	UGU	7	0	6
CCC	10	0	7	UGC	3	40	6
CCA	3	0	8	Phe			
CCG	26	21	19	UUU	13	10	30
Ala				UUC	39	40	22
GCU	13	0	15	Ile			
GCC	33	21	28	AUU	7	10	33
GCA	13	10	22	AUC	20	60	35
GCG	10	31	42	AUA	0	0	1
Gly				Met			
GGU	23	0	32	AUG	36	21	25
GGC	42	21	37	Trp			
GGA	7	0	4	UGG	16	30	6
GGG	7	31	5				

Table 6.3 Putative Codon Usage Table for *M. methylotrophus*

Adapted from the codon usage table from the metapyrocatechase gene on the TOL plasmid (Nikai et al., 1983), the *E. coli* mixture is from Green, P.J., Gupta, M., Boyer, H., Brown, W.E., and Rosenberg, J.M. (1981) *J. Biol. Chem.* , 256 , 2143-2153.

orientation. both in the mm1 sequencing data, table 6.1, and one termination site at 345 bp in the mm2 data, table 6.2. neither site directly relates to an open reading frame.

CHAPTER 7

Discussion

7.1 Genetic Considerations Involved in using M. methylotrophus for Genetic Manipulation Experiments.

The application of the traditional techniques of microbial genetics has proved difficult when dealing with M. methylotrophus. The availability of genetic variants is limited, and their production and isolation proved to be an arduous task.

The primary carbon source for the organism, methanol, is used highly efficiently, and the genes governing its assimilation initially represented a prime target for genetic analysis. However, although mutants had previously been constructed which contained no functional methanol dehydrogenase, the isolation of genetic sequences corresponding to these mutational deficiencies has proved a fruitless task (Burt, D., pers. comm.; this work, unpublished results). It is possible that the organism is so efficient in the conversion of methanol to formaldehyde that the introduction of a cloned methanol dehydrogenase gene on a high copy number plasmid may actually prove to be lethal event, due to the accumulation of high levels of toxic formaldehyde within the cell, or due to an excessive drain on the cells available RNA polymerase. The trimethylamine pathway provided gratuitous carbon sources with

which to apply similar genetic techniques. The pathway has several advantages over the methanol-assimilation route (Chapter 1), which provided a greater chance to isolate a M. methylotrophus gene. The techniques used for the genetic analysis of bacteria, initially exemplified in Escherichia coli K12, are increasingly available for a number of different bacteria. The techniques do however require adaption for each species of bacteria if optimal results are to be obtained. For successful genetic studies to be carried out with any given bacterium it is useful to have (a) sufficient mutagens for the generation of genetic variants, (b) a system of efficient genetic exchange between strains of bacteria. The use of transposon mutagenesis for the generation of genetic variants proved valuable in this work. Not only did In5 provide a readily selectable phenotypic marker (kanamycin resistance), but the 5.2 kb element provided a prominent physical marker in the later analysis of the mutants. The transposon donor, the plasmid pLG221 (Boulnois et al., 1985), was particularly useful as a system for mobilising and integrating the transposon into M. methylotrophus. All mutants obtained were shown to contain the transposon, but the original plasmid containing the transposon is not inherited by the methylotroph. However, multiple insertions were shown to occur quite frequently (Figure 4.2), which could make subsequent genetic analysis difficult and confusing. Providing an efficient system of genetic exchange in the methylotroph proved more difficult. There is now an increasing interest in the exchange of genetic material between unrelated bacteria (Atherton et al., 1979). In many cases this requires

selected segments of chromosomal DNA to be inserted into particular recipient bacteria; recombinants of this type can be achieved by in vitro cloning of prokaryotic and eukaryotic DNA to suitable plasmid vectors. Although, in the past, the recipient bacterium was usually E. coli, today there is a requirement for more flexible interspecies and intergeneric exchange, particularly where commercial organisms are involved. In such cases an initial consideration must be for any restriction/modification systems present within the recipient bacterium. M. methylophilus possesses two restriction enzymes, Mme I and Mme II (Boyd, 1983), initially demonstrated by the observation that P. aeruginosa strains which contained conjugative plasmids were unable to transfer them directly into M. methylophilus, but required intermediate passage through E. coli before transfer into the methylotroph. The passage through E. coli is required to enable the DNA to be methylated by a dam-type methylase which P. aeruginosa lacks (Boyd 1983). Suitable vectors available for cloning in Methylophilus have been developed with this in mind (Sharpe, 1982), including the vector used in this work, the plasmid pGSS33. This vector was found to be stable within the methylotroph as long as a continual selection pressure was applied. As these vectors specifically designed for M. methylophilus cannot initiate their own transfer into the recipient bacterium, mobilisation was obtained by R64drd II, a plasmid of the IncIX group capable of promoting conjugation between E. coli and M. methylophilus.

7.2 The Induction Response.

The response given by M. methylotrophus to the introduction of methanol and the methylated amines to its surrounding environment was studied in chapter 3. Methanol dehydrogenase was seen to be constitutively synthesised within the cell, while the enzymes required for the metabolism of the methylated amines appeared to be induced as a result of the presence of these amines in the growth medium. The levels of trimethylamine dehydrogenase, and methylamine dehydrogenase remained barely detectable in the absence of the amines (table 3.2).

Great variation often exists between the amount of a protein present at its induced level and that present when the environment conditions dictate that it would serve no useful purpose. For example there are some 3000 β -galactosidase molecules in each E. coli cell growing in the presence of B-galactosides such as lactose, but less than one thousandth this number in cells growing on other carbon sources.

This phenomenon of induction is familiar in many microorganisms, to conserve energy by not producing the proteins unnecessary under those conditions. In addition, it is often seen that the phenomenon of induced enzyme synthesis involves an increase in a level of enzyme activity which is already present at a low level in uninduced cells of the same type. This 'basal' level of enzyme is demonstrated with trimethylamine dehydrogenase in M. methylotrophus (table 3.2); a basal level approximately 4% of the total, induced,

level. There is the possibility that this basal enzyme activity could be due to the presence of a small proportion of mutant cells which constitutively produce a high level of trimethylamine dehydrogenase even in the absence of a suitable inducer. Although such mutant cells are probably present within a population, their small numbers would be unlikely to account for this significant basal enzyme level.

It is possible that basal enzyme levels also exist for dimethylamine monooxygenase and methylamine dehydrogenase, but that the assays used in this work were unable to detect them. Alternatively, the transcription of their messenger RNA could be linked with that of trimethylamine dehydrogenase. All three enzymes in the pathway appear to be induced together, even when the production of the first two enzymes of the pathway would be superfluous, as with growth on methylamine. In addition there are probably several ancillary proteins produced that are involved in the uptake and utilisation of the amines. It is likely that some of these are also inducible, in a manner similar to the β -galactoside permease and transacetylase proteins in the β -galactoside system. It seems that all three substrates act as efficient inducers of the system (Table 3.3). It is not unusual for a gene system to respond to several, albeit similar, inducers; for example, the β -galactosidase operon responds to several growth substrates apart from lactose to initiate transcription of the operon (Jacob and Monod, 1961).

7.3 Regulation of the Pathway.

That methylotroph cells do not contain all the enzymes they require for the catabolism of all the substrates they can utilise, means that the entire bacterial genome is not blindly transcribed into mRNA sequences, and further translated into proteins. Gene expression can be controlled by many sophisticated mechanisms. Several genes are always expressed in growing bacteria, for they require certain enzymes present for growth under all conditions. Other, inducible, enzymes are switched on and off as they are required by a particular physiological condition. The variation in the amount of a particular protein in a cell must reflect its rate of de novo synthesis from transcriptional or translational levels.

In M. methylotrophus the increase in the rate of methylamine utilisation in complemented mad 1 cells is related to the available number of mRNA templates identified by the isolated Mad + gene, which increases with the introduction of methylamine. Control of this gene, and possibly the rest of the pathway, is thus at the transcriptional level, and not a translational or post-translational effect.

The synthesis of RNA appears to be regulated at all levels of the transcription process (Von Hippel et al. 1984). The ultimate objective of the control of gene expression is to provide the cell with the correct quantities of proteins and structural nucleic acids at the relevant times in cell growth. Transcriptional regulation can occur at the promotor-RNA polymerase stage (vis strong or weak promoters), as well as

at the initiation, elongation, and termination stages. There are also several common patterns of regulation which use ancilliary proteins to control transcription. These control systems invariably depend on the type of metabolic activity of the system being regulated, and although the molecular mechanisms for each of the regulatory patterns vary quite widely, they usually fall into one of two major categories; negative regulation and positive regulation. In negative regulation, an inhibitor or repressor protein is present within the cell. All known repressors bind at specific sites (operators) on their respective DNA molecules, blocking the initiation of transcription of the corresponding mRNA molecules. To relieve this binding the repressor must combine with an appropriate inducer (anti-inhibitor), where the attachment of the inducer inactivates repressor binding, allowing transcription to proceed. In positive regulation, an effector molecule (which may be a protein, a small molecule or a molecular complex) is required to stimulate the rate of transcription. Transcription is thus regulated at each phase of RNA synthesis by a complex series of instructions involving the RNA polymerase, the gene itself, and the adjacent DNA together with a variety of regulatory proteins and small molecule ligands. The trimethylamine pathway, by nature of its induction response, must involve some method of regulation. The genes involved in the pathway are induced together, possibly coordinately, which implies that these genes contain similar, or are controlled by communal, regulatory regions.

In addition, the regulation of the pathway seems unlikely to be a response dependent purely on the presence of methylated

amines; other factors affect the regulation of the pathway, as indicated by the cells' ability to assimilate methanol in preference to an amine in a mixed substrate media. It seems reasonable and economic for M. methylotrophus to use methanol preferentially as a substrate, for which it produces methanol dehydrogenase constitutively, to the methylated amines, the assimilation of which requires an expenditure of energy to produce the necessary enzymes for substrate-utilisation. This preferential utilisation of methanol may become important to the bacterium in its natural environment, where substrates and conditions may quickly change. Other bacteria show a similar diauxic or catabolic response when growing on a mixed substrate medium, where one metabolite constitutes a better energy source. In E. coli many of its substrates (galactose, maltose, arabinose, sorbitol) are metabolised by pathways encoded by catabolite sensitive operons (Adhya and Garges 1982). For these operons a metabolite-mediated positive control (cyclic adenosine monophosphate) is required in sufficient concentration for it to form a complex with a catabolite activator protein to enable transcription to proceed. At this stage we cannot decipher if such an ancilliary metabolite is required for transcription of the genes involved in trimethylamine assimilation. The isolation and characterisation of the DNA preceding the structural genes may provide some indication of this, as many positively controlled genes contain regions of homology required for the identification and binding of the CAP protein.

7.4 Structural Implications.

The mutations affecting the assimilation of trimethylamine(tmd 3) and methylamine(mad 1), can give some indication of the possible positioning of these genes relative to one another on the bacterial chromosome. The mutation tmd 3 not only prevents the utilisation of trimethylamine, but also inhibits the assimilation of methylamine. It is unlikely that this is due to a double transposon insertion into two relevant genes, and is most likely to be the effect of one mutational event. Three main possibilities exist. Firstly, that a mutation in one of the genes necessary for trimethylamine assimilation not only prevents the utilisation of trimethylamine, but through a polar event inactivates a gene required for methylamine assimilation. Secondly, that the mutation tmd 3 is in a regulatory gene required for the joint transcription of the trimethylamine dehydrogenase and methylamine dehydrogenase structural or regulatory genes. Thirdly, that the gene encodes a permease required for the uptake of trimethylamine, and that a product of this is required for methylamine gene induction. This latter possibility can be tested by using radioactively labelled trimethylamine as a substrate, to measure incorporation of the label into the cell.

The first suggestion relies on both affected genes lying close to one another in the DNA, probably as part of the same operon. A transposon integration early in the operon would produce a truncated polycistronic mRNA, preventing the translation of later genes. Most transposons do exert strong polar effects on the expression of genes in an operon which lie distal to the site of the inserted element. It is possible

that these polar events may occur by similar transcription mechanisms which are thought to cause other types of polarity in bacterial operons (Adhya and Gottesman 1978). The polarity of some insertion elements involves the transcription termination factor rho, and can be fully or partially relieved in rho strains (Das et al. 1977), and polar effects of insertions in lambda are also suppressed by the phage anti-termination function (Kleckner et al. 1978). It is also known that In5 contains termination sites at either end of the transposon which could produce premature termination of transcription. To determine if polycistronic mRNAs are involved in this gene system, the isolation of the mRNA transcribed from genes relieving the mutational events and the sizing of these RNA(s) in both the wild-type and mutant cells would prove useful. Alternatively, it may be possible that the genes are physically very close but structurally separate, whereupon, if strong enough, the polarity of the In5 insertion in one gene may interfere with the initiation of transcription of the second gene.

It is equally possible that the transposon insertion has occurred in a regulatory gene of region common to the genes required for the pathway. This does not require the genes to be structurally connected, but if not they must contain similar regulatory regions which respond to the same stimuli. Such mutated regulatory genes might include a repressor gene or region (such that the repressor was rendered immune to the effects of the inducer), or a positive regulatory region actively required for transcription to proceed (such as the Ara C protein in arabinose regulation, Epstein and

Beckwith 1968). In addition, if the genes affected were part of a unit of function and regulation, a transposon insertion into the operator, promotor, or -35 binding sites might all give similar effects.

The mad 1 mutation also provides some information. In this case the transposon could be in either a structural or regulator gene, but unconnected with the genes involved in trimethylamine assimilation. The genes may still be connected together in an operon, but the mutation must then be distal to the genes involved in trimethylamine assimilation.

It is unlikely that the mad 1 mutation is in a gene coding for a repressor protein, as the introduction of the wild-type gene (Mad +) into the methylamine mutant cell relieves the mutational effect, leaving the pathway fully inducible (Table 5.3). This means that the mad 1 mutation is recessive. Since the Mad + product complements in a trans-configuration, it must either be a diffusible molecule, or the 11 kb fragment must contain the complete gene along with any necessary regulatory regions. Because the 2.3 kb sub-clone does not relieve the mad 1 mutation it is likely that part of the gene, or its associated regulatory regions, might be contained on another of the sub-clones. This would require further sequence analysis of the 11 Kb fragment 5' and/or 3' to the 2.3 kb isolate.

7.4 Obtaining and Analysing the DNA Sequencing Data.

The sequencing data were obtained by two methods; the deletion method of Lin, lei, and Wilcox 1979, and the extension of sequencing data using oligonucleotide primers. The deletion method contributed very little to the final sequence data, and was abandoned in the early stages. The main reason for this was the difficulty in obtaining subclones containing sizeable deletions. The oligonucleotide internal primer method, although more time consuming, provided a far more reliable method of sequencing. In addition the method relies on only two templates from which the sequencing data were obtained, resulting in a higher level of confidence that the data contains no large (deletions, additions, rearrangements) sequencing errors. In addition the oligonucleotide primer method ensures that the sequence is contiguous, without any gaps.

Although we know the site of transposon mutagenesis is within 300 bp of one end of the 2.3 kb subclone, the Mad + gene may not be situated at this point, but due to a polar effect or a mutation in a regulatory sequence, the gene may be situated slightly further away. The direct site of mutagenesis in mad 1 could be determined by using the 2.3 kb subclone as a probe to isolate the mutated gene from a mad 1 gene bank, and sequencing the site of insertion of In5. Once the site of insertion has been established the nature of the mutational effect will become clearer. There are, in the sequencing data, several candidates for the mutated open reading frame or

associated region, which cannot at present be resolved.

In studying the sequence of a molecule of DNA, for a gene or regulatory region for which the sequence or position is unknown, several considerations must be taken into account. A structural gene requires a start (AUG) codon to initiate translation, and a termination codon (UAA, UAG, UGA) to end translation, but not all open reading frames depicted by these parameters will code for proteins; other factors must be taken into account before an open reading frame can be identified as a gene. In addition to these stop and start sites a gene requires other features in the sequence which will help initiate and regulate the transcriptional and translational processes. RNA polymerase requires certain signals within the DNA sequence to enable it to bind to the correct place on the DNA molecule. The identification of, and binding to, these sites is enhanced by the sigma (σ) factor complexing with the RNA polymerase (Burgess et al., 1969, McClure 1985). The DNA sequences of more than one hundred E. coli promoter regions have been reported (Hawley and McClure 1983), and homologies between such regions have been noted (Pribnow 1975, Schaller et al., 1975), and consensus sequences are continually being revised (Rosenberg and Court 1979). The current consensus sequence dictates that the TTG at -35 and the IA---I near -10 are both highly conserved.

Deviations from the 'perfect' consensus sequences of TTGACA and IAIAAI may be related to the range of initiation frequencies encountered in promoters. The notion that the consensus promoter is likely to be near maximal is supported by the construction of the semisynthetic promoter (TAC) in

which the -35 and -10 sequences are precisely homologous to the consensus sequences. These sequences promote RNA chain initiation at a very high frequency (Amman et al. 1983).

Those promoters with poor homology to the consensus in the -35 region are frequently controlled by dissociable positive factors (Raibaud and Schwartz 1984), while poor -10 homology may indicate a different sigma factor. The lack of promoter-like structures before the main open reading frames designated by Testcode and Frames, do not necessarily exclude these regions from being genes; but may indicate positive control or a dissimilar sigma factor to that usually used by E. coli. Some recognition features must however remain the same, as M. methylophilus is known to recognise some E. coli promoter sequences to an acceptable level of gene expression (Hennam et al. 1982) It would not be construed as unusual if M. methylophilus contained its own distinct sigma factor, and in addition a sigma factor resembling the E. coli σ -70 factor. At least two sigma factors are known to exist in E. coli (Grossman et al. 1984), and several species are known in B. subtilis (Gilman and Chamberlin 1983, Johnson et al. 1983). In addition several viruses redirect the transcriptional machinery of their hosts by substituting their own sigma factors (Lee and Pero, 1981).

The TATAAC sequence identified in the sequencing data (Figure 7.1a) may indicate an E. coli like sigma factor, since the sequence is a good candidate for a promoter region, containing homology at both the putative -10 and -35 sites (last chapter). The lack of an open reading frame immediately after these sites may not be significant, as some Pseudomonad

genes are known to contain promoter regions some distance from the structural genes (Brammar, W. J et al. 1986, unpublished results) RNA polymerase protection studies and S₁ mapping could possibly clarify this point.

In addition to the promoter region, the DNA may contain other identifiable features. These may include a sequence complementary to the 3' end of ribosomal RNA. It is possible that such sequences in M. methylotrophus may bear some relation to the 3' end of Pseudomonas ribosomal RNA (Shine and Dalgarno 1975), or to E. coli 16s rRNA (Shine and Dalgarno 1974). In addition to these sequences, there are often termination (UAA, UGA) codons closely preceding the translation-initiation codon (Atkins 1979). The absence of such sequences before Methylophilus coding regions may indicate a difference in the 3' region of the ribosomal RNA species in this organism. However, because E. coli genes are transcribed in this organism (Windass et al. 1980, Kennam et al. 1982), the 3' complementary sequence is likely to have some homology to the E. coli consensus.

The open reading frame, ORF-6, identified by both 'Frames' and 'Testcode', contains two ATG codons in frame with each other. 13 base pairs behind the first start codon lies a putative ribosome binding site which has homology with both the E. coli and Pseudomonas 16s rRNA, Figure 7.1b). The open reading frame ORF-1 also contains an ATG codon behind which lies a putative ribosomal binding site, Figure 7.1c.

Using both Testcode and Frames to identify likely protein coding regions it is possible to build up a picture of a putative codon usage table for M. methylotrophus, using

Codon frequency to generate the table from the open reading frame ORF-6, (Table 6.3). From this it seems that the codon usage in M. methylophilus may be more like the Pseudomonads than E. coli, due to the general bias towards G or C nucleotides, which is most marked in the wobble position.

Towards the 3' end of the open reading frame itself, there may be identifiable termination sites in addition to the termination codons. Many bacterial transcripts have a series of uridine residues at the 3' end, preceded by a GC-rich region of dyad symmetry in the DNA, and such sites are generally efficient terminators (Platt 1981). The program 'Search' identified three such sites (Section 6.4), but none of these corresponded to any frames identified by Testcode or Frames, and so are unlikely to be significant.

Attempts to identify rho-dependent termination sites are complicated by several factors. Relatively few examples of known rho-dependent termination sites are known at the sequence level. For these known sequences the rho termination sites vary considerably with the type of gene being regulated and the organism in which it resides (Platt and Bear 1983, Morgan et al. 1985).

Conclusions

Through the use of transposon mutagenesis this work has isolated and characterised two mutants of M. methylotrophus, called tmd 3 and mad 1. These mutants are deficient respectively in the ability to utilise trimethylamine (tmd 3) and the ability to utilise methylamine. Using the mad 1 mutation an 11 kb fragment of M. methylotrophus DNA, which relieved the mutant phenotype, was isolated from a wild type gene bank. After sub-cloning of this fragment to 2.3 kb, a Southern blot revealed the transposon insertion to be within a few hundred base pairs of one end of this sub-clone. Subsequent sequencing of this fragment uncovered several features of potential interest.

Future work could include S-1 mapping of putative start sites, RNA polymerase protection studies, and investigations into the strength of any isolated promoter regions within the methylotroph. With the isolation and characterisation of M. methylotrophus -10 and -35 binding sites, we take a step towards understanding what is required for high-level expression of genes in the organism, all of which will be essential if M. methylotrophus is used to express foreign prokaryotic or eukaryotic coding sequences at a commercially viable rate.

APPENDIX 1

It was found in this work that the enzyme assays for methylamine monooxygenase produced far lower reading than expected, Table A1. This was later found to be due to contamination of the dimethylamine source, Figures A1-A3.

Table A1:

Grown On.	DMA				
	1	2	3	4	AM
MeOH	0.0	0.0	0.0	0.0	0.0
MeOH/ IMA	13.0	84.2	29.1	16.1	35.6
IMA	14.7	27.5	31.3	20.1	23.4

Figures A1, A2, A3.

Nuclear magnetic Resonance studies of trimethylamine, dimethylamine, and methylamine show that although methylamine is relatively pure, giving a spectra comparable with the pure compound (inset), trimethylamine contains a small trace of methylamine, and dimethylamine seems to contain not only small traces of methylamine, but the compound is altered (no peak at 9.3 ppm) possibly to an extent which makes it unrecognisable to the enzyme dimethylamine monooxygenase.

Figure A1 NMR Spectra of Trimethylamine used in this work,
Spectra of the Pure Compound is Inset.

START OF SWEEP

END OF SWEEP

Trimethylamine hydrochloride, 98%
 (trimethylammonium chloride)
 $(CH_3)_3N \cdot HCl$
 MW 95.57 m.p. 283-284° (dec) Bell 4.43
 IR 1376 HYGROSCOPIC

10ppm

5ppm

2ppm

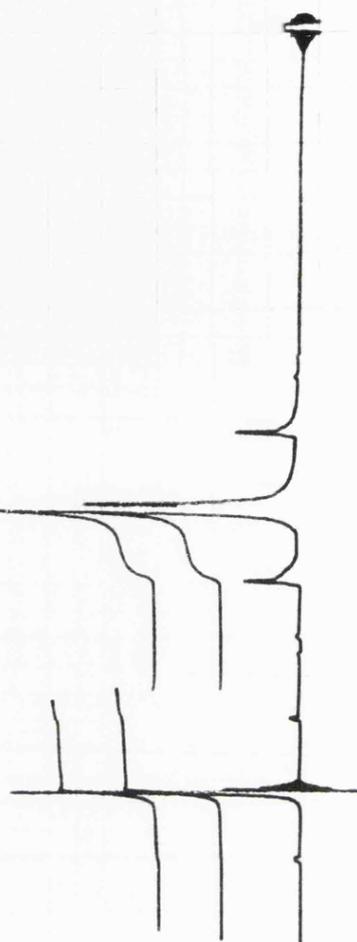
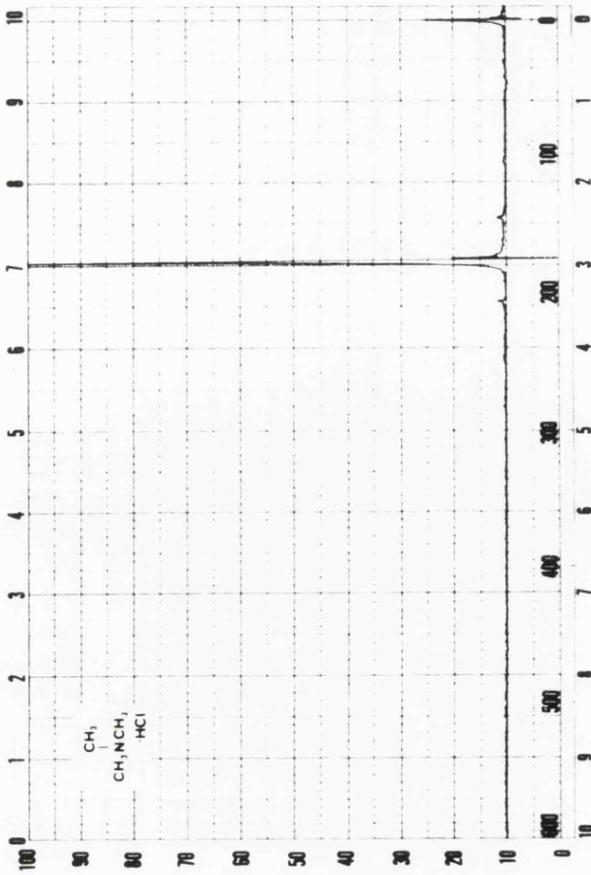


Figure A2 NMR Spectra of Dimethylamine used in this work,
Spectra of the Pure Compound is Inset.

END OF SWEEP

Dimethylamine hydrochloride, 97%
(CH₃)₂NH·HCl
M.W. 81.55, m.p. 170-173° Beil 4.39 IR 135G
HYGROSCOPIC

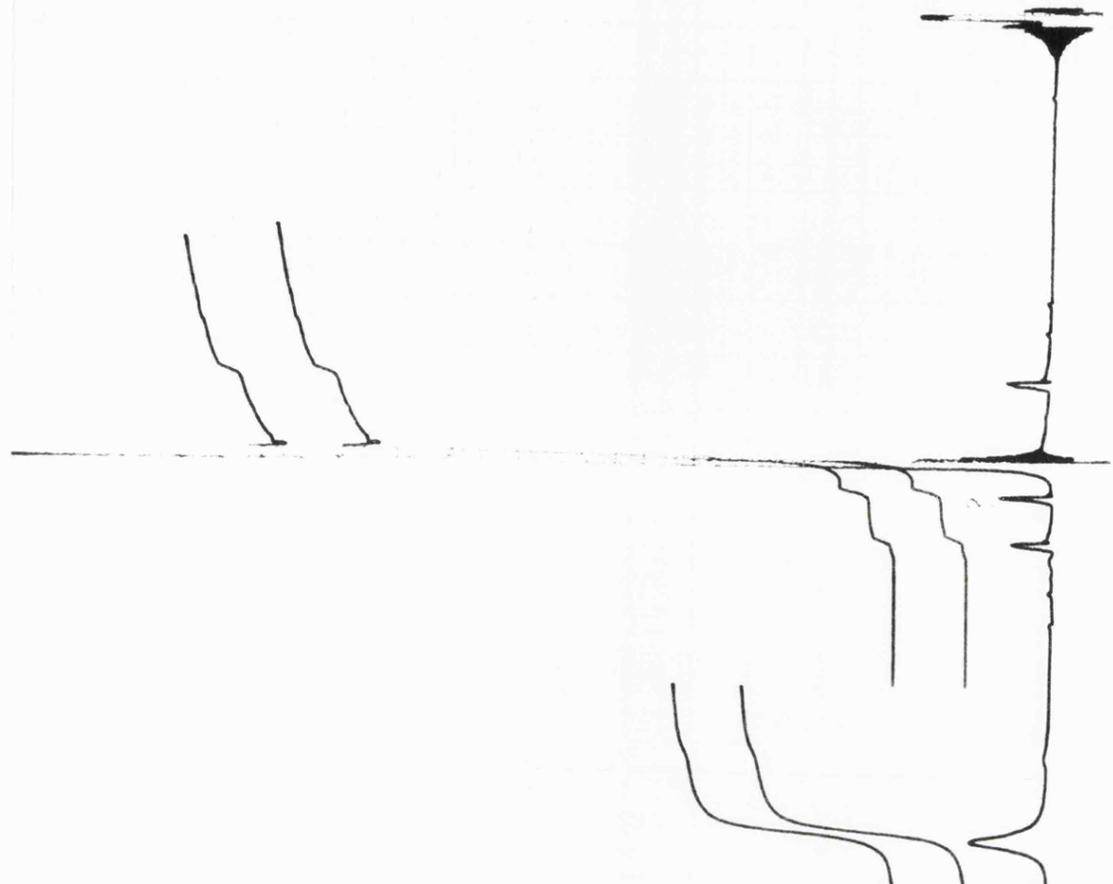
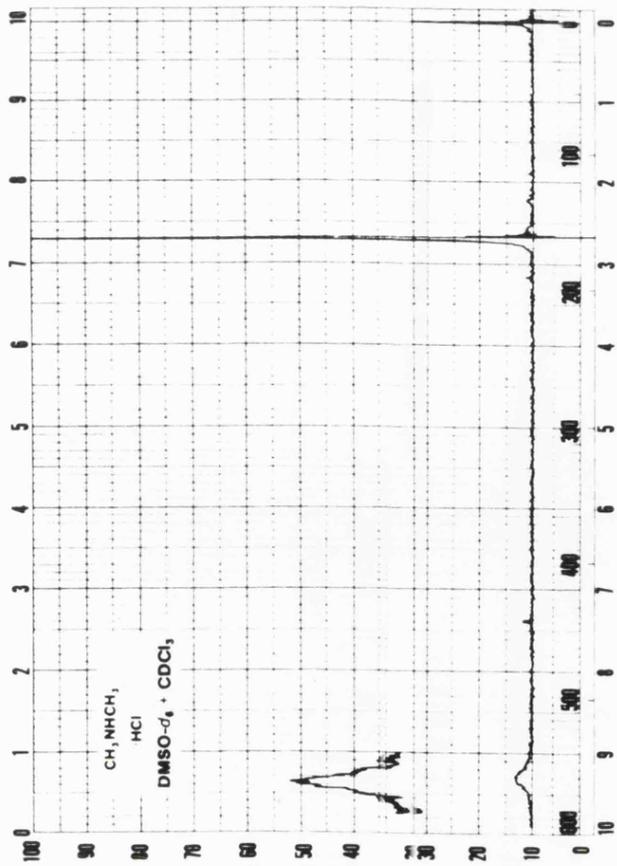
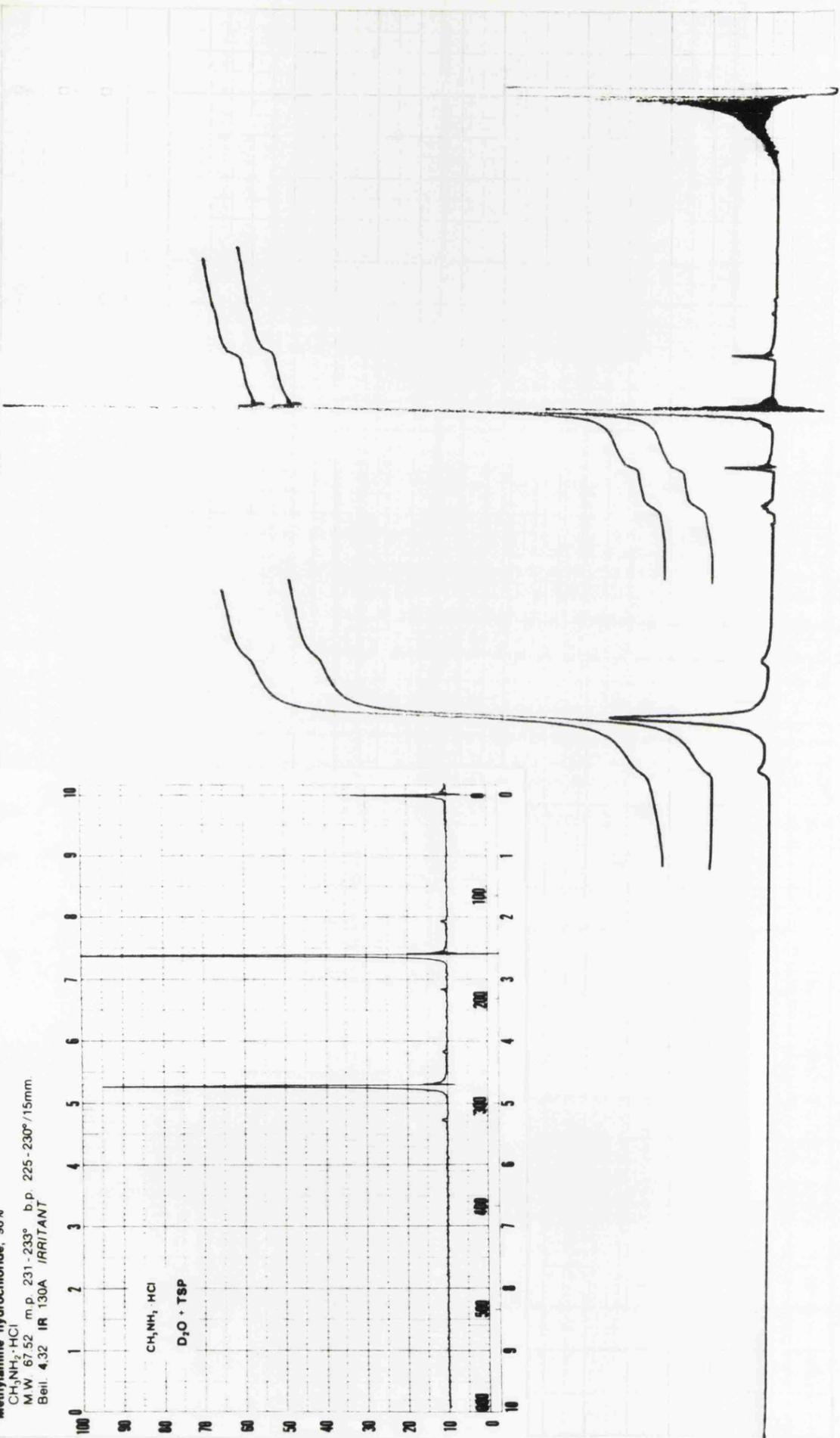


Figure A3 NMR Spectra of Methylamine used in this Work, Spectra
of the Pure Compound is Inset.

START OF SWEEP

END OF SWEEP

Methylamine hydrochloride, 98%
 $\text{CH}_3\text{NH}_2 \cdot \text{HCl}$
 MW 67.52 m.p. 231-233° b.p. 225-230°/15mm
 Beil. 4.32 IR 130A IRRITANT



10ppm
 5ppm
 2ppm

Product UK

... Instrument Ltd., Argent House, Fenner Lane, Boreham, Essex, SSO 9 5JG, England

MM3



APPENDIX 2

Calculation of the growth rate constants;

If N^0 is the population size at a certain time, and N its size at a subsequent time, t , the number of generations that has occurred may be calculated from;

$$k = \frac{\log N^0 - \log N}{0.301 \cdot t}$$

where k is the exponential growth rate constant, defined as the number of doublings per unit time, and usually expressed as the number of doublings per hour. It is often convenient to express the growth rate as the time required for the population to double, this is the reciprocal of the growth rate, $1/k$.

APPENDIX 3

According to Clark and Carbon, 1976

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

Where:-

N is the number of recombinants required

f is the fractional representation of the genome,
(insert size/size of the total genome- 3×10^6 for
M.methylotrophus)

P is the probability of obtaining any one given
gene from the gene bank.

To rearrange this equation to obtain a value for P, we
multiply both sides of the equation by: $\ln(1-f)$.

$$\text{Therefore: } \ln(1-P) = N \cdot \ln(1-f)$$

Taking logs:

$$1-P = e^{-N \cdot \ln(1-f)}$$

Subtracting 1 from both sides, and taking the negative:

$$P = 1 - e^{-N \cdot \ln(1-f)}$$

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Physiological and Genetic Aspects of the Utilisation
of Methylated Amines in M. methylophilus .

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M. methylophilus is a Gram negative obligate methylotroph depending on the presence of reduced carbon compounds containing one or more carbon atoms, but containing no carbon-carbon bonds. This organism can synthesize all its cellular constituents from methanol, trimethylamine, dimethylamine, or methylamine. Conversion of methanol and the methylated amines to cell carbon involves the ultimate oxidation to formaldehyde and ammonia. While the methanol dehydrogenase is produced constitutively, the enzymes involved in the assimilation of the methylated amines are inducible. All three enzymes of the trimethylamine pathway are always induced regardless of the methylated amine substrate.

Transposon mutagenesis was used to generate mutations in M. methylophilus and an antibiotic selection procedure used to isolate mutants defective specifically in the trimethylamine pathway. Two mutants were characterised and subjected to further study. The mutant tmd 3 was unable to utilise trimethylamine, dimethylamine or methylamine as substrates and was shown to lack trimethylamine dehydrogenase by polyacrylamide gel electrophoresis. Enzyme studies confirmed the lack of the dehydrogenase within the mutant cells.

The mutant mad 1, unable to use methylamine as a substrate, was shown via enzyme studies to contain trimethylamine dehydrogenase, and dimethylamine dehydrogenase, but to lack methylamine dehydrogenase activity.

Molecular cloning of wild-type M. methylophilus DNA in a broad host-range plasmid vector was used to isolate DNA fragments that could replace the mad 1 defect. An 11kb fragment was isolated that fully restored methylamine dehydrogenase activity to mad 1 cells. A 2.5kb fragment was subcloned and shown, by Southern blotting with ³²P-labelled In5 DNA as a probe, to contain the site of integration of the In5 insertion, located to within a few hundred base pairs of the end. DNA sequencing, now in progress, has generated 600 base pairs of sequence from either end of the subcloned fragment, within which several regions of interest were noted.

