THE DEVELOPMENT OF GENETIC TECHNIQUES FOR THE OBLIGATE METHANOTROPH, *METHYLOCOCCUS CAPSULATUS* (BATH)

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Finally, I would like to thank my friends and relatives outside the department who provided my means of escape.

Declaration

The work presented in this thesis is the result of original research conducted by myself under the supervision of Dr J.C. Murrell. In the instances where others have contributed to the work, specific references have been made.

None of the information contained in this thesis has been used in any previous application for a degree.

Sylvia Davidson

Summary

In order to further the study of the molecular biology and biochemistry of the obligate methanotroph *Methylococcus capsulatus* (Bath) several genetic techniques where investigated.

A system for transfer of plasmids from *E.coli* to *M.capsulatus* by conjugation using a filter-mating technique was developed, and a variety of broad-host-range plasmids were transferred. Several parameters which could effect the efficiency of plasmid transfer were assessed, including the possible presence of a restriction/modification system, and it was found that transfer was at its' most efficient when *M.capsulatus* was in early logarithmic growth, transfer took place over 24 hours at 37^0C, and the ratio of donor to recipient was at least 1:5. It was also possible to transfer the plasmids RP4 and pULB113 from *M.capsulatus* to *E.coli*. The initial development of a vector for analysis of promoter expression in *M.capsulatus* was attempted.

Several plasmids were also investigated for their ability to act as mutagenesis vectors in *M.capsulatus*. pSUP2021 was found to transfer into *M.capsulatus* and Tn5, present on the vector, was shown to be inserted into the chromosome. The entire pJFF350 vector was found to be transferred into the chromosome, including Omegon-kanamycin fragment, IS1 ends and RP4-*mob* fragment. This vector was used to produce novel plasmids containing pJFF350 DNA and *M.capsulatus* chromosomal DNA. Two vectors, developed for marker-exchange mutagenesis of glutamine synthetase, were tested and found to be unsuccessful. A further vector was developed from pJFF350 and was found to transfer into the chromosome, but as yet its exact position of insertion is unclear

Electroporation was investigated, however, despite the alteration of electrical parameters and the pretreatment of cells no success was achieved. Results appeared to indicate a problem with a restriction/modification system.

The mobilization of the chromosome using RP4 prime plasmids and pULB113 was attempted but was unsuccessful.

ABBREVIATIONS LIST

A	Absorbance
Ap	Ampicillin
ATP	Adenosine triphosphate
Ci	Curie
Cm	Chloramphenicol
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetra-acetic acid
g	gramme
Gm	Gentamicin
h	hour
Inc	Incompatibility group
IPTG	Isopropyl-thiogalactoside
Km	Kanamycin
Kb	Kilobase
kV	kilovolts
l	litre
MDH	Methanol dehydrogenase
μF	microfarad
min	minute
MMO	Methane monooxygenase
mSec	millisecond
NMS	Nitrate mineral salts
nm	nanometre
NTG	Nitrosoguanidine

OD	Optical density
Ω	Ohms
ONPG	O-nitrophenyl ß-D-galactopyranoside
PQQ	Pyrroloquinoline quinone
rpm	revolutions per minute
SDS	Sodum dodecyl sulphate
sec	second
Sm	Streptomycin
Sp	Spectinomycin
Tc	Tetracycline
Tn	Transposon
Tris	Tris-hydroxymethylaminomethane
uv	ultra violet
v/v	volume/volume
w/v	weight/volume

INTRODUCTION

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Methylococcus capsulatus strain Bath is a Gram-negative, aerobic coccus which grows on methane as its sole source of carbon and energy.

A general term for the group of organisms that *M.capsulatus* belongs to, is the C_1-utilizers. These are organisms that have the ability to use as their sole source of carbon, compounds that are more reduced than carbon dioxide and contain no carbon-carbon bonds. Such compounds include methane, methanol, N-methyl and S-methyl compounds. The C_1-utilizers can be divided into two categories, the C_1-utilizing autotrophs, as defined by Quayle and Ferenci (1978), that oxidize C_1 compounds to CO_2 and then assimilate the CO_2 formed, and the methylotrophs that obtain energy from the oxidation of C_1-compounds and assimilate carbon as formaldehyde or a mixture of formaldehyde and carbon dioxide (Colby & Zatman, 1973; Quayle & Ferenci, 1978).

Methylococcus capsulatus belongs to the methylotrophs, a group which can be sub-divided into, obligate methylotrophs, restricted facultative methylotrophs and facultative methylotrophs. The obligate methylotrophs can be further sub-divided into the obligate methane utilizers and the obligate methanol/methylated amine utilizers. *M.capsulatus* is an obligate methane utilizer.

1.2 METHANE AND METHANOTROPHS-OCCURENCE AND HISTORY

Approximately 10^3 megatons of methane are produced globally each year from anaerobic methanogenic activity due to the degradation of organic matter. This production combined with an equal volume from natural gas wells makes methane the most abundant reduced carbon compound in nature. It therefore represents a vast reserve of carbon and energy for bacterial growth. It is chemically very stable and its oxidation yields a large amount of energy. This abundance of methane is reflected by the ubiquitous nature of methanotrophs and they can represent up to 8% of the total 'heterotrophic' population (Whittenbury *et al.*, 1976).

Methane produced in anaerobic environments diffuses into aerobic habitats, e.g, soils, surface layers of sediments and natural waters where it may consequently be oxidized by methanotrophs. The ecology of these organisms has been reviewed by Hanson (1980) and Rudd & Taylor (1980).

Methane-oxidizing bacteria have been known for decades, Söhngen isolated what he named *Bacillus methanicus* in 1906. This strain does not remain in any culture collections but it was well described, and is probably identical to *Pseudomonas methanica* isolated by Dworkin & Foster (1956). Up to 1970 only three methanotrophs had been isolated, including *Methylococcus capsulatus* (Foster & Davis, 1966). In 1970, Whittenbury and colleagues (Whittenbury *et al.*, 1970) devised new enrichment and isolation procedures and isolated over 100 strains of

methanotroph. From this work a basic scheme of classification could be devised (Davies & Whittenbury, 1970, Whittenbury *et al.*, 1970). Since this time reports of 'new' species of methanotroph have been reported from Russia and Japan (Galchenko *et al.*, 1975; Romanovskaya *et al.*, 1980; Urakami & Komagata, 1986, 1987; Malashenko *et al.*, 1972, 1975; Galchenko, 1977; Galchenko & Andreev, 1984)

There have been reports of the isolation of facultative methanotrophs, *Methylobacterium organophilum XX* by Patt *et al.*,(1974) and *Methylobacterium ethanolicum* and *Methylobacterium hypolimneticum* by Lynch *et al.*,(1980). The authenticity of these facultative methanotrophs is in doubt following the discovery that *M.ethanolicum* was in fact a syntrophic association of an obligate methane oxidiser strain POC, which is similar to *Methylocystis* species, and a *Xanthobacter* species, strain H414, which can fix N_2 and can grow on a variety of substrates including methanol, formate, ethanol, succinate and H_2 + CO_2 (Lidstrom *et al.*, 1983).

1.3 CLASSIFICATION OF OBLIGATE METHANOTROPHS

The isolation of over 100 strains of obligate methanotroph by Whittenbury and colleagues made it possible to devise a simple scheme of classification. The scheme divided the obligate methanotrophs into two types, type I and type II, based mainly on the internal membrane arrangements, cell shape and resting stage. Five groups or 'genera' were suggested: *Methylomonas, Methylobacter,*

Methylococcus, Methylosinus, and *Methylocystis* and these groups still remain today as major groups in the taxonomy of methanotrophs.

This classification scheme was refined further by Whittenbury and Dalton (1983) to include an anomaly, the organism *Methylococcus capsulatus*. This organism was assigned to the group type X. A detailed review of the taxonomic criteria which have been used to identify methanotrophs and assign them to the groups is outlined by Green, (1992). The subject has become steadily more complicated as organisms that have been assigned species names on the basis of a few characteristics now appear to be improperly characterized, in particular this is the case with the genus *Methylococcus* (Green, 1992). There are many groups or 'species' of methanotroph whose taxonomic affiliations are uncertain.

The basic classification scheme of Whittenbury & Dalton (1983) can be summarised as follows:-

Type I:- Utilize the ribulose monophosphate pathway (RuMP) for formaldehyde assimilation, lack a complete tricarboxylic acid (TCA) cycle, possess bundles of vesicular disc-shaped intracytoplasmic membranes and contain DNA with a 50-54% G+C content. e.g, *Methylomonas* and *Methylobacter* spp.

Type X:- e.g, *Methylococcus capsulatus*. The majority of formaldehyde is assimilated via the RuMP pathway and small

amounts via the serine pathway (Dalton & Whittenbury, 1976). They are the only methanotrophs known to assimilate CO_2 via the Benson-Calvin cycle utilized by autotrophic bacteria (Taylor *et al.*, 1981). They contain DNA with 61.7-63.1% G+C content.

Type II:- Utilize the serine pathway for formaldehyde assimilation. They possess a complete TCA cycle, contain paired intracytoplasmic membranes around the cell periphery and contain DNA with approximately 62.5% G+C content.

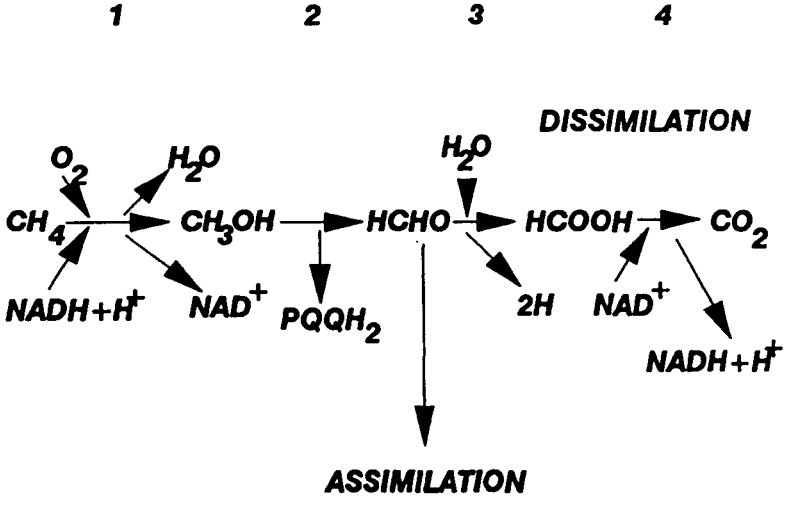
There are limitations in these classical approaches to taxonomy and recently a molecular biological approach has been taken. This includes DNA-DNA homology studies (Doronina *et al.*, 1989). Another approach is that of ribosomal RNA sequencing. The rRNAs are universally distributed, evolutionarily homologous, functionally important, conserved in nucleotide sequences and overall secondary structure, and lack artifacts of lateral transfer between contemporary organisms (Pace *et al.*, 1986). Structural analysis of 16S rRNAs from 12 methylotrophic bacteria was carried out by Tsuji *et al.* (1990). They developed a phylogenetic tree that summarised the relationship among methylotrophic bacteria and other representatives of purple eubacteria. They found that the *Methylococcaceae* (Whittenbury & Krieg, 1984) were not an evolutionarily coherent group but scattered among the purple

eubacteria. Woese (1987) divided the purple eubacteria into 4 subdivisions, α, β, γ and δ. Tsuji and his colleagues found that the type I methanotrophs fell into the β and γ subdivisions as did *Methylococcus capsulatus* (γ subdivision), whereas the type II methanotrophs were all in the α-sub-division. They postulate that unique sequences within the 16S rRNA molecules of methylotrophs could be utilized to construct specific probes which would have great taxonomic, commercial and environmental importance.

1.4 THE PHYSIOLOGY AND BIOCHEMISTRY OF OBLIGATE METHANOTROPHS

1.4.1 Carbon assimilation

The pathway of methane oxidation is outlined in figure 1.4.1. It is at formaldehyde that a branch in the pathway occurs to either, assimilation into cell carbon or dissimilation to formate then carbon dioxide. These pathways operate simultaneously. The assimilation of formaldehyde into cell carbon can occur by two possible routes, by either the ribulose monophosphate pathway (Type I methanotrophs) or the serine pathway (Type II methanotrophs). In *Methylococcus capsulatus* both these routes may be used and there is also the possibility of incorporation of CO_2 via the Benson-Calvin cycle.



1 - Methane monooxygenase (MMO)

2 - Methanol dehydrogenase (MDH)

3 - Formaldehyde dehydrogenase

4 - Formate dehydrogenase

From Anthony, (1982)

Figure 1.4.1: The pathway of methane oxidation and dissimilation

1.4.1.1 Methane oxidation

The enzyme methane monooxygenase (MMO) is responsible for the initial O_2 and NAD(P)H-dependent hydroxylation of methane to methanol. This enzyme has been found to exist in two forms, a soluble form and a particulate form. The first reports of cell extracts capable of this conversion were by Ribbons & Michalover (1970). Tonge *et al.* (1975) observed a particulate MMO in *Methylosinus trichosporium* but it was not until 1976 that a soluble MMO was reported for *Methylococcus capsulatus* (Colby & Dalton, 1976). The best characterised systems to date are the soluble MMOs of *M.capsulatus* (Bath) and *M.trichosporium* OB3b.

These two organisms are now known to produce both forms of the enzyme. The production of the enzymes was found to be dependent upon the copper concentration perceived by the organism. At high copper concentrations, generally present at low biomass, the particulate form of the enzyme is produced. As the biomass increases and the copper concentration is reduced, there is some form of switch, as yet unknown, whereby the production of the particulate form is reduced and the soluble form is produced (Stanley *et al.*, 1983). These two enzymes have been found to be distinct and unrelated (Smith & Dalton, 1989). The particulate and soluble enzymes differ markedly in several features in both *M.capsulatus* and *M.trichosporium*. The soluble enzyme is capable of inserting oxygen into a wide range of aromatics,

alicyclic and heterocyclic compounds, higher n-alkanes and branched alkanes, whereas, the particulate enzyme is more limited in substrate specificity. This suggests that the hydroxylase component of the two enzymes differ. The particulate enzyme can obtain reducing equivalents from an electron transport chain or a NADH-independent hydrogenase, e.g, methanol dehydrogenase (Leak & Dalton, 1983) and the soluble form of the enzyme is capable only of gaining electrons from NAD(P)H.

The biochemistry of methane utilization has been reviewed by Anthony (1986) and Fox (1990) and therefore, I will merely outline the basics. The information about the enzyme methane monooxygenase was initially gathered by studies on the soluble MMO of *M.capsulatus* now, however, there is a substantial amount of data on the soluble MMO of *M.trichosporium* OB3b (Burrows *et al.*, 1984; Fox *et al.*, 1989; Fox *et al.*, 1991). Little information exists on any particulate MMO due to the difficulties found in the preparation of a pure and active enzyme from the membrane.

The soluble MMO of *M.capsulatus* and *M.trichosporium* OB3b are both three component systems: component A, the hydroxylase, component C, the NADH-driven reductase and component B, a regulatory protein.

Component A is a non-haem iron protein composed of three subunits of 54, 42, and 17 kDa in an $(\alpha\beta\gamma)_2$ arrangement. From electron paramagnetic resonance studies

component A was thought to be the site of substrate hydroxylation (Woodland & Cammack, 1985).

Component B has a molecular mass of around 16 KDa in *M.capsulatus* and *M.trichosporium* OB3b. It is a small unstable protein susceptible to protease degradation, it appears to contain no prosthetic groups. In *M.capsulatus* this protein is absolutely necessary for MMO activity (Green & Dalton, 1985), whereas in *M.trichosporium* OB3b it only gives a ten-fold stimulation in activity (Fox *et al.*, 1989), and it is not present in *Methylobacterium* CRL-26 (Patel & Savas, 1987). The role of protein B in *M.capsulatus* appears to be to split up high potential electron pairs from NAD(P)H and transfer them to the oxygenase component of protein A in one electron steps at a constant redox potential. In this manner it switches the oxidase activity of component A to an hydroxylase activity (Green & Dalton, 1985). It therefore has a regulatory role in the enzyme.

Component C is an iron-sulphur flavoprotein of 38 KDa. It contains one flavin adenine nucleotide (FAD) and one Fe_2S_2 centre per molecule (Colby & Dalton, 1978; Colby & Dalton, 1979). Protein C accepts electrons from NADH which are then passed on to protein A (Lund *et al.*, 1985). Protein C is able to pass electrons to protein A in the absence of protein B, but this transfer is not coupled to substrate oxidation.

The pathway of electron transfer between proteins A, B and C of the MMO complex is shown in figure 1.4.2.

Figure 1.4.2: The proposed molecular mechanism of soluble MMO in *M.capsulatus*.

1) Proteins A and C catalyze the novel four electron reduction of oxygen to water in the presence or absence of a hydroxylate substrate.

2) Addition of protein B switches the enzyme complex from an oxidase to an oxygenase and the reduction of oxygen to water is no longer catalyzed. In the absence of CH_4, steady-state electron transfer between proteins A and C is shutdown.

3) Addition of CH_4 to the complete methane monoxygenase complex restores inter-protein electron transfer and the oxygenase reaction is catalyzed to the complete exclusion of the oxidase reaction.

Dotted lines indicate very low reaction or no reaction.

Taken from Green and Dalton, (1985).

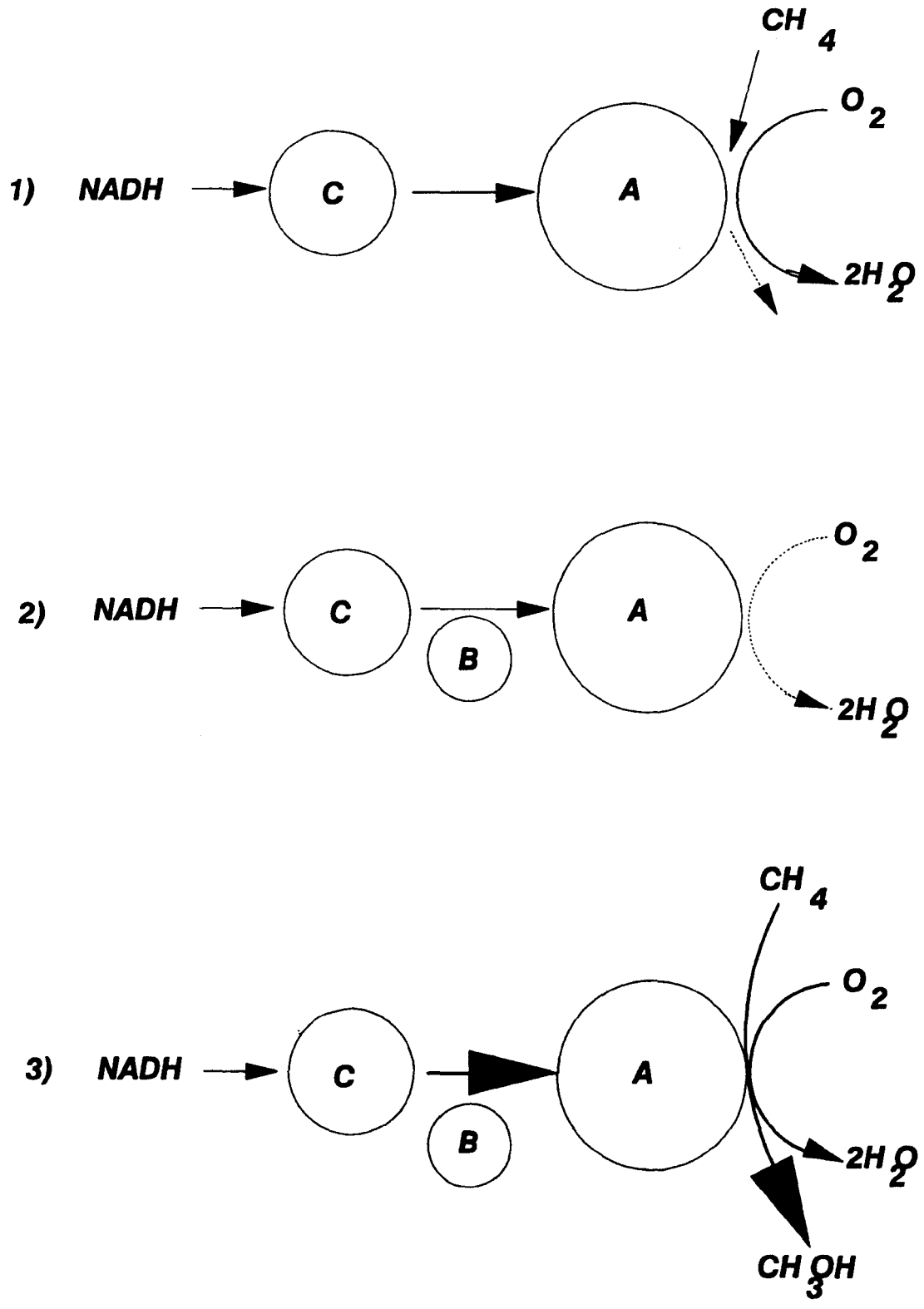


Figure 1.4.2: The proposed molecular mechanism of soluble MMO in M.capsulatus

1.3.1.2 Methanol oxidation

The catalysis of methanol to formaldehyde is carried out in all methylotrophs examined so far by a NAD(P)-independent methanol dehydrogenase (reviewed by Anthony, 1986). The enzyme is part of a periplasmic system that is coupled to at least one soluble cytochrome c also in the periplasm (Anthony, 1982).

Methanol dehydrogenase (MDH) was found to contain a novel prosthetic group a pyrrolo quinoline quinone (PQQ) which has been characterised extensively (Duine *et al.*, 1980; Salisbury *et al., 1979*) and is reviewed by Duine (1986). Each subunit of MDH is associated with a single PQQ group.

1.4.1.3 Formaldehyde and formate oxidation

Formaldehyde produced by the oxidation of methanol can be either dissimilated to carbon dioxide or assimilated into cell carbon. There are two possible pathways for dissimilation to CO_2. The first is by successive dehydrogenase action, initially to formate, thence to CO_2 (Figure 1.4.1). The second is by a series of cyclic reactions involving the ribulose monophosphate pathway enzymes.

The first route involves the enzyme formaldehyde dehydrogenase of which there are two groups, one NAD(P) dependent and one NAD(P)- independent. Stirling and Dalton (1978) isolated a NAD(P)-dependent formaldehyde

dehydrogenase from *M.capsulatus* and also found evidence to suggest there was a similar enzyme present in *M.trichosporium* OB3b (Stirling & Dalton, 1979). The formate oxidation is by a soluble NAD-dependent formate dehydrogenase (Stirling & Dalton, 1978).

The second route involves a cyclic series of reactions involving hexulosephosphate synthase and 6-phosphogluconate dehydrogenase (Strom *et al.*, 1974; Colby & Zatman, 1975). Two molecules of NAD(P)H are produced by each turn of the cycle.

1.4.1.4 Pathways of formaldehyde fixation

Carbon for the production of cell components is obtained from the diversion of formaldehyde from the oxidative pathway to assimilatory pathway. There are two possible routes for formaldehyde assimilation; the ribulose monophosphate (RuMP) pathway (Figure 1.4.3) and the serine pathway (Figure 1.4.4). The possession of these pathways has been used as a taxonomic feature, type I methanotrophs utilize the RuMP pathway and type II utilize the serine pathway (Colby *et al.*, 1979). The detection of these pathways is generally by assaying for the presence and activity of certain key enzymes in each pathway, however, it has been found that the presence of one or two enzymes is not conclusive proof of the dominance of that pathway. (Bamforth and Quayle, 1977). The biochemistry of methanotroph carbon assimilation pathways is reviewed by

several authors (Quayle, 1980; Higgins *et al.*, 1981; Anthony, 1982) and will not be discussed further here.

Figure 1.4.3

The RuMP pathway and its variations

Ru5P: ribulose-5-phosphate
Hu6P: D-erythro-L-glycero-3-hexulose-6-phosphate
F6P: fructose-6-phosphate
FDP: fructose-1,6-diphosphate
G3P: glyceraldehyde-3-phosphate
DHAP: dihydroxyacetone phosphate
E4P: erythrose-4-phosphate
Xu5p: xylulose-5-phosphate
S7P: sedoheptulose-7-phosphate
R5P: ribulose-5-phosphate
G6P: glucose-6-phosphate
6PG: 6-phosphogluconate
pyr: pyruvate

1 3-hexolosephophate synthase
2 phospho-3-hexuloisomerase
3 6-phosphofructokinase
4 fructose diphosphate aldolase
5 transketolase
6 transketolase
7 ribulose phosphate epimerase
8 ribulose phosphate isomerase
9 fructose diphosphatase
10 triosephosphate isomerase
11 glucose phosphate isomerase
12 glucose-6-phosphate dehydrogenase
13 6-phosphogluconate dehydratase + phopho-2-keto-3-deoxygluconate aldolase
14 transketolase + triokinase

Taken from Colby, Dalton and Whittenbury (1979)

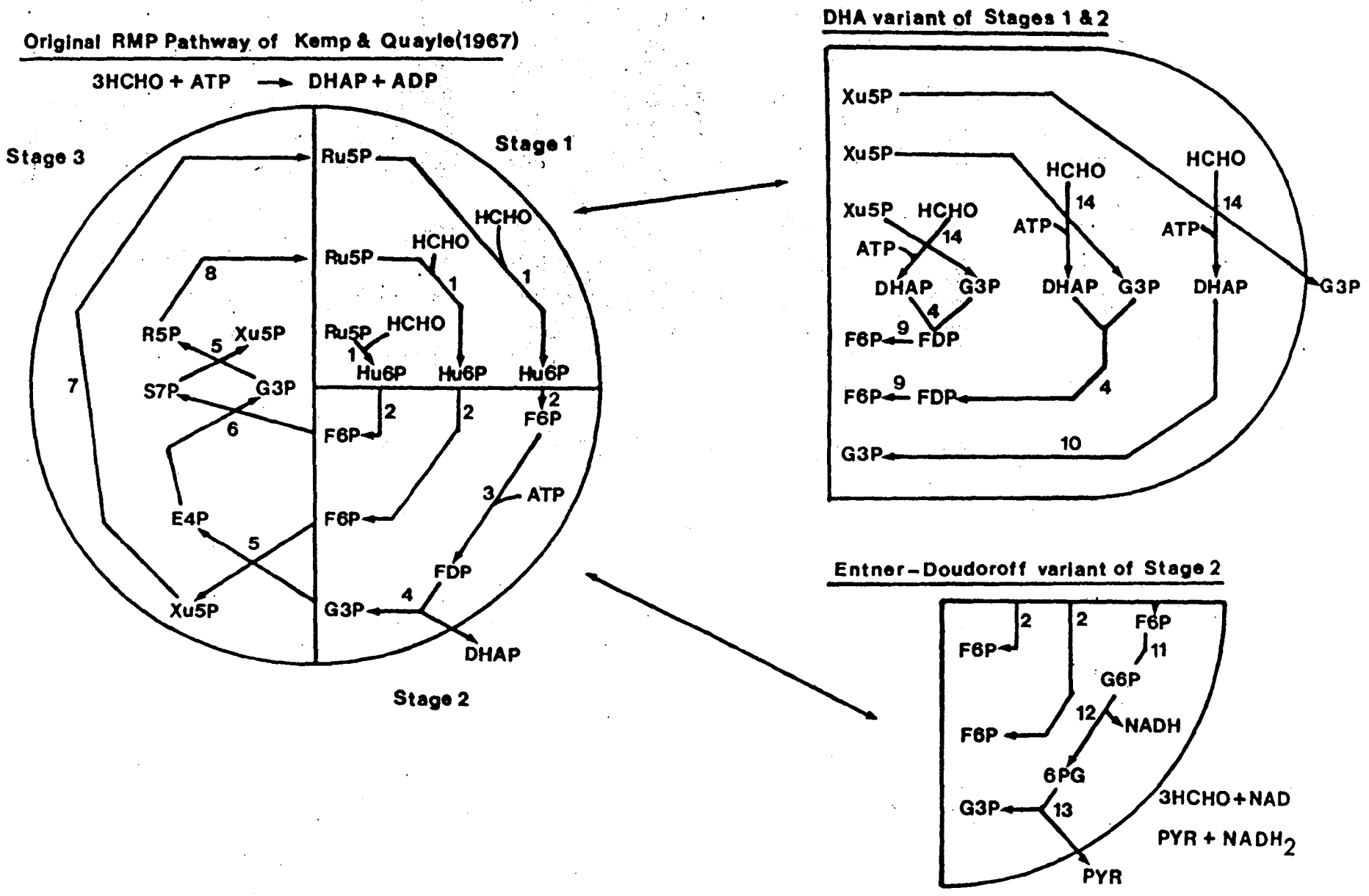

Original RMP Pathway of Kemp & Quayle(1967)
3HCHO + ATP → DHAP + ADP
Stage 3
Stage 1
Stage 2
Ru5P
HCHO
Hu6P
R5P
Xu5P
S7P
G3P
E4P
F6P
FDP
ATP
DHAP
Xu5P
DHA variant of Stages 1 & 2
Xu5P
HCHO
ATP
DHAP
G3P
F6P
FDP
Entner-Doudoroff variant of Stage 2
F6P
G6P
NADH
6PG
PYR
3HCHO+NAD
PYR + NADH2

Figure 1.4.4

The Serine Pathway

a) Serine transhydroxymethylase (EC 2.1.2.1)

b) Serine glyoxylate amino-transferase

c) Hydroxypyruvate reductase (EC 1.1.1.29)

d) Glycerate kinase (EC 2.7.1.31)

e) Phosphopyruvate hydratase (EC 4.2.1.11)

f) Phosphoenol-pyruvate carboxylase (EC 41.1.31)

g) Malate dehydrogenase (EC 1.1.1.37)

h) Malate thiokinase (EC 6.2.1.-)

i) Malyl-CoA lyase (EC 4.1.3.24)

j) Isocitrate lyase (EC 4.1.3.1)

- - - - unknown reactions

OHOPYR: hydroxypyruvate

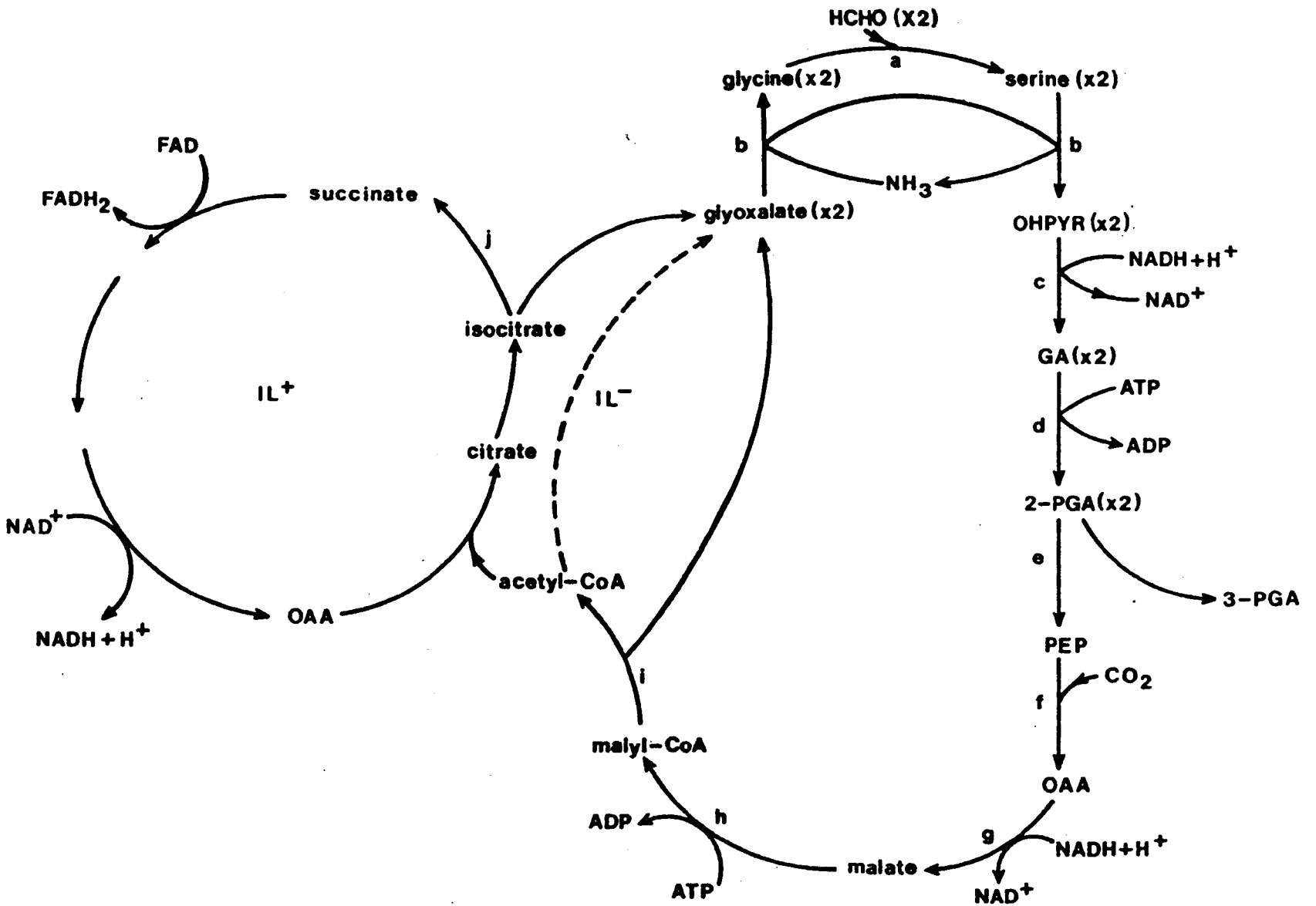
GA: glycerate

PGA: phosphoglycerate

PEP: phosphoenolpyruvate

OAA: oxaloacetate

Taken from Colby, Dalton and Whittenbury (1979)


HCHO (X2)
a
glycine(x2)
serine (x2)
b
b
NH3
glyoxalate(x2)
OHPYR (x2)
NADH+H+
c
NAD+
GA(x2)
ATP
d
ADP
2-PGA(x2)
e
3-PGA
PEP
CO2
f
OAA
g
NADH+H+
NAD+
malate
h
ADP
ATP
malyl-CoA
i
acetyl-CoA
citrate
isocitrate
j
succinate
FAD
FADH2
IL+
IL−
NAD+
NADH+H+
OAA

1.5 THE GENETICS AND MOLECULAR BIOLOGY OF THE METHANOTROPHS

1.5.1 Mutagenesis

There are several difficulties faced in the study of the genetic systems of methanotrophs. The least of which is the slow growth rate as it is possible to grow methanotrophs to good turbidity in batch culture in two to three days and to single colonies on nitrate minimal salts in 3-4 days.

In the study of the genetics of methanotrophy one of the major hurdles is the lack of any system for the production of mutants in their carbon metabolism, or in any other system for that matter. The substrate capabilities of methanotrophs is very restricted, i.e, methane. However, it is possible to grow some methanotrophs on methanol as a sole source of carbon and energy. The organisms often need a period of adaptation where the methanol concentration is slowly increased. This has been carried out successfully with *Methylocystis parvus* OBBP (Hou *et al.*, 1978), *Methylosinus trichosporium* OB3b (Best & Higgins, 1981) and *Methylomonas albus* (McPheat *et al.*, 1987a). The organisms will grow on 1% (v/v) high quality methanol and, in the case of *M.trichosporium* OB3b, the concentration could be increased to 4% (v/v). Other reports have stated that several methanotrophs can be adapted to grow on methanol vapour (Green, 1992). Growth on methanol allows the mutation of the enzyme methane monooxygenase.

Another commonly cited difficulty associated with genetic studies in methanotrophs is the sensitivity of the organisms to exogenous amino acid sources (Bainbridge, 1983; Lidstrom *et al.*, 1984), and hence the difficulty in obtaining auxotrophs. This however has found not to be the case. Eccleston and Kelly (1972) working with *Methylococcus capsulatus* (Foster and Davis strain), showed that out of 21 amino acids tested only threonine, phenylalanine, histidine, tyrosine and homoserine inhibited exponential growth when supplied at 1.0 mM. The organism was shown to have a broad specificity amino acid transport system. It has also been shown that a number of amino acids will serve as nitrogen sources for methanotrophs (Murrell, 1981).

1.5.1.1 Conventional mutagenesis

The frequency of spontaneous mutation in *Methylococcus capsulatus* (Foster and Davis strain), as judged by the frequency of resistance to antibiotics, amino acid analogues and other compounds, was assessed by Harwood *et al.*(1972). The frequency was found to be similar to that of other bacteria (<1 in 1 x 10^8). Attempts to increase this frequency using conventional mutagens such as ultra-violet light, nitrosoguanidine and ethyl methane-sulphonate failed. This failure was attributed to lack of an error-prone SOS repair system, as the compounds caused cell death but did not affect the frequency of mutation (Williams *et al.*, 1977; Williams & Bainbridge, 1976) but this observation has not

been adequately studied and there may be other reasons why these mutagens do not effect methanotrophs. It is possible to induce filamentation in methanotrophs using uv light (Williams & Shimmin, 1978), and the sensitivity of the methanotrophs *Methylosinus* sp and *Methylobacter* sp to mutagens is increased in this state. Due to this phenomenon, mutants for which no direct selection exists are very difficult to isolate and would require the screening of millions of colonies (Harwood *et al.*, 1972). Drug resistant mutants have been isolated, along with one leaky p-aminobenzoic acid requiring auxotroph of *M.capsulatus* which was a spontaneous mutant (Harwood *et al.*,1972).

1.3.1.2 Dichloromethane mutagenesis.

Dichloromethane (DCM) is fortuitously oxidised by the enzyme methane monooxygenase (MMO) to carbon monoxide, a build up of which is lethal to the cell . This 'suicide substrate' can be used to isolate methanotrophs defective in the first step of methane metabolism. The method relies on the ability of some methanotrophs to grow on methanol but still produce MMO. Therefore, only cells defective in MMO will survive incubation with DCM. This method has been successfully used to isolate mutants of *Methylomonas albus* (McPheat *et al.*, 1987) and *Methylosinus trichosporium* OB3b (Nicolaidis & Sargent, 1987). The success of the method has also been attributed to the inherent mutagenicity of DCM (Jongen *et al.*, 1978) and its ability to permeate the cells

and/or increase the permeability of the cells. A mutation frequency of 1 x 10^{-4} to 1 X 10^{-5} for *Methylomonas albus* was reported (McPheat *et al.*, 1987) with a reversion frequency of 1 x 10^{-8}. The mutants were stable and failed to oxidize propylene oxide (Leak & Dalton, 1983), a standard method for detecting active MMO. It was found that on polyacrylamide gels the mutants could be split into 3 groups dependent on bands missing from the gels which corresponded to four polypeptides thought to be the MMO. Since *Methylomonas albus* does not contain a soluble MMO the mutations were all in the particulate MMO. The mutations in *Methylosinus trichosporium* OB3b could have been in the particulate or soluble form of the enzyme but due to the medium used for growth (a high concentration of copper compared to biomass) they were more likely to be expressing the particulate form and so still contained an active soluble form (Nicolaidis & Sargaent, 1987).

Recently a method has been devised to determine the presence of the soluble form of MMO while the organism is growing on plates. This relies on the ability of soluble MMO to convert naphthalene to 1-naphthol and 2-naphthol, a conversion which cannot be performed by particulate MMO. The napthols can then be reacted with tetrazotised o-dianisidine to form purple diazo dyes with large molar absorptivities (Brusseau *et al.*, 1990). This assay will obviously be very important for the rapid detection of mutants defective in soluble MMO on plates.

1.5.2 Gene transfer

Any successful genetic study of an organism relies on the presence of a system whereby homologous and non-homologous DNA can be introduced into the organism. A system for gene transfer is needed for certain types of mutagenesis, e.g, transposon mutagenesis, expression studies and complementation studies. An attempt to produce such a system began with a study of plasmid and bacteriophage biology in the methanotrophs.

1.5.2.1 Plasmids and bacteriophages

The first screening of obligate methanotrophs for plasmid DNA was carried out by Warner *et al.*(1977). They screened three obligate methanotrophs; *Methylomonas methanica, Methylococcus capsulatus* (NCIMB 11083) and *Methylosinus trichosporium* OB3b, along with a facultative methylotroph; *Pseudomonas* AM1 (now *Methylobacterium extorquens*). Plasmid DNA was found only in *Pseudomonas* AM1 (three distinct plasmids). A later screening of obligate methanotrophs for plasmids by Lidstrom & Wopat (1984) resulted in the isolation of several plasmids from nine out of ten strains. Only *M.capsulatus* (Bath) was found to contain no plasmids. They used several techniques for their isolations, the most successful being a harsh alkaline-SDS

Strain	Size (kb)		
Type I			
Methylomonas albus **BG8**	**55**		
Methylobacter capsulatus **Y**	**94**		
Methylomonas methanica **S1**	**186**		
Type X			
Methylococcus capsulatus	**none detected**		
Type II			
Methylosinus trichosporium **OB3b**	**186**	**159**	**145**
Methylosinus trichosporium **OB3bH**	**186**	**159**	**145**
Methylosinus trichosporium **OB5b**	**186**	**159**	**145**
Methylosinus sporium **5**	**170**	**108**	
Methylocystis parvus **OBBP**	**186**	**159**	
Methylocystis **POC**	**176**	**152**	**75**

Table 1.5.1: Plasmids detected in methanotrophs

Taken from Lidstrom *et al.*, (1984)

lysis then absolute minimum handling after lysis. Table 1.5.1 shows the plasmids isolated. They are all large, the smallest being 55 kb and the largest 186 kb, and difficult to obtain in large quantities, which made restriction analysis difficult.

The plasmids present in different species of obligate methanotroph appeared to be distinct. Varying in size and restriction pattern. In hybridization experiments. homology occurred only when plasmids from *M.trichosporium*were used to probe restriction digests of *M.sporium* plasmids (and vice versa). A small region of homology was found which could have represented a common function such as plasmid replication. Screening for antibiotic and heavy metal resistances showed that none of these characteristics could be assigned to any of the plasmids. Despite their large size, often indicative of encoding conjugative functions, they did not appear to perform conjugal transfer but there were however, no suitable markers to check for transfer. The 55 kb plasmid from *M.albus* BG8, the easiest to isolate, was chosen for further study and was partially restriction mapped. This plasmid has possibilities for the construction of cloning vectors for methanotrophs.

Prior to this extensive screening of methanotrophs plasmids had been detected by Haber *et al.*(1983) in the organism known as SB-1, a type II methanotroph. Two plasmids were present of 130 and 210 kb. The smaller plasmid, pR6-1,

was restriction mapped, fragments sub-cloned into pBR322, and it was stably maintained in *Escherichia coli* HB101. Haber *et al.* also carried out conjugation experiments to ascertain whether these plasmids would mediate chromosomal mobilization. Crosses were carried out with a variety of *Pseudomonas aeruginosa* auxotrophs. One leucine requiring auxotroph was complemented at a frequency of 2×10^{-4} transconjugants per recipient cell. The plasmid contained in this strain upon analysis was found to exhibit homology to both pR6-1 and the resident *Ps.aeruginosa* plasmid (Haber *et al.*, 1983).

Methane oxidation does not appear to be encoded on plasmids. The production of methane oxidation mutants of *M.albus* BG8 using DCM (section 1.3.1.2) was not accompanied by loss of the 55 kb plasmid. The genes coding for MMO have been cloned from *M.capsulatus* (Bath) (Mullens & Dalton, 1987; Stainthorpe *et al.*, 1989, 1990) and from *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991) and the genes were found to be located on the chromosome.

There appears to be no suitable natural plasmids present in the obligate methanotrophs from which a gene transfer system could be devised.

There have been a number of reports of the isolation of bacteriophages specific for certain strains of methanotroph (Tyutikov *et al.*, 1976; Tyutikov *et al.*, 1980; Tyutikov *et al.*, 1983; Tikhonenko *et al.*, 1982). They have been isolated from a wide range of environments including

groundwater, soils, cattle, fish and oil and gas installation waters. The most common appeared to be bacteriophages specific for *Methylosinus* and *Methylocystis* species. These bacteriophages however did not exhibit any transducing ability.

1.5.2.2 Transformation and electroporation

The successful transformation of DNA into an obligate methanotroph was reported by Williams and Bainbridge (1971) working with *Methylococcus capsulatus*. This procedure required long contact times and large amounts of DNA and was therefore highly inefficient. Although a variety of transformation protocols were tested for *M.capsulatus* (Bath) by Cardy (1988), none were successful.

The technique of electroporation has been successfully used on many organisms generally refractory to normal transformation methods, and has been used with some success for transformation of *M.trichosporium* OB3b. This method will be discussed further in section 1.8.

1.5.2.3 Conjugation systems

The first report of the transfer of plasmids by conjugation into obligate methanotrophs was by Warner *et al.*(1980). They transferred, by filter-mating, the broad host range plasmid, R68.45 (Inc P1), from *Pseudomonas aeruginosa* PAO8 to *Methylosinus trichosporium* OB3b. Kanamycin resistance was selected for and the frequency of

transfer was 10^{-2} to 10^{-3} transconjugants per donor. They also transferred the broad-host range plasmid RP4. However, they were unable to transfer either of these plasmids to *Methylococcus* sp NCIB 11083 by patch matings or plate mating.

Since this report several potentially useful gene transfer systems have been reported for methanotrophs and methylotrophs. Lidstrom *et al.*(1984) developed a filter mating system for the transfer of the Inc P1 cosmid pVK100 (Knauf & Nester, 1982) by mobilization with the RP4 derivative pRK2013 (Figurski & Helsinki, 1979) into three different species of obligate methanotroph. The frequencies of transfer were 10^{-2} per donor for both *Methylomonas albus* BG8 and *Methylocystis* POC, a frequency high enough to allow direct complementation of mutants. The frequency of transfer into *Methylosinus* 6 was considerably lower, 10^{-8} per donor, but this would still allow for the transfer of specific cosmid derivatives into the cell.

McPheat *et al.*(1987) studied the transfer of several plasmids into *M.albus* BG8. The IncP1 plasmid RP4 and the IncQ plasmid R300B were transferred at a frequency of 10^{-3} per donor. The IncW plasmid pS-a, IncP1 plasmids R68.45 and R751 and derivatives containing the bacteriophage Mu and/or transposons were transferred at a lower frequency of 10^{-7} to 10^{-8}. They concluded that the Mu-associated suicide effect did not occur in *M.albus* and these plasmids could, therefore, not be used for transposon mutagenesis. A

possible reason for the low frequency of transfer into *M.albus* was thought to be the presence of a restriction system. This theory was tested by comparison of the frequencies of transfer into heat-shocked and normal cells. Although there was a 5-6 fold increase in plasmid transfer into the heat-shocked cells, this was not thought to be significant enough to indicate a restriction system having a major impact on transfer.

The RP4 derivative pULB113 (RP4::mini-mu) was used by Al-Taho and Warner (1987). This plasmid was shown to be transferred from *E.coli* to *Methylosinus trichosporium* OB3b and into two facultative methylotrophs, *Methylobacterium* AM1 and *Methylobacterium extorquens*. The plasmid was also transferred between the species of methylotroph and back to *E.coli*. The plasmid however, was only able to mobilize the chromosome of the facultative methylotrophs, and no formation of R-primes was shown for *M.trichosporium* OB3b.

1.5.2.4 Transposon and marker exchange mutagenesis

The discovery of broad-host range transfer systems for use with methanotrophs opened up the possibilities for transposon mutagenesis and marker exchange mutagenesis. Lidstrom *et al.*(1984) attempted transposon mutagenesis with a range of suicide plasmids including pJB4JI (Beringer *et al.*, 1978), hybrid IncP-Mu-Tn5 plasmids, and several ColE1 plasmids containing Tn5, Tn10 and Tn7, transferred into the three methanotrophs *M.albus*, *Methylocystis* POC and

Methylosinus 6. All these plasmids proved unsuccessful possibly due to the low frequency of transfer, and/or transposition, which made detection within the background of spontaneous resistance impossible.

Lidstrom *et al.*(1984) decided to look at the possibility of marker exchange mutagenesis, initially described by Ruvkun and Ausubel (1981) for use in *Rhizobium*. Lidstrom *et al.*(1984) created a *Hin*dIII gene library of the *Methylosinus* 6 chromosome in pBR325. This library was probed with a heterologous probe of the genes concerned with nitrogen fixation from *Klebsiella pneumoniae* (Ruvkun & Ausubel, 1980). A 2.3 kb homologous fragment of DNA was isolated, which was then mutagenized by insertion of Tn5. This hybrid plasmid could be transferred by conjugation to *Methylosinus* 6 using the helper plasmid pRK2013. The plasmid would be unstable and selection for kanamycin would identify organisms in which the mutated *nif* genes had undergone homologous recombination with the chromosomal *nif* genes to produce an organism unable to fix nitrogen, but resistant to kanamycin (Figure 1.5.1). It was found that this was one of four possibilities, the others being; insertion of the entire plasmid in the *nif* genes, partial plasmid insertion, and insertion of the Tn5 at a site other than the *nif* genes. These possibilities could be identified by screening the chromosome of kanamycin resistant colonies with Tn5, pBR325 and the 2.3 kb *nif* fragment. The mutants were stable, and analysis of the polypeptides associated with nitrogen

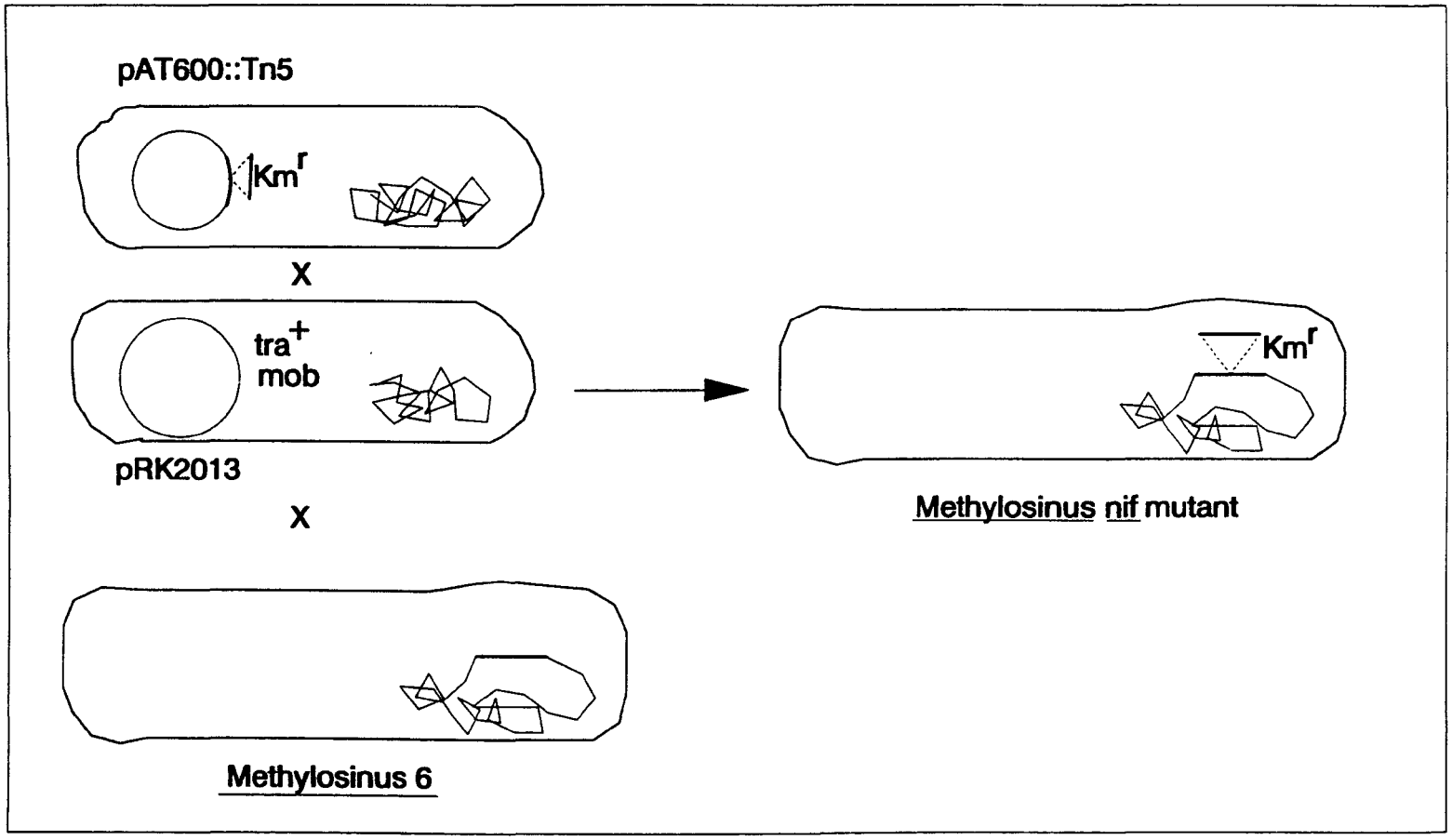


Figure 1.5.1: Marker exchange mutagenesis of Methylosinus 6

fixation showed that three polypeptides were missing. It was concluded that this one step marker exchange was a suitable method for the production of mutants, however, care would have to be taken in the screening of the mutants to determine the exact events at the molecular level.

1.5.3 The molecular biology of the methanotrophs

It is relatively recently that the molecular biology of the methanotrophs has received attention. Studies on their biochemistry and physiology are fairly advanced in the area of carbon metabolism and information is available on the nitrogen metabolism. Therefore, these two systems were chosen for the study of the molecular biology in an attempt to produce a more complete picture of these systems. The techniques developed would also be of use in the study of other methanotroph systems.

1.5.3.1 Nitrogen metabolism genes

a) Nitrogen fixation genes

Nitrogen fixation is carried out by all type II and type X methanotrophs (Murrell & Dalton, 1983). The *nif* genes required for nitrogen fixation have been found to be highly conserved among the diazotrophs and the *Klebsiella pneumoniae* genes *nif*HDK have been used to identify *nif* gene clusters in several organisms (Ruvkun & Ausubel, 1980; Mazur *et al.*, 1980; Hennecke, 1981; Normand *et al.*, 1988). The *Klebsiella pneumoniae nif*HDK cluster on plasmid pSA30

(Cannon *et al., 1979*) was used by Toukdarian and Lidstrom (1984) to identify the *nif*HDK genes in *Methylosinus* 6, a type II methanotroph. The genes were chromosomally located and a 2.3 kb *Hin*dIII fragment was cloned onto pBR325 to produce plasmid pAT600 and pAT601 (reverse orientation). *E.coli* Maxi-cell analysis of the products of these plasmids identified two polypeptides of molecular weight 57,000 and 34,000. The proteins were thought to be expressed from vector promoters, not cloned *Methylosinus* promoters. The *nif*H, D and K genes of the facultative methylotroph *Xanthobacter* H4-14 were also identified. The hybridization patterns found with both methylotrophs were dissimilar indicating that the *nif* region was not physically conserved (Toukdarian & Lidstrom, 1984).

The 2.3 kb fragment isolated from *Methylosinus* 6 was then used in a marker exchange experiment (Toukdarian & Lidstrom, 1984), which has been outlined in section 1.5.2.4. The mutants produced, unable to grow without a source of combined nitrogen, lacked three out of the five major polypeptides associated with nitrogen fixation.

The *Klebsiella pneumoniae nif*H gene was used to identify *nif* genes in other methanotrophs. Oakley and Murrell (1988) surveyed 13 strains of methanotrophs, 6 type I, 6 type II, and *M.capsulatus* (Bath) for the presence of *nif*H homologes in the chromosome. Although only the type II and type X methanotrophs are known to fix nitrogen (Murrell & Dalton, 1983) *nif*H homologes were also found in the type I

non-diazotroph, *Methylomonas methanica*. They postulated that this indicated an evolutionary loss of nitrogen fixing ability. This work continued with *M.capsulatus* (Bath). Using the *nif*HDK probe from *K. pneumoniae* the identification and subsequent cloning of a 10 kb fragment was carried out. The fragment contained the structural *nif* genes in the order *nif*H, D and K as has been found in many other diazotrophs (Murrell & Oakley, 1991). The *nif*H gene has subsequently been sequenced and shown to contain a high degree of homology with other *nif*H genes (Murrell & Oakley, unpublished observations). A *M.capsulatus* cosmid bank was probed with *nif*B, A, L and *nif*V, S and homologous sequences were identified.

b) Ammonia assimilation genes

Chromosomal DNA from a number of Type I and Type II methanotrophs was screened with probes of the nitrogen regulatory genes *ntr*A, B and C, and the glutamine synthetase structural gene (*gln*A), obtained from *Klebsiella* and *Azotobacter*. *gln*A homologues were found in the chromosome of all the Type I and Type II methanotrophs tested. Homologues to the *ntr*C and *ntr*A genes of *K.pneumoniae* were also found (Cardy, 1989). This is evidence for the presence of the *ntr* system for the control of nitrogen metabolism. This system is well reviewed for the enterobacteriacae (Merrick, 1988).

The *gln*A gene was isolated using a heterologous probe from *Klebsiella pneumoniae* (Cardy & Murrell, 1990). It was found to be present on a 5.2 kb *Eco*RI genomic DNA

fragment separated from *ntrC* by some 8.5 kb, a situation found in some other non-enteric organisms (Szeto *et al.*, 1987; Pawlowski *et al.*, 1987; Haselkorn, 1986). The *glnA* gene was analysed in detail (Cardy & Murrell, 1990). The gene was sequenced and the protein product expressed in a Zubay-type *in vitro* transcription/translation system. Two types of promoter-like sequences were found to be present, one resembling an *E.coli* -10, -35 promoter and the other resembling an *E.coli* NtrA dependent promoter with conserved sequences at -12 and -24. The promoter arrangement was similar to that found in the Enterobacteriaceae *glnA* gene. The gene was able to complement *E.coli glnA* mutants and its expression in this organism was regulated by nitrogen levels (Cardy & Murrell, 1990).

1.5.3.2 Carbon metabolism

a) Methane monooxygenase genes

Mullens and Dalton (1987) used the first 39 amino acids of the N-terminal of the γ sub-unit of protein A of the soluble MMO of *Methylococcus capsulatus* (Bath) to construct a degenerate oligonucleotide probe, with which they isolated a plasmid containing a 0.64 kb *Sal1-Pst1* fragment, which, when restriction mapped, showed good homology to the original γ probes. The identity of this fragment was confirmed by DNA sequencing of the 5′ end.

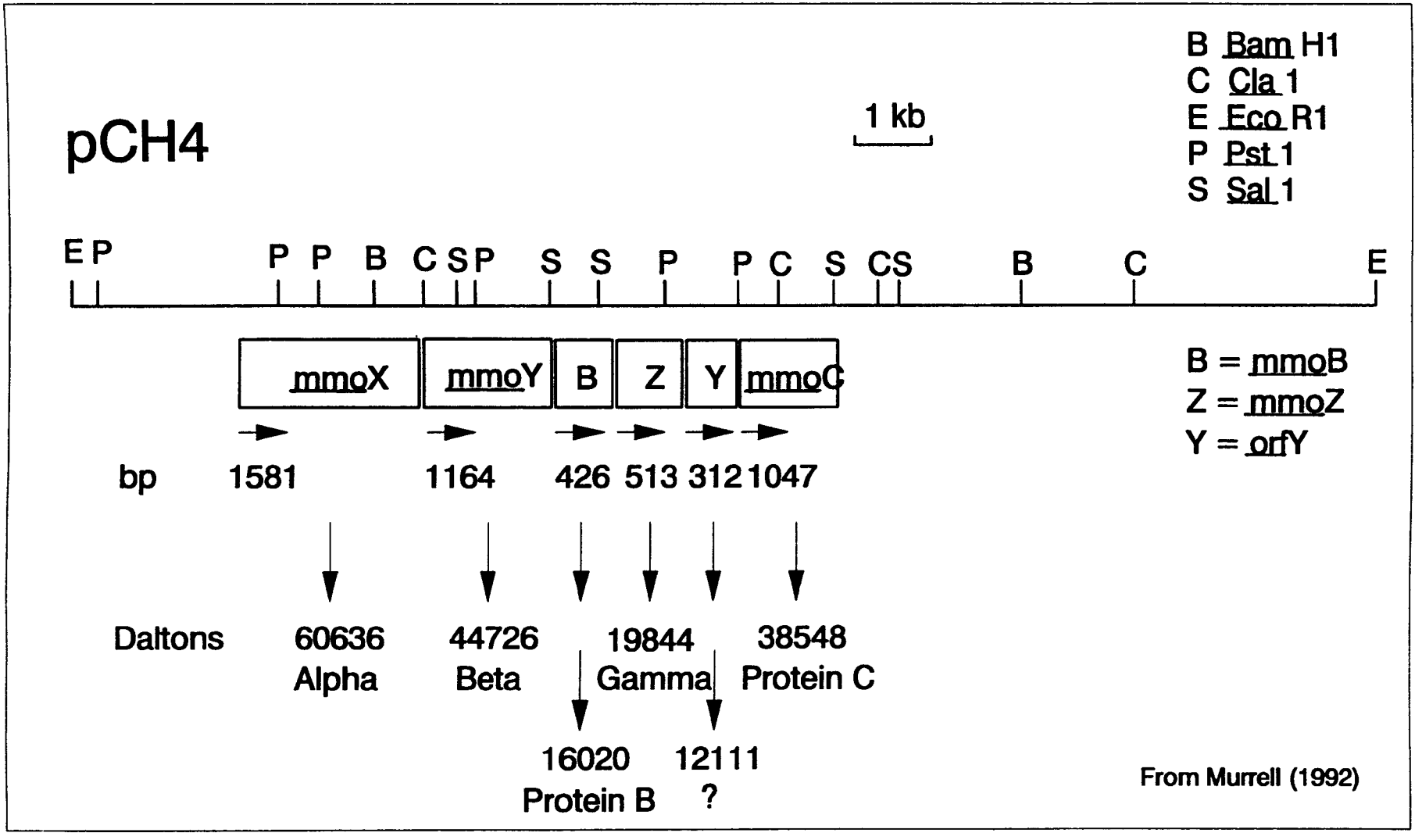


Figure 1.5.2: Organization of the M.capsulatus MMO genes

This method was also used by Stainthorpe *et al.*(1989). The N-terminal amino acid sequence for theß-subunit of protein A from *M.capsulatus* (Bath) was used to design a short (14 nucleotides) mixed oligonucleotide probe, which was used to isolate a recombinant plasmid, pCH4. Sequencing of the pCH4 plasmid has shown that the 11.9 kb fragment contains the entire sMMO gene cluster, α, ß, and γ subunits of protein A, *mmo*X, Y and Z, protein B (*mmo*B) and protein C (*mmo*C) (Stainthorpe *et al.*, 1989, 1990). These are all linked and their organisation can be seen in figure 1.5.2.

*mmo*X, which codes for theα-subunit of protein A, was found to be very similar in size to the published size of the polypeptide (Woodland & Dalton, 1984). The protein did not show significant homologies to any protein present in current databases there were, however, some similarities to conserved regions of the ribonucleotide reductase B_2 proteins of *E.coli* and other organisms. The ribonucleotide reductases contain a μ-hydroxobridge dinuclear iron centre (Nordland *et al.*, 1990) which is vital for catalytic activity. This type of structure may also be associated with protein A, where it is thought the active site resides, and it is hoped through site-directed mutagenesis of the protein to elucidate the residues that bind the iron and their importance in activity of the enzyme.

*mmo*Y, the ß-sub-unit of protein A, is separated from *mmo*Z by the gene *mmo*B (Pilkington *et al.*, 1990). The

polypeptides encoded by these genes showed no significant homologies with any proteins in the current databases.

The *mmo*C gene encodes the reductase component of sMMO. It is an iron-sulphur protein and shows homology to bacterial and plant ferredoxins. Sequencing has confirmed that protein C has a plant-like ferredoxin 2Fe-2S centre already suggested by its absorbance and EPR spectra (Lund *et al.*, 1985).

Of the promoters found on the gene cluster only *mmo*X is preceded by a consensus sequence homologous to the -35 and -10 sequence of *E.coli*. *Nif* and *ntr*-like consensus sequences were also found preceding *mmo*X. The regulation sequences present on this cluster of genes may be entirely new due to their regulation by copper and methane, and S1 nuclease mapping and primer extension experiments are being carried out to elucidate the control of transcription. In order to start a program of site-directed mutagenesis on the MMO proteins the polypeptides have been expressed in *E.coli*. A DNA fragment containing *mmo*B was cloned into a T7 expression vector, pT7-5 (Tabor & Richardson, 1985), and the protein expressed was found to be functional. Another fragment containing *mmo*C was cloned into pT7-7, a similar expression vector but containing its own ribosome binding site and ATG start codon, and this was also expressed as a fully functional protein (C.West, Pers. Communication).

A 5.8 kb *Bam*H1 fragment of pCH4 was used as a probe to screen a variety of type I and type II

methanotrophs (Stainthorpe *et al.*, 1990). The type II methanotrophs *Methylosinus sporium* 5, *Methylosinus sporium* 12 and a variety of *Methylosinus trichosporium* strains showed significant homology to the probe. No homology was found with *Methylocystis parvus* OBBP (type II) or any type I strains. This is consistent with the biochemical information that indicates that these strains do not possess a soluble MMO but only the particulate form.

The *mmo* gene cluster of *M.trichosporium* OB3b has also been cloned and sequenced, (Figure 1.5 3) (Cardy *et al.*, 1990, 1991). Sequence comparison of the *mmo*X genes of *M.capsulatus* and OB3b showed a 94% similarity, a high degree of conservation between the species. The promoters of the genes have been studied using S1 nuclease mapping and primer extension analysis. *M.trichosporium* OB3b can be adapted to grow on methanol and is thus, much more amenable to genetic analysis than *M.capsulatus*. Biochemical analysis of the sMMO by Fox *et al.*, (1989,1990) and recent studies on genetic transfer techniques should make this organism ideal for the study of obligate methanotrophy.

a) Methanol dehydrogenase (MDH)

A study of *Methylobacterium* sp. strain AM1 mutants defective in growth on methanol by Nunn and Lidstrom (1986a,b) showed that at least 10 genes were involved in the production of active MDH. This number was then increased to 12 by studies on what was thought to be two linked genes coding for the MDH structural gene and

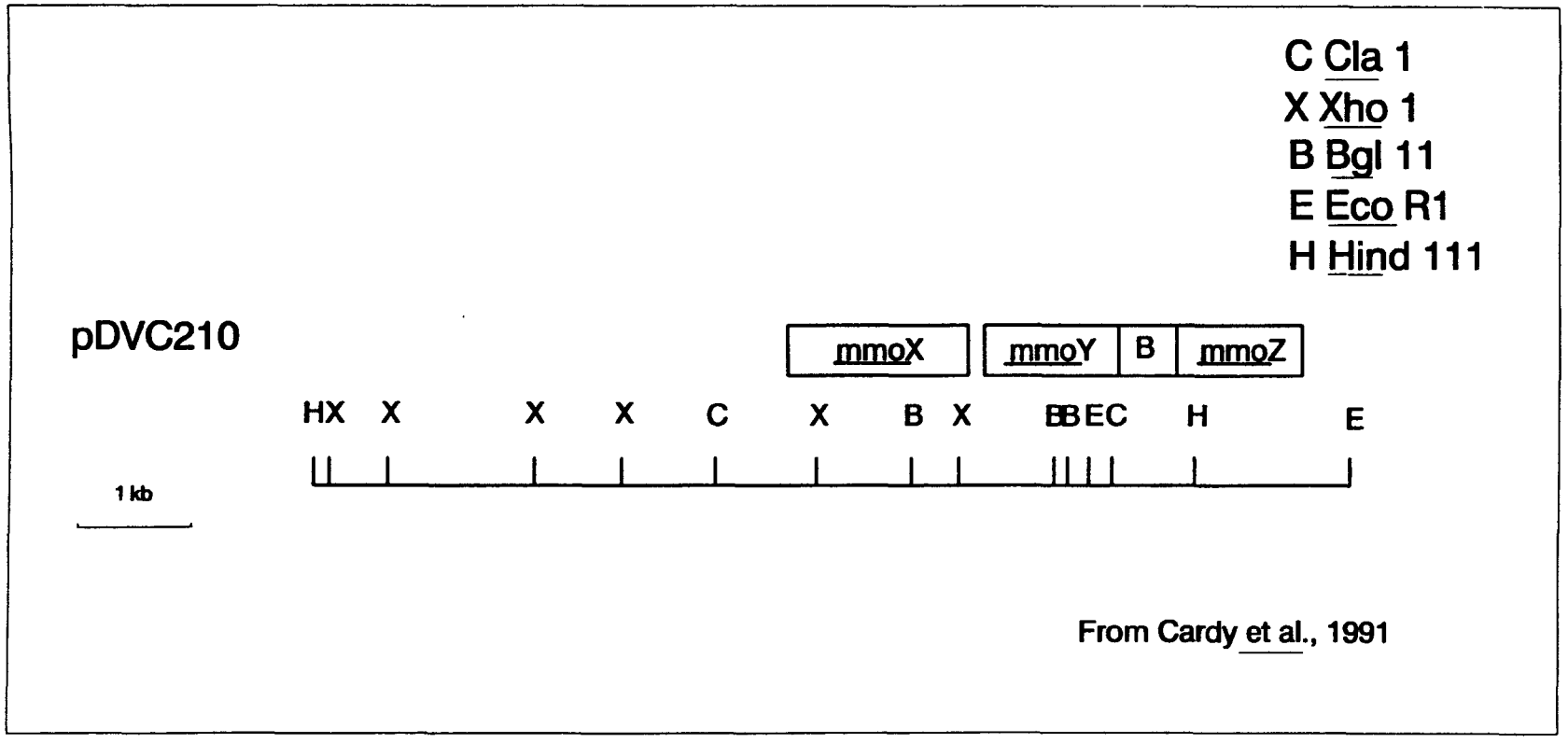


Figure 1.5.3: Organization of M.trichosporium OB3b genes

cytochrome C_L. The DNA was in fact found to code for four protein products (Anderson & Lidstrom, 1988). Further reports brought this number up still further to a possible 20 for *Methylobacterium* sp. strain AM1, and 14 for *Methylobacterium organophilium* XX (Bastien *et al.*, 1989; Lidstrom, 1990). The molecular biology of methanol dehydrogenase in the methylotrophs will be discussed in more detail in the next section.

The *mox*F gene isolated by Nunn and Lidstrom (1986b), coding for the large subunit of MDH was used to identify putative *mox*F genes in obligate methanotrophs (Stephens *et al.*, 1988). Mutations in MDH in obligate methanotrophs are lethal which makes the isolation of mutants extremely difficult. The study of the expression of the genes involved in methanol oxidation has to be carried out in an alternative host system such as *Methylobacterium* AM1. The success of this approach is dependent on the amount of biochemical and genetic conservation in the *mox* genes.

Stephens *et al.*(1988) used an internal fragment of the *Methylobacterium* AM1 *mox*F gene to screen a range of type I, type II, and type X (*Methylococcus capsulatus*) obligate methanotrophs. The probe showed virtually no homology to non-methylotrophs. The homology was such that it was possible to identify chromosomal fragments in a variety of type I, II and X obligate methanotrophs, together with restricted and facultative serine pathway methanol utilizers. The results suggest that the *mox*F gene is broadly

conserved among methylotrophs. The putative *mox*F of *Methylomonas albus* BG8 and *Methylococcus capsulatus* (Bath) were selected for further study. They were cloned using cosmid banks, then subcloned into the plasmid pRK310 to produce the plasmids pRS2117 (*M.capsulatus*) and pRS8604 (*M.albus*). The presence of MDH was determined using two methods; a T7 expression system in *E.coli* and expression in a MDH^- mutant of *Methylobacterium* AM1. In the first system, using *E.coli*, the presence of MDH was determined using western blot analysis and antibody to *M.albus* BG8 MDH. Bands of 60 kDa were found for both *M.albus* BG8 and *M.capsulatus* (Bath) which corresponded to the large subunit of MDH. Active MDH can not be detected in *E.coli* as the co-factor PQQ is not produced. The second system, using the *Methylobacterium* AM1 mutant, avoided this problem. The plasmids were mobilized into the mutant using pRK2013. A high frequency of methanol positive colonies was produced which contained MDH activity (Stephens *et al.*, 1988). The activity was much lower than in the wild-type, but it was interesting that the MDH was active in this heterologous host, due to the number of genes known to be involved in MDH activity.

The structural gene from *Methylobacterium organophilium* XX was used to identify the *mox*F and other *mox* genes in the obligate methanotroph *'Methylosporovibrio methanica* 81Z', which were then shown to function in *Methylobacterium* sp. AM1 (Bastien *et al.*, 1989)

Lidstrom and colleagues have identified a number of *mox*A genes in methanotrophs using heterologous *Methylobacterium mox*A probes. *Mox*A genes are involved with the association of MDH and the co-factor PQQ.

Another approach to the identification of *mox*F genes from methanotrophs is to use degenerate oligonucleotide probes designed by examining the sequence of the highly conserved 5′ end of *mox*F (Waechter-Brulla & Lidstrom, 1990). This method avoids the problem of varying G+C ratios and codon bias present in different methanotrophs. This method has been used to clone the *mox*F gene from *Methylomonas* A4 which shows considerable sequence homology with promoters of other *mox*F genes.

The MDH from the type II methanotroph, *Methylosinus trichosporium* OB3b, has been isolated using an entirely different method. A genomic library of *M.trichosporium* was constructed in the expression vector, gt11. This was then screened with antibody raised against purified MDH from *M.trichosporium* (Al-Taho *et al.*, 1990). Lysates were prepared from positive plaques after growth of the phage in the presence of the inducer IPTG, then western blotted. A large molecular weight protein cross reacted with the antibody, this was thought to be a fusion protein. After subcloning and expression studies a 2.1 kb DNA fragment was isolated which expressed a protein of a smaller molecular weight which cross-reacted with MDH antibody. The inducer

IPTG was not required for expression which was therefore thought to occur from an unidentified methanotroph promoter.

These three different methods for the isolation of genes from methanotrophs will be of great importance in the study of the molecular biology of the obligate methanotrophs. The degree of conservation of proteins in the methylotrophs is also interesting as this will hopefully make it possible to use methylotroph mutants as heterologous hosts for the expression of methanotroph genes instead of *Escherichia coli* which has many drawbacks.

1.6 THE GENETICS AND MOLECULAR BIOLOGY OF METHYLOTROPHIC BACTERIA

The genetics of methylotrophy is far more advanced than that of methanotrophy for several reasons, one of the most important being the discovery that a wide range of broad-host range plasmids could be transferred to methylotrophs and stably maintained. These plasmids have been used for a number of applications. It has also been possible to produce a wide variety of mutants using various techniques, including marker exchange and transposon mutagenesis, in both obligate and facultative methylotrophs. Genes from methylotrophs have also been expressed in *Escherichia coli* making analysis of the products possible. The study of Gram-positive methylotrophs is much slower due to a lack of suitable cloning vectors. The successful use of shuttle vectors derived from coryneform bacteria has been

recently reported by Nesvera *et al.*(1991) for protoplast transformation of *Brevibacterium methylicum*, a facultative Gram-positive methylotroph, and this should enable the study of methylotrophy in Gram-positives. Another technique that will advance the study of methylotrophy is that of electroporation. The technique will be discussed in more detail in section 1.7.2. It has already been successful with *Methylobacterium extorquens* NR-2 (Ueda *et al.*, 1991). The broad-host range plasmid pLA2917 was electro-transformed into *M.extorquens* NR-2 at an efficiency of 8 X 10^3 transformants per μg of plasmid DNA. The success of this method opens up the possibility of using a much wider range of plasmids for methylotrophs. The important techniques used in the study of the molecular biology of methylotrophy will be discussed in the following sections.

1.6.1 Plasmids from methylotrophic bacteria.

Many plasmids have been isolated from methylotrophs but so far they have all remained cryptic. One of the first isolations was of three plasmids from *Methylobacterium extorquens* AM1 of 23, 29 and 32.5 kb (Warner *et al.*, 1977) but these were later shown to be larger, 33, 40 and 65 kb (Kim & Lidstrom, 1989). Problems have often been found with the isolation of plasmids from methylotrophs but several variations of commonly used techniques have increased yields. The addition of an acetone step by Kim and Lidstrom (1989) visibly increased yields and

susceptibility of the plasmid to restriction analysis. The plasmids found in *M.extorquens* AM1 could not be assigned any function and the loss of the middle-sized plasmid in the laboratory strain did not correspond with any biochemical or genetic characteristic of the bacteria (Kim & Lidstrom, 1989), however, further study of the organism may yet reveal differences between the laboratory strain and that kept at the NCIMB which contains all three plasmids.

The obligate methylotroph *Methylomonas methylovora* contains a very large plasmid of 133 kb which was thought, due to its' size, to be possibly conjugative (Monteiro *et al.*, 1982). One of the most detailed studies of the biology of methylotroph plasmids is that of two plasmids present in *Methylomonas clara*. These plasmids are 46 kb (pBE-2) and 16 kb (pBE-3), the 16 kb plasmid is however a deletion derivative of the 46 kb plasmid (Marquardt *et al.*, 1984). Sequences homologous to these plasmids were also found to occur in the chromosome of a *M.clara* strain which contained no plasmids. The plasmid replicon did not function in *E.coli*. Marquardt *et al.* (1984) identified a region of 7 kb that contained the origin of replication and they suggested also the possible presence of insertion element-like sequences which could account for the frequency of spontaneous deletion mutants, and the presence of plasmid sequences in the chromosome. The plasmid pBE-3 has been shown to be transcriptionally active in *E.coli* but the products have not been identified. Two divergent overlapping

promoters were found which have homology to the classic *E.coli* -10, -35 sequence (Metzler *et al.*, 1988). Kues (1989) studied the basic replicon of pBE-2 and pBE-3 in detail and localized the origin to 1.3 kb of DNA and tentatively suggested a rolling circle mode of replication. So far it does not appear that genes involved with methanol metabolism are plasmid encoded.

1.6.2 Conventional mutagenesis

The use of conventional mutagens to produce mutants in the obligate methylotrophs has only really been successful with *Methylobacillus flagellatum*. Although temperature sensitive mutants of *Methylophilus methylotrophus* defective in glutamate synthase were isolated using NTG by Windass *et al.*(1980), this method was not optimized. A method for NTG mutagenesis was optimized for *Methylobacillus flagellatum* and both auxotroph and C_1 mutants were isolated (Tsyganokov & Kazakova, 1987; Kletsova *et al.*, 1988). Tsyganokov and Kazakova found that the isolation of auxotrophs was affected by plating of the NTG treated culture onto complete, rich media. *M.flagellatum* was very sensitive to the presence of many amino acids and other supplements. They plated the cultures onto minimal media supplemented with only one amino acid, often at a high concentration to overcome any poor affinity uptake system. This method produced much higher frequencies and a greater variety of auxotrophs.

Another approach was taken by Kletsova *et al.*(1988) to look for mutants defective in RuMP cycle enzymes. The same method of NTG mutagenesis was used as for the earlier isolation of auxotrophs but this time, because mutations in the C_1 pathways are lethal, they isolated temperature sensitive mutants, those capable of growth at 30^0C but not at 42^0C. They isolated 4000 NTG temperature sensitive mutants, 500 of which were screened further. They isolated 9 mutants defective in phosphoglucoisomerase and 2 in glucose 6-phosphate dehydrogenase. The enzyme activity was completely lost when assayed at 50^0C.

More success has been achieved with the facultative methylotrophs. Nunn and Lidstrom (1986) devised a method for the isolation of mutants in methanol metabolism using the 'suicide' substrate allyl alcohol. Methanol dehydrogenase (MDH) converts allyl alcohol to the toxic acrylic aldehyde. They grew cultures of *Methylobacterium* AM1 on methanol minimal medium then, following treatment with UV or nitrous acid, they were plated onto nutrient agar, allyl alcohol and methanol (the methanol was required to induce production of MDH). In this manner they managed to isolate 10 distinct classes of MDH mutants. Machlin *et al.* (1988) used this method to mutate *Methylobacterium organophilum* XX and found that the uv or nitrous acid treatment served to enhance the spontaneous mutation rate found on allyl alcohol. Earlier attempts to use uv and NTG mutagenesis on this organism resulted only in mutants defective in the

synthesis of serine pathway enzymes (Anthony, 1982; DeVries, 1986). This method was used again, using ethyl-methanesulphonate to enhance mutation, to produce *M.organophilum* XX defective in PQQ synthesis (Machlin *et al.*, 1988). This method has proved invaluable in elucidation of the genetics of methanol metabolism.

1.6.3 Transposon mutagenesis and marker exchange.

The lack of success in isolating mutants of obligate methylotrophs (and facultative methylotrophs to some extent) using conventional methods has led to the use of alternative methods. One of the first successful transposon delivery systems for methylotrophs was the plasmid pMO75 and its use with *Methylobacterium* AM1 (Whitta *et al.*, 1985). PMO75 is a derivative of the *Pseudomonad* conjugative plasmid R91-5 (IncP-10), containing the kanamycin resistance transposon Tn5. Using filter matings to transfer this plasmid, 6 mutants in methanol metabolism were isolated and 6 auxotrophs. Prior to this report, Mazodier *et al.*(1982) reported transfer of Tn5 to *Methylobacterium organophilum* but no mutants were reported. Bohanon *et al.*(1988) successfully transferred Tn5 to the obligate methylotroph *Methylophilus methylotrophus* AS1. The anthranilate synthase gene (*trpE*) of *M.methylotrophus* was cloned and mutated with Tn5 when in *E.coli*, then transferred to the suicide vector pSUP202 (Simon *et al.*, 1983a). This plasmid was transferred to *M.methylotrophus* from the *E.coli*

strain S17-1 (possesses RP4 mobilization functions integrated into the chromosome making it a 'high frequency of transfer' strain). Kanamycin resistant colonies were screened for a requirement for tryptophan but none were found, despite the screening of thousands of colonies. They found that there was a problem with the stability of Tn5, this was remedied by removal of the IS50R end. When the procedure was repeated with the modified Tn5, stable tryptophan requiring mutants were isolated. The chromosomal *trpE* gene had undergone homologous recombination with the mutated *trpE* gene, a double cross-over event between the homologous DNA. There was then loss of the vector plasmid pSUP202 from the cell.

A similar marker exchange process was used to produce *recA* mutants of *Methylomonas clara* (Ridder *et al.*, 1991). The *M.clara recA* gene was mutated by the insertion of a kanamycin resistance cassette. This was cloned into pBR322, the 'suicide' vector, which was mobilized using the two plasmids R751 and colK/Km^r. They obtained stable *recA* mutants *M.clara* resulting from homologous recombination and a double cross-over event. The production of this strain will help in the development of stable host-vector systems for this organism.

1.6.4 Chromosomal mapping

Chromosomal mapping is usually accomplished by the isolation of mutants and the determination of the spatial relationships of these mutants, however, due to the difficulties in obtaining mutants in methylotrophs this method cannot be widely used. However, it has been possible to employ broad-host range plasmids for gene mapping in methylotrophs. Some mutants of methylotrophs have been used in mapping experiments but the mapping of the chromosome has largely been dependent on complementation mapping. This method obviously relies on functionally similar genes and adequate expression in the heterologous host. Three different systems that have been used with this method will be described.

1.6.4.1 R-prime mapping

R-primes are produced when the plasmid integrates into the chromosome and when mobilized from the organism takes with it a portion of the host chromosome, usually in the range of 65-140 kb. The plasmid R68.45, an R68 derivative, has been particularly successful for the production of prime plasmids in methylotrophs. R68.45 was first shown to transfer into *Methylobacterium organophilum* XX and *Methylobacterium* AM1 by Jeyaseelan and Guest (1979). Moore *et al.*(1983) used a derivative of R68 with enhanced chromosome mobilizing ability, pMO172, to map the chromosome of *Methylophilus methylotrophus* AS1, an obligate

methylotroph. They constructed a variety of prime plasmids which they transferred individually to auxotrophs of *Ps. aeruginosa*. The complementation of two or more functions by the same prime plasmids indicated that the *M.methylotrophus* AS1 genes were on the same chromosomal fragment and linked. They identified four groups of linked markers. Kearney and Holloway (1987) also transferred prime plasmids of *M.methylotrophus* AS1 to auxotrophs of *Ps.aeruginosa* and *E.coli* and concluded that it was not possible to study the regulation of the transferred genes in any meaningful way in the heterologous hosts. Tatra and Goodwin (1985) used R68.45 to transfer part of the chromosome of *Methylobacterium* AM1 to auxotrophs of *Methylobacterium* AM1 isolated by NTG mutagenesis. They studied the linkage of antibiotic resistance markers and the genes of C_1 metabolism. R68.45 has also been used to produce prime plasmids in *Methylophilus viscogenes* and *Methylophilus methylotrophus* AS1 (Lyon *et al.*, 1988). pULB113, another RP4 derivative, has been used to produce prime plasmids in *Methylobacterium* AM1, *Methylobacterium extorquens* (Al-Taho & Warner, 1987), and *Methylobacillus flagellatum* KT, in which 7 groups of linked markers were identified using a library of prime plasmids (Tsyganokov *et al.*, 1990).

1.6.4.2 Cosmid gene banks

When Tatra and Goodwin (1985), working with *M.methylotrophus* AS1, found that not all regions of the chromosome could be mapped using R-

prime plasmids, gene banks were produced using broad host range cosmids which contained 40-50 kb of chromosome (Lyon *et al.*, 1988). This method had previously been used with *Methylobacterium* AM1 and *Methylobacterium organophilum* XX, where randomly generated cosmid genomic libraries were used to complement carbon utilization mutants in methylotrophs (Allen & Hanson, 1985; Fulton *et al.*, 1984; Nunn & Lidstrom, 1986). Lyon *et al.*(1988) used the cosmid pLA2917 to produce a random gene bank which should have contained a comprehensive representation of the genome in fewer than 1000 clones. With this approach they compared gene arrangements in the obligate methylotrophs *Methylophilus viscogenes* and *Methylophilus methylotrophus* AS1. With up to 7 auxotrophic markers found on 1 cosmid containing 25 kb of chromosome they concluded that there was clustering of auxotrophic markers in the overall genome arrangement, and that this clustering was similar in both strains of methylotroph. They also confirmed 5 of the 6 linkage groups found by Moore *et al.*(1983) working on *M.methylotrophus* AS1, and identified 5 additional *M.viscogenes* and 2 more *M.methylotrophus* AS1 linkage groups. With the smaller insert size found in cosmids, Lyon *et al.* were able to determine the relative order of some markers, assuming that no internal rearrangements had taken place during transfer. From this genetic linkage data from the two obligate methylotrophs, a high degree of conservation of gene

arrangement was found but no restriction identity, and very little DNA-DNA homology (Lyon *et al.*, 1988).

1.6.4.3 Hfr strains

Broad host range plasmids can be used to produce Hfr-like donors that can transfer chromosomal markers in a polar fashion dependent upon the site of insertion and the direction of transfer. Hfr-like strains are capable of transferring and linking much larger sections of the chromosome than R-prime plasmids and it is possible, theoretically, to map the entire chromosome.

Hfr-like donors have been constructed in *Methylobacillus flagellatum* KT using the RP4 derivative pAS8-121 (Serebrijski *et al.*, 1989). One Hfr-like donor was found to mobilize the chromosome in a polarized manner from a single transfer origin and it was possible to construct a preliminary time-of-entry map of the *M.flagellatum* chromosome. The time of entry of six randomly dispersed markers, four of which were in known groups of linked markers, was determined in interrupted mating experiments by transfer to a variety of *Ps.aeruginosa*, *E.coli*, and *M.flagellatum* mutants (Tsyygankov *et al.*, 1990). In this manner a linear map of *M.flagellatum* chromosome consisting of 44 markers was constructed.

1.6.5 The molecular biology of the methylotrophs

1.6.5.1 The *mox* genes

The elucidation of the molecular biology of the genes involved with methanol oxidation (*mox* genes) has been possible due to the positive selection method involving allyl alcohol for *mox* mutants already described in section 1.6.2. This technique has been widely used to produce mutants of *Methylobacterium* AM1 and *Methylobacterium organophilum* XX (Nunn & Lidstrom, 1986a; Machlin *et al.*, 1988). This method, however, can not be used for the autotrophic methanol utilizers such as *Paracoccus denitrificans* as the methanol dehydrogenase is suppressed when the organisms are grown on alternative substrates (Harms *et al.*, 1985). The most detailed studies have taken place on *M.extorquens* AM1 and *M.organophilum* XX. Studies on the Gram-positive methanol utilizers are considerably behind those of the Gram-negative methylotrophs due to the lack of cloning vectors but these are now becoming available.

a) Regulation of methanol oxidation

The expression of methanol dehydrogenase (MDH) is not repressed in *Methylobacterium* strains by the presence of other carbon substrates (Anthony, 1982; O'Connor, 1981), whereas MDH is only expressed in *P.denitrificans* in the presence of methanol (Merrick *et al.*, 1987). The inducer of MDH for the *Methylobacterium* strains is not known.

b) The genes of the *mox* system

The genes involved in methanol oxidation have been cloned by complementation using clone banks constructed in broad host range plasmids such as pVK100 (Knauf & Nester, 1984). A minimum of 20 genes are thought to be required in *M.extorquens* AM1 and 14 in *M.organophilum* XX (Machlin *et al.*, 1987; Allen & Hanson, 1985; Nunn & Lidstrom, 1986a,b; Bastien *et al.*, 1989). The genes are clustered and show similar organisation in both strains. Figure 1.6.1 shows the organisation of some of the known *mox* genes for *M.extorquens* AM1. Not all of the genes have been assigned definite functions. The cluster *mox*FJGI encodes the structural genes for MDH. The gene functions were determined by western blotting and sequencing. The *mox*F gene codes for a protein of 60 kDa, the large sub-unit of MDH and *mox*I codes for the small subunit of MDH, 10 kDa. *Mox*G codes for the periplasmic cytochrome C_L of 21 kDa and *mox*J is a protein of 30 kDa, this has been expressed in *E.coli* and sequenced but no function can be assigned as yet (Nunn & Lidstrom, 1986ab;Anderson & Lidstrom, 1988; Nunn & Anthony, 1988a; Nunn & Anthony, 1988b).

Seven other *mox* genes are involved with the co-factor PQQ. The genes *mox*AKL are closely clustered and involved with the assembly or modification of MDH with PQQ. Mutants in *mox*CPO and H (not shown on Figure 1.6.1) will grow in the presence of PQQ (Lidstrom, 1990). Other *mox*

mutants exhibit pleiotropic phenotypes and are possibly involved in regulation, stability or processing functions.

c) Expression and sequence analysis of *mox* genes

Tn5 insertion analysis of the *mox*FJGI cluster in *Methylobacterium* AM1 indicated that these were co-transcribed as a large transcript, but there was also a smaller transcript of the first two genes (Nunn & Lidstrom, 1986). In *M.organophilum* XX the transcripts appeared to be all mono-cistronic, except *mox*N and D which were co-transcribed.

Nucleotide sequence data is available for several *mox* genes and comparisons of amino acid and nucleotide sequences show a high degree of conservation. The *mox*F gene is highly conserved showing 96% similarity at the amino acid level between *M.extorquens* AM1 and *M.organophilum* XX, and *P.denitrificans* exhibits 75-80% similarity to the *Methylobacterium* strains (Lidstrom, 1990). The three *mox*F sequences also have signal peptides of 27-32 amino acids which are virtually identical in the *Methylobacterium* strains, but these differ considerably to the sequence in *P.denitrificans*.(Lidstrom, 1990). It has been possible to use the *mox*F gene from the *Methylobacterium* strains to screen a variety of other methane and methanol utilizing organisms indicating that there is a sufficient degree of sequence conservation (Stephens *et al.*, 1988; Tsuji *et al.*, 1989). This would be expected from the similar nature of the MDH protein (Anthony, 1982). Sequence data is also available

(Acetyl CoA recycle)-(*mox*Q,E,F,J,G,I,A,K,L,B)-

(*ppc*, *mcl*, *mox*C,P,O,M,N,D)-(*glk*).

Mox = Methanol dehydrogenase

ppc = PEP carboxylase

mcl = Malyl CoA lyase

glk = Glycerate kinase

Figure 1.6.1 - The proposed order for C_1 and *mox* genes in *M.extorquens* AM1. The distance between each gene cluster is not known.

From Lidstrom, (1990).

for the *mox* G and *mox*I genes of *M.extorquens* AM1 which also have signal sequences.

The transcriptional start site has been mapped for *mox*F of *M.extorquens* and *M.organophilum* XX and found to differ by only one base (Machlin & Hanson, 1988; Lidstrom, 1990). Some similarities to the *E.coli* -10, -35 consensus sequence have been found, but the most conserved *E.coli* bases are not present and the protein is not expressed from these promoters in *E.coli* (Machlin *et al.*, 1987; Anderson & Lidstrom, 1988; Harms *et al*, 1987). Just downstream of the *mox*F gene in the *Methylobacterium* strains a strong putative stem-loop structure has been found which may be involved in regulation (Lidstrom, 1990).

1.6.5.2 PQQ genes

The co-factor PQQ is essential for methanol dehydrogenase (MDH) activity. It was first discovered in MDH but is now known to be present in a variety of other enzymes, collectively known as quinoproteins (Duine *et al.*, 1986). Studies on the biosynthetic pathway of PQQ in *Hyphomicrobium* X and *Methylobacterium* AM1 have shown that the starting substrates are tyrosine and glutamate (Van Kleef *et al.*, 1988; Houck *et al.*, 1988).

Mazodier *et al.*(1988) located a gene involved in PQQ biosynthesis when cloning *mox*F from *Methylobacterium organophilum* DSM 760. This gene was *pqq*A and could complement *M.organophilum* DSM 760 PQQ mutants. They produced

a *pqq*A mutant by insertion of a kanamycin resistance cassette from pUC4K into the gene and marker exchange mutagenesis with the chromosomally located *pqq*A. Further studies on EMS induced PQQ mutants of *M.organophilum* DSM 760 found, by complementation analysis, six genes involved with PQQ biosynthesis (Biville *et al., 1989*). The genes *pqq*ABCDE were clustered with *pqq*A about 30 kb away from *mox*F. The gene *pqq*F is located about 19 kb away from the cluster. The precise functions of the genes have not yet been determined. The genes could not be expressed in *E.coli* or *Ps.testosteroni*. The genes may correspond to the *mox*C,O,P,H or T genes in *M.extorquens* AM1 (Lidstrom, 1992).

1.6.5.3 Serine cycle genes.

The serine cycle has been studied in greatest detail in the facultative methylotrophs *Methylobacterium extorquens* and *Methylobacterium organophilum* XX, in which mutants in serine cycle genes have been isolated (DeVries, 1986). Studies on the regulation, induction and repression, of six enzymes of the serine pathway in *Methylobacterium extorquens* have suggested that these activities may be coded by a genetic regulon. In *M.extorquens* AM1 the genes coding for malyl-CoA lyase, acetyl-CoA-independent PEP carboxylase, glycerate kinase and the acetyl-CoA recycle pathway have been cloned by complementation of mutants with cosmid clone banks (Fulton *et al.*, 1984; Lidstrom *et al.*, 1987; Stone & Goodwin, 1989). Tatra & Goodwin (1983,1985) used R68.45, the

broad host range plasmid derived from RP4, to mobilize the chromosome and map mutants defective in the serine cycle. A gene order has been proposed and is shown in figure 1.6.1. The genes coding for malyl-CoA lyase and PEP carboxylase are located within 15 kb of the *mox*CD gene cluster (Fulton *et al.*, 1984; Lidstrom *et al.*, 1987), and the genes for glycerate kinase and acetyl-CoA oxidation pathway are located 10-15 kb from each other and from other C_1 genes (Stone & Goodwin, 1989).

In *M.organophilum* XX, the genes for malyl-CoA lyase, glycerate kinase and the acetyl-CoA recycle pathway have been cloned and found to be separated by at least 30 kb (Allen & Hanson, 1985). None of these genes are on cosmid clones that contain cytochrome c and the *mox* system (Machlin *et al.*, 1988). It is still conceivable that a similar gene organisation is present in both *Methylobacterium* strains but more mapping needs to be carried out.

1.6.5.4 Ribulose monophosphate cycle genes

Most methylotrophs that utilize the RuMP pathway for formaldehyde assimilation are obligate methanol-utilizers for which it is very difficult to obtain mutants. To overcome this problem Levering *et al.*(1987) studied the Gram-positive facultative methylotroph *Arthrobacter* P1 in which they obtained four different classes of mutants. There is however no suitable genetic system for this organism.

The pathway in the obligate strains is essential for growth therefore only conditional mutants can be isolated. Two temperature sensitive mutants of *Methylobacillus flagellatum* have been isolated but these have not been mapped (Kletsova *et al.*, 1988).

1.6.6 The expression of foreign DNA in methylotrophs

The ability of the methylotrophs to grow well on relatively cheap substrates such as methanol and ammonia has made them attractive as heterologous hosts for the production of foreign proteins. A variety of foreign genes have been expressed in methylotrophs and it appears that expression is not a problem. The glutamate dehydrogenase gene of *E.coli* has been expressed in *Methylophilus methylotrophus* and was found to complement glutamate synthase mutants, although the enzyme activity per gene copy was 10-20 fold lower than that found with *E.coli* (Windass *et al.*, 1980). Two eukaryotic cDNA's, chicken ovalbumin and mouse dihydrofolate reductase, have been expressed in *M.methylotrophus* (Hennam *et al.*, 1982). The expression in all these cases was from vector promoters and, although less than that found with *E.coli*, it was significant. The broad host range vectors used were both based on R300B and appeared to be stable. The pyruvate dehydrogenase (PDH) gene from *E.coli* has been cloned into the restricted facultative methylotroph *Hyphomicrobium* X. The organism was then able to

grow on pyruvate but at a low growth rate and it contained very low PDH complex activity (Dijkhuizen *et al.*, 1984).

The highly efficient *E.coli* promoters *tac*, *lac* and *trp* have been used for expression in methylotrophs. The *lac* promoter was used for the expression of chicken ovalbumin in *M.methylotrophus* (Hennam *et al.*, 1982) and the *tac* promoter was used to express cDNA encoding the human interferon αF (Chistoserdov *et al.*, 1987). The expression obtained was 2-3 fold higher than that found in *E.coli*. Byrom (1984) reported the expression of a streptomycin resistance gene from the *tac* promoter and complementation of a tryptophan mutant was dependent upon the *trp* promoter. The majority of antibiotic resistance genes are expressed adequately in most methylotrophs, although it has been reported that the ampicillin resistance gene on pBR322 is not expressed in *M.extorquens* AM1 (Lidstrom, 1992).

1.7 GENETIC TECHNIQUES FOR NON-ENTERIC GRAM-NEGATIVE BACTERIA

1.7.1 Conjugation and broad host range plasmids

Conjugation is the process that allows efficient gene transfer from one bacterial cell to another through plasmid encoded functions. The molecular mechanism for conjugation is mainly unknown. The process of gene transfer between bacteria has been reviewed by Heinemann, (1991) and Willets and Wilkins (1984). The majority of the work on the mechanism of conjugation has been carried out on the narrow

host range F plasmid however, recent work has shown that there are differences between this system and the broad-host range system employed by RP4/RK2 (IncP) (Guiney & Lanka, 1989). The process of conjugation can be affected by a number of factors, such as, the cell growth phase, and a number of environmental parameters, i.e, ionic strength, temperature and anaerobiosis.

Conjugative plasmids have been classified into at least 20 incompatibility groups and some of these groups particularly Inc N, P and W have broad host ranges, they are able to conjugate to and stably replicate in a wide range of bacteria. The IncP plasmids are reviewed by Thomas and Smith (1987) and the replication of the IncP and IncQ plasmids is reviewed by Kues and Stahl (1989). The *mob* and *oriT* mobilization functions of a broad-host range plasmid have also been shown to promote transfer into plant cells (Buchanan-Wollaston *et al.*, 1987). Transfer can also occur between Gram-negative and Gram-positive organisms: from *E.coli* to corynebacteria (Schafer *et al.*, 1990), from *E.coli* to *Streptomyces* sp. (Mazodier *et al.*, 1989), and from *Enterococcus faecalis* to *Escherichia coli* (Trieu-Cuot *et al.*, 1988).

The use of conjugation and the broad-host range plasmids has been the basis for gene transfer systems for bacteria for which no other method of transformation is possible. The plasmids not only transfer themselves into cells but are able to mobilize other non-conjugative

plasmids into cells so enabling the introduction of a number of types of vector into cell and a range of genetic manipulations to take place.

1.7.1.1 Stable plasmids

The majority of stable broad host range vectors are based upon the natural plasmid RP4 (IncP) or IncQ plasmids RSF1010 (Figure 1.7.1) and R300B. There have also been vectors developed upon the use of pVS1 a Pseudomonads plasmid and the Inc W plasmids.

The plasmid RP4 is probably identical to plasmids RP1, RK2, R18 and R68 (Thomas & Smith, 1987). The genetic basis for the plasmids' promiscuity has been studied and they possess an unusual replicon of great complexity (Figurski *et al.*, 1985; Thomas & Smith, 1987; Goncharoff *et al.*, 1991; Walter *et al.*, 1991). The drawback to the use of RP4 as a cloning vector is its large size, 56 kb, and therefore, derivatives have been produced for use in cloning systems. A system was developed that separated the replication and transfer functions of RK2 to produce pRK290 and pRK2013. pRK290 is non-selftransmissable and encodes tetracycline resistance but has the replication host range of RK2 (Ditta *et al.*, 1980). pRK2013 is the helper plasmid, consisting of the RK2 transfer functions cloned onto a ColE1 replicon (Figurski & Helsinki, 1979). This binary plasmid system has been highly successful, e.g, in the study of *Rhizobium* spp (Ausubel & Ruvkun, 1981; Ditta *et al.*, 1980;

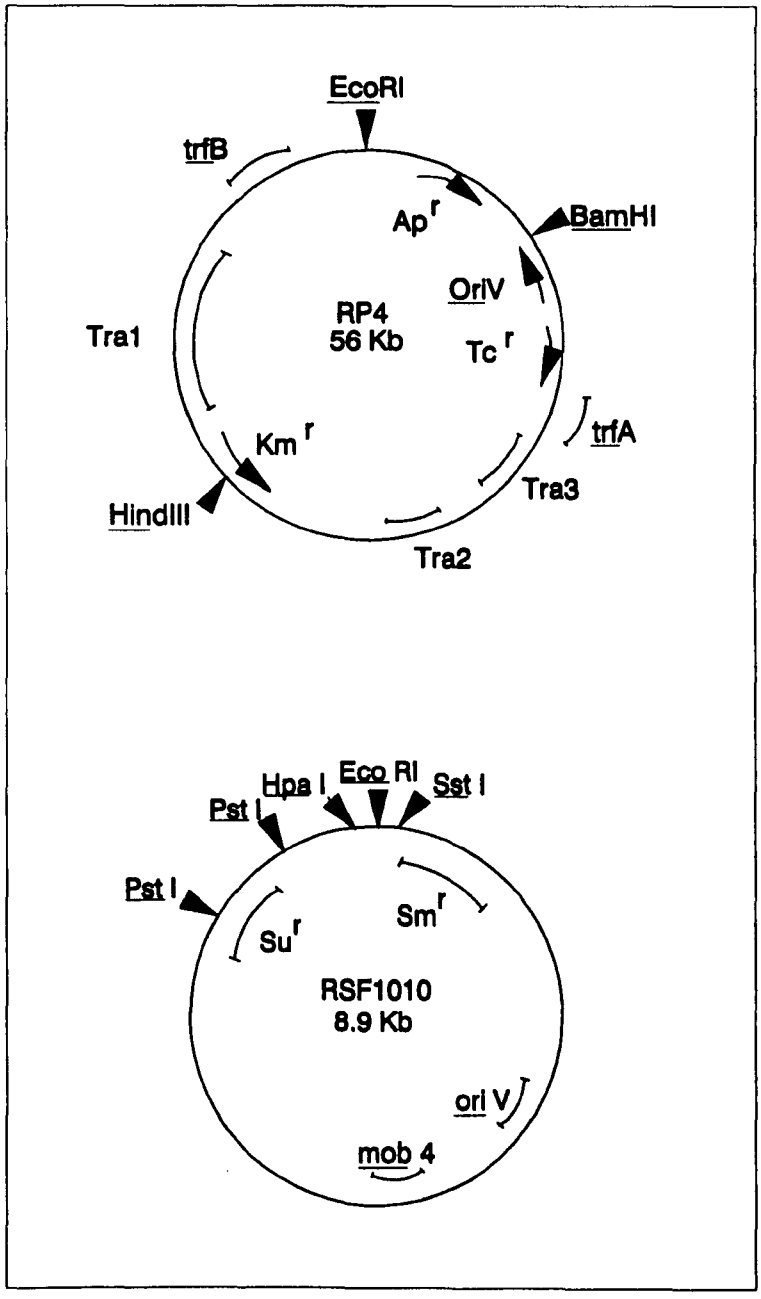


Figure 1.7.1: Broad-host range plasmids RP4 and RSF1010. The genes trfA, trfA and oriV are concerned with replication and maintenance. Tra1,2, and 3 are involved in conjugal transfer and mob4 is concerned with plasmid mobilization.

Ditta *et al.*, 1981), *Agrobacterium* spp (Garfinkel *et al.*, 1981), *Rhodopseudomonas capsulata* (Klug & Drews, 1984) and *Gluconobacter oxydans* (Condon *et al.*, 1991). From this initial pRK290/pRK2013 system has developed other useful vectors, such as a range of cosmids, including pVK100 and pVK102 (Knauf & Nester, 1982) which contain tetracycline and kanamycin resistance and the smaller pRK290 derivative pRK404 (Ditta *et al.*, 1985).

Other natural plasmids that have been used for the development of broad-host range vectors are the IncQ plasmids RSF1010 and R300B. These are non self-transmissible but can be mobilized by RP4 and pRK2013 and have broad-host range replicative functions. Detailed studies on RSF1010 can be found in Frey & Bagdasarian, (1989), Haring & Scherzinger, (1989) and Scholz *et al.*,(1989). Although RSF1010 and related IncQ plasmids, such as R300B, are of a small size (RSF1010 is 8.89 kb) and encode sulphonamide and streptomycin resistance these are not usable in many bacteria and they contain few suitable restriction sites for cloning purposes, therefore derivatives have been developed.

The derivatives have been developed include basic cloning vectors, expression vectors and promoter probe vectors. These derivatives are produced by the addition or substitution of genes from other plasmids, and some examples are listed in table 1.7.1. along with examples of vectors produced from broad-host range plasmids from other Inc groups.

1.7.1.2 Chromosomal mobilization vectors

The broad-host range plasmid RP4 has been shown for a long time to mobilize the chromosome of certain bacteria, albeit at a very low frequency (Stanisich & Holloway, 1971; Beringer, 1974). It has been found that the chromosome mobilizing ability of RP4 can be enhanced by the insertion of various types of DNA. Denarie *et al.*(1976) inserted mu to enhance chromosome mobilization in *Klebsiella pneumoniae* and Van Gijsegem and Toussaint (1982) inserted a defective mini-mu to produce pULB113. This plasmid has been successful in a range of bacteria including *Proteus mirabilis* and *Salmonella typhimurium* (Van Gijsegem & Toussaint, 1982), *Alcaligenes eutrophus* and *Pseudomonas fluorescens* (Lejeune *et al.*, 1983), and two facultative methylotrophs (Al-Taho & Warner, 1987). Another method to enhance mobilizing ability has been to insert homologous DNA into RP4 at a unique restriction site. This has been successful with *E.coli* (Barth, 1979) and *Proteus morganii* (Beck *et al., 1982*).

Another derivative of RP4, R68.45, was found to have enhanced mobilizing ability (Haas & Holloway, 1976). This has been successfully used with several bacteria, including *Rhodopseudomonas sphaeroides* (Tucker & Pemberton, 1979) and *Methylobacterium* AM1 (Jeyaseelan & Guest, 1979; Tatra & Goodwin, 1985).

Series	Inc	Derived from	Reference
pGSS	Q	pBR322/pBR328 R300B	Sharpe 1984
pMMB	Q	pKT231/pHC79	Frey *et al.*, 1983
pDSK	Q	pUC19/Tn903/ RSF1010	Keen *et al.* 1988
pKT	Q	RSF1010/p-Sa	Bagdasarian *et al.*,1981
pSa1	W	pBR322/p-Sa	Tait *et al.*, 1983
pGV	W	pBR325/p-Sa	Leemans *et al.* 1983
pME	-	pVS1/Tn801/ pACYC177	Itoh & Haas, 1985
pVK	P1	pHC79/pRK290	Knauf & Nester 1982

Table 1.7.1

Yet another approach was adopted by Simon *et al.*(1983b). They produced a variant of the transposon Tn5 containing the *mob* functions of RP4, known as the *mob* or *oriT* fragment. This Tn5-*mob* fragment when present on an unstable plasmid, such as pBR325, will transpose into the chromosome or any stable plasmid present in the cell. The Tn5-*mob* DNA provides a point at which the chromosome can be mobilized by RP4. In this manner it was possible to mobilize the mega-plasmids of *Agrobacterium tumefaciens* and the chromosome of *E.coli* (Simon *et al.*, 1983b).

1.7.1.3 Mutagenesis vectors

Vectors used for mutagenesis are generally not based on the broad-host range plasmids already mentioned as a desired characteristic is that they be unstable in the cell and deliver the transposon or some other mutagenized gene into the chromosome and then are lost from the cell. However, with the genetic manipulation of non-enteric organisms there is often the problem of introduction of the plasmids to the cell. When no transformation mechanism is available the plasmids have to be transferred to the cell by conjugation and therefore the transfer genes from the broad-host range plasmids have been cloned into narrow host-range vectors to make them suitable for a variety of mutagenesis techniques. These vectors are transposon delivery vehicles for generalized transposon mutagenesis or have been developed for more precise mutagenesis, such as marker exchange mutagenesis.

The narrow host range cloning vector pBR322 can be mobilized at a very low frequency, a process which requires the presence of a conjugative plasmid and a ColK plasmid. Simon *et al.* (1983a) identified the broad host range mobilization functions of RP4 on a discrete fragment of DNA, the *mob* fragment. This fragment when cloned into vectors allowed their efficient transfer by conjugation with RP4 or derivatives. The *mob* fragment was used to produce the pSUP series of vectors, designed for a variety of purposes. Simon *et al.* (1983a) cloned the *mob* fragment into pBR325 and then loaded this vector with Tn5. To increase the transfer frequency of the vector and simplify the conjugation technique they developed a 'high frequency of transfer' strain of *E.coli*. This contained the transfer functions of RP4 needed to mobilize plasmids incorporated into the chromosome which avoided triparental matings and the complication of a second plasmid being transferred. This series of vectors could be used for random transposon mutagenesis or marker exchange mutagenesis

Another series of pSUP vectors can be used for identification of promoters in the chromosome. These have the promoterless reporter genes *lacZ* or *luc* attached to Tn5-*mob* and placed on narrow host range vectors such as pACYC184. The plasmids are transferred to the organism by conjugation and the reporter gene and Tn5 transposes into the chromosome. If this transposition is downstream of an active promoter an operon fusion results and the

promoterless reporter gene is expressed (Simon *et al.*, 1989).

1.7.2 Electroporation

Electroporation is the production of pores in the cell membrane through which macromolecules including DNA can enter the cells by the passage of an electrical current across the cell. The entry of DNA is transformation of the cell hence the term electro-transformation. This technique was first used for eukaryotes (reviewed Knight & Scrutton, 1986) and has since become widely used for prokaryotes. The first report of bacterial electro-transformation was by Shivarova *et al.* (1983), who transformed protoplasts of *Bacillus cereus*. With the advent of more powerful electroporation apparatus it was possible to transform untreated bacteria, the first report was by Fiedler and Wirth (1988) who transformed *Escherichia coli* and *Pseudomonas putida* with no prior treatment. Since then the list has continued to grow steadily and many bacteria for which no transformation procedure exists have been electro-transformed. Wirth *et al.* (1989) studied the electro-transformation ability of various species of Gram-negative bacteria belonging to 11 different genera, many of which were recent isolates from natural sources, as well as laboratory strains. They found that this method of transformation was successful with a wide variety of species.

ORGANISM	EFFICIENCY	REFERENCE
Citrobacter freundii	1×10^3	Wirth *et al.*, 1989
Enterobacter aerogenes	5×10^1	"
Erwinia carotovora	4×10^5	"
Serratia plymuthica	1.8×10^3	"
Yersinia pestis	2.7×10^7	Conchas & Carniel, 1990
Azospirillum brasilense	1.5×10^4	Van de Broeck *et al.*, 1989
Bacteroides uniformis	10^5	Thomson & Flint, 1989
Escherichia coli	10^9-10^{10}	Dower *et al.*, 1988
Bordetella pertussis	10^6	Zealey *et al.*, 1988
Aquaspirillum itersonii	3×10^4	Eden & Blakemore 1991
Methanococcus voltae	Not determined	Micheletti *et al.*, 1991
Caulobacter crescentus	3×10^8	Gilchrist & Smit, 1991

Figure 1.7.2

The exact process by which the DNA enters the cells is not known but it is generally accepted that when a high voltage electrical pulse of exponential decay waveform is applied to a mixture of DNA and cells, transient membrane permeabilization occurs. This permeabilization is reversible if the duration or magnitude of the electric field does not exceed a certain limit (Shigekawa *et al.*, 1988). Some studies indicate that entry is by passive diffusion (Dower *et al.*, 1988) and theoretically, on a purely physical basis, all bacteria should be electro-transformable. In practice a large number of factors influence the electro-transformation of bacteria and the method has to be optimized for each strain of bacteria used. Electroporation has been successful for both Gram negative and Gram positive organisms. Table 1.7.2 lists a selection of bacterial strains that have been electro-transformed and the frequencies obtained. In some cases particularly with the Gram positive organisms, which have tougher cell walls, the bacteria have to be treated in some way prior to electro-transformation. The method for the production of *E.coli* cells for electro-transformation is outlined in materials and methods section 2.2.6. This method developed initially by Dower *et al.*(1988) has been used as a starting point for the development of methods for other bacteria. The bacteria are harvested, washed and resuspended at a high cell concentration in a buffer of low ionic strength.

The parameters effecting electroporation include:

electrical factors, such as strength of the electrical field and duration of the pulse, DNA concentration and pre and post pulse incubation with DNA, the presence of restriction/modification systems, the growth phase and growth medium of the cells, recovery procedure after electro-transformation, the buffer used to resuspend the cells for electro-transformation, and the mechanism of antibiotic resistance on the plasmid. Several of these factors will be discussed at greater length in the results section 3.3.

The process of electro-transfer was first described by Summers and Withers (1990) and is the direct transfer of plasmids between strains by an electric pulse being applied to a mixture of the two strains. in a procedure identical to electro-transformation. This has been shown to be successful between *E.coli* strains (Summers & Withers, 1990) and between *E.coli* and *Salmonella typhimurium* (Pfau & Youderian, 1990). This makes the introduction of DNA into cells even easier as the DNA no longer has to be purified. It is also possible to cure plasmids by application of an electric pulse, this has been successful for *E.coli* (Heery *et al* .,1989).

MATERIALS

&

METHODS

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial Strains

All bacterial strains used are listed in table 2.1.1.

2.1.2 Plasmids and Cosmids

All plasmids and cosmids used are listed in table 2.1.2.

2.1.3 General Growth Media

2.1.3.1 *Escherichia coli*

E.coli was routinely grown on Luria-Bertani broth (Maniatis *et al.*,1984).

E.coli auxotrophs were grown on M9 minimal medium.

2.1.3.2 *Methylococcus capsulatus* (Bath)

The organisms were routinely grown on nitrate minimal salts (NMS) (Dalton & Whittenbury, 1976)

2.1.4 Antibiotics

The antibiotics were prepared as a 100X concentrate and filter sterilized. The final concentrations used are stated in table 2.1.3.

Antibiotic	concentration	
	E.coli	*M.capsulatus*
	µg/ml	
Kanamycin	25	50
Gentamicin	10	10
Streptomycin	25	25
Tetracycline	12.5	-
Ampicillin	50	-
Chloramphenicol	10	-
Erythromycin	5	-
Rifampicin	20	20

Table 2.1.3

2.1.5 Chemicals

All chemicals were supplied by BDH, Fisons or Sigma. All restriction enzymes were supplied by Amersham or BRL. DNA polymerase I and T4 ligase were supplied by BRL.

NAME/NCIMB No.	PHENOTYPE	REFERENCE
S17-1	F-; *thi; pro* (r- m+) ::RP4-2 (Tc::Mu) (Km::Tn7)	Simon *et al.*,1983
C600	F-; *thi-1; thr-1; leu*B6; *lac*Y1; *ton*A21 *sup*E44	Appleyard 1954
HB101	F-; *hsd*S20 (r-,m-) *rec*A13; *ara*-14; *pro*A2 *lac*Y1; *gal*K2; *rps*L20 (sm); *xyl*-5; *mtl*-1 *sup*E44;	Boyer & Rowland-Dussoix, 1969.
DH1	F-; *rec*A1; *end*A1; *gyr*A96; *thi*-1; *hsd*R17 (r-,m+); *sup*E44;	Low, 1968
9482	F-; *thr; leu; lac; mtl thi; str*-r; T1-r; T2-r T6-r	NCIMB
10241	F-; *pro; trp; his; lac; str*-r	NCIMB
11865	F-; *pro*A2; *rec*A13; *ara*-14; *lac*Y1; *gal*K2; *xyl*-5; *mtl*-1; *rps*L20(sm) *sup*E44; *hsd*S20.	NCIMB
ET8045	*rbs*,*lac*Z::IS1,*gyr*A *hut*Ck, *ntr*A208::Tn10	MacNeil *et al.*, 1982.

Table 2.1.1 ***E.coli*** **strains**

Plasmid/ cosmid	Inc group	Relevant features	Markers	Reference/source
RP4	P1	Tra$^+$	Km;Ap;Tc	NCIMB
RSF1010	Q/P4		Sp;Sm	NCIMB
pKT231	Q/P4	Tra$^+$	Sm;Km	Bagdasarian *et al.*, 1981
pLG221	I	Tn5	Km	Boulnois *et al.*, 1985
pMMB33/34	Q	Tra$^+$; cosmid	Km	Frey *et al.*, 1983
pRK2013	colE1	Tra$^+$	Km	Figurski & Helinski, 1979
pVK100	P1	cosmid	Km;Tc	Knauf & Nester, 1982
pJFF350	colE1	Omegon Km	Km	Fellay *et al.*, 1989
pULB113	P1	RP4::mini-mu; Tra$^+$	Km;Tc;Ap	Van Gijsegem & Toussaint, 1982
pGSS33	Q	Cm	Ap;Sm;Tc;	Sharpe, 1984
pDSK509	Q/P4		Km	Keen *et al.*, 1988
pDSK519	Q/P4	*lacZ*	Km	Keen *et al.*, 1988
pME277	ND	Tn501	Hg	Itoh *et al.*, 1984
pUB307	P1	Tra$^+$	Km;Tc	Bennet *et al.*, 1977
pMD100	colE1	pRK2013::Tn501	Hg;Km	Ely, 1985
pSUP2021	colE1	Tn5	Km	Simon *et al.*, 1983
pSUP::Tn5 B10,11,12 20,22,40	colE1	Tn5;*mob*	Km;Gm	Simon *et at.*, 1989

Plasmid/ cosmid	Inc group	Relevant features	Markers	Reference/source
pDC2	colE1	pBR325:: *glnA*	Sm;Ap;Tc	D Cardy, 1988
pSD1	colE1	pBR329:: *mob*	Ap;Tc	This work
pSD10	colE1	pSD1:: *glnA*	Ap;Sm	This work
pSD100	colE1	pJFF350:: *glnA*	Km;Sm	This work
pBR329	colE1		Ap;Tc;Cm	University of Warwick
pBEE132	colE1	pRK2013:: Tn5-132	Km	Ely, 1985
pSDZ1	colE1	pDSK509:: *lacZ*	Km;Sm	This work

ND=not determined

Table 2.1.2

2.2 METHODS

2.2.1 Growth and Maintenance of Microorganisms

2.2.1.1 Methanotrophs.

Growth was routinely achieved using mineral salts medium (MS) (Dalton & Whittenbury, 1976), containing 1 g/litre potassium nitrate (NMS). Other supplements used are stated in the appropriate Methods or Results section. Sterile phosphate stock solution was added aseptically to sterile NMS when cool. The methane used as the carbon source contained 5% (v/v) carbon dioxide.

Growth on solid media was achieved using NMS solidified with 2% (w/v) Bacto agar (Difco). Growth was in anaerobic gas jars to which methane was added to give an approximate mixture of 50% (v/v) methane in air. Batch cultures were in 250 ml flasks sealed with suba seals. 50 ml air was extracted by syringe and replaced with 100 ml methane. Large scale liquid culture was achieved using a 2 litre LH fermenter at pH 8.5, supplied with air (200 ml/min), and CH_4 50 ml/min) and run as batch or continuous culture, at 45^0C. Dissolved oxygen tension (DOT) was typically 20% of air saturation.

2.2.1.2 *Escherichia coli*.

Routine growth of all *E.coli* strains was in Luria Bertani broth (LB) or M9 minimal medium (Maniatis *et al.*, 1982) SOC medium used for electroporation experiments is described by Hanahan (1983). *E.coli* strain ET8045 was grown

on M9 medium containing no NH_4Cl but 0.02% (w/v) arginine plus the appropriate antibiotics.

E.coli stocks were maintained on LB plates and as glycerol stocks (-20^0C).

2.2.2 Screening for antibiotic resistance

Antibiotic discs (Mast laboratories Ltd) were placed onto plates spread with 100 µl of *M.capsulatus*. Plates were then incubated at 45^0C for up to two weeks. *M.capsulatus* was also screened for mercury resistance using a range of concentrations of mercuric chloride from 5-50 mg/ml.

2.2.3 Isolation of antibiotic resistant mutants of *M.capsulatus*

100 µl of *M.capsulatus* (approximately 10^9 cells per ml) were spread onto plates containing either gentamicin (10 µg/ml), kanamycin (25 µg/ml), erythromycin (5 µg/ml), rifampicin (20 µg/ml) or streptomycin (15 µg/ml) and incubated for two weeks. Any resistant colonies found were then tested in liquid medium with the appropriate antibiotic.

2.2.4 Conjugation experiments

2.2.4.1 Transfer of plasmids from *Escherichia coli* to *Methylococcus capsulatus*

The wild-type and the streptomycin resistant mutant of *M.capsulatus* were both used in conjugation experiments. The *M.capsulatus* recipient was grown in liquid medium (50 ml), to a cell concentration of 10^8 per ml (A_{600} between 0.2-0.3). The donor *E.coli* were grown in LB (10 ml) with the appropriate antibiotic to a cell concentration of 10^8 per ml. For each filter mating, 50 ml of recipient *M.capsulatus* and 1 ml of donor *E.coli* were used. When a triparental conjugation was being carried out, 1 ml of each plasmid containing strain was used. Donor cells were centrifuged, washed with NMS and resuspended in the same volume of NMS (1 ml). The recipient and donor were mixed and filtered onto a pre-wetted 0.22 μm nitrocellulose filter (Millipore). This was then placed onto a NMS plate containing 0.02% (w/v) Proteose-Peptone. This allowed growth of both the donor and recipient when methane was present. Incubation was at 37^0C for 24 h. After incubation, the cells were washed off the filter with 10 ml of NMS, vortexed, then centrifuged and resuspended in 1 ml of NMS. Cells were plated onto selective NMS plates or transferred into NMS flasks and incubated at 45^0C for up to 2 weeks.

2.2.4.2 Transfer of plasmids from *M.capsulatus* to *E.coli*

The method was almost identical to that outlined in section 2.2.4.1. However, only 10 ml of *M.capsulatus*, the donor, was used in each conjugation experiment. *E.coli* transconjugants were plated onto selective LB plates and incubated at 37^0C.

2.2.4.3 Liquid mating

A 50 ml culture of *M.capsulatus* , A_{600} between 0.2 and 0.3, was centrifuged and the resultant cell pellet resuspended in 0.5 ml NMS containing 0.02% (w/v) Proteose-Peptone. 1 ml of an overnight culture of the donor *E.coli*,(1 ml of each *E.coli* strain if a "helper plasmid" was needed), was centrifuged and the cell pellet washed with NMS, as described above. The final cell pellet was mixed with the *M.capsulatus* culture. Cells were transferred to a Bijou and the top sealed with a suba seal. 3 ml of methane was added and the solution was gently shaken at 37^0C overnight. The culture was then plated onto NMS plates containing the appropriate antibiotics.

2.2.5 Transformation of *Escherichia coli*

Transformation of *E.coli* was carried out using one of two methods depending on the size of the plasmid. Plasmids of 15-20 Kb in size were electro-transformed into *E.coli*. This procedure is outlined in section 2.2.6. Large

plasmids were transferred into *E.coli* using the calcium chloride method of Cohen *et al.*, (1972).

2.2.6 Electroporation of *Escherichia coli*

2.2.6.1 Preparation of cells.

The method used is that recommended by Bio-rad Laboratories, the manufacturer of the Gene pulser and Pulse controller used.

1 l of LB broth was inoculated with 10 ml of a fresh overnight culture of *E.coli*. The cells were then grown at 37^0C with vigorous shaking to an OD_{600} of between 0.5 and 0.8. Cells were chilled on ice for 15-30 min then centrifuged at 4^0C for 15 min at 10,000 rpm (Beckman J2-21). The cell pellet was resuspended in a total volume of 1 l cold sterile distilled water and centrifuged again as above. This cell pellet was then resuspended in 0.5 l of cold sterile distilled water (pH 7.5) and centrifuged as before. The cell pellet was resuspended in 20 ml of cold sterile 10% (v/v) glycerol, transferred to Oakridge tubes and centrifuged for 15 min at 15,000 rpm. The resulting cell pellet was resuspended in 2-3 ml of 10% glycerol, 50 μl aliquots frozen in dry ice, and stored for up to 6 months. The final cell concentration was at least 1 x 10^{10} cells/ml.

2.2.6.2 Electro-transformation of *E.coli*.

The Gene pulser apparatus was set to 25 μF capacitance, 2.5 kV and the pulse controller unit to 200 .

Cuvettes (0.2 cm gap between electrodes) were chilled on ice and *E.coli* cells allowed to thaw out slowly at room temperature then placed on ice. To a cold Eppendorf tube was added 40μl of cell suspension and 1-5 μl of DNA solution in a low ionic strength buffer such as TE (10:1 Tris-EDTA, pH 8.0). This was mixed well and allowed to stand on ice for 1 min. The cell mixture was transferred to the cuvette and the suspension moved to the base of the cuvette by tapping lightly on the base. One pulse of 12.5 kV/cm was then applied to the cuvette at the above setting with a time constant of 4 to 5 msec. Immediately 1 ml of SOC (room temperature) was added and the mixture resuspended with a Pasteur pipette, then incubated in a sterile test tube at 37^0C with shaking (200 rpm) for 1 h. Cells were then plated onto selective media.

2.2.7 Electroporation of *Methylococcus capsulatus*

2.2.7.1 Preparation of cells.

Methylococcus capsulatus used for electroporation experiments were either grown in a fermenter or in 250 ml shake flasks to an A_{600} of between 0.2 and 0.3. Due to the high cell density that could be obtained using cells grown in the fermenter it was usually only necessary to use 50 ml volumes to obtain cells at the cell density required for electrotransformation. If shake flask cultures were being used, at least 200 ml of culture was needed. Cells were washed as for *E.coli* (2.3.6) and the final resuspension was

in 2-3 ml of 10% glycerol, if the cells were from the fermenter or 0.5 ml if the cells were from shake-flasks. Cells could be stored at -70^{0}C.

2.2.7.2 Electro-transformation

Cells were normally used straight after preparation (kept on ice). A variety of conditions were used and these will be outlined in the appropriate Results section. Cells were mixed with DNA solution in an Eppendorf tube but immediately transferred to a pre-cooled cuvette and the pulse applied to the cells. Straight after the pulse, 1 ml of NMS containing 0.02% (w/v) Proteose-Peptone was added and cells resuspended with a Pasteur pipette. The cell culture was then added to 50 ml NMS containing 0.02% (w/v) Proteose-Peptone in a 250 ml flask, methane was added and cells shaken at 200 rpm overnight to allow recovery. After this recovery period, kanamycin (50 μg/ml) was added and cells incubated for a further 5 days. Cells were subsequently plated onto NMS containing kanamycin (50 μg/ml). This method is discussed further in the appropriate results section.

2.2.8 Determination of plasmid entry into *Methylococcus capsulatus*.

The method is based on one devised by D.Rawlings (pers. comm). Cells were prepared for electroporation, DNA added and pulsed as outlined in the previous section.

Immediately 1 ml of 10% (v/v) glycerol, 100 μl X 10 DNAase buffer (5mM $MgSO_4$) and 5 μl of DNAase (5 mg/ml) were added, the solution transferred to an Eppendorf tube and left at room temperature for 30 min. The solution was then centrifuged, and the cell pellet resuspended in 1 ml NMS. Cells were re-centrifuged and the pellet resuspended in 100 μl of Solution 1 used for the small-scale isolation of plasmid DNA (section 2.3.10). The cell solution was then used for plasmid isolation. The resulting DNA pellet was washed with 70% ethanol then resuspended in 20 μl T.E (10:1 pH 8.0). When the pellet was resuspended, 5 μl were used to electrotransform *E.coli* cells which were then plated onto selective plates.

Control experiments using *E.coli* are discussed in section 3.3.

2.2.9 Preparation of Plasmid DNA from *E.coli*

The method of Birnhoim & Doly (1979) as modified by Maniatis *et al.*, (1982) was used. Slight modifications were made to the large scale preparation of plasmid so that the procedure could be carried out in 45 ml Oakridge tubes. Solution 2 was also not put on ice prior to use and the DNA pellet was not washed with 70% ethanol. The final DNA pellet from the large scale preparation was resuspended in 30 ml of T.E (pH 8.0) to which was added 30 g CsCl and then 3 ml ethidium bromide (10 mg/ml). The density gradient was centrifuged overnight at 45k rpm (Beckman L8-70). The

plasmids pDSK509 and pDSK519 could only be prepared from *E.coli* by the boiling method outlined in Maniatis *et al.*, (1982).

2.2.10 Preparation of plasmid DNA from *Methylococcus capsulatus*

The method was essentially that used for *E.coli* (2.2.9) but with certain modifications. The cell pellet was washed prior to addition of lysozyme in Solution 1. The lysis time was increased to 15 min at 37^0C. The DNA pellet from the large scale preparation was resuspended in 3.8 ml T.E to which was added 4.4 g CsCl and 0.3 ml ethidium bromide and the solution was centrifuged in a Vti65.2 rotor at 60K rpm (Beckman L8-70) for 16 h.

2.2.11 Preparation of *Methylococcus capsulatus* chromosomal DNA

This method is based on a method of Oakley & Murrell (1988).

2.2.11.1 Large-scale

The method was used for 500 ml of cells at an OD_{600} > 7. Cells were centrifuged and resuspended in 10 ml EDTA-saline, treated with 50 µg/ml lysozyme and placed at 37^0C until cell distortion was apparent under the microscope. 3.5 ml of 0.25 M EDTA (pH 8.0) were added and the cells placed at 37^0C for a few minutes. 0.6 ml of a 35%

(v/v) solution of Sarcosyl were then added and the solution mixed gently by inversion. Proteinase K was added (200 µl of a 10 mg/ml stock solution) and the solution mixed and incubated at 37^{0}C until the solution cleared. 3.5 ml of 5 M sodium perchlorate were added and the solution transferred to 60^{0}C and mixed intermittently for 15 min. An equal volume of phenol/ chloroform/isoamylalcohol (25:24:1) was added and the solution gently mixed to produce a milky solution. The mixing was continued for 15-30 min then the solution was centrifuged (Beckman J2-21, 18000 rpm) for 30 min at room temperature. The top layer was transferred to a clean tube and extraction carried out with chloroform as above until the interface obtained after centrifugation was clean. 1/50th volume of 5 M NaCl was added to the resulting aqueous phase and the solution was then cooled on ice. 2 volumes of chilled (-20^{0}C) ethanol were added. At this stage, the DNA could be spooled using a glass rod, washed in 70% ethanol and dried in a vacuum dessicator. The DNA pellet was resuspended in 10 ml of TE and treated with RNAase A (heat-treated as in Maniatis *et al.*,1982) at a final concentration of 100 µg/ml (37^{0}C for 30 min). The final volume was adjusted to 30 ml, 30 g CsCl added and then 3 ml of ethidium bromide. The gradient was centrifuged at 45K rpm for 16 h (Beckman L8-70).

2.2.11.2 Small-scale

This method was used for small volumes of cells, 100 ml at an OD_{600} of approximately 0.4.

Cells were centrifuged, resuspended in 5 ml EDTA-saline and treated with lysozyme (50 µg/ml) at 37^0C for 15-30 min. 1.75 ml EDTA (0.25 M) and 0.3 ml of 35% (v/v) Sarcosyl were added and the solution mixed gently. Proteinase K (50 µl of a 10 µg/ml stock) was added, the solution incubated at 37^0C for at least 30 min until the solution cleared. The volume was increased to 7.6 ml with TE (10:1, pH 8.0) then split into two 3.8 ml portions. To each were added 4.4 g CsCl and 0.3 ml ethidium bromide. These were then centrifuged in a Vti65 rotor overnight at 60K rpm (Beckman L8-70).

2.2.11.3 Extraction and purification of chromosomal DNA from CsCl gradients

Extraction of the DNA from gradients was essentially as in section 2.3.12.8 The resulting chromosomal DNA was dialysed 3 times against 3 l of TE (10:1, pH 8.0) at 4^0C.

2.2.12 Routine DNA manipulation methods

2.2.12.1 Agarose gel electrophoresis.

DNA samples were electrophoresed routinely on horizontal agarose (medium type II, Sigma) gels. Gels were prepared in and run using 1 X tris-borate EDTA (TBE)

electrophoresis buffer (0.89 M Tris-base, 0.89 M boric acid, 0.002 M EDTA (pH 8.0)). The loading buffer was 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene blue and 15% (w/v) Ficoll (Type 400) in distilled water. Mini-gels (50 ml of gel) were run at 60 mA for about 1.5 h, midi-gels (100 ml volume of gel) at 100 V for 2 h and large gels (400 ml volume of gel) at 60 V usually overnight. Gels were stained with ethidium bromide and DNA visualized by transillumination with short-wave uv light. Polaroid 665 film was used to record gels.

2.2.12.2 Extraction of DNA from agarose gels.

Two methods were used for the extraction of DNA fragments from gels. The first method was electroelution into dialysis tubing. The DNA fragment within the agarose was placed in dialysis tubing (prepared as in Maniatis *et al.*, 1982) with a small volume of 0.5 X TBE electrophoresis buffer and the tubing sealed with dialysis clips. This was then placed in a gel tank containing 0.5X TBE buffer. The DNA electroluted at 100 V. DNA was released from the dialysis tubing by reversing the current. DNA was then recovered in 0.5X TBE with a Pasteur pipette. Ethidium bromide was removed by extraction with TE-saturated butan-1-ol.

The second method was to use Geneclean II (supplied by Stratech Scientific Ltd.). This method was

routinely used for extraction of DNA to be used in ligations.

2.2.12.3 Use of Restriction Enzymes

Restriction enzymes and buffers were used according to the manufacturers (BRL and Amersham) instructions. If partial digest of DNA was required, the enzyme was inactivated by the methods of Maniatis *et al.*,(1982).

2.2.12.4 Extraction with phenol/chloroform and precipitation with ethanol

DNA solutions were purified by extraction with phenol/chloroform/isoamyl-alcohol (25:24:1). An equal volume was added to the solution, mixed gently then centrifuged in an Eppendorf or Oakridge tube at room temperature. The top layer was removed and DNA precipitated by addition of 1/10th volume 3 M sodium acetate and 2 volumes ethanol, (-20^0C overnight or an hour in a dry ice/ethanol bath). After centrifugation at 4^0C the resulting DNA pellet was dried briefly in a vacuum dessicator then resuspended in T.E (pH 8.0).

2.2.12.5 Dephosphorylation of DNA.

The removal of the 5′ phosphate group of vector DNA was carried outprior to ligation to prevent recircularization of the vector. To each 10 μl of DNA

solution (containing approximately 1 μg DNA) the following were added:-

1.5 μl 0.5 M glycine pH 9.4

1.5 μl MgCl (10 mM), ZnCl (1 mM).

1.5 μl sterile distilled water

0.5 μl Calf intestinal phosphatase (CIP) (Amersham)

The mixture was incubated at 37^{0}C for 15 min a further 0.5 μl CIP was added and the incubation continued for another 15 min. This was again repeated. This method worked best for overhanging 5'ends produced by endonuclease digestion. Endonuclease digestion can also produce recessed 5' ends, if this was the case the conditions for phosphorylation were addition of 0.5 μl CIP, 15 min at 37^{0}C, 15 min at 56^{0}C, further addition of 0.5 μl CIP and the incubations repeated. In both cases, CIP was inactivated by addition of 2.0 μl 0.25mM EDTA and 2.5 μl 20% SDS (sodium dodecyl sulphate) and heating at 68^{0}C for 15 min.

2.2.12.6 Ligation.

DNA to be ligated was cut with compatible restriction enzymes. Vector DNA was dephosphorylated and vector and insert DNA was mixed in a ratio of 1 vector : 5 insert. DNA solutions were mixed and the volume increased to at least 100 μl. A phenol/chloroform extraction was

performed followed by a chloroform extraction. DNA in the top layer (aqueous) was then precipitated with ethanol and salts for 1 h in a dry ice/ethanol bath. DNA was centrifuged at 4^0C and the resulting pellet was briefly dried then resuspended in 14 μl of distilled water, 4 μl X 5 ligase buffer and 2 μl ligase. Ligations were carried out at 16^0C for at least 18h.

2.2.12.7 Quantitation of DNA

DNA was quantified on an agarose gel by running alongside markers of known concentration. The markers used were Lambda DNA cut with *Hin*dIII which produced fragments of 23.17, 9.46, 6.75, 4.26, 2.2, 1.92 and 0.58 Kb.

2.2.12.8 Extraction of DNA from CsCl gradients

Plasmid DNA on CsCl gradients was visualized using a hand-held uv lamp and removed through a wide-bore needle. Ethidium bromide was removed by repeated extractions with butan-1-ol saturated with TE (pH 8.0). Plasmid DNA was precipitated by addition of 2 volumes of water and 6 volumes of ethanol (-20^0C overnight). The solution was centrifuged at 4^0C, the resulting pellet was dried briefly in a vacuum dessicator and then resuspended in 1 ml TE (pH 8.0).

2.2.13 Quantitative assay for ß-galactosidase activity using ONPG.

2.2.13.1 Assay for activity in *Escherichia coli* (Maniatis *et al.*, 1982)

An overnight culture of *E.coli* was diluted 1:50 in 10 ml of LB, grown to an $O.D_{600}$ of 0.4 then placed on ice to prevent further growth.

Cells were lysed by the addition of 0.9 ml Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M ß-mercaptoethanol, pH 7.0), 2 drops of chloroform and 1 drop of 0.1% SDS to 0.1 ml of cell culture. The mixture was vortexed for 10 sec and allowed to equilibrate to 28^0C.To equal portions of this mixture was added 0.2 ml of ONPG (O-nitrophenyl ß-D-galactopyranoside), dissolved in sterile distilled water, at 1 min intervals. When a yellow colour developed, the reaction was stopped by the addition of 0.5 ml 1 M Na_2CO_3. The time at which the yellow colour developed was recorded and units of B-galactosidase activity (ie conversion of ONPG per minute, arbitrary units) can be determined using the following equation:-

$$= \frac{1000 \times A_{420} - (1.75 \times A_{550})}{t \times 0.1 \times A_{600}}$$

t= time in minutes

The absorbance of the mixture was measured at A_{420} and A_{550}, and the A_{600} of the culture was measured.

2.2.13.2. Assay for activity in *Methylococcus capsulatus*

The method for the assay of ß-galactosidase activity in *M.capsulatus* differs only in the method of cell lysis and the quantity of cells used. The strain containing the plasmid was grown overnight to an A_{600} of 0.2 to 0.3 and 10ml of this culture was centrifuged and the supernatant removed to leave the cell pellet as dry as possible. The cell pellet was then resuspended in 1 ml Z buffer. Cells were lysed by sonication using an MSE sonicator. The sonication was carried out on ice with 3 X 6 sec 18 micron peak-to-peak bursts with a 30 sec cooling period between each burst. The culture was centrifuged and the cell-free extract removed and used for the assay.

2.2.14 Southern transfer of DNA

The method used was that outlined in Maniatis *et al.* (1982) except that 20 X SSC and not 10 X SSC was used as transfer solution. DNA was transferred onto nitrocellulose (Amersham, Hybond-C).

2.2.15 Nick Translation of DNA

2.2.15.1 Probe preparation

The method was that outlined in Maniatis *et al.*, (1982). The recipe used was:-

x μl DNA (usually about 500 ng)

1 μl TTP

1 μl ATP

1 μl CTP

1 μl GTP* (^{32}P labelled, 10 μCi/μl)

4 μl X20 nick translation buffer

1 μl DNA polymerase I

1 μl DNAase (1 μl of 1 mg/ml stock in 50 μl H_2O then 1 μl of above in 50 ml H_2O)

y μl sterile distilled water

x and y were adjusted according to the concentration of DNA to give a final volume of 20 μl. The mixture was incubated at 16^0C for at least 3 h. The reaction was stopped by the addition of 5 μl EDTA (0.25 M).

2.2.15.2 Probe purification

The radiolabelled probe was separated from unincorporated nucleotides using a Sephadex G50 column. The probe was boiled for 15 min then cooled on ice immediately prior to use.

2.2.16 Hybridisation of probe to nitrocellulose filters

Nitrocellulose filters were placed in a plastic bag. An appropriate volume of hybridization solution was added to the bag, usually 10-50 ml, the bag was sealed and

incubated at 65^{0}C for at least 1 h. The hybridization solution contained 30 ml 20 X SSC, 1 ml Denhardt solution (Denhardt, 1966), 100 µl sheared and heat-denatured Herring sperm DNA and 69 ml distilled water. The hybridization solution was poured off and fresh hybridization solution and nick-translated probe were added. The bag was heat sealed and then sealed within a second bag. Hybridization was overnight at 65^{0}C. The filter was then washed under conditions requiring a certain stringency of DNA binding using varying concentrations of SSC and different temperatures. e.g, 500 ml of 2 X SSC for 20 min at 80^{0}C. See appropriate results section for conditions.

2.2.17 Autoradiography

Filters were air-dried and autoradiography took place at -70^{0}C. The autoradiograph was developed using Kodak X-ray developer and Unifix fixer.

2.2.18 Colony hybridizations with *Escherichia coli*

2.2.18.1 Growth of colonies on nitrocellulose filters

Colonies were transferred by tooth-pick onto duplicate plates containing the appropriate antibiotic. The colonies on one plate were picked onto a nitrocellulose filter (Amersham, Hybond-C) marked out with a grid for easy identification of the colonies. The plates were grown as normal, inverted at 37^{0}C overnight.

2.2.18.2 Treatment of filters

This method was that of Grunstein & Hogness (1975) as modified by Maniatis *et al.*,(1982). DNA released from bacterial colonies was fixed to nitrocellulose filters by baking at 80^0C for 2 h.

2.2.18.3 Hybridization of probe to filter

Radiolabelled probes were prepared as in section 2.2.15 and the filters were treated as in section 2.2.16.

2.2.19 Colony hybridisation of *Methylococcus capsulatus*

The method was essentially the same as for *E.coli*, however the colonies were grown on agar plates and transferred to the filter for lysis andthe cell lysis time was increased with 10% SDS (from 3 min to 10 min). The resulting filter was then treated in an identical manner.

RESULTS

CONJUGAL TRANSFER OF PLASMIDS TO *METHYLOCOCCUS CAPSULATUS*

3. RESULTS

3.1 CONJUGAL TRANSFER OF PLASMIDS TO *METHYLOCOCCUS CAPSULATUS* (BATH).

The development of a reliable method for the transfer of plasmids into *Methylococcus capsulatus* was important for several reasons. The regulation of genes that have been cloned from the organism can only be studied at present by expression in a heterologous host such as *E.coli* and regulatory factors may not be present in *E.coli*. Methods for the production of mutants by transposon mutagenesis and marker-exchange mutagenesis rely on an efficient gene transfer system. A primary aim therefore, was to devise an efficient method for the transfer of heterologous and homologous genes into *M.capsulatus*.

3.1.1 Natural antibiotic resistance and isolation of antibiotic resistant mutants of *M.capsulatus*.

For a successful gene transfer system, transfer of plasmid must be detected by the acquisition of a new antibiotic resistance or a new metabolic process, e.g, ß-galactosidase activity, in the host organism. Most plasmids used as cloning vectors encode several antibiotic resistances. *Methylococcus capsulatus* (Bath) was screened to assess suitable markers for gene transfer as outlined in section 2.2.2. An ideal marker for plasmid transfer would be resistance to an antibiotic for which *M.capsulatus* has no natural resistance.

Methylococcus capsulatus was resistant to a large number of antibiotics, thereby decreasing the number of potential cloning vectors that could be used (table 3.1.1). The first screening did however, show that *M.capsulatus* was sensitive to gentamicin, erythromycin, streptomycin, kanamycin, carbenicillin, and rifampicin. A second screening was undertaken, this time incorporating the antibiotic into agar plates at the concentration used in Mast rings. Only kanamycin, gentamicin and carbenicillin totally inhibited growth. Spontaneous mutants resistant to erythromycin or rifampicin were isolated at too high a frequency ($>10^{-5}$) to make these useful markers for plasmid transfer. Spontaneous streptomycin resistance mutants were isolated but at a fairly low frequency ($<10^{-4}$) and, therefore, streptomycin was also considered as a possible marker for gene transfer experiments. *M.capsulatus* was also sensitive to mercury chloride, resistance to which is carried on several potentially useful cloning vectors and could therefore be used as an indication of gene transfer.

In a conjugation system, a selection against the donor organism is needed, often antibiotic resistance possessed by the recipient but not the donor, obviously many antibiotics could be used for this purpose. It was possible to isolate streptomycin (50 μg/ml) and gentamicin (20 μg/ml) resistant mutants of *M.capsulatus* but no kanamycin resistant mutants were found. Attempts to isolate spontaneous mercury chloride resistant mutants were unsuccessful.

Antibiotic/ Compound	Concentration µg	Effect
Gentamicin	10	++
Chloramphenicol	25	-
Fuscidic Acid	10	-
Trimethoprin	1.25	-
Ampicillin	10	-
Penicillin G	1 unit	-
Sulphatriad	200	-
Cloxacillin	5	-
Novobiocin	5	-
Erythromycin	5	+
Tetracycline	25	-
Cephaloridine	5	-
Streptomycin	10	++
Lincomycin	2	-
Clindamycin	2	-
Co-trimoxazole	25	-
Kanamycin	50	++
Rifampicin	10	+
Carbenicillin	5	++
Mercury Chloride	5 mg/ml	++

Table 3.1.1: Antibiotic sensitivity profile of *M.capsulatus*

++ = very sensitive + = high rate of spontaneous resistance
- = resistant

3.1.2 Development of a filter mating technique

The final method adopted is outlined in section 2.2.4 and shown in figure 3.1.1. This was developed from a method used by Whitta *et al.* (1983), originally used for *Methylobacterium* AM1. Although *M.capsulatus* is inhibited by rich media, it was possible to incorporate 0.02% w/v Proteose-Peptone in NMS plates. The presence of Proteose-Peptone and methane allowed the growth of both the donor(*E.coli*) and *M.capsulatus* and therefore, the transfer of plasmids. Placing the filters at 37^0C would reduce the growth rate of *M.capsulatus* and filters were left for 24 hours for transfer to occur to compensate for this reduction in growth rate. Often, using conjugation as a method for gene transfer, there are difficulties due to the subsequent growth of the donor organism and it is necessary to inhibit this growth. At first antibiotics were used to which only *M.capsulatus* was resistant. Subsequently, it was noted that the lack of complex carbon source in selective NMS plates and a growth temperature of 45^0C were sufficient to inhibit the growth of *E.coli*. Some growth of *E.coli* did occur when 0.02% (w/v) Proteose-Peptone was incorporated in selective plates but the growth temperature of 45^0C was sufficient to reduce this growth to a thin lawn upon which it was easy to detect the *M.capsulatus* colonies.

The first plasmid to be transferred was the cosmid pVK100 (Knauf & Nester, 1984) (Figure 3.1.2). The transfer of pVK100 was subsequently used to investigate a variety of

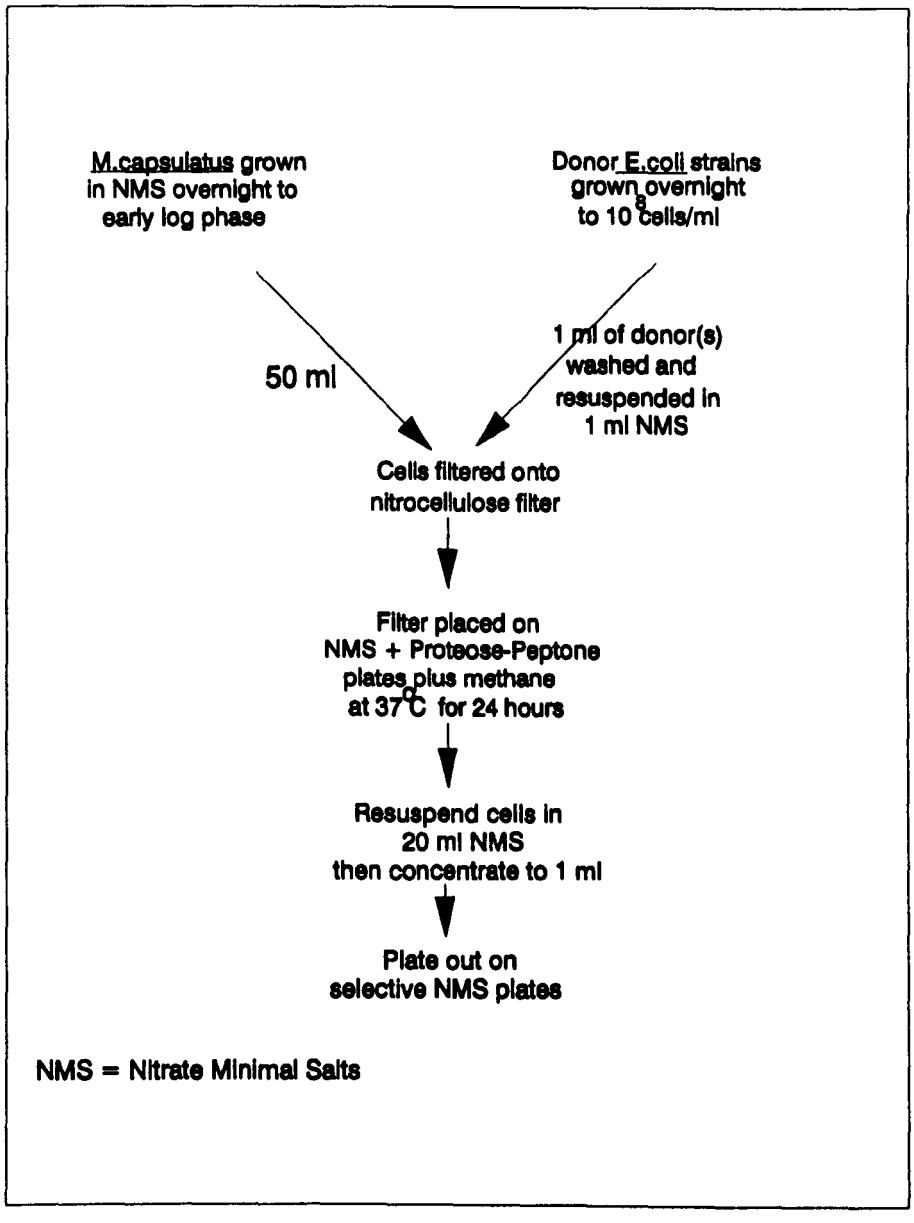


Figure 3.1.1: The technique for the transfer of plasmids to M.capsulatus by filter-mating.

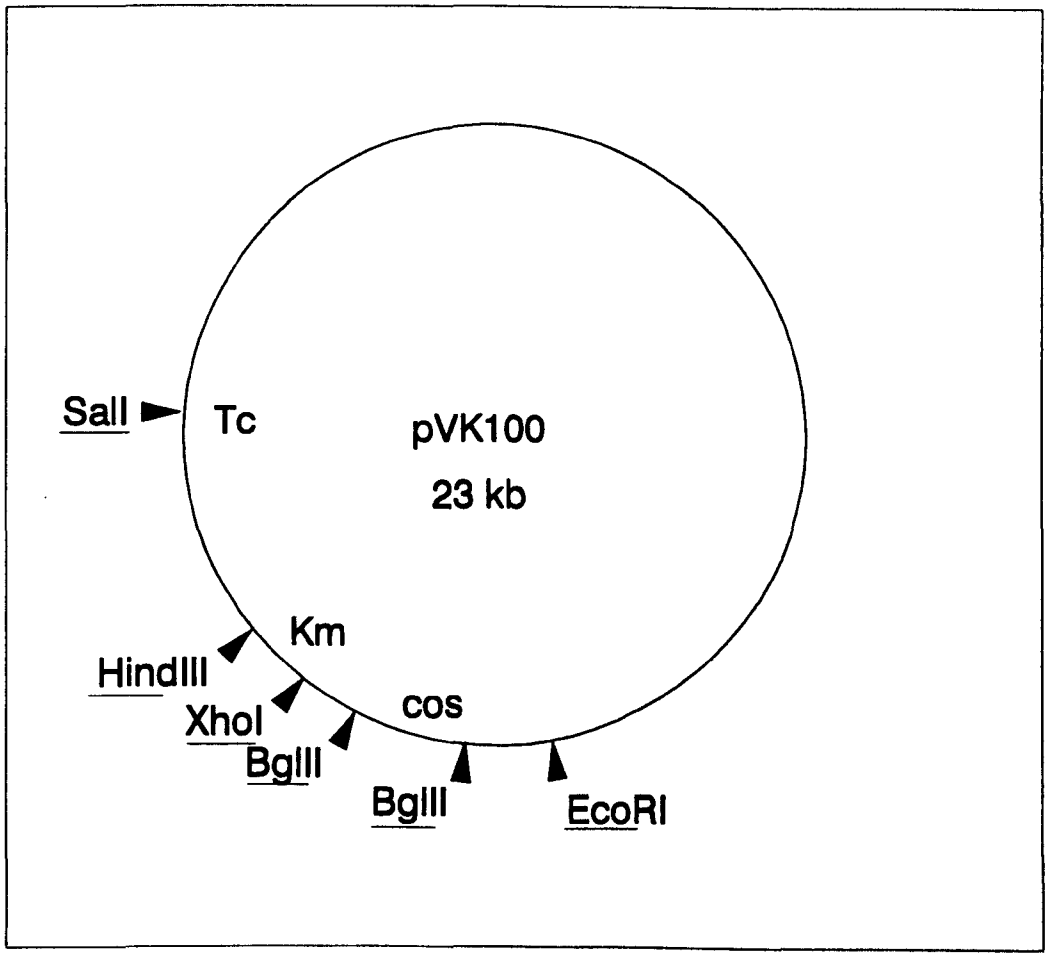


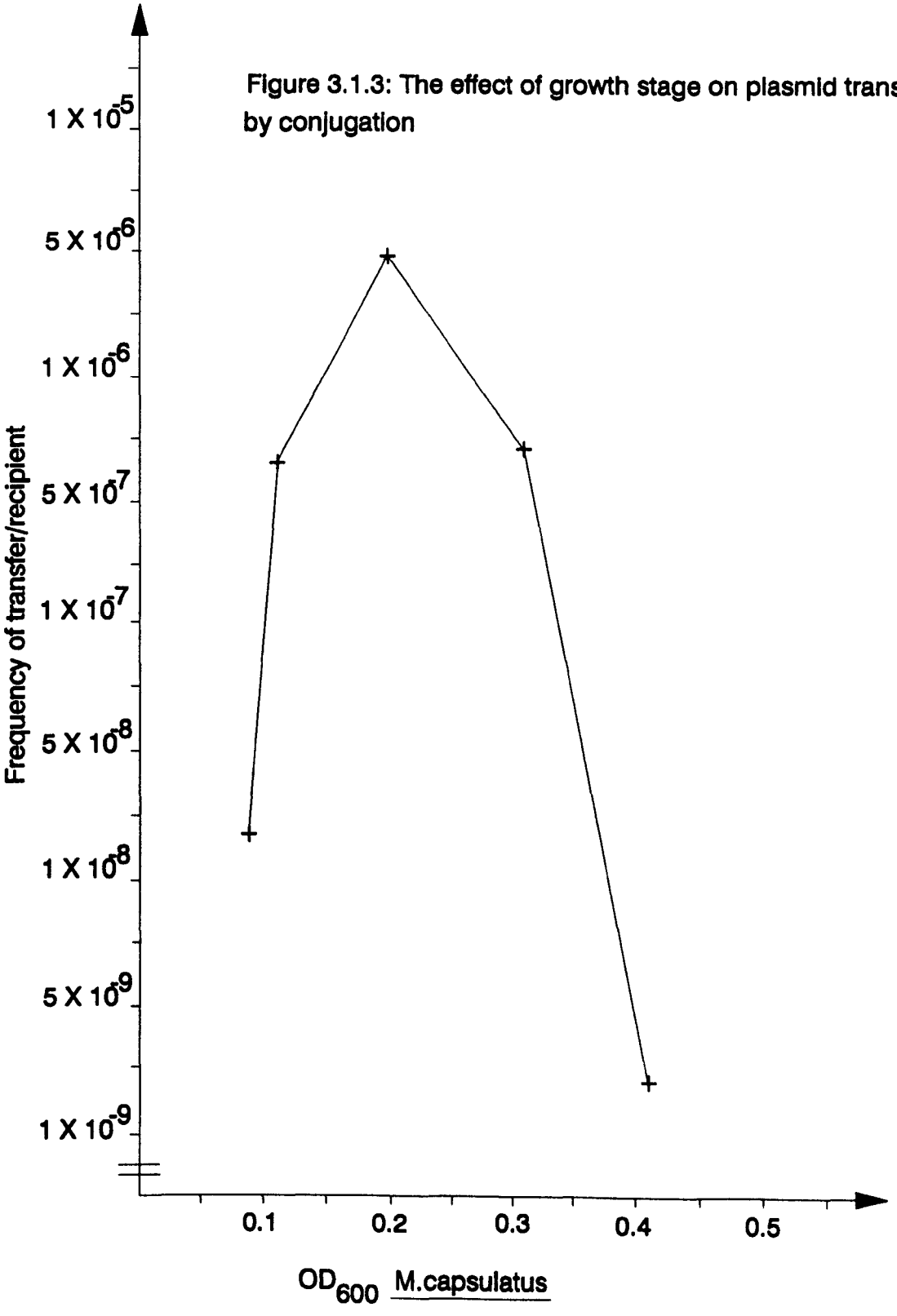
Figure 3.1.2: Restriction map of pVK100

parameters that may affect the efficiency of transfer. Transfer was achieved using a triparental conjugation with the RP4 derivative pRK2013 (Figurski & Helinski, 1979) as the mobilizing or 'helper' plasmid. PRK2013 contains the RP4 genes necessary for transfer and mobilization of itself and other plasmids but does not contain the RP4 broad-host range replication functions, having only a narrow-host range *col*E1 replicon.

The effect of the growth phase of the recipient, *M.capsulatus*, was studied. Cells were used from early, mid and late logarithmic growth and stationary phase. The number of viable cells present in culture was determined by plating a dilution series. The OD_{600} of the culture was equated with the number of viable cells and also a growth curve was constructed. Figure 3.1.3 shows that the number of transconjugants per recipient present was greatest at early to mid-logarithmic growth which corresponded to an OD_{600} of 0.15 to 0.25.

The second parameter examined was that of the time that cells were left on filters before they were transferred to kanamycin containing NMS plates. Figure 3.1.4 shows that there was a rapid drop in the number of transconjugants produced if cells were left on the filter for longer than 24 hours. The cultures used for each conjugation were in mid-logarithmic growth.

Figure 3.1.3: The effect of growth stage on plasmid transfer by conjugation



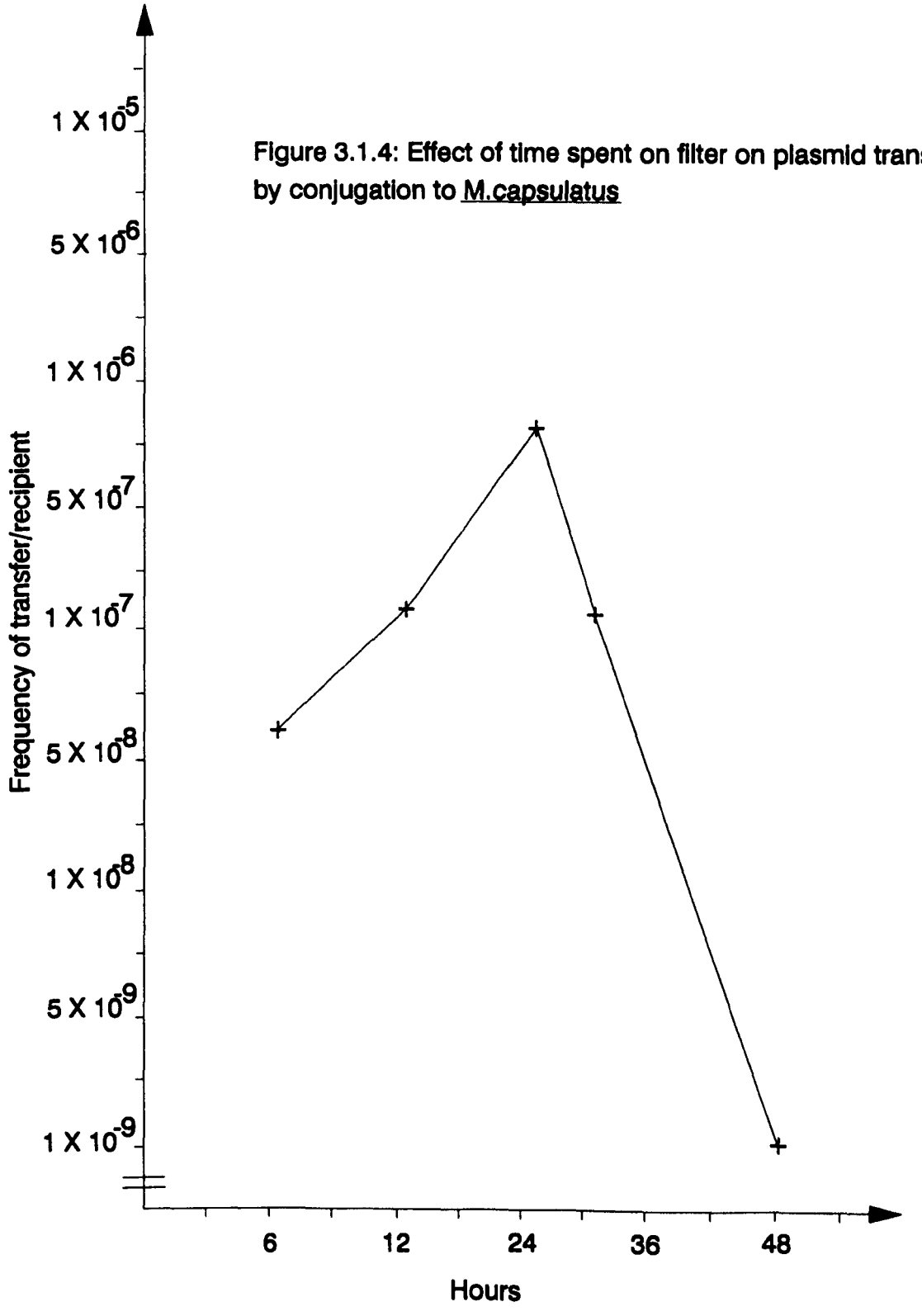


Figure 3.1.4: Effect of time spent on filter on plasmid transfer by conjugation to M.capsulatus

The ratio of donor to recipient was a factor that was also considered. The first conjugation experiments had a ratio of 1 donor to 5 recipient (with triparental conjugations this was 1:1:5). This often meant using 50 ml of *M.capsulatus* and 1 ml of the donor *E.coli*. Conjugations were attempted with a ratio of 1:0.5, 1:1, 1:2, 1:3.5, 1:7 and 1:10 approximately. Table 3.1.2 shows the effect on the frequency of transconjugants obtained. The *M.capsulatus* cultures used were in mid-logarithmic phase of growth.

Ratio *E.coli*:	*E.coli*:	*M.capsulatus*	Transfer Frequency (per recipient) (average of 5 experiments)
1	1	0.5	None
1	1	1	2×10^{-10}
1	1	2	4.2×10^{-9}
1	1	3.5	4×10^{-8}
1	1	5	6.6×10^{-7}
1	1	7	6×10^{-7}
1	1	10	6.2×10^{-7}

Table 3.1.2

In all conjugations the *E.coli* used were from an overnight culture containing approximately 1×10^8 viable cells per ml.

Washing of *E.coli* was found to be important. If the *E.coli* cells were washed only once, the frequency of transconjugant production was reduced, often to zero.

Transfer of plasmids in liquid mating experiments was attempted using the method outlined in section 2.2.4.3, but these were unsuccessful.

3.1.3 Transfer Frequencies

Table 3.1.3 lists all the plasmids that were tested for transfer into *M.capsulatus*. The number of viable *E.coli* and *M.capsulatus* were determined by serial dilution and plating onto LB or NMS agar (section 2.2.1).

The success of the conjugation was assessed by the number of transconjugants produced per viable recipient cell.

$$\frac{\text{No. of Transconjugant}}{\text{No. of Viable Recipient Cells Present}} = \text{Frequency of transfer}$$

This way of determining the frequency of the conjugation makes it possible to assess differences between cultures that have an identical numbers of viable cells, as determined by serial dilutions, but may have a very different number of cells competent for conjugation. Colonies of *M.capsulatus* were easily distinguishable on selective plates. The frequency of plasmid transfer calculated for plasmids where transfer is determined by streptomycin resistance on table 3.1.3, has been adjusted to allow for spontaneous streptomycin resistant mutants. Prior to conjugation, the culture was plated onto selective plates

Plasmid	Helper plasmid	Marker	Frequency of transfer per recipient	Size (kb)
Stable plasmids				
pVK100	pRK2013	Km	6.6×10^{-7}	23
pDSK509	pRK2013	Km	2×10^{-3}	9.1
pULB113	-	Km	1.5×10^{-6} (4×10^{-4})	60
RP4	-	Km	4.3×10^{-8} (3.22×10^{-5})	56
pGSS33	pRK2013	Sm	2.7×10^{-6}	13.4
RSF1010	pRK2013	Sm	5.2×10^{-7}	8.8
pSDS1	pRK2013	Km	5.2×10^{-7}	17
pKT231	pLG221	Sm,Km	0	13
pDSK519	pRK2013	Km	$1.2 \times 10_{-6}$	8.1
pME277	pUB307	Hg	0	14.2
pMMB33	pLG221	Km	0	13.75
Mutagenesis Vectors				
pJFF350	RP4	Km	5.2×10^{-8}	5.3
pSUP2021	RP4	Km	2×10^{-9}	ND
pLG221	-	Km	0	ND
pDC2	pRK2013	Sm	0	17.2
pSD10	pRK2013	Sm	0	13.3
pSD100	pRK2013	Km,Sm	ND	12.5
pBEE132	-	Km	0	ND
pMD100	-	Hg	0	ND

ND=not determined

Table 3.1.3: Plasmids tested for transfer into *N.capsulatus*

and the frequency of spontaneous resistance determined, (1-2 colonies per 10^6 cells).

The numbers in brackets are the transfer frequencies from *M.capsulatus* to *E.coli*. The majority of conjugations were triparental conjugations, i.e., a third plasmid was needed to provide mobilization functions. The 'helper' plasmid was usually pRK2013 (Figurski & Helinski, 1979). In other cases, e.g, pJFF350, the plasmid was present in the *E.coli* S17-1 (Simon *et al.*, 1983) which contains the functions of RP4 incorporated into the chromosome. This strain of *E.coli* has been found to transfer plasmids at a much higher frequency than strains where RP4 is present as an autonomous plasmid and also without the inconvenience of the possible transfer of RP4 (Simon *et al.*, 1983).

The low frequencies of transfer were thought possibly to be due to the effect of a restriction system in *M.capsulatus*. Subjecting cells to a heat-shock has been shown to reduce the effect of restriction systems (Uetake *et al.*, 1964). The conjugations were therefore performed, with pVK100, after *M.capsulatus* cells had been subjected to a range of temperatures for varying lengths of time. Table 3.1.4 shows the results of the treatments. The frequency of transconjugant production was not significantly affected by heat-shock. There was no difference in the frequencies with treatment of up to 50^0C and 3 minutes. Any further increase in temperature and/or time of exposure led to a decrease in transconjugants. When the heat-treated *M.capsulatus* were

plated onto non-selective plates it was found that there was a significant reduction in the number of viable cells (i.e., approximately 100 colonies after 100 μl was plated onto non-selective plates, pre heat-treatment cells produced confluent growth).

^{0}C	Time (min)	Frequency of transfer
48	2	5.0×10^{-7}
48	3	6.0×10^{-7}
48	5	5.8×10^{-7}
50	2	5.6×10^{-7}
50	3	5.4×10^{-7}
50	5	3.2×10^{-8}
55	2	1.1×10^{-9}
55	3	0
55	5	0

Table 3.1.4

Another test for the effect of restriction and modification on transfer of plasmids was the frequency of transfer of pVK100, that had previously been present in *M.capsulatus*, from *E.coli* to *M.capsulatus*. The basic scheme for this experiment is outlined in figure 3.1.5, together with the relevant transfer frequencies (underlined). The transfer frequencies are expressed as transconjugants per recipient.

COLUMN 1	COLUMN 2
Transfer of pVK100 to *M.capsulatus* (Bath) from *E.coli* by conjugation 9.5×10^{-7}	
Isolation of pVK100 from *M.capsulatus* by alkaline-lysis mini-prep.	Isolation of pVK100 from *E.coli* by alkaline-lysis mini-prep.
Transfer to *E.coli* (DH1) by electroporation	Transfer to *E.coli* (DH1) by electroporation
Transfer of pVK100 to *M.capsulatus* by conjugation 5×10^{-9}	Transfer of pVK100 to *M.capsulatus* by conjugation 9.5×10^{-7}
16% survival of kanamycin resistant transconjugants upon subsequent plating onto kanamycin.	64.5% survival of kanamycin resistant transconjugants upon subsequent plating onto kanamycin.

Figure 3.1.5

A comparison could be made between pVK100 that had been replicated in *M.capsulatus* and *E.coli*, and pVK100 that had only undergone replication in *E.coli*. It was found that pVK100 originally isolated from *M.capsulatus* (column 1, Figure 3.1.5) was transferred at a thousand fold lower frequency (5×10^{-9}) from *E.coli* to *M.capsulatus* than pVK100 that had only replicated in *E.coli*. 200 transconjugants resulting from each conjugation were plated onto further plates containing kanamycin (50 μg/ml). Not every colony containing pVK100 survived, the average (4

separate conjugation experiments) percentage survival was 64.5% for pVK100 replicated only in *E.coli* and 16% for pVK100 replicated in both *M.capsulatus* and *E.coli*(Figure 3.1.5). The loss of kanamycin resistance was assumed to indicate loss of plasmid.

The presence of pVK100, isolated from *M.capsulatus* then subsequently transformed into *E.coli*, was by selection for kanamycin resistance. The plasmid pVK100 also encodes resistance to tetracycline but only 25% (110 colonies tested) of the *E.coli* colonies, containing pVK100 from *M.capsulatus*, were resistant to both tetracycline (12.5 μg/ml) and kanamycin (50 μg/ml). Replication of pVK100 in *M.capsulatus* appeared 75% of the time to cause loss of tetracycline resistance, indicating perhaps some rearrangement of the DNA

3.1.4 Detection of plasmids in *M.capsulatus*

3.1.4.1 Presence of antibiotic resistances

Initial detection of plasmid transfer was by the acquisition of antibiotic resistance. However, this was not relied upon totally to indicate that transfer had taken place. In most cases it was only possible to use a single antibiotic resistance, kanamycin, to indicate transfer which was not ideal. The isolation of antibiotic resistant colonies and their growth on subsequent antibiotic plates, was followed by testing their ability to grow in liquid

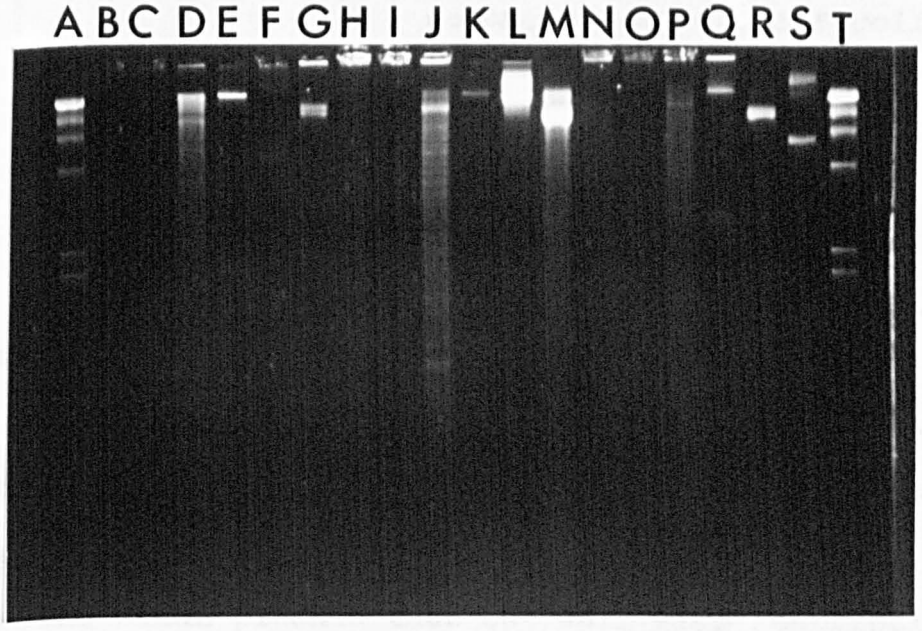
culture in the presence of the antibiotic. These liquid cultures were used to isolate the relevant plasmid.

3.1.4.2 Isolation of plasmid from *M.capsulatus*.

The plasmid preparation method finally chosen is outlined in section 2.2.10. Three different methods of plasmid preparation were tried, boiling-lysis (Holmes & Quigley, 1981), alkaline-lysis (Birnboim & Doly, 1979) and the method of Kado and Lui (1981). The plasmid solutions resulting from the boiling method and the alkaline-lysis method are shown in figure 3.1.6. The boiling method was found to produce plasmid contaminated with proteins and large amounts of chromosome, and often no plasmid at all. With the alkaline-lysis technique, plasmid was consistently produced but it was often heavily contaminated with chromosomal DNA which made the detection of plasmid on agarose gels difficult, particularly after restriction digests, which produced masking chromosomal DNA fragments. Plasmid present at very low amounts in *M.capsulatus* would have been difficult to detect on a gel. To overcome this problem the plasmid was isolated from *M.capsulatus* and subsequently electroporated into *E.coli* (section 2.2.6). From the resulting antibiotic resistant *E.coli* transformants the original plasmid could be isolated. Controls were carried out each time to ensure that the plasmid had originated from the *M.capsulatus* culture.

Figure 3.1.6: Comparison of the DNA solutions obtained from *M.capsulatus* by the alkaline-lysis and the boiling-lysis method

A = *Hin*dIII cut λ
B = pSDZ1 isolated from *M.capsulatus* by boiling-lysis-cut with *Bam*H1
C = pSDZ1 isolated from *M.capsulatus* by boiling-lysis-uncut
D = pSDZ1 isolated from *M.capsulatus* by alkaline-lysis-cut with *Bam*H1
E = pSDZ1 isolated from *M.capsulatus* by alkaline-lysis-uncut
F = pSDZ1 isolated from *E.coli*-uncut
G = pSDZ1 isolated from *E.coli*-cut with *Bam*H1
H = pDSK509 isolated from *M.capsulatus* by boiling-lysis-cut with *Eco*R1
I = pDSK509 isolated from *M.capsulatus* by boiling-lysis-uncut
J = pDSK509 isolated from *M.capsulatus* by alkaline-lysis-cut with *Eco*R1
K = pDSK509 isolated from *M.capsulatus* by alkaline-lysis-uncut
L = pDSK509 isolated from *E.coli*-uncut
M = pDSK509 isolated from *E.coli*-cut with *Eco*R1
N = pDSK519 isolated from *M.capsulatus* by boiling-lysis-cut with *Eco*R1
O = pDSK519 isolated from *M.capsulatus* by boiling-lysis-uncut
P = pDSK519 isolated from *M.capsulatus* by alkaline-lysis-cut with *Eco*R1
Q = pDSK519 isolated from *M.capsulatus* by alkaline-lysis-uncut
R = pDSK519 isolated from *E.coli*-uncut
S = pDSK519 isolated from *E.coli*-cut with *Eco*R1
T = *Hin*dIII cut λ


A B C D E F G H I J K L M N O P Q R S T

This method also allowed a rudimentary comparison of the effectiveness of the plasmid preparation methods used. Equal volumes of the plasmid preparations (5 μl), alkaline-lysis, boiling-lysis, and Kado & Lui, were used to electro-transform equal numbers of *E.coli* cells present in 40 μl of cells prepared for electroporation (Table 3.1.5). The alkaline-lysis method was the most successful.

	colonies per 100 μl *E.coli*		
Plasmid	**Boiling**	**Alkaline**	**Kado & Lui**
pSDZ1	70	200	0
pDSK519	28	80	0
pDSK509	100	200	0

Table 3.1.5

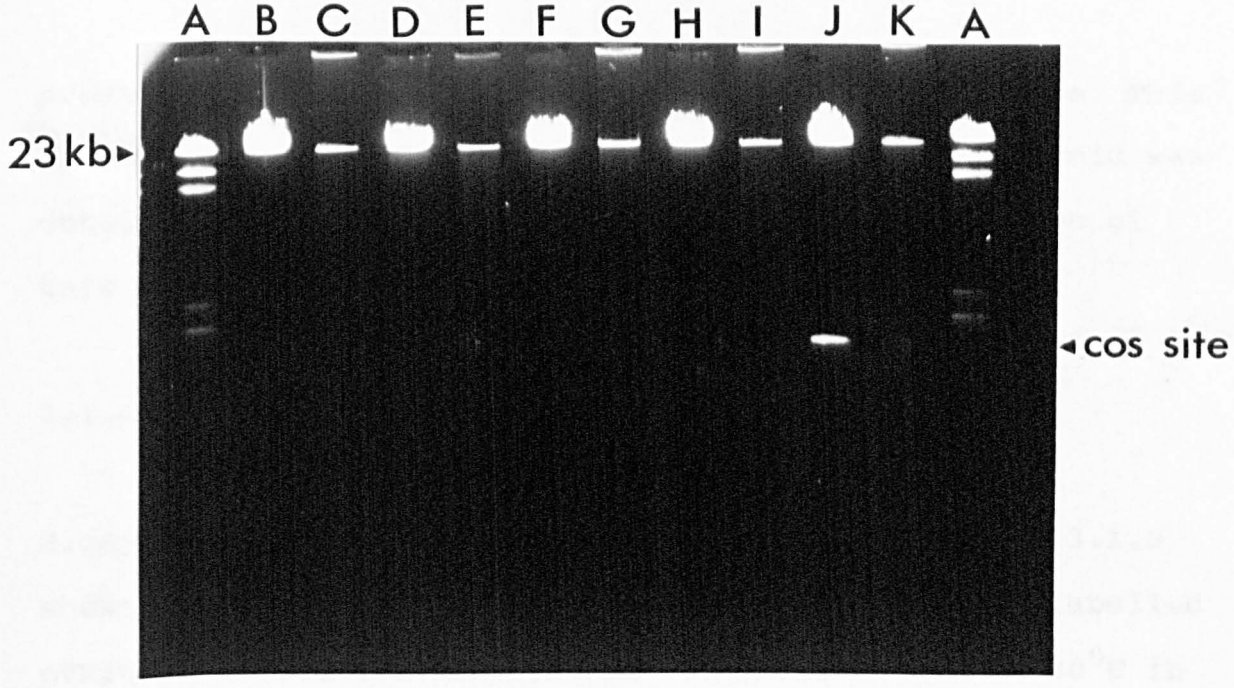
The alkaline-lysis method was used to isolate plasmid from *M.capsulatus* on a large scale as outlined in methods section 2.2.10. The method worked extremely well producing clean plasmid that cut well with restriction enzymes. Figures 3.1.7 and 3.1.8 show pVK100 and pDSK509 isolated from *M.capsulatus* compared with the plasmids isolated from *E.coli*. It is interesting to note that both the cosmid pVK100 and the plasmid pDSK509 were easier to isolate from *M.capsulatus* than from *E.coli*. The plasmid pDSK509 could not be easily isolated from *E.coli* using the alkaline-lysis method, plasmid yield was higher using the boiling method (Maniatis *et al., 1982*) but this also

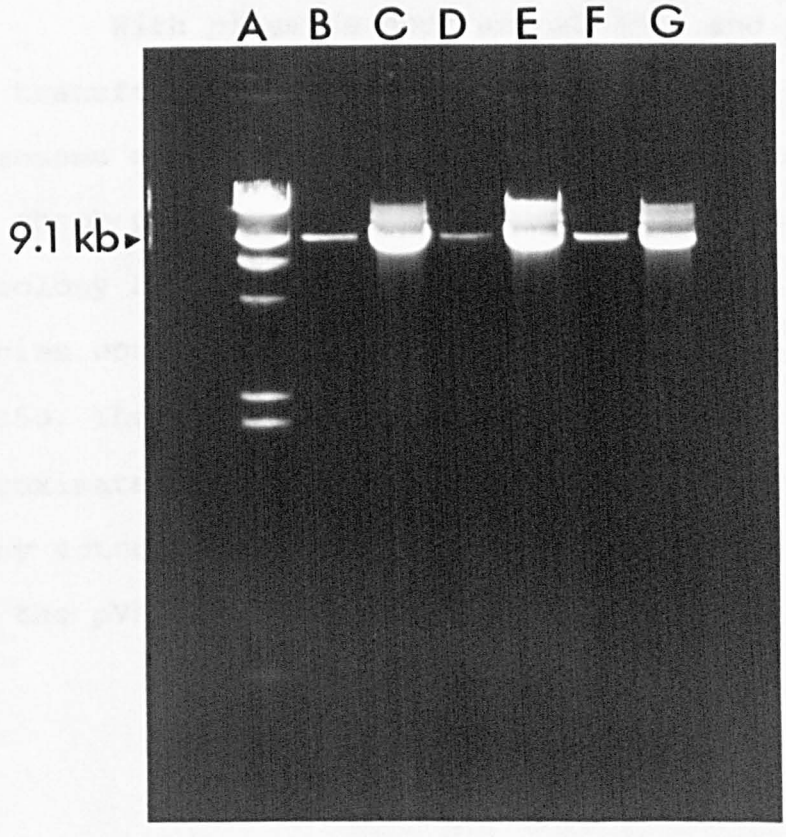
Figure 3.1.7: pVK100 isolated by the large-scale alkaline-lysis method from *M.capsulatus* and *E.coli*.

A = *Hin*dIII cut λ
B = pVK100 from *E.coli* cut with *Sal*1
C = pVK100 from *M.capsulatus* cut with *Sal*1
D = pVK100 from *E.coli* cut with *Xho*1
E = pVK100 from *M.capsulatus* cut with *Xho*1
F = pVK100 from *E.coli* cut with *Hin*dIII
G = pVK100 from *M.capsulatus* cut with *Hin*dIII
H = pVK100 from *E.coli* cut with *Eco*R1
I = pVK100 from *M.capsulatus* cut with *Eco*R1
J = pVK100 from *E.coli* cut with *Bgl*1
K = pVK100 from *M.capsulatus* cut with *Bgl*1

Figure 3.1.8: pDSK509 isolated by the large-scale alkaline-lysis method from *M.capsulatus* and isolated from *E.coli* by the boiling-lysis method.

A = *Hin*dIII cut λ
B = pDSK509 from *M.capsulatus* cut with *Eco*R1
C = pDSK509 from *E.coli* cut with *Eco*R1
D = pDSK509 from *M.capsulatus* cut with *Sal*1
E = pDSK509 from *E.coli* cut with *Sal*1
F = pDSK509 from *M.capsulatus* cut with *Bam*H1
G = pDSK509 from *E.coli* cut with *Bam*H1


A B C D E F G H I J K A
23 kb
cos site


A B C D E F G
9.1 kb

produced plasmid slightly contaminated with chromosome. This phenomenon was noted by Keen *et al* from whom the plasmid was obtained. No such problem was found with the isolation of this plasmid from *M.capsulatus*.

3.1.4.3 Colony hybridization

Another check for presence of plasmid in *M.capsulatus* was to use colony hybridization. Figure 3.1.9 shows colonies of *M.capsulatus* probed with the radiolabelled pVK100. The filters were washed twice for 20 min at 80^0C in 0.1 X SSC. Since the probe was homologous, highly stringent conditions could be used. The wild type *M.capsulatus* colony acted as a negative control.

With plasmids such as pSUP2021 and pJFF350, which upon transfer to *M.capsulatus* may insert DNA into the chromosome and can not replicate as autonomous plasmids, the only check on transfer, apart from antibiotic resistance, was colony hybridization. Figure 3.1.10 shows *M.capsulatus* colonies containing pJFF350 probed with radiolabelled pJFF350. The washing conditions were as above, (approximately 90% "stringency"). The wild-type *M.capsulatus* colony acted as a negative control and it did not 'light-up' with the pVK100 or the pJFF350 probes.

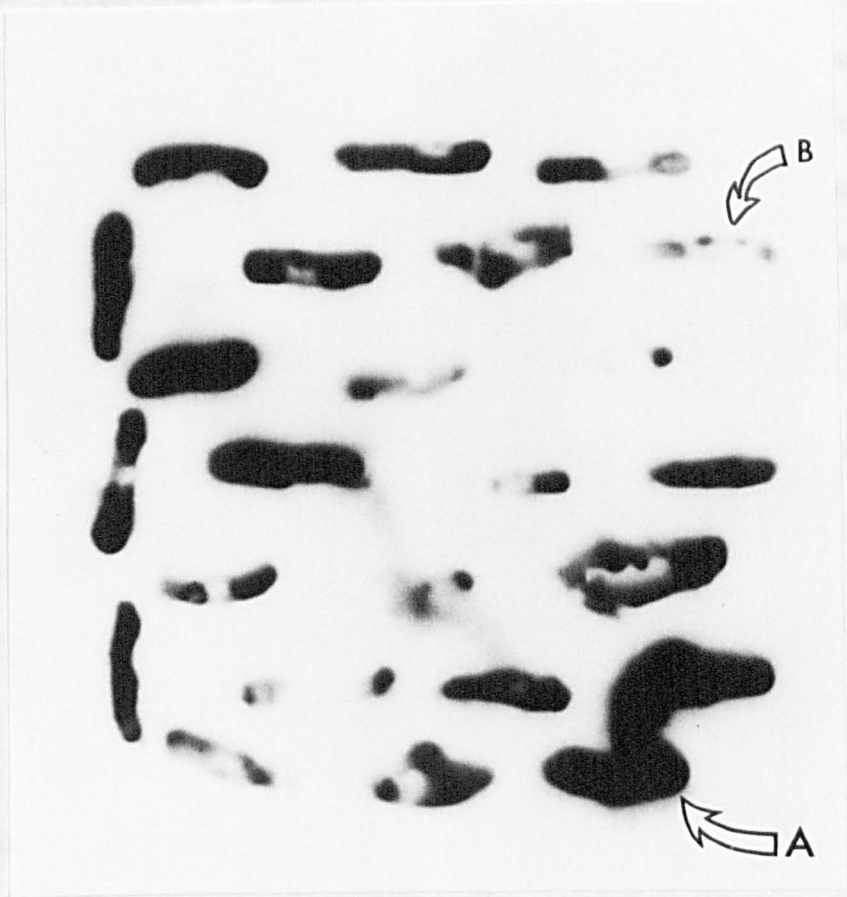
Figure 3.1.9: Hybridization of *M.capsulatus* colonies containing pVK100 with radiolabelled pVK100. Washing conditions required approximately 90% 'stringency'.

A = *E.coli* positive control.
B = negative control

Figure 3.1.10 (and 3.2.7): Hybridization of *M.capsulatus* colonies containing pJFF350 with radiolabelled pJFF350. Washing conditions required approximately 90% 'stringency'.

A = *E.coli* positive control.
B = negative control


B
A


B
A

3.1.5 Transfer of plasmids from *M.capsulatus* to *E.coli*

Plasmids could be transferred from *M.capsulatus* to *E.coli* by a very similar method to that outlined in section 3.1.2. The ratio of *M.capsulatus* to *E.coli* was altered to 1:1, but the time on filter and the growth phase of the *M.capsulatus* culture were unaltered. The frequency of transfer per recipient, of RP4 and pULB113, is shown in brackets on table 3.1.3. RP4 and pULB113, transferred from *M.capsulatus* to *E.coli*, exhibited their full range of antibiotic resistances in *E.coli*.

3.1.6 Growth of *M.capsulatus* containing plasmids

Since *M.capsulatus* does not naturally contain plasmids (Lidstrom & Wopat, 1984) the effect of the presence of plasmids on growth was studied. Table 3.1.6 shows growth rates for the wild-type at 45^0C and 37^0C and the growth rates in shake flasks for *M.capsulatus* containing pDSK509 at these temperatures. The wild-type has a slower growth rate at 37^0C than at 45^0C. *M.capsulatus* containing pDSK509 grew slower than the wild-type at both temperatures but the growth of *M.capsulatus* containing pDSK509 was slower at 45^0C than at 37^0C.

Temperature	Growth rate (h^{-1}) Wild-type	plus pDSK509
37^0C	0.55	0.40
45^0C	0.75	0.35

Table 3.1.6

3.1.7 Production of pSDS1: a promoter probe vector for *M.capsulatus*

One problem encountered with the vectors that could be transferred into *M.capsulatus* was the lack of suitable marker-genes. The majority of the vectors that were reliably transferred at a reasonable frequency contained only one usable marker-gene,ie., kanamycin resistance. pDSK519 (Figure 3.1.11) had kanamycin and *lacZ* (fragment). It could be transferred into *M.capsulatus* but at a 1000-fold lower frequency than pDSK509 (Figure 3.1.11), the plasmid from which it was derived. *M.capsulatus* was assayed for the presence of ß-galactosidase as outlined in section 2.2.13. However, it was discovered that the plasmid pDSK519 did not appear to contain an active *LacZ* as ß-galactosidase could not be detected when the plasmid was present in *E.coli* DH1. pDSK509 was used to produce a promoter-probe vector by the insertion of a promoterless *lacZ*.

The promoterless *lacZ* used was obtained from the plasmid pGS100, obtained from D.Cardy (University of Warwick) which contained the drug resistance markers from pSa. The procedure for the construction of the vector is outlined in figure 3.1.12. The promoterless *lacZ* was obtained on a 7.9 kb *BamH1* fragment of pGS100. The insertion of this fragment in the multiple cloning site of pDSK509 retained 3 unique restriction sites where DNA fragments could be inserted in front of the promoterless *LacZ*. The pGS100 *BamH1* fragment could insert either way into pDSK509.

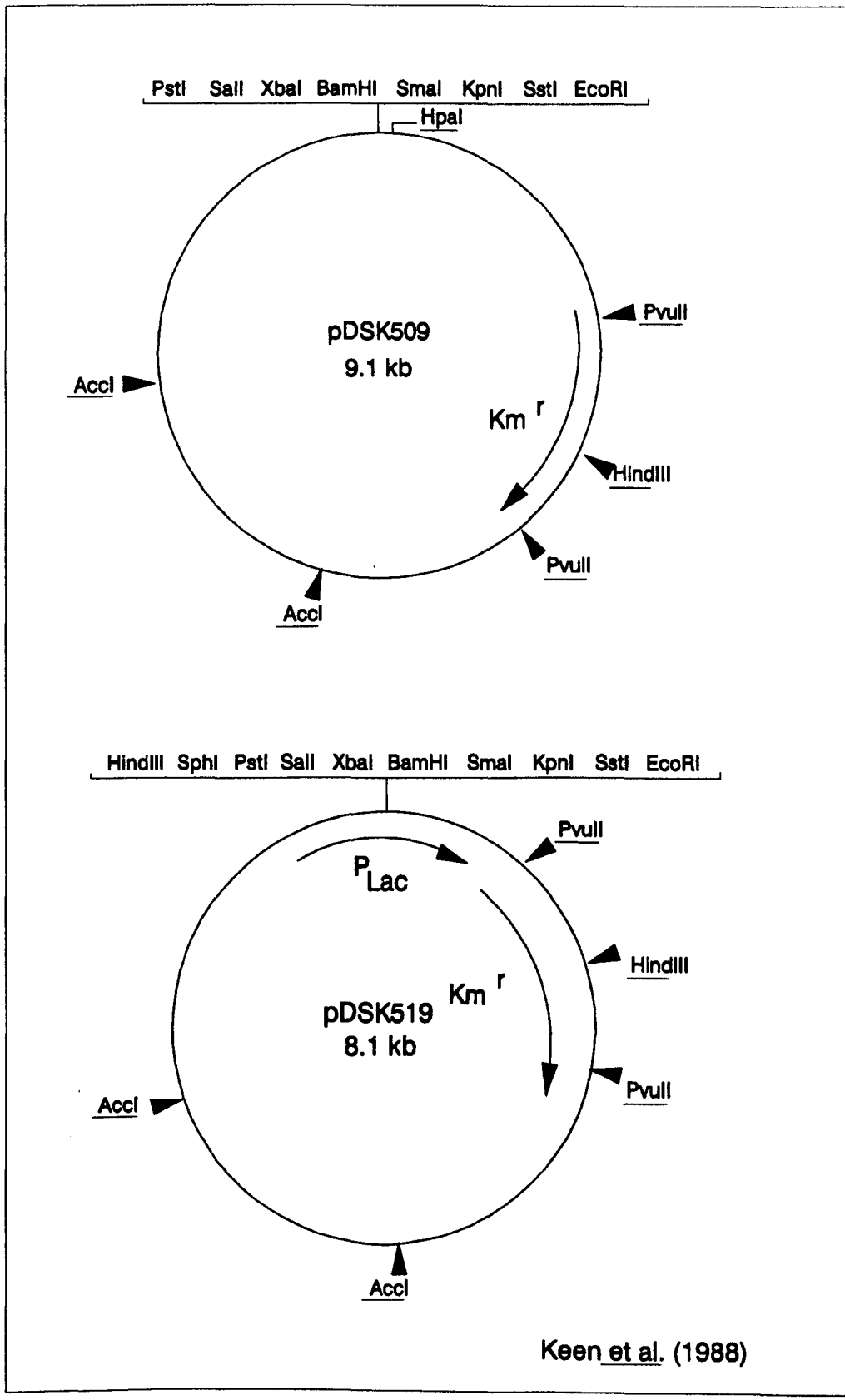


Figure 3.1.11 : Restriction maps of pDSK509 and pDSK519

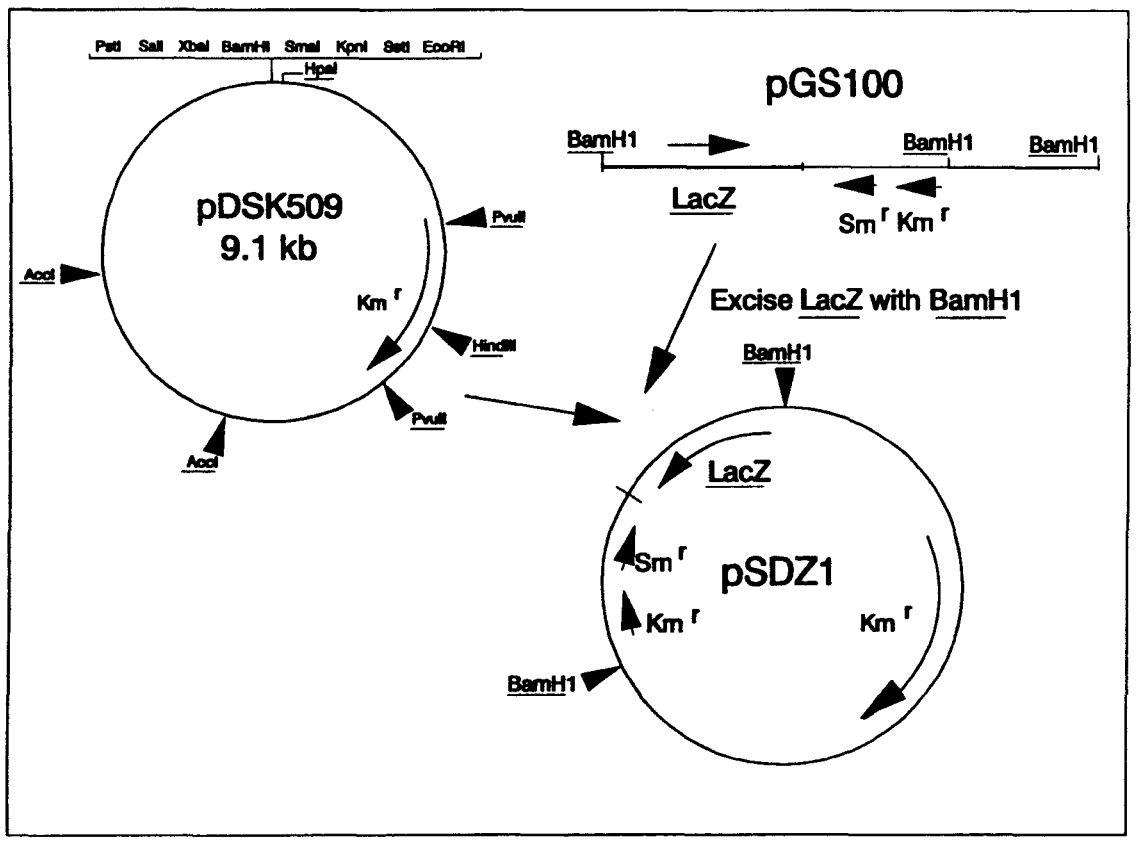


Figure 3.1.12: The production of pSDZ1

The best way was considered to be with the start of the *lacZ* gene next to the *Sma*1 site, and thus the gene would hopefully not be transcribed by the promoter from the antibiotic resistances which transcribe in the opposite direction. DNA fragments could be inserted into any of the three single sites, *Hpa*1, *Kpn*1 and *Sst*1. If a promoter was present on the DNA a transcriptional fusion would result in expression of ß-galactosidase. This vector would allow the study of promoter expression within *M.capsulatus* in the presence of all necessary regulatory factors.

This plasmid was transferred into *M.capsulatus*, at a frequency of 5.2×10^{-7} transconjugants per recipient and both streptomycin and kanamycin resistance were expressed. The background expression of ß-galactosidase by this plasmid in *E.coli* was compared with *E.coli* containing plasmid pDAH274, obtained from C.West (University of Warwick). pDAH274 is a promoterless *lacZ* vector for use in *Myxococcus*, but which has background expression in *E.coli*. Table 3.1.7 gives the figures for ß-galactosidase activity. The units of activity is the conversion of ONPG by ß-galactosidase per minute (arbitary units)

Plasmid	units of activity (average of 4 assays)
pDAH274	2324.76
pSDS1	479.9

Table 3.1.7

An equivalent number of cells were used for each assay. *M.capsulatus* containing pSDZ1 was also assayed for ß-galactosidase activity but none was found, without a positive control no conclusions about the expression of *lacZ* in *M.capsulatus* and the usefulness of this vector as a promoter probe vector could be drawn.

3.1.8 Discussion

3.1.8.1 Antibiotic resistance of *M.capsulatus*

The observed resistance of *M.capsulatus* to such a wide range of antibiotics was perhaps not due to any inherent resistance mechanisms, but in part possibly due to the breakdown of the antibiotics with prolonged exposure, e.g, 2 weeks, at 45^0C. This phenomenon of antibiotic instability at high temperatures, has been investigated by Peteranderl *et al.* (1990). They found that tetracycline was unstable at 50^0C but kanamycin showed no significant loss of activity at temperatures up to 72^0C. The length of time, up to two weeks, necessary for growth of some transconjugants would mean that an antibiotic such as tetracycline would be useless as a marker. This decrease of activity at 50^0C, assessed in a modified MIC (minimum inhibitory concentration) test using *E.coli*, *Staphylococcus aureus* and *Bacillus megaterium* as the test organisms, was observed also for erythromycin, streptomycin, ampicillin and penicillin G. Chloramphenicol was however, found to increase in activity at 50^0C when assessed in MIC test, and the resistance of

M.capsulatus to this antibiotic was therefore due to some inherent resistance mechanism. The tests carried out by Peteranderl and colleagues were carried out under anaerobic conditions and it is possible that the presence of oxygen may even decrease the time it takes for the antibiotic to breakdown. Since the optimum growth temperature for transconjugants of 45^0C conveniently prevented growth of *E.coli*, selection at 37^0C was not used, even though it may have enabled other markers to be used. Kanamycin resistance appeared to be an ideal marker for transfer due to the total lack of spontaneous mutants.

3.1.8.2 Development of a conjugation system

The growth stage of the *M.capsulatus* culture is of great importance in the process of conjugation. The process of conjugation requires energy for a variety of processes, such as DNA synthesis of the second strand of plasmid and protein production, although the exact energetic requirements of the conjugation process are unknown. Cells that have entered stationary phase due to starvation undergo certain metabolic changes and slowing down of oxidative phosphorylation. It has also been noted that organisms in stationary phase may possess slightly altered membrane properties (reviewed by Brown & Williams, 1985), this would affect a process such as conjugation, which involves the membrane in a large number of processes; starting with the formation of multicellular unions and finishing with the

passage of DNA through the membrane via some sort of pore. In a culture in stationary phase, the number of cells "competent" for conjugation are reduced even though the number of viable cells may be higher when the culture is plated onto non-selective medium. This reduction in "competence" for conjugation may be more pronounced for an organism such as *M.capsulatus* due to the potential production of a cyst, which must entail a great change in membrane metabolism, and of course the the production of a cyst would effectively prevent the entry of DNA. The decrease in conjugation frequency if cells in stationary phase cultures are used for conjugation has also been noted for *E.coli* (Curtiss *et al.*, 1969).

The length of time spent on the filter is important; the time must be long enough for transfer, replication of the plasmid, and expression of antibiotic resistances to occur. The presence of the plasmid in *M.capsulatus* may be a drain on cell resources, especially as this organism has no natural plasmids (Lidstrom & Wopat, 1984). If the organisms are left on the filter under no selective pressure there may be a tendency for loss of plasmid to occur from *M.capsulatus*. The other possibility, particularly as the transfer is taking place at the optimum growth temperature for *E.coli*, is that the rapid growth of *E.coli* over the slower growing *M.capsulatus* may adversely effect *M.capsulatus*. *M.capsulatus* is susceptible to a variety of complex carbon compounds, and it may well be the

case that the *E.coli* are producing compounds that are either inhibiting the *M.capsulatus* or are lethal to it. Whatever is the case, if the donor and recipient were left on the filter for longer than 24 hours, there was a substantial drop in the number of transconjugants produced, and after 48 hours on the filter there were few transconjugants produced.

Increasing the ratio up to 1 donor to 10 recipient did not significantly effect the frequency of transconjugant production, a plateau was reached. One particular experiment which was carried out, with just 5 ml of *M.capsulatus* as the recipient, produced no transconjugants (ratio of approximately 1 donor : 0.5 recipient).

The transfer of plasmids in liquid culture was unlikely to be successful. The broad-host range plasmids used in the conjugation experiment were all R-type plasmids and these are known to produce rigid pili. The rigid pili, unlike the flexible pili produced by the F plasmids, are sensitive to mechanical damage and multicellular unions are very easily disrupted if mating occurs in liquid (Viljanen & Boratynski, 1991). There is also evidence, in some organisms, that the properties of the cell membrane alter on solid media (Brown & Williams, 1985) which may influence the uptake of DNA via conjugation.

The plasmids that have been successfully transferred into *M.capsulatus* all possessed the RP4 based transfer system and relied on IncP or IncQ replication systems. Other plasmids which contained transfer systems

based on *Pseudomonas* plasmids were unsuccessful (see table 3.1.3). The RP4 system is well known to transfer to a wide number of Gram-negative organisms. It was noted however, that despite the identical transfer and replication systems of the plasmids, there were quite pronounced differences between the frequency of transconjugant production, i.e, frequency of transfer of pDSK509 was 2×10^{-3}, pDSK519, 1.2×10^{-6}, which were both based on RSF1010, (frequency = 5.2×10^{-7}). To understand the possible reasons for these differences the molecular events surrounding the process of conjugation must be considered. For example, how are these plasmids able to have such a broad host range, and what factors determine transfer and stability of the plasmid? These processes are reviewed by Thomas and Smith (1987), Kues and Stahl (1989), Willets and Wilkins (1984), Viljanen and Boratynski (1991), and Heinemann (1991). The majority of the work has been on the F plasmids of *E.coli*, and it is only recently that the transfer and replication functions of the broad host range plasmids have been analysed. The conclusion was, that there was a complex regulation network of genes organizing the adaptation of the RK2 (RP4) basic replicon to the prevailing host conditions and to autonomous maintenance in different Gram-negative bacteria.

Conjugative transfer requires both *a cis*-acting site, the origin of transfer (*oriT*), and a number of trans-acting functions which are necessary for mating-pair formation, initiation and continuation of DNA transfer, and

control of these processes. The molecular mechanisms for the initiation of transfer replication are poorly understood. The general model for conjugative DNA synthesis proposes that only one of the plasmids' strands is transferred to the recipient cell. This single strand is created by cleavage at the 'nick'(or nic) site within *oriT*, and subsequent strand displacement through rolling circle type replication. Relaxosomes are plasmid DNA-protein relaxation complexes, thought to function as intermediates in the initiation reaction. Once the single strand of plasmid DNA is transferred to the recipient, the complimentary strand must be synthesized and the plasmid establish replication etc.(reviewed by Willets & Wilkins, 1984 and Heinemann, 1991).

The copy numbers of RP4 and RSF1010 vary between bacterial species and the plasmid encoded replication functions are very complex. The stable maintenance of RK2 (identical to RP4) requires certain regulator genes. The replication of RK2 varies between species in the number and type of proteins that it requires and shows great versatility in replication (Thomas & Smith, 1987). There is a complex network of genes organizing the adaptation of the RK2 replicon to the host conditions and to autonomous replication in different bacteria. The IncQ plasmid RSF1010 relies on some host functions for replication, such as host DNA pol III and gyrase, but has gained a certain degree of independence (Kues & Stahl, 1989).

There are a number of points during conjugation and establishment of stable replication in the cell where processes can be unsuccessful, such as DNA replication and coordination of plasmid replication and partition at bacterial division. The lack of transfer or replication of certain plasmids may have been due to the lack of host factors compatible with that particular plasmids mode of replication ,e.g, DNA polymerases, DNA gyrases. Other systems that can affect the observed frequency of transfer are the hosts' restriction and modification systems.

The possibility of a restriction system in *M.capsulatus* affecting conjugal transfer was studied by observing the effect of a heat-shock on the efficiency of transfer. Heat-shock, and also exposure to ultra-violet light, temporarily reduces the activity of the restriction system (Uetake *et al.*, 1964), and thus transfer of plasmid would increase following a heat-shock if the restriction system played a significant role in preventing plasmid stabilization. One problem with attempting to subject *M.capsulatus* to a heat-shock is that it is very sensitive to temperatures above 48^0C (S. Stanley personal communication.) which is very close to its optimum growth temperature of 45^0C. This leaves a very small window of temperatures to work in. The exposure of the *M.capsulatus* culture to 50^0C for 3 minutes was assumed to have subjected *M.capsulatus* to a heat-shock. There was no rise in the number of transconjugants after any heat-shock regimes

tested, and so it appeared that any restriction system, if present, did not affect conjugal transfer. It would have perhaps been easier to use a variety of doses of uv light and then determine the effect on conjugation.

Similar experiments were carried out for the conjugal transfer of plasmids to *Methylomonas albus* (McPheat *et al.*, 1987) and to *Gluconobacter oxydans* (Condon *et al.*, 1991). Both sets of researchers came to the same conclusion, that restriction systems did not effect conjugal transfer of plasmids. Eden and Blakemore (1991) compared the transfer of plasmids RP4 and pSa151 by conjugation and electroporation into the genus *Aquaspirillum* and they concluded that, plasmids transferred by conjugation were somehow immune to the restriction system, whereas plasmids transferred by electroporation were not. The generally accepted method of plasmid transfer during conjugation (reviewed by Willets & Wilkins, 1984) is that of a single strand being transferred to the recipient, and it is thought that single strand DNA is not attacked by restriction enzymes thus making the process of conjugation immune to restriction barriers.

The possibility that pVK100 underwent structural alterations during transfer to and propagation in *M.capsulatus* and *E.coli* was studied. The reduction in transfer frequency, and the loss of tetracycline resistance from 75% of pVK100 plasmids isolated from *M.capsulatus*, would point towards some kind of modification and deletion of DNA occurring which perhaps affected the transfer

functions and tetracycline resistance. It was impossible to investigate loss of kanamycin resistance as this was the only selectable marker on pVK100 in *M.capsulatus*. PVK100 that had undergone replication in *M.capsulatus* was less stable upon conjugation back into *M.capsulatus* than pVK100 that had not, only 16.5% of transconjugants survived as opposed to 65% of transconjugants containing pVK100 that had only undergone replication in *E.coli*. These results would point towards structural modifications occurring during transfer and propagation, but the exact point could not be determined. When RP4 was transferred by conjugation into *Alcaligenes* H12 (Schwab *et al.*, 1983). It was found that following transfer of RP4 to *Alcaligenes* H12, a number of the transconjugants were only tetracycline resistant and no longer kanamycin resistant. Subsequent analysis showed that deletions had occurred around the kanamycin resistance gene, and also in the *tra 1* region, rendering the plasmids defective for transfer.

The plasmids had a variety of deletions. Of 15 analysed, only two contained identical deletions. This deletion of DNA occurred only when transfer was from *E.coli* to *Alcaligenes* and not *vice versa*. It also occurred when transfer was between different strains of *Alcaligenes*. It was concluded that the deletion process was random within a certain region of the plasmid and was associated with the conjugation process. The appearance of kanamycin sensitive deletion derivatives has also been reported for *Pseudomonas putida* (Chakrabarthy *et al.*, 1975) and *Agrobacterium tumefaciens* (Van Fliet *et al.*, 1978).

Schafer *et al.* (1990) found that subjecting Coryneform bacteria to a heat-shock markedly enhanced the conjugal transfer of plasmids from *E.coli* to these bacteria, indicating restriction barriers were present. They also found that mutation of the restriction system enhanced transfer.

These observations appear to contradict the accepted theory of single strand DNA immunity to restriction enzymes, but of course there is the possibility that conjugal plasmid transfer mechanisms vary between organisms. Most work has been carried out on the F plasmids of *E.coli* and perhaps the molecular mechanisms for this plasmid are not identical to the broad-host range plasmids considered here.

The broad-host range plasmids have very few restriction sites for enzymes, possibly due to the high %G+C

content of their DNA. This is thought to enable them to be stably maintained in a wide variety of hosts. The sections of DNA that have the most susceptible sites are the antibiotic resistance genes, presumed to have been acquired later in the plasmids evolution (Thomas & Smith, 1987).

The activity of a restriction system may explain differences in transfer frequencies between plasmids that are not very different in size and contain the same transfer system, i.e, pDSK509, pSDZ1 and pDSK519, also RP4 and pULB113.

The high rate of transfer of pDSK509, a derivative of RSF1010, is probably due to either an inherent stability or lack of susceptibility to the putative restriction system, or the very good expression of kanamycin resistance from Tn903. The kanamycin resistances of the other plasmids are derived from other sources, ,e.g, Tn5 in RP4. The lack of stability of pDSK519, which is identical to pDSK509 apart from the *lac*Z gene, must therefore be due to the presence of the LacZ gene. pDSK509 is also one of the smallest plasmids used, and will therefore have the lowest energy burden on the cell.

M.capsulatus possesses no natural plasmids and one could speculate that the appearance of extrachromosomal elements in the cell may cause a 'panic' and random restriction take place. The energy burden on the cell may force the organism to try to reduce the size or number of proteins that the plasmid is producing. This could account

for the loss of tetracycline resistance which is not being selected for, and the loss of mobilization functions which would also not be needed.

A better understanding of the molecular events occurring in *M.capsulatus* containing pVK100 would be obtained upon the isolation of pVK100 from a number of transconjugants and a thorough restriction analysis and determination of their transfer capacities.

The transfer of the non-stable plasmids, i.e, the transposon mutagenesis vectors, will be discussed in the next section. It is sufficient to say here, that the factors which determine the frequency of transconjugant production are many, including the ultimate insertion of the DNA into the chromosome.

3.1.8.3 The detection of plasmids in *M.capsulatus*

The detection of plasmids in *M.capsulatus* to verify that plasmids had indeed been transferred, was carried out in a number of ways. Isolation of plasmid using the standard *E.coli* mini-preps was carried out, the most successful of which was the alkaline-lysis procedure. This contained slight alterations from the method outlined in Maniatis *et al.* (1984) which improved the cell-lysis and plasmid preparation. The plasmids isolated by any of the methods were very difficult to visualize on gels, even with the alkaline-lysis method , due to contaminating chromosome. The most sensitive way of detecting the plasmid rapidly was

to electroporate the plasmid preparation into *E.coli*. This proved to be a reliable method for the detection of plasmids but obviously not as rapid as the use of a gel. However, the alkaline-lysis method worked well on a large scale and clean plasmid was produced after centrifugation through caesium-chloride. This plasmid restricted well, was produced in large amounts, and could subsequently be restriction mapped. It was interesting to note that the RSF1010 derivative pDSK509 was extremely difficult to isolate from *E.coli* using the alkaline-lysis technique, the boiling lysis technique had to be used, which produced a much dirtier plasmid preparation, the plasmid however, could be isolated from *M.capsulatus* using the alkaline-lysis technique with no problem at all. This could indicate differences in the state of pDSK509 within the cell and its association with membranes or chromosome. A similar, but not so dramatic a difference, was seen for pVK100. This cosmid, when isolated on a large scale from *E.coli*, often contained contaminating chromosomal DNA but this was never the case with pVK100 isolated on a large scale from *M.capsulatus*.

3.1.8.4 The expression of foreign DNA in *M.capsulatus*

The presence of plasmids within a cell represent a drain on the cells resources and it is not surprising that there is a reduction in growth rate. What is interesting is that the growth rate is greater for the plasmid containing cell at 37^0C than at 45^0C. This could be due to the

temperature adversely affecting the proteins produced by the plasmid, such as the kanamycin resistance determinant. This may mean that more of this protein may have to be produced to cope with the antibiotic at 45^0C than at 37^0C. The protein may be denatured at a greater rate at 45^0C.

Several genes have been cloned from *M.capsulatus* (see section 1.5.3), but these genes have so far only been studied in heterologous hosts, ie, *E.coli*. With the finding that several broad host plasmids have been transferred to and stably maintained in *M.capsulatus*, there is a possibility for the production of vectors allowing a study of the expression of genes within *M.capsulatus*. In order to study such gene expression, a suitable marker is needed. The plasmids that have been transferred to *M.capsulatus* contain only kanamycin resistance as a marker. Although a promoterless kanamycin gene has been used to produce a promoter-probe vector for Corynebacteria (Cadenas *et al.*, 1991) this type of system would be of no use if the promoter was weak, or under the control of regulatory factors, and selection was not made under conditions that resulted in expression of the promoter. There is the need for an additional gene to act as a reporter gene, along with the kanamycin resistance determinant to select for the presence of the plasmid. It was decided to use *lacZ* as a reporter gene, since ß-galactosidase is easily assayed and its expression is not a pre-requisite for cell survival. *M.capsulatus*, due to its limited carbon metabolism, does not

contain ß-galactosidase. The only obvious problem is that of import of the substrate for the colorimetric assay of colonies. *M.capsulatus* would not contain the necessary permease (*LacY*). ß-galactosidase can however be assayed after breakage of the cells. This requires growth of *M.capsulatus* in liquid, but the promoter activity can be quantified. One drawback to many such expression vectors is the amount of read-through that occurs, i.e, ß-galactosidase production from other powerful promoters present on the plasmid, (often the antibiotic resistance promoters). This problem tends to be more pronounced in *E.coli* and may not occur in *M.capsulatus*.

The promoter-probe vector pSDZ1 contained a promoterless *LacZ*, and if a section of DNA containing a promoter was inserted in front of the gene a transcriptional fusion would be produced. This vector, when tested in *E.coli*, did produce ß-galactosidase but at a quarter of the level seen for a similarly designed vector for *Myxococcus*. This vector is now ready for use as a promoter-probe vector for *M.capsulatus*. Unfortunately no positive control was available as pDSK519 does not produce LacZ, except in certain backgrounds and there is therefore no way as yet to determine whether *LacZ* will be functionally expressed in *M.capsulatus* and whether it is possible to assay the enzyme produced.

RESULTS

MUTAGENESIS TECHNIQUES FOR *M.CAPSULATUS* AND THE PRODUCTION OF A PUTATIVE *GLNA* MUTANT

3.2 MUTAGENESIS TECHNIQUES FOR M.CAPSULATUS AND PRODUCTION OF A PUTATIVE GLNA MUTANT.

An important aim of the work was to devise a system for the production of mutants of *M.capsulatus* (Bath). Previous attempts at mutagenesis of *M.capsulatus* by chemical treatment have been unsuccessful (section 1.5.1). Therefore, I concentrated on the use of transposons for random mutagenesis and vectors for marker-exchange mutagenesis. Both these methods rely on a gene transfer system and the more efficient the system the fewer the number of colonies that have to be screened, as the insertion of such extrachromosomal elements tends to be a low frequency event. The vectors that were used are listed in table 3.2.1.

Plasmid	Transposable Element	Resistance Marker	Transposition Frequency*
pSUP2021	Tn5	Km	1×10^{-9}
pLG221	Tn5	Km	0
pJFF350	ΩKm	Km	5.2×10^{-8}
pMD100	Tn501	Hg	0
pDC2Ω	ΩSm	Sm	0
pSD10	ΩSm	Sm	0
pSD100	ΩKm	Km	ND

ND = Not determined * = per recipient *M.capsulatus*

Table 3.2.1

3.2.1 pSUP2021; A Generalized Transposon Mutagenesis Vector

The vector pSUP2021 (Simon *et al.*, 1984) (Figure 3.2.1), is based on pBR325 containing the broad host range transfer system of RP4, the *mob* site. The vector has a narrow host-range replication system, and once inside a cell where it can not replicate the plasmid is lost. The Tn5, however, can transpose into the chromosome and this event can be detected by the acquisition by the cell of kanamycin resistance.

The vector, in *E.coli* S17-1 (Simon *et al.*, 1983), was transferred by conjugation to *M.capsulatus*. The rate of kanamycin resistant *M.capsulatus* was 1 X 10^{-9} per recipient cell. This is the rate of Tn5 insertion into the chromosome at a point where disruption of the chromosome did not result in cell death. The initial rate of plasmid transfer and the absolute rate of Tn5 insertion could not be determined.

The kanamycin resistant *M.capsulatus*, produced from such conjugations, grew in liquid media with kanamycin (50 μg/ml) present. Plasmid could not be detected in the cells by the alkaline-lysis method which had been used successfully for the isolation of other plasmids (section 3.1.4.2).

Colonies were transferred onto a nitrocellulose filter and colony blots carried out. Wild type *M.capsulatus* and *E.coli* containing pSUP2021 were present as controls. The filter was probed with the entire pSUP2021 plasmid (radiolabelled) and then the filter was washed with 1 X SSC

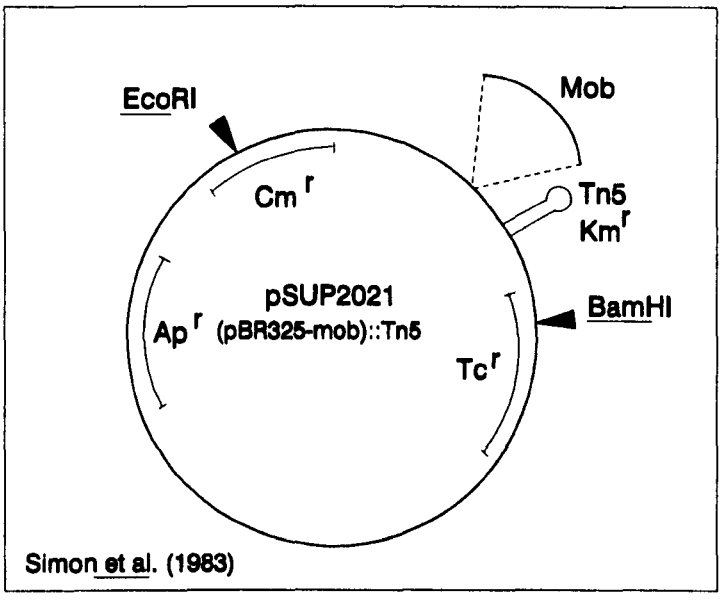


Figure 3.2.1

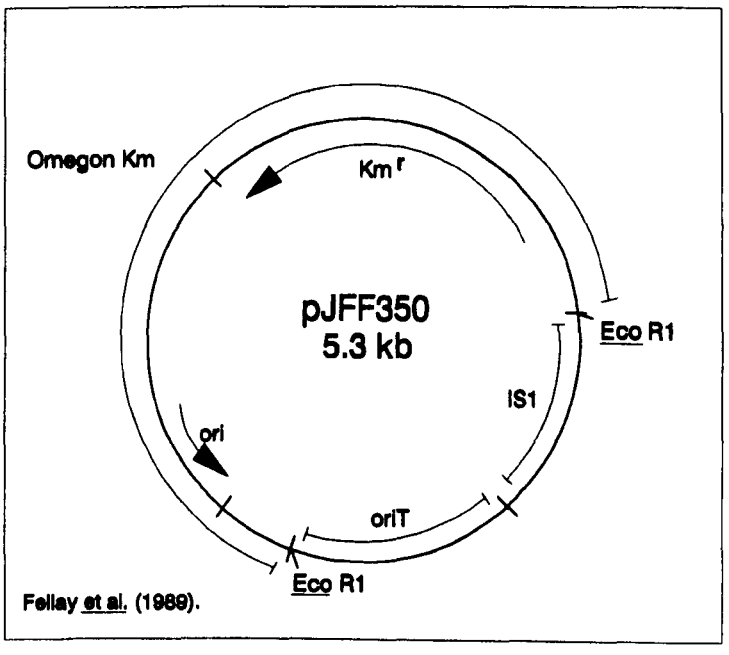


Figure 3.2.5

at 75^{0}C (2 X 20 min washes). These washing conditions allowed for 85% homology and above. The resulting autoradiograph (Figure 3.2.2), after overnight exposure, showed that DNA from the wild-type *M.capsulatus* colony had no homology with pSUP2021.

Chromosomal DNA was isolated from a 100ml culture of kanamycin resistant *M.capsulatus*. The chromosome was cut with *Eco*R1, which does not cut within Tn5, and two identical gels were run (Figure 3.2.3). Wild-type chromosome cut with *Eco*R1 and pSUP2021 cut with *Eco*R1 were run alongside as controls. After Southern blotting, one gel was probed with pBR325 and the other gel with pSUP2021. The gel probed with pSUP2021 showed a homologous band in the kanamycin resistant *M.capsulatus* DNA at approximately 13 kb, a large enough fragment to contain the 5.7 kb of Tn5, but no homology was found to the wild-type DNA (Figure 3.2.4). The pBR325 probe showed no homology with *M.capsulatus* DNA. It was concluded therefore, that Tn5 had indeed inserted into the *M.capsulatus* chromosome and that kanamycin resistance of Tn5 was being expressed.

Tn5 can be used as a generalized transposon mutagenesis vector as with many organisms it has been found to insert into the chromosome randomly and not have any "hot-spots" for insertion (Berg & Berg, 1983). Over 800 colonies of kanamycin resistant *M.capsulatus* resulting from several conjugations were picked onto NMS containing 0.02% (w/v) Proteose-Peptone. Individual colonies were then

Figure 3.2.2: Hybridisation of *M.capsulatus* colonies containing pSUP2021 with radiolabelled pSUP2021. Washing conditions allowed for 85% homology and above.

A = *E.coli* positive control.
B = negative control


B
A

Figure 3.2.3:

Gel I and Gel II

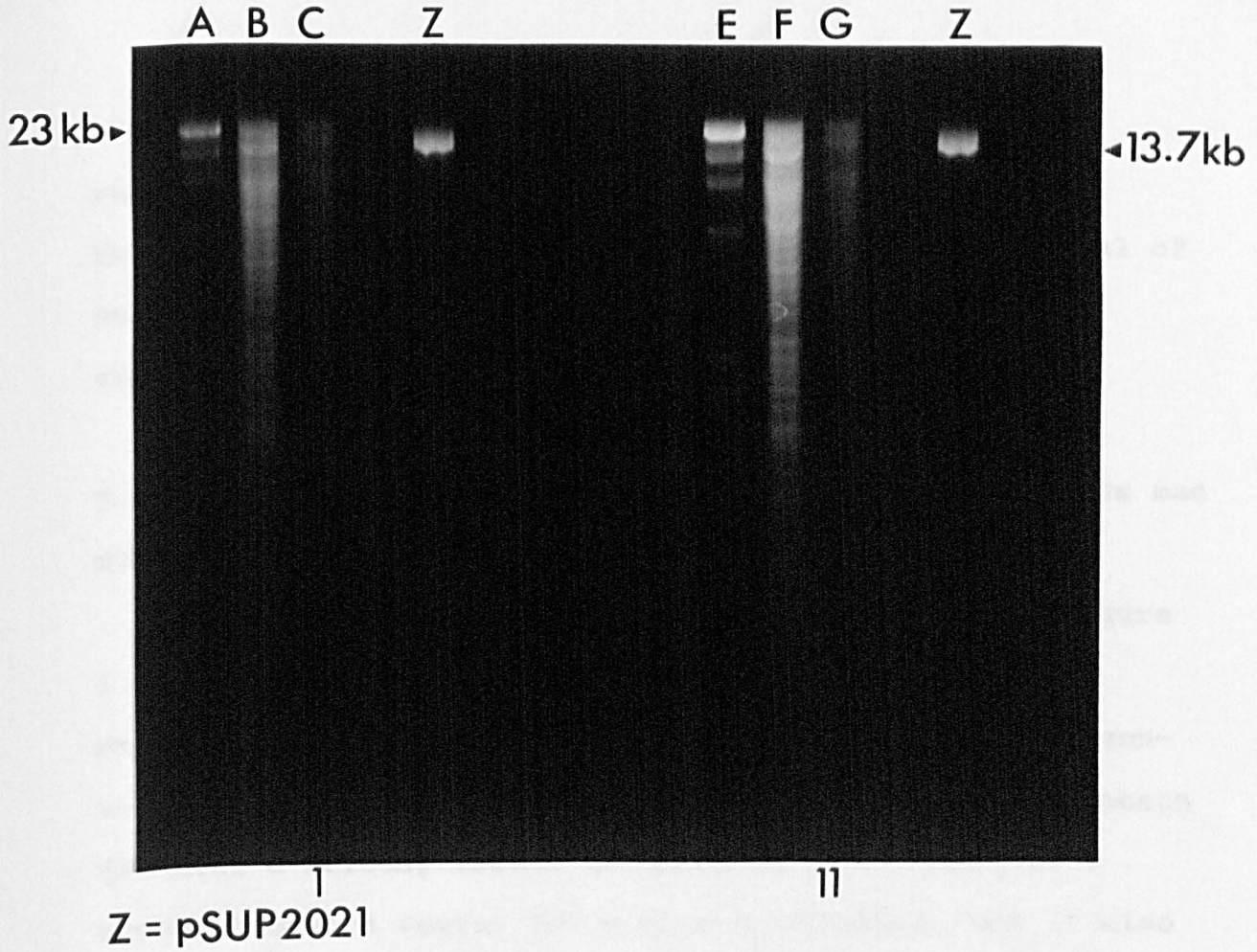
A & E = *Hin*dIII cut λ
B & F = Wild-type *M.capsulatus* **chromosome cut with** *Eco*R1
C & G = Kanamycin-resistant *M.capsulatus* **chromosome cut with** *Eco*R1.

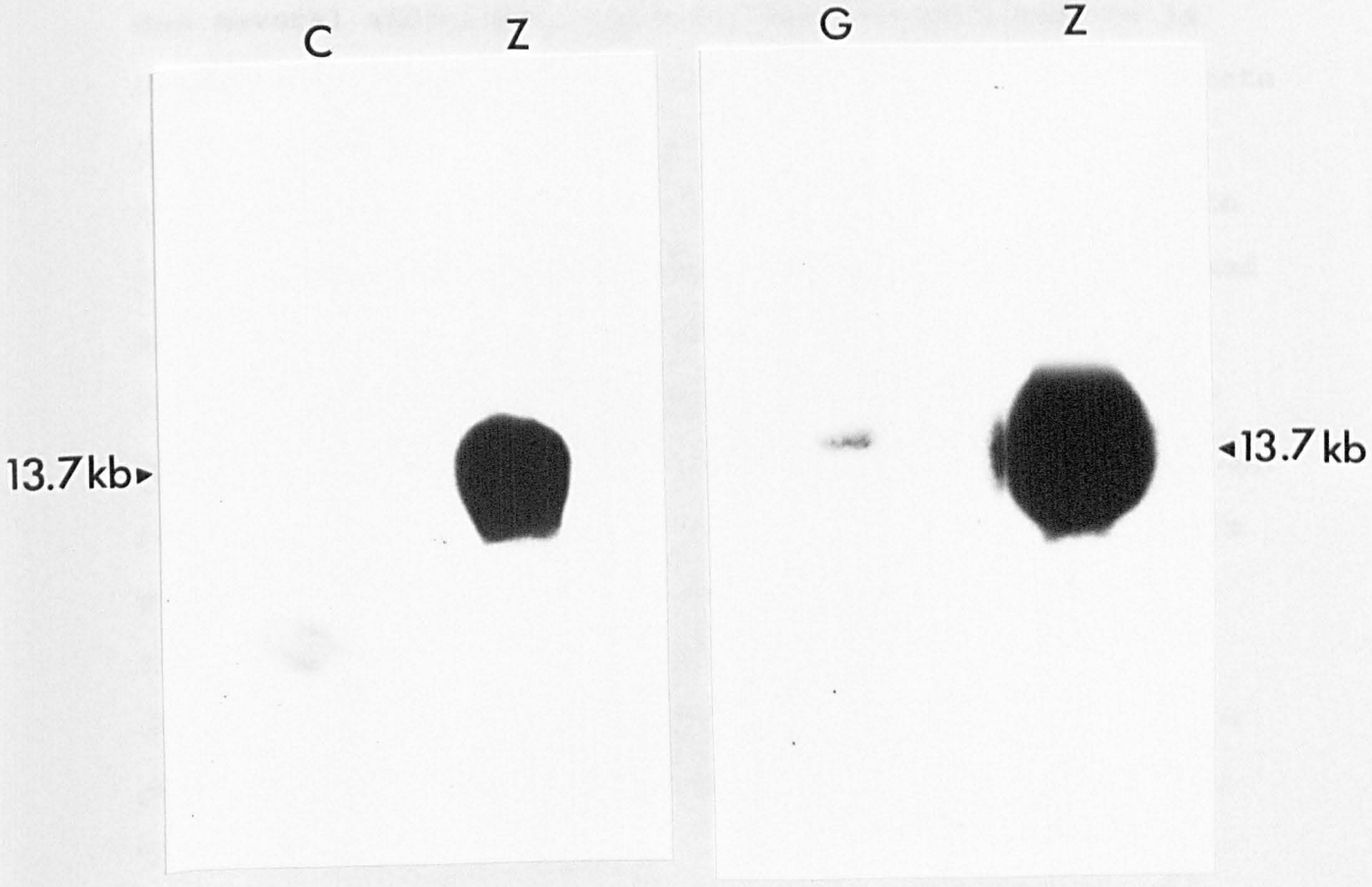
Figure 3.2.4. Screening of kanamycin-resistant *M.capsulatus* **chromosome and wild-type** *M.capsulatus* **chromosome for the presence of Tn5. Washing conditions allowed for 85% homology and above.**

Gel I probed with radiolabelled pBR325.

Gel II probed with radiolabelled pSUP2021 (pBR325::Tn5).

Ladels A to G as for figure 3.2.3.


A B C Z
E F G Z
23 kb
13.7kb
1
11
Z = pSUP2021


C Z
G Z
13.7kb
13.7 kb

replica-plated onto NMS alone and NMS containing 0.02% Proteose-Peptone. No colonies were found, out of a total of 800, to have any requirement for Proteose-Peptone, suggesting that no auxotrophs were present.

3.2.2 pJFF350; Transfer of the plasmid into *M.capsulatus* and subsequent insertion into the chromosome.

The plasmid pJFF350 (Fellay *et al.*, 1989)(Figure 3.2.5) contains the Omegon-kanamycin fragment and was designed for *in vitro* insertional mutagenesis. The Omegon-kanamycin fragment is a derivative of the Omegon interposon (Prentki & Krisch, 1984). The plasmid can be used as a straightforward vector for random mutagenesis, but it also has several additional features. The Omegon-kanamycin is flanked, in inverted orientation, by synthetic 28 bp repeats derived from the ends of IS1. The IS1 transposition functions are located on the donor plasmid but external to the Omegon-Kanamycin. The insertions are therefore presumed to be extremely stable because they lack the ability for further transposition. Also present alongside the Omegon-Kanamycin and concomitantly transposed, is a *col*E1 replicon. Once insertion of the Omegon-Kanamycin has occurred, it is possible to isolate the DNA surrounding the point of insertion from the organism by production of a plasmid (Figure 3.2.6). Marker-exchange mutagenesis vectors can be produced by the insertion of specific DNA into pJFF350 to target insertion.

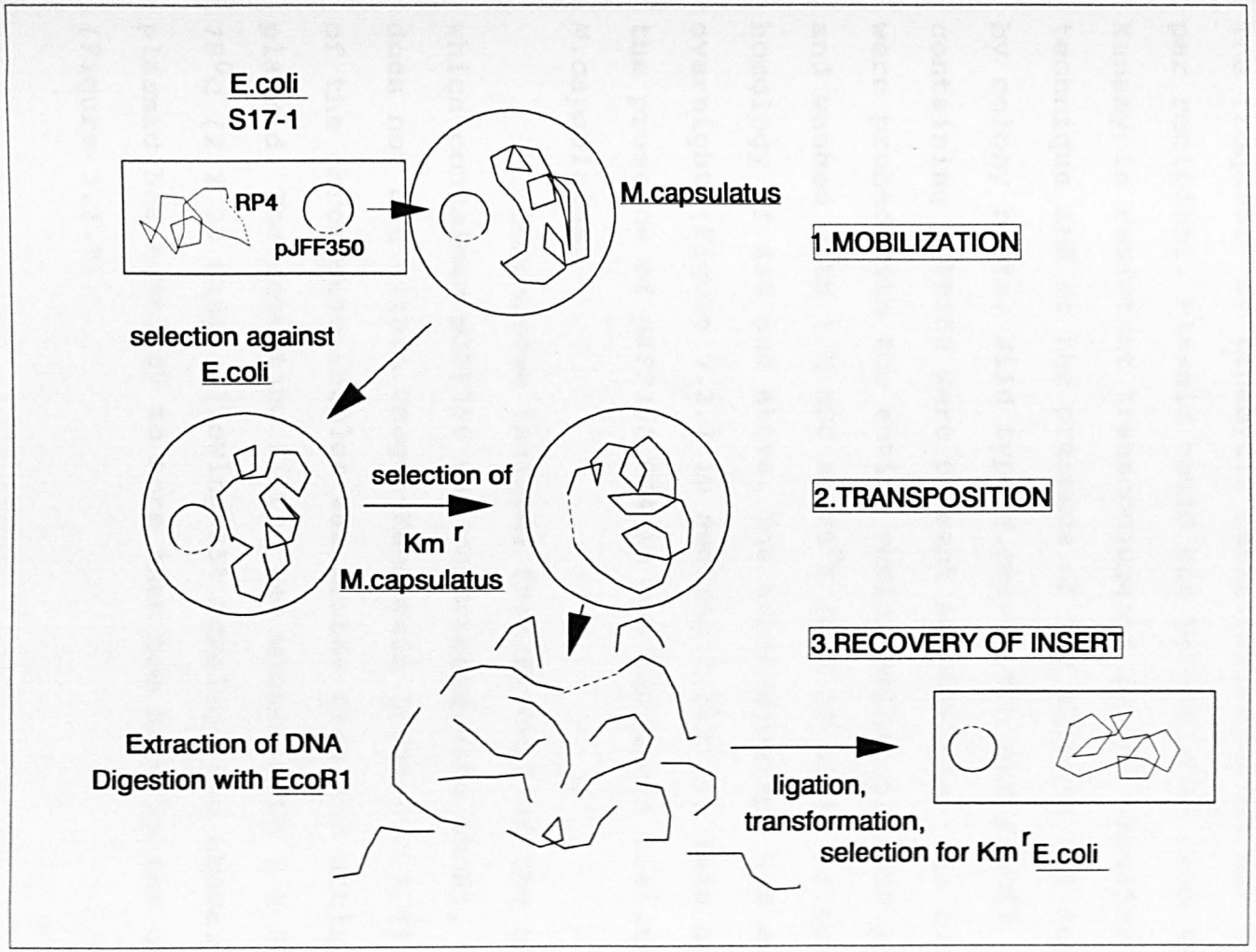


Figure 3.2.6: General strategy for in vivo mutagenesis using pJFF350

The plasmid pJFF350, in *E.coli* strain S17-1, was transferred to *M.capsulatus* by the standard conjugation technique and the transconjugants were selected for on NMS plus 50 μg/ml kanamycin and 0.02% (w/v) Proteose-Peptone. The frequency of kanamycin resistant colonies was 5.2×10^{-8} per recipient. Plasmid could not be isolated from the kanamycin resistant transconjugants by the alkaline-lysis technique and so the presence of the plasmid was determined by colony blots. Wild type *M.capsulatus* and *E.coli* containing pJFF350 were present as controls. The filters were probed with the entire radiolabelled pJFF350 plasmid and washed with 1 X SSC at 75^0C (2 X 20 min), allowing homology of 85% and above. The autoradiograph was exposed overnight (Figure 3.2.7 in section 3.1.4.3). This confirmed the presence of pJFF350 DNA in the kanamycin resistant *M.capsulatus*.

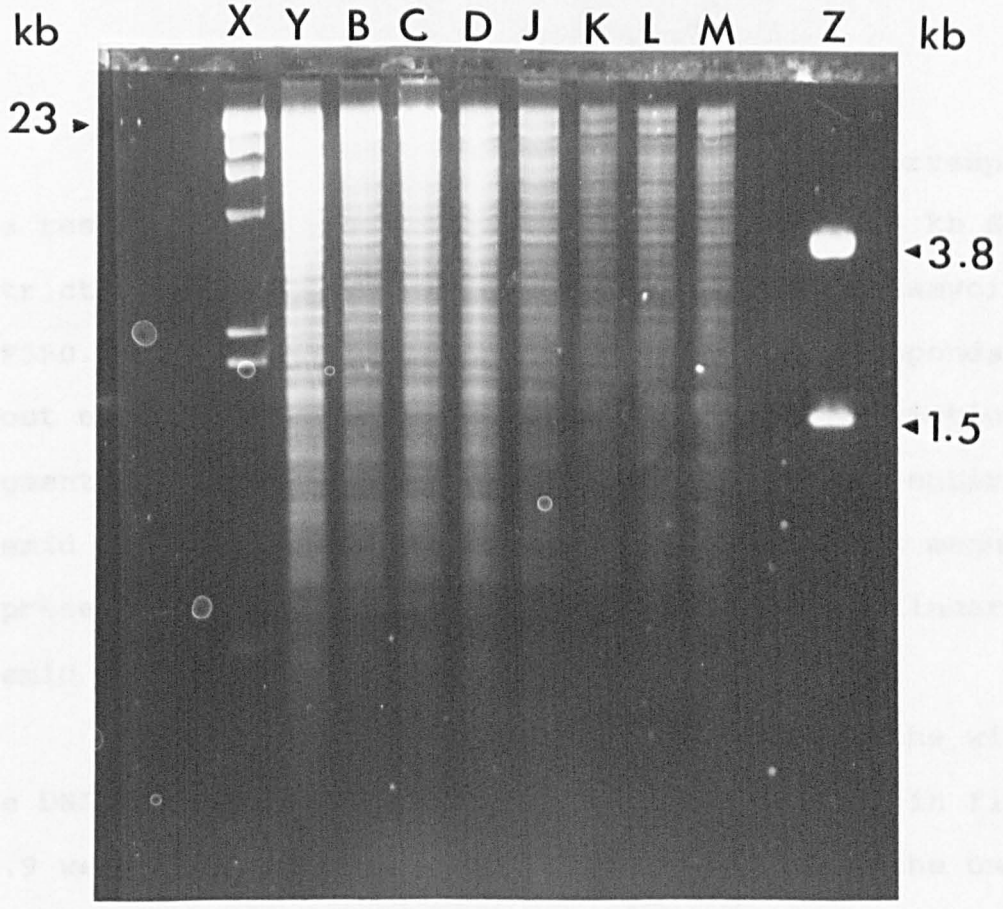
Chromosome isolated from several of the colonies which contained pJFF350 was restricted with *Eco*R1, which does not cut within Omegon-Kanamycin (Figure 3.2.8). A gel of the chromosome samples was probed with the entire pJFF350 plasmid. The resulting filter was washed with 1 X SSC at 75^0C (2 X 20 min), allowing 85% homology and above. The plasmid had homology to more than one band in the chromosome (Figure 3.2.9).

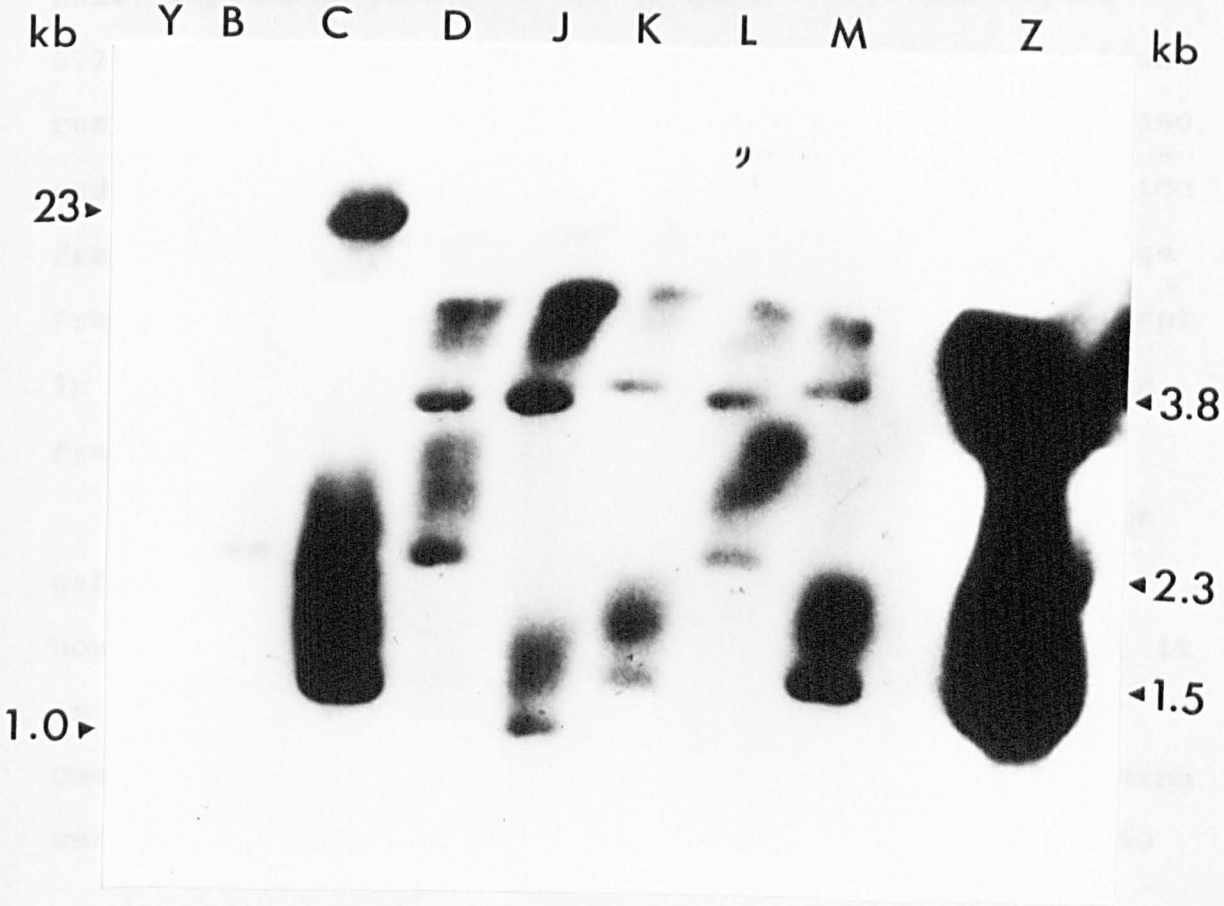
Figure 3.2.8: Chromosome isolated from kanamycin-resistant *M.capsulatus* cut with *Eco*R1.

X = *Hin*dIII cut λ
Y = Wild-Type *M.capsulatus* chromosome cut with *Eco*R1
B to M = Kanamycin resistant *M.capsulatus* chromosme cut with *Eco*R1.
Z = pJFF350 cut with *Eco*R1.

Figure 3.2.9: Screening of chromosome of kanamycin-resistant *M.capsulatus* for the presence of pJFF350 DNA. Washing conditions allowed 85% homology and above.

X = *Hin*dIII cut λ
Y = Wild-Type *M.capsulatus* chromosome cut with *Eco*R1
B to M = Kanamycin resistant *M.capsulatus* chromosme cut with *Eco*R1.
Z = pJFF350 cut with *Eco*R1.


kb
X Y B C D J K L M Z
kb
23
3.8
1.5


kb
Y B C D J K L M Z
kb
23
3.8
2.3
1.5
1.0

The larger of the bands (except in C) corresponds to a restriction fragment the same size as the 3.8 kb *Eco*R1 restriction fragment which contains the Omegon-Kanamycin of pJFF350. In M and K the second smaller band corresponds almost exactly with the smaller 1.5 kb *Eco*RI restriction fragment of pJFF350. This could indicate that the entire plasmid has inserted into the chromosome in such a manner as to preserve the *Eco*R1 sites at either end of the linearized plasmid with no deletion.

As no homology to pJFF350 was found in the wild-type DNA, it was assumed that all the bands shown in figure 3.2.9 were parts of pJFF350, ie, DNA external to the Omegon-Kanamycin had transposed into the chromosome. To verify this, duplicate gels were run (Figure 3.2.10 and Figure 3.2.11). One gel was probed with the Omegon-Km, on an *Eco*R1 restriction fragment of 3.8 kb (Figure 3.2.12) from pJFF350, and the other was probed with the smaller *Eco*R1 restriction fragment of 1.5 kb (Figure 3.2.13) from pJFF350. The large fragment showed homology to DNA fragments of 3.8 kb (except in C). The 1.5 kb fragment showed homology to the smaller fragments of DNA (Figure 3.2.13).

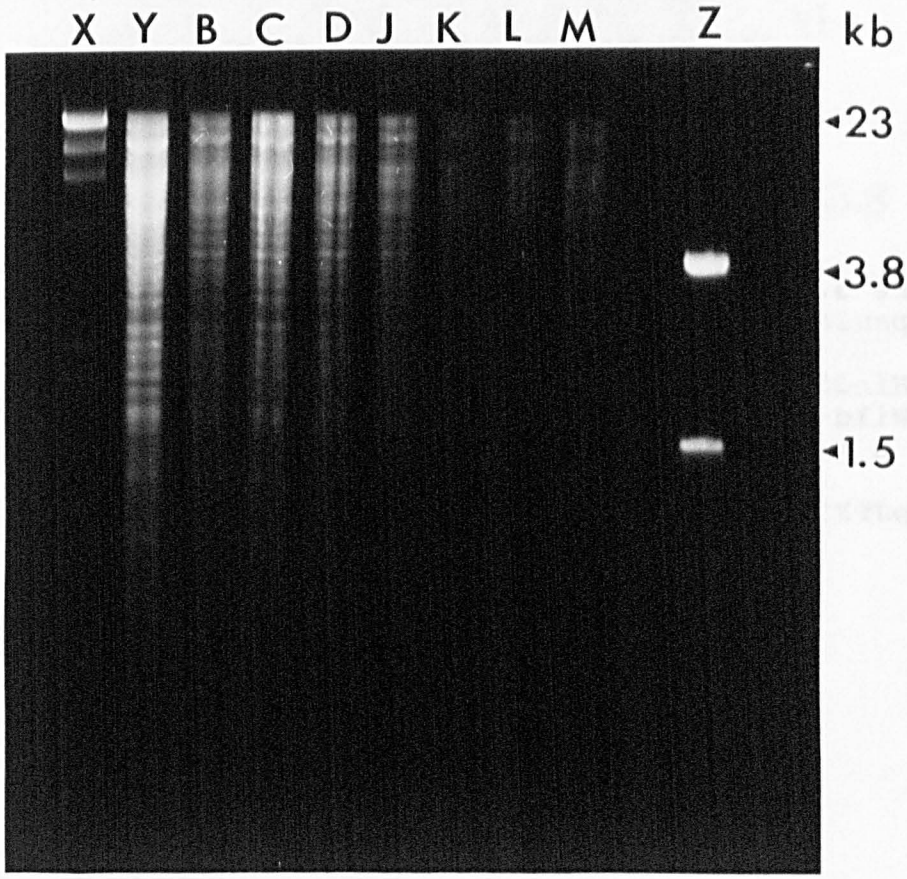
The insertion of the entire plasmid (or a large part of the plasmid) has probably occurred. It cannot however be assumed that the entire plasmid has inserted, it is possible that only some of the plasmid outside the Omegon-Km has inserted. Four different restriction patterns were produced when the chromosome was probed with pJFF350

Figure 3.2.10: Chromosome isolated from kanamycin-resistant *M.capsulatus* cut with *Eco*R1.

X = *Hin*dIII cut λ
Y = Wild-type *M.capsulatus* chromosome cut with *Eco*R1
B to M = Chromosome isolated from kanamycin-resistant *M.capsulatus* cut with *Eco*R1.
Z = pJFF350 cut with *Eco*R1.

Figure 3.2.12: Screening of chromosome isolated from kanamycin-resistant *M.capsulatus* with the 3.8 kb fragment of pJFF350 containing the Omegon-kanamycin fragment. Washing conditions allowed 85% homology and above.

X = *Hin*dIII cut λ
Y = Wild-type *M.capsulatus* chromosome cut with *Eco*R1
B to M = Chromosome isolated from kanamycin-resistant *M.capsulatus* cut with *Eco*R1.
Z = pJFF350 cut with *Eco*R1.


X Y B C D J K L M Z
kb
23
3.8
1.5

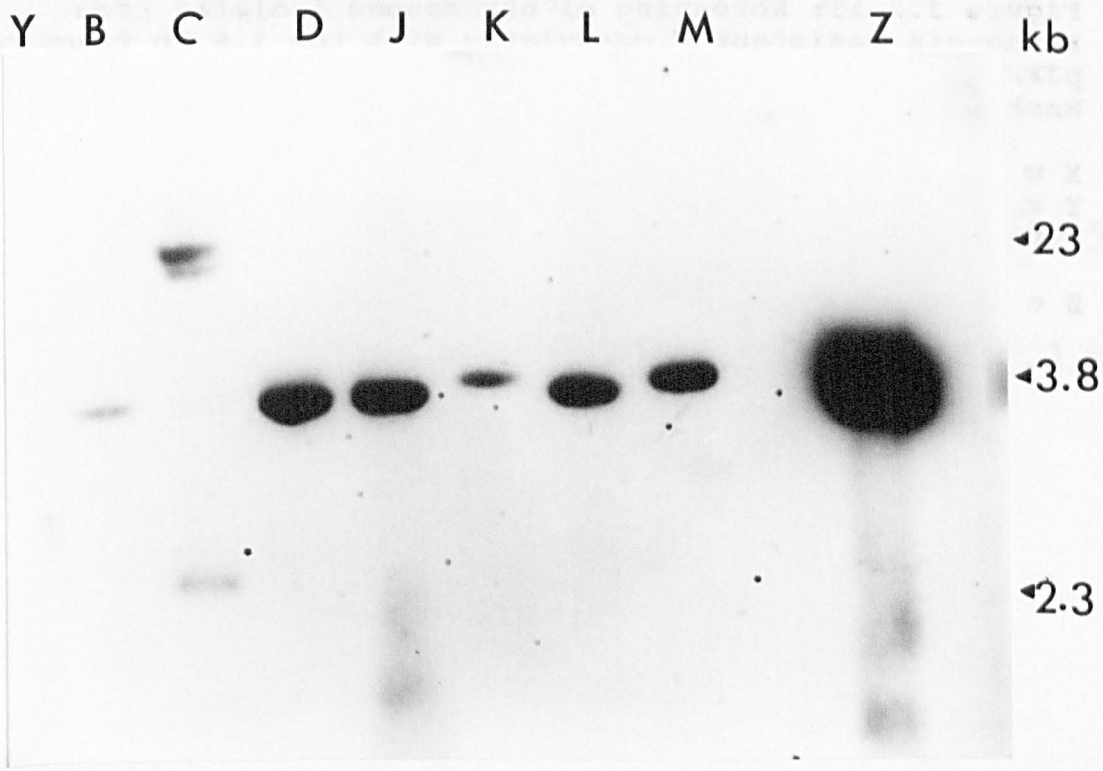
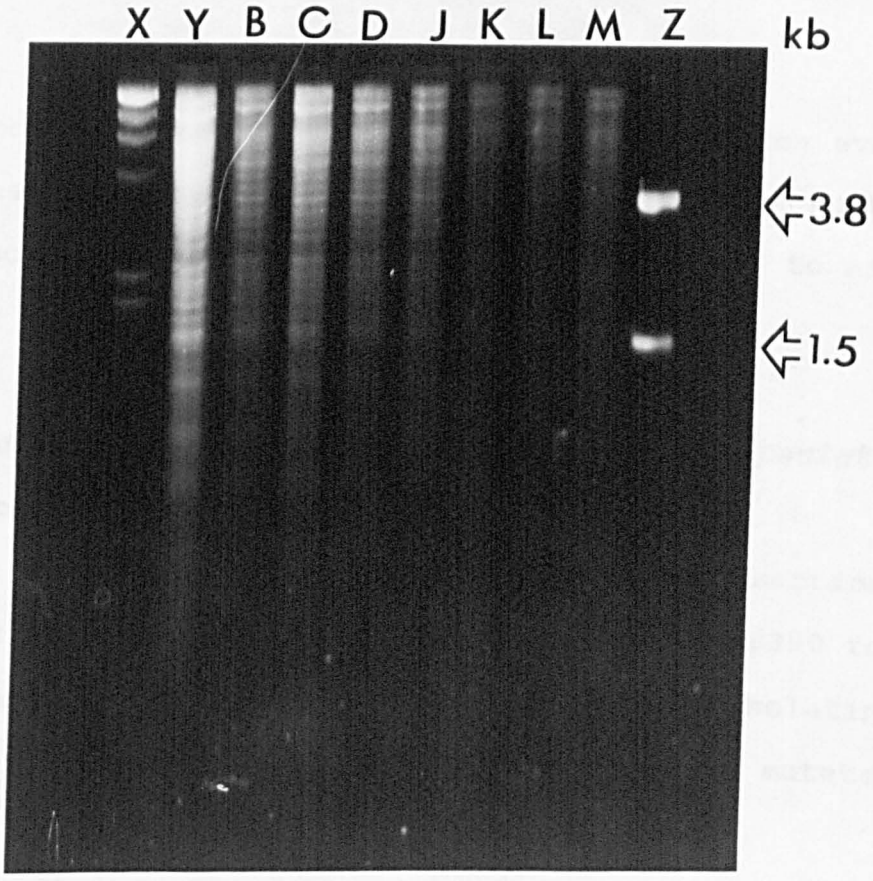

Y B C D J K L M Z
kb
23
3.8
2.3

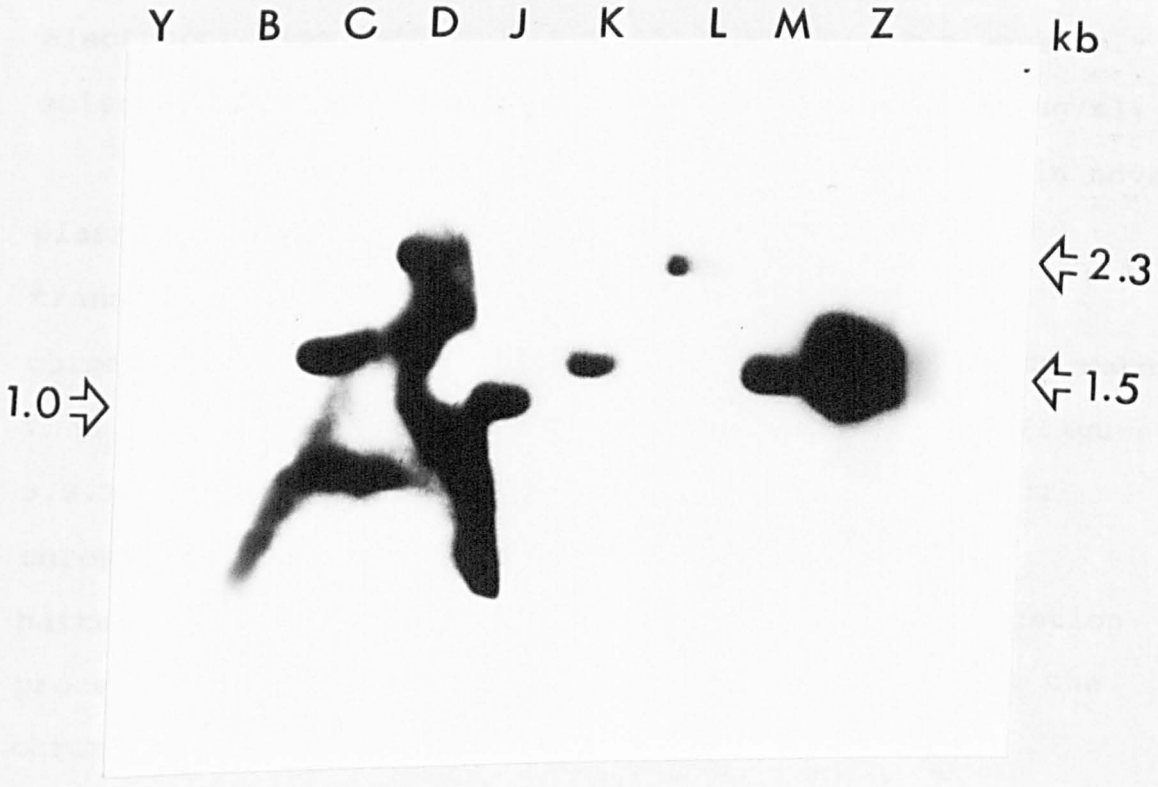
Figure 3.2.11: Chromosome isolated from kanamycin-resistant *M.capsulatus* cut with *Eco*R1.

X = *Hin*dIII cut λ
Y = Wild-type *M.capsulatus* chromosome cut with *Eco*R1
B to M = Chromosome isolated from kanamycin-resistant *M.capsulatus* cut with *Eco*R1.
Z = pJFF350 cut with *Eco*R1.

Figure 3.2.13: Screening of chromosome isolated from kanamycin-resistant *M.capsulatus* with the 1.5 kb fragment of pJFF350 containing the IS1 ends and RP4-*mob* fragment. Washing conditions allowed 85% homology and above.

X = *Hin*dIII cut λ
Y = Wild-type *M.capsulatus* chromosome cut with *Eco*R1
B to M = Chromosome isolated from kanamycin-resistant *M.capsulatus* cut with *Eco*R1.
Z = pJFF350 cut with *Eco*R1.


X Y B C D J K L M Z
kb
3.8
1.5


Y B C D J K L M Z
kb
2.3
1.5
1.0

which could correspond to four types of insertion event. More restriction digests and probing of a larger number of chromosome from transconjugants would be needed to ascertain exactly what has occurred.

3.2.3 pJFF350; production of plasmids from *M.capsulatus* chromosome.

As already outlined in the preceding section (and figure 3.2.6) one of the main advantages of pJFF350 for insertional mutagenesis is the possibility of isolating DNA surrounding the insertion and hence isolating a mutated gene.

Chromosome samples obtained from *M.capsulatus*, containing pJFF350, were restricted with *Eco*R1, the fragments ligated and the entire mixture transformed by electroporation into *E.coli* (DH1). Transformants were selected for on Luria Broth agar plus kanamycin (50 μg/ml).

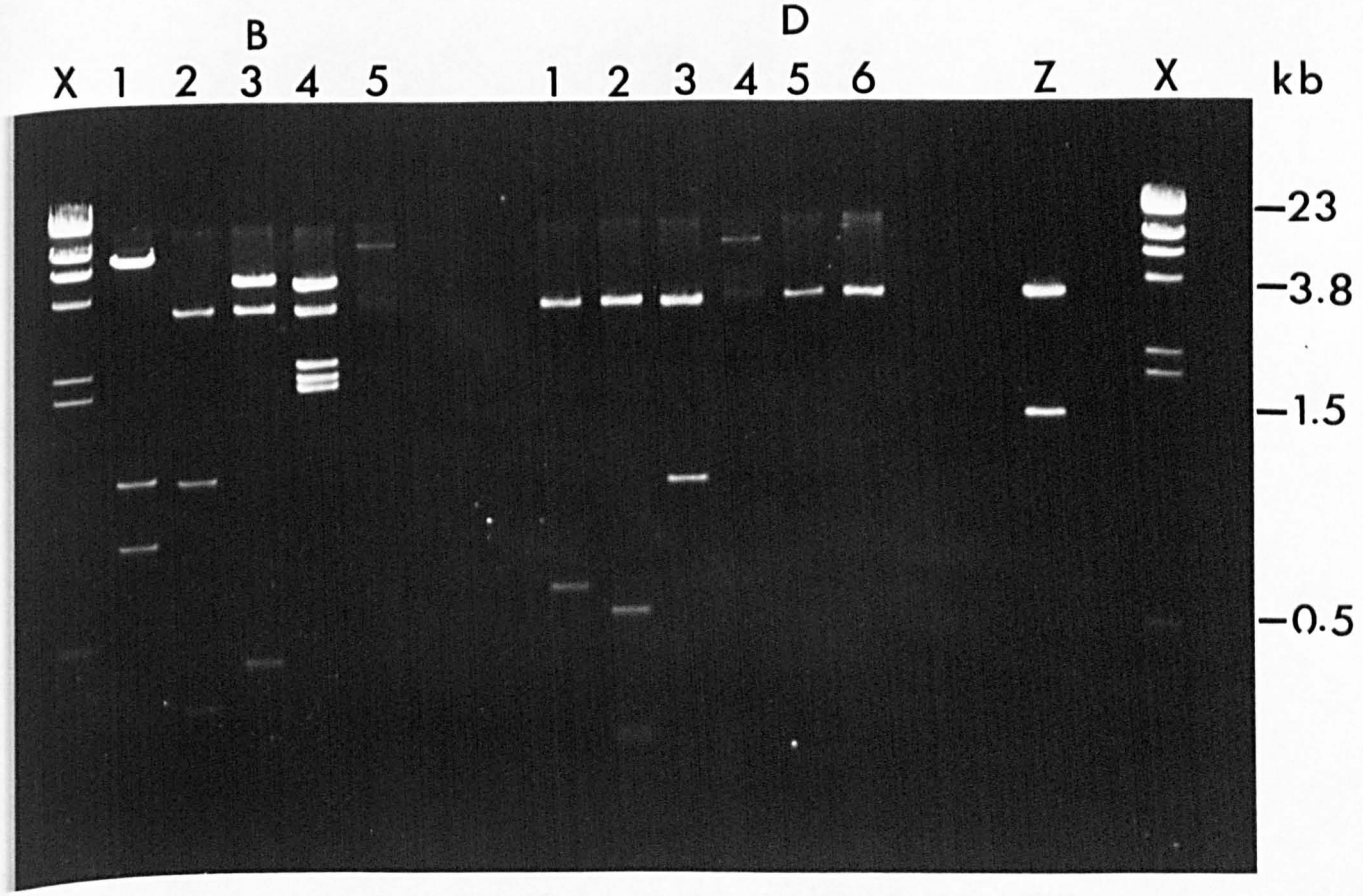
Transformants obtained were found to contain novel plasmids. These plasmids were isolated from several transformants, five of which had been produced from chromosome B and six from chromosome D. These plasmids were then restricted with *Eco*R1 (Figure 3.2.14) and *Pst*1 (Figure 3.2.15). The plasmids, although obtained from identical chromosome, gave a variety of different restriction patterns. This indicates the random nature of the ligation process and the differing amounts of restriction that the chromosome had undergone.

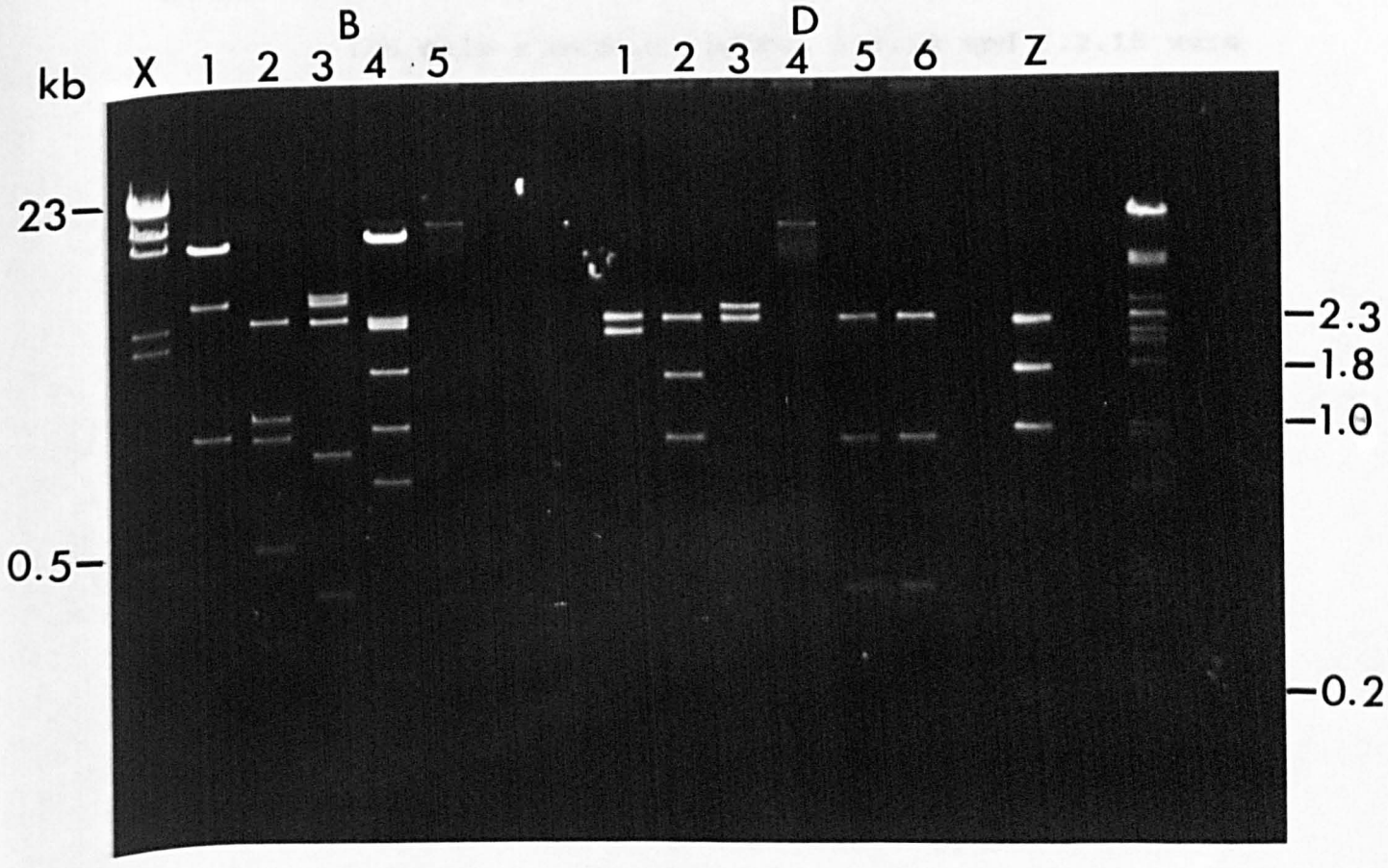
Figure 3.2.14: Novel plasmids cut with *Eco*R1, produced by ligation of chromosome cut with *Eco*R1 from *M.capsulatus* containing pJFF350.

X = *Hin*dIII cut λ
B1 to B5 = Plasmids produced from chromosome of *M.capsulatus* B
D1 to D6 = Plasmids produced from chromosome of *M.capsulatus* D
Z = pJFF350 cut with *Eco*R1.
X = *Hin*dIII cut λ

Figure 3.2.15: Novel plasmids cut with *Pst*1, produced by ligation of chromosome cut with *Eco*R1 from *M.capsulatus* containing pJFF350.

X = *Hin*dIII cut λ
B1 to B5 = Plasmids produced from chromosome of *M.capsulatus* B
D1 to D6 = Plasmids produced from chromosome of *M.capsulatus* D
Z = pJFF350 cut with *Pst*1.


B
D
X 1 2 3 4 5 1 2 3 4 5 6 Z X kb
23
3.8
1.5
0.5


B
D
kb X 1 2 3 4 5 1 2 3 4 5 6 Z
23
0.5
2.3
1.8
1.0
0.2

The plasmids cut with *Eco*R1 almost all yielded a band of approximately 3.8 kb (B1 being the exception). This band is the same size as the Omegon-kanamycin fragment shown in the control pJFF350 cut with *Eco*R1. The plasmids cut with *Pst*1 almost all yielded (B1 again being the exception) a 2.3 kb band, which is probably the internal *Pst*1 fragment of the Omegon-kanamycin.

If in the original insertion event pJFF350 had behaved as described in section 3.2.2 (Figure 3.2.6) the bands in the *Eco*R1 digest other than the 3.8 kb band could be assumed to be parts of *M.capsulatus* chromosome. However, since the entire pJFF350 has been inserted into the chromosome it is possible that parts of the plasmid external to the Omegon-kanamycin were present in these new plasmids.

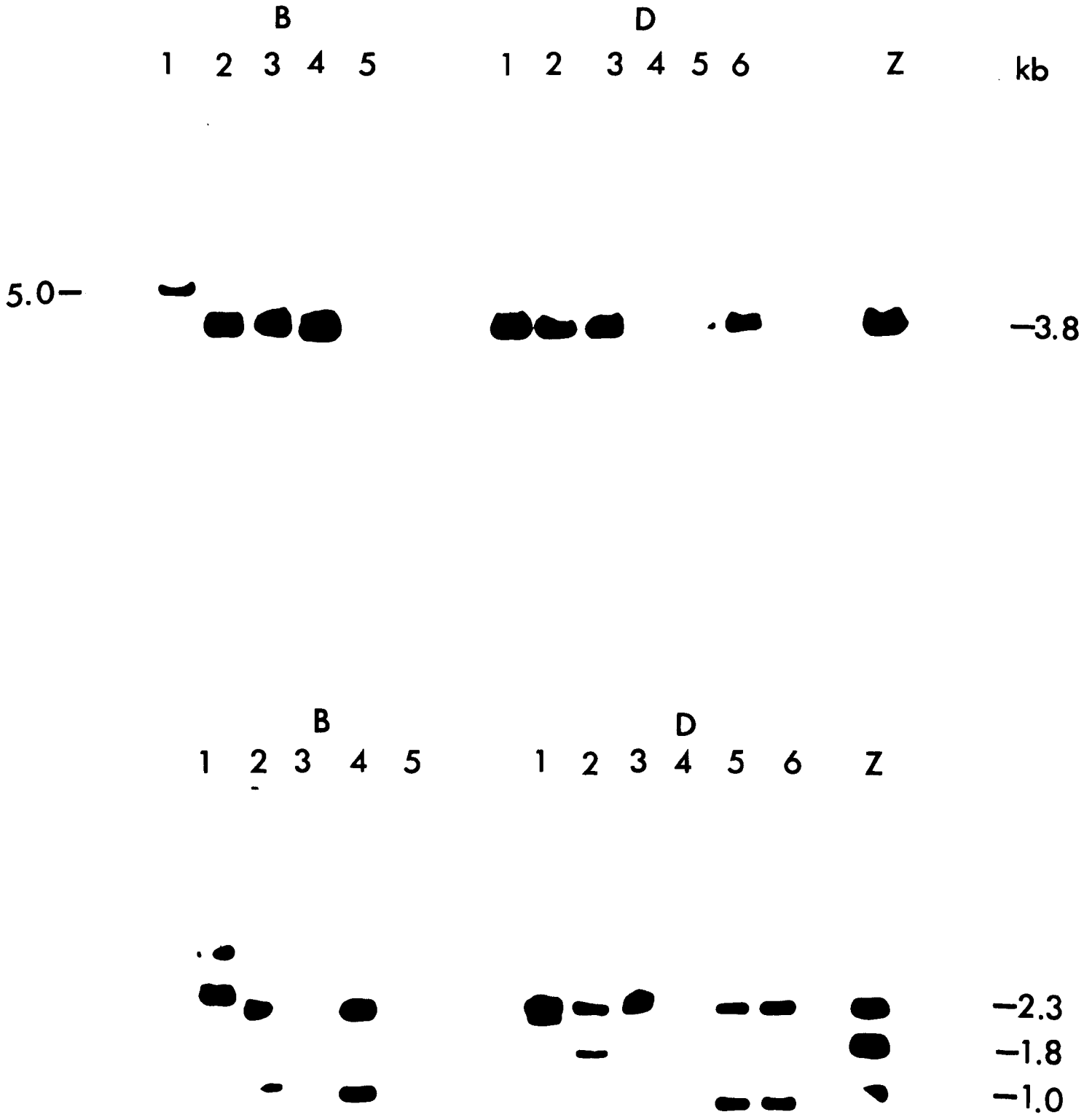
The gels shown in figures 3.2.14 and 3.2.15 were probed. The *Eco*R1 digested plasmids were probed with a radiolabelled *Pst*1 fragment of 2.3 kb internal to the Omegon-kanamycin. The filter was washed in 1 X SSC at 85^0C (2 X 20 min) which allowed 85% homology and above. This probing indicated that the 3.8 kb bands did contain the Omegon-kanamycin (Figure 3.2.16). The *Pst*1 digests were probed with the entire radiolabelled.pJFF350 plasmid. The filter was washed in an identical manner as the *Eco*R1 filter. This probing indicated that several of the bands contained DNA homologous to pJFF350 (Figure 3.2.17). The bands that did not show homology could now be assumed to be *M.capsulatus* chromosomal DNA, but it was probably the case

Figure 3.2.16: Screening of novel plasmids cut with *Eco*R1 with a radiolabelled 2.3 kb *Pst*1 fragment internal to the Omegon-kanamycin fragment of pJFF350. Washing conditions allowed for 85% homology and above.

X = *Hin*dIII cut λ
B1 to B5 = Plasmids produced from chromosome of *M.capsulatus* B
D1 to D6 = Plasmids produced from chromosome of *M.capsulatus* D
Z = pJFF350 cut with *Eco*R1.
X = *Hin*dIII cut λ

Figure 3.2.17: Screening of novel plasmids cut with *Pst*1 with radiolabelled pJFF350.

X = *Hin*dIII cut λ
B1 to B5 = Plasmids produced from chromosome of *M.capsulatus* B
D1 to D6 = Plasmids produced from chromosome of *M.capsulatus* D
Z = pJFF350 cut with *Pst*1.


B
D
1 2 3 4 5
1 2 3 4 5 6
Z
kb
5.0–
–3.8
B
D
1 2 3 4 5
1 2 3 4 5 6
Z
–2.3
–1.8
–1.0

that bands that showed homology contained both pJFF350 DNA and *M.capsulatus* DNA.

Two plasmids D1 and J1 were produced from the restriction and ligation of chromosome from different *M.capsulatus* transconjugants containing pJFF350. Gels were run of the plasmids, the original chromosome (D and J), the wild-type chromosome and pJFF350, all digested with *Eco*R1 (Figure 3.2.18). The plasmids D1 and J1 were then used as radiolabelled probes to probe the chromosome from which they originated. The filters were washed in 1 X SSC (2 X 20 min) at 85^0C which allowed for homology of 85% and above. The plasmids contained homology to the chromosome from which they were obtained (two bands) and also homology to the wild-type chromosome (one band) (Figures 3.2.19 and 3.2.20). This indicated that plasmids D1 and J1 did contain *M.capsulatus* DNA.

The procedure for the production of plasmids could also be carried out when the chromosome B was cut with *Xho*1, which also did not cut within the Omegon-kanamycin or *col*E1 replicon. *Xho*1 does not cut as frequently within the *M.capsulatus* chromosome and therefore it should be possible to produce larger plasmids containing more *M.capsulatus* chromosome. The plasmids produced were cut with *Eco*RI (Figure 3.2.21) and produced a number of different restriction patterns. The plasmids contained a larger number of bands indicating a large amount of *M.capsulatus* chromosome present in the plasmids.

Figure 3.2.18: Novel plasmids cut with *Eco*R1, produced by ligation of *M.capsulatus* chromosome containing pJFF350 cut with *Eco*R1

Gel I and II

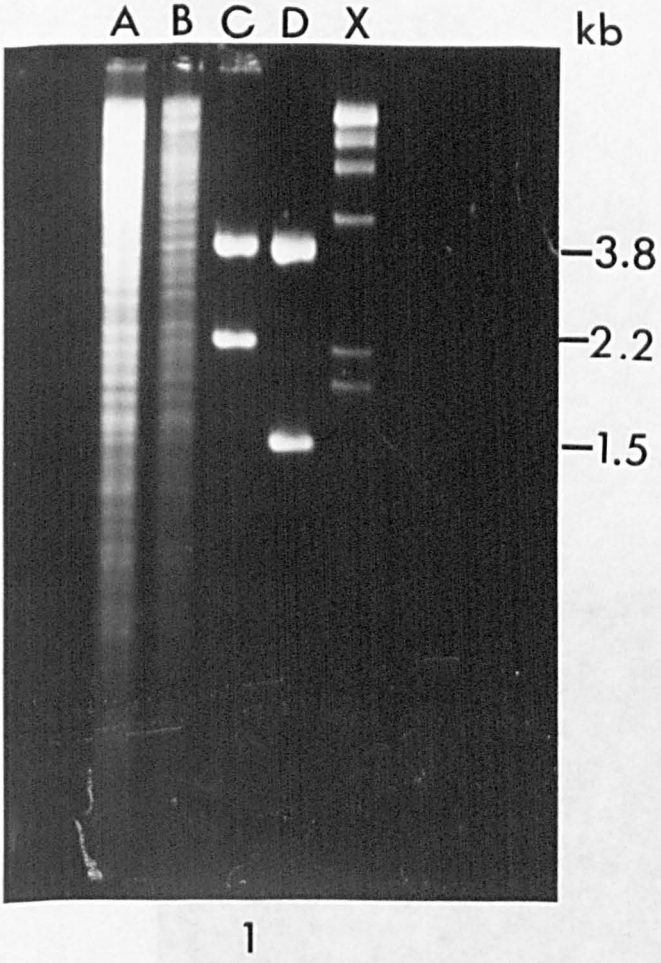
A & E = Wild-type *M.capsulatus* chromosome cut with *Eco*R1
B = *M.capsulatus* chromosome D cut with *Eco*R1
C = Plasmid D1 cut with *Eco*R1
D & H = pJFF350 cut with *Eco*R1
F = *M.capsulatus* chromosome J cut with *Eco*R1
G = Plasmid J1 cut with *Eco*R1
X = *Hin*dIII cut λ

Figure 3.2.19: Screening of gel I with radiolabelled D1 plasmid. Washing conditions allowed for 85% homology and above.

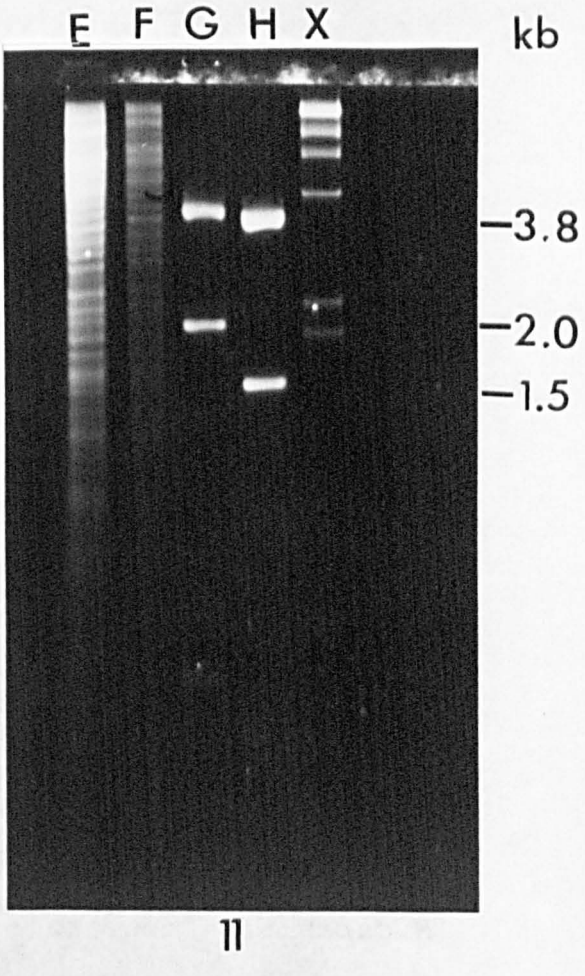
A = Wild-type *M.capsulatus* chromosome cut with *Eco*R1
B = *M.capsulatus* chromosome D cut with *Eco*R1
C = Plasmid D1 cut with *Eco*R1
D = pJFF350 cut with *Eco*R1

Figure 3.2.20: Screening of gel II with radiolabelled J1 plasmid. Washing conditions allowed for 85% homology and above.

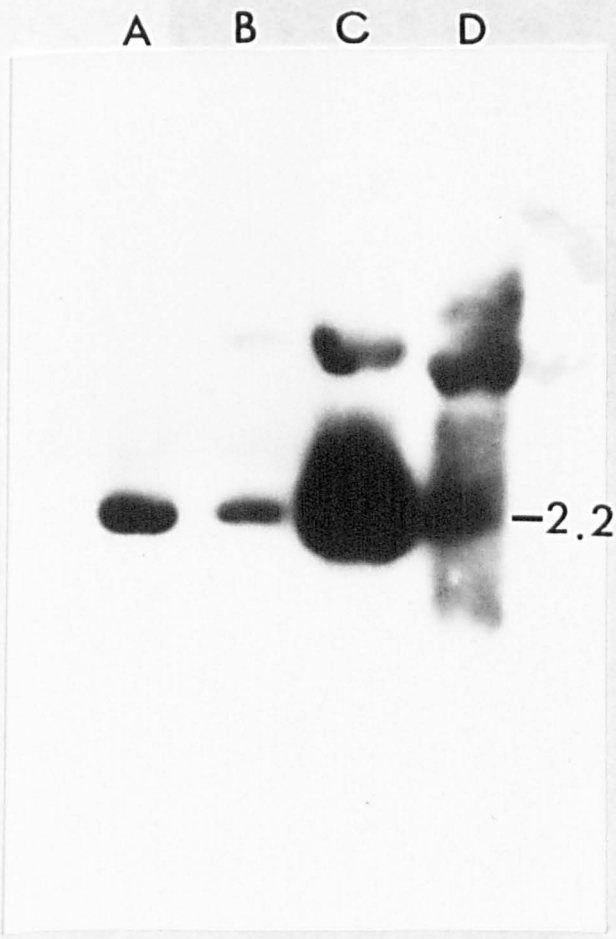
E = Wild-type *M.capsulatus* chromosome cut with *Eco*R1
F = *M.capsulatus* chromosome J cut with *Eco*R1
G = Plasmid J1 cut with *Eco*R1
H = pJFF350 cut with *Eco*R1
X = *Hin*dIII cut λ



1



11



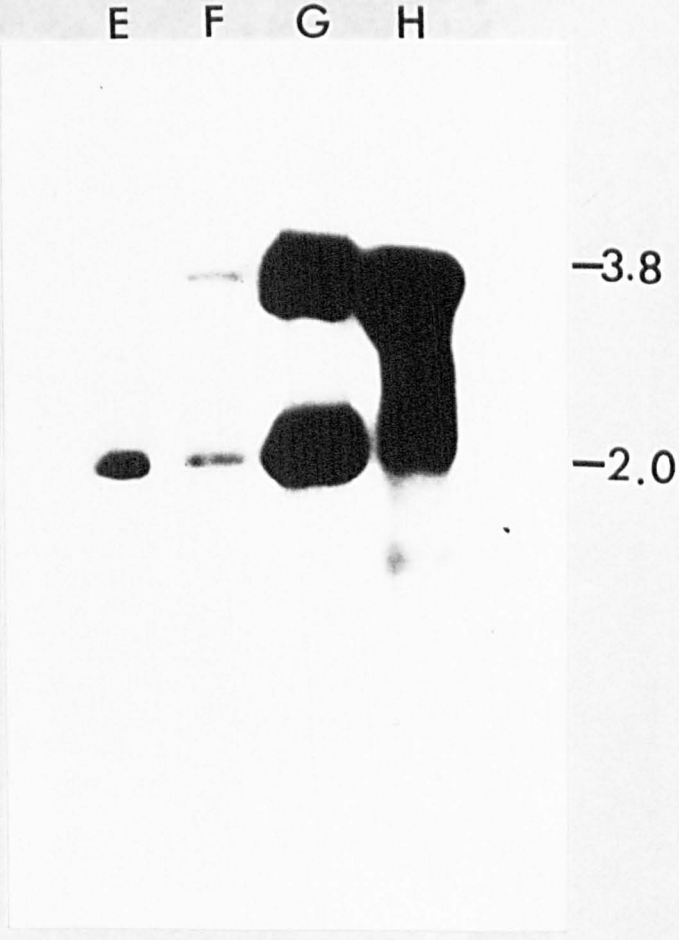
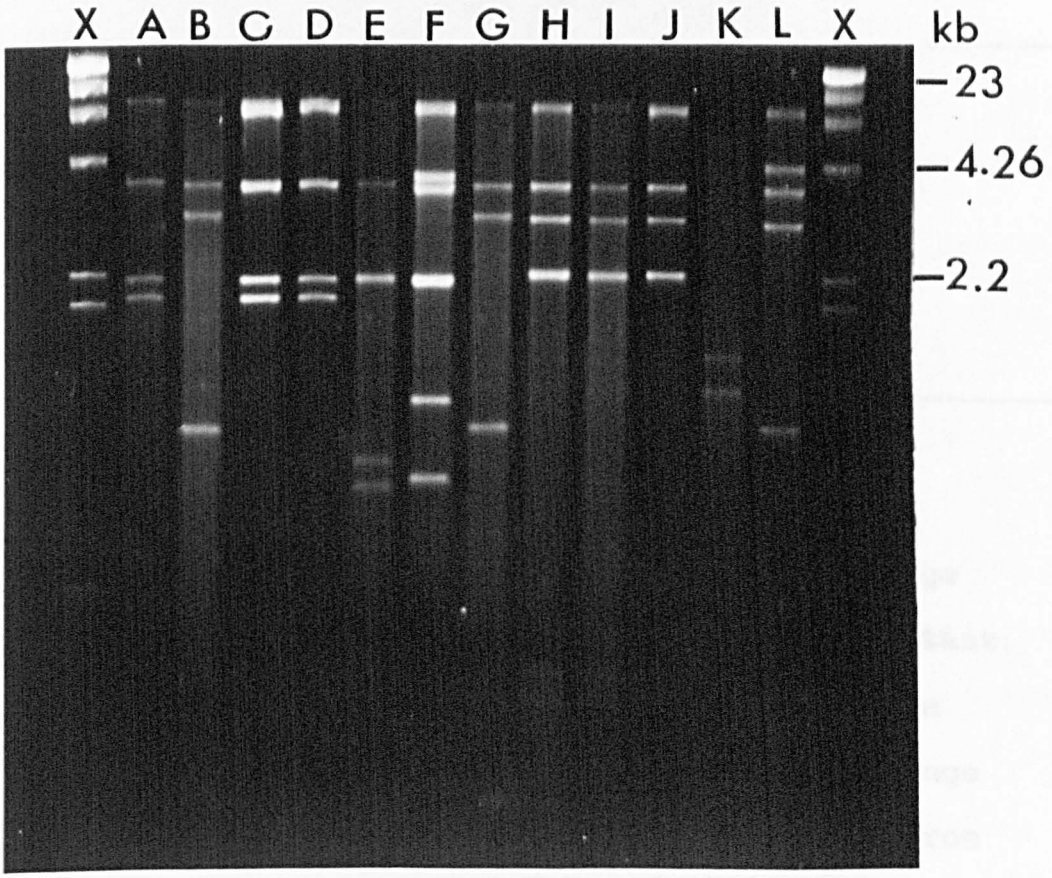


Figure 3.2.21: Novel plasmids cut with *Eco*R1, produced by ligation of chromosome cut with *Xho*1 isolated from *M.capsulatus* containing pJFF350

X = *Hin*dIII cut λ
A to L = Novel plasmids cut with *Eco*R1.


X A B C D E F G H I J K L X
kb
23
4.26
2.2

Plasmids obtained from both *Eco*RI digests of the chromosome and *Xho*1 digests were transformed into a range of *E.coli* auxotrophs (Table 2.1.2). The amino acid supplements required are outlined in table 3.2.2. No plasmids were found however that complemented any of the auxotrophs.

E.coli	Amino Acids required in M9 medium for growth					
	thr	leu	thi	pro	his	arg
9482	+	+	+	-	-	-
10241	-	-	-	+	+	-
11865	-	-	-	+	-	-
ET8045	-	-	-	-	-	+

Table 3.2.2

3.2.4 pSD100; a pJFF350 derivative for Marker-Exchange Mutagenesis and the production of a putative glnA mutant.

pJFF350 contains a unique *Nde*I site for the insertion of DNA to produce vectors for marker-exchange mutagenesis. However, few genes have been isolated from *M.capsulatus* (section 1.5.3). Of those genes that have been characterized, the gene coding for glutamine synthetase, *glnA*, was considered to be the most suitable for use in the development of a marker-exchange system, for the following reasons: the gene has been cloned and sequenced (Cardy, 1988), the inactivation of *glnA* in *M.capsulatus* is presumed to produce straightforward glutamine auxotrophy and such

auxotrophs can be simply isolated by the incorporation of glutamine in the medium, and the *glnA* of *M.capsulatus* has already been inactivated by insertion of the Omegon-streptomycin fragment and is present in pDC2Ω on a 7.2 Kb *Eco*R1 fragment (Cardy, 1988)(Figure 3.2.22). As the *glnA* gene has no suitable *Nde*I or compatible restriction site for straight-forward insertion of a fragment into pJFF350, I decided to merely use pJFF350 as a delivery vehicle for the mutated *glnA*. It was hoped that there would be homologous recombination between the mutated *glnA* and the chromosomal *glnA* and such mutants could be isolated by selection for the kanamycin resistance which would be transferred concomitantly into the chromosome, adjacent to the mutated *glnA*. The Omegon-kanamycin should give a more reliable indicator than streptomycin resistance.

Production of pSD100 (Figure 3.2.23) was by the partial digestion of pJFF350 at only one *Eco*R1 site and the insertion of the 7.2 kb *Eco*R1 fragment from pDC2Ω to give a final plasmid of 12.5 kb encoding both kanamycin and streptomycin resistance. This plasmid was transformed into *E.coli* DH1 from where it could be transferred by conjugation in a triparental mating with pRK2013 as the helper plasmid.

Several conjugations were undertaken with this plasmid. Transconjugants were selected for on NMS containing 0.02% w/v Proteose-Peptone, 25 μg/ml streptomycin and 25 μg/ml kanamycin. Three transconjugants were obtained that showed resistance to both kanamycin and streptomycin. These

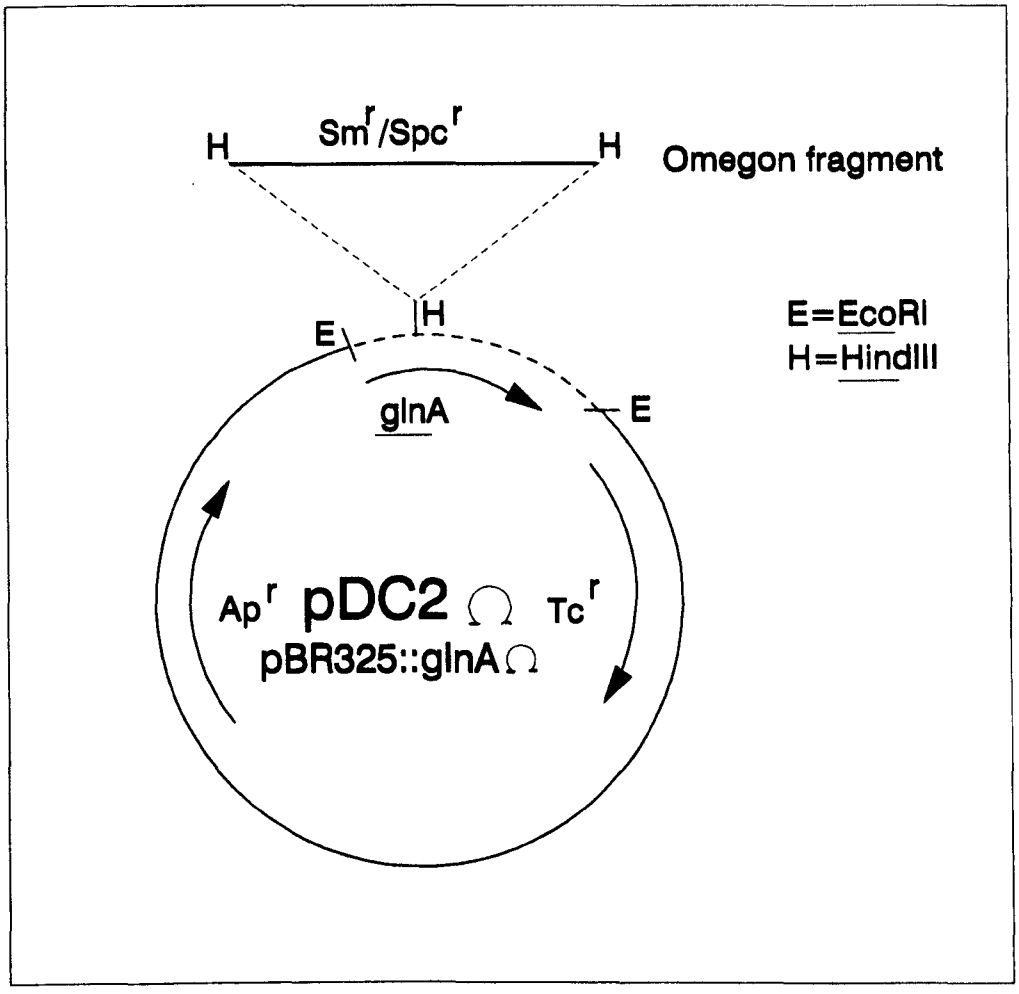

H
Sm^r/Spc^r
H
Omegon fragment
E=EcoRI
H=HindIII
H
E
glnA
E
Ap^r pDC2 Ω Tc^r
pBR325::glnAΩ

Figure 3.2.22

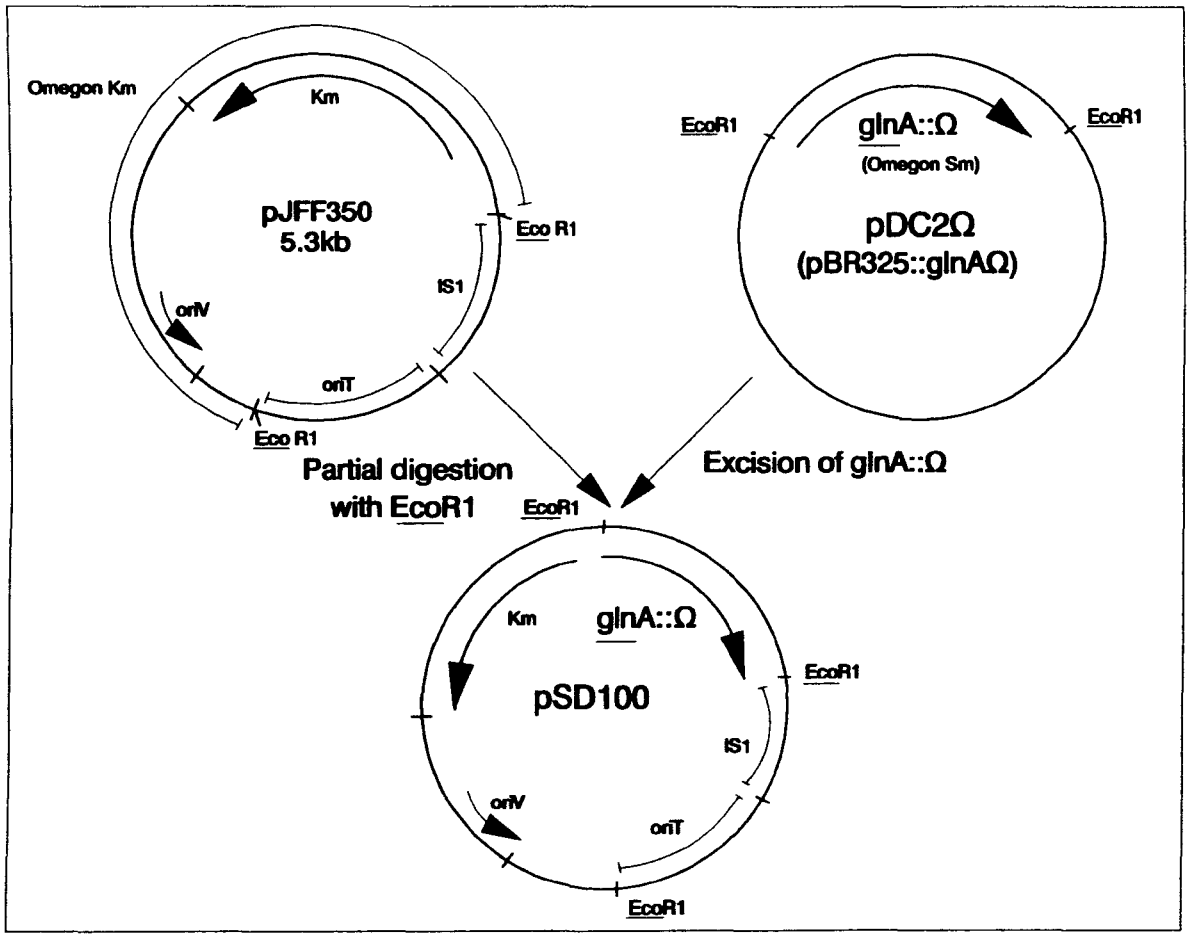


Figure 3.2.23: Production of pSD100

were plated onto NMS +/- Proteose-Peptone (0.02% w/v). None of the colonies required Proteose-Peptone for growth. Subsequent platings eliminated the possibility that growth was due to the carry-over of Proteose-Peptone from the first plates.

This result appeared to indicate that the mutated *glnA* had not inserted into the chromosomal *glnA* and the colonies were not glutamine auxotrophs. One transconjugant was used in further tests. This *M.capsulatus* transconjugant (putative *glnA* mutant) grew in liquid media containing streptomycin (25 μg/ml) and kanamycin (50 μg/ml) without addition of glutamine. Wild-type *M.capsulatus* and the mutant *M.capsulatus* were inoculated into liquid media under nitrogen-fixing conditions. 0.5 ml from overnight liquid cultures of wild-type and mutant were transferred to 50 ml of liquid media which contained no fixed nitrogen source. Flasks (250 ml capacity) were sealed with a Suba seal then flushed with oxygen-free nitrogen, this would have considerably lowered the partial pressure of oxygen in the flask. 150 ml of nitrogen was removed and replaced by 100 ml of air and 100 ml of methane. Under these conditions *M.capsulatus* would fix N_2 (Murrell & Dalton, 1983). The wild-type grew to an OD_{600} of 0.8 in 2 days, whereas the mutant *M.capsulatus* was unable to grow under these conditions.

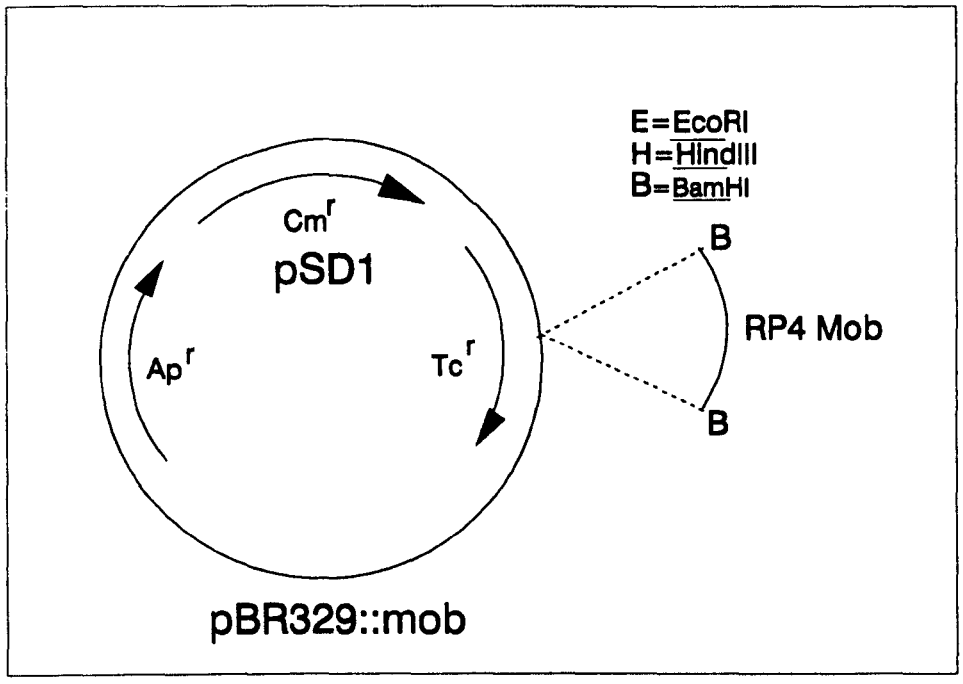


Figure 3.2.24

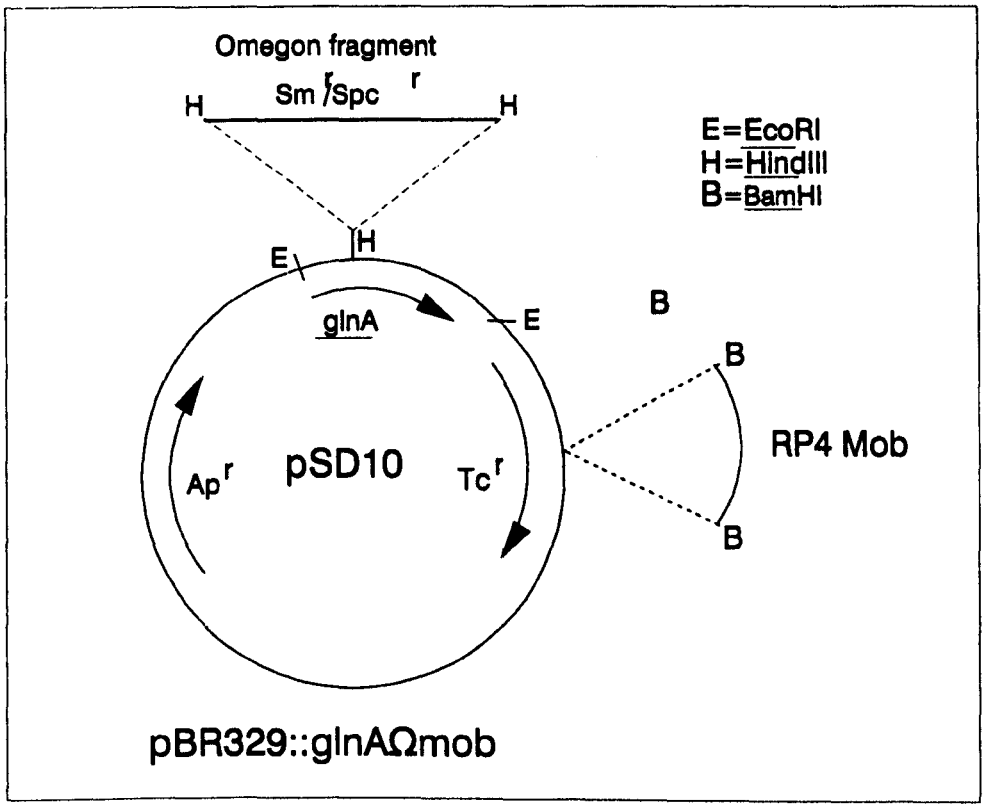


Figure 3.2.25

A preliminary probing of chromosome isolated from the mutant showed that it contained DNA homologous to pSD100, whereas, the wild-type chromosome only contained DNA homologous to *glnA* (results not shown). Further analysis would be needed to ascertain exactly where the plasmid had inserted and what was the precise effect upon the *M.capsulatus*.

3.2.5 pDC2Ω and pSD10 as Marker-Exchange Mutagenesis Vectors

The marker-exchange vector pDC2Ω (Figure 3.2.22) (Cardy, 1988) contains the *glnA* gene of *M.capsulatus* in pBR325. The *glnA* has been inactivated by insertion of the Omegon-streptomycin fragment. This plasmid, as a derivative of pBR322, can be transferred by conjugation due to the presence of the *bom* site (the site on *colE1* plasmids that is recognised by the a *mob* gene product, and it is at or near this site that transfer is initiated) from pMB1. It does however require the presence of two helper plasmids. The donor strain needs to contain an I -type plasmid such as R64drd11 which can provide the *tra* functions and another plasmid must provide the *colE1* *mob* functions (van Haute, 1983). This is a complicated conjugation so to increase the frequency of transfer and also to simplify the procedure, the *mob* site from RP4 was isolated from pSUP2021 (Simon *et al.*, 1983) on a 1.9 kb *BamH1* fragment. The vector pSD1 (Figure 3.2.24) was created by the insertion of this *mob* fragment into the *BamH1* site in pBR329. This vector was then

used to produce pSD10 by insertion of the *glnA* fragment from pDC2Ω into the *Eco*R1 site (Figure 3.2.25). This vector was used in conjugations. The transconjugants were selected for on NMS plus 0.02% w/v Proteose-Peptone and streptomycin (25 μg/ml). Despite the screening of over 700 streptomycin resistant colonies, none were found that required glutamine or Proteose-Peptone for growth.

3.2.6 Discussion of Mutagenesis Experiments

The transfer of plasmid DNA has been shown to be possible using the method of conjugation outlined in section 3.1. This has allowed the introduction of several types of vectors designed for insertional mutagenesis of Gram-negative bacteria.

Two vectors, pSUP2021 and pJFF350, have potential for the isolation of mutants of *M.capsulatus*. Both vectors rely on the RP4 mobilization system and were transferred from the S17-1 strain of *E.coli* that is a 'high frequency of mobilization strain' (Simon *et al.*, 1983). In both cases the acquisition of kanamycin resistance indicated insertion of the transposable element into the chromosome. The frequency of kanamycin resistant exconjugants using pSUP2021 was 2×10^{-9} and using pJFF350 was 5.2×10^{-8} per recipient cell. The actual transposition frequency is probably higher because a significant fraction of the total transposition events are not isolated because they take place in genes that are essential for cell viability. The frequency also

depends upon the mobilization efficiency into *M.capsulatus*, which is unknown. The frequencies found with both vectors are much lower than those found for other species. The transposition of Tn5 from pSUP2021 into the *Rhizobium meliloti* chromosome was 10^{-5} per recipient cell (Simon *et al.*, 1983), and when Tn5 was transferred, on a different vector, into *Proteus mirabilis* the frequency was 4.5×10^{-6} per recipient cell (Belas *et al.*, 1991). The transposition frequencies for pJFF350 for *Pseudomonas putida*, *Paracoccus denitrificans*, *Rhizobium leguminosarum* and *Agrobacterium tumefaciens* are 5×10^{-5}, 5×10^{-7}, 2×10^{-7} and 3×10^{-9} per recipient cell respectively (Fellay *et al.*, 1989). Only *A.tumefaciens* has a lower transposition frequency than *M.capsulatus*. The difference in the transposition frequencies between species is due either, to host restriction barriers, poor mobilization of the vector or a different frequency of transposition of Tn5 and Omegon-kanamycin.

pSUP2021 has been shown to insert Tn5 into the chromosome of *M.capsulatus*, as determined by homology to Tn5 present in chromosome isolated from kanamycin resistant transconjugants.

Tn5 has been widely used for transposon mutagenesis due to several advantageous properties that can be summarized as follows: i) it has a satisfactorily high frequency of transposition in many different Gram-negative species, ii) it generally inserts with little target

specificity, and iii) it exhibits a low probability of genome rearrangements upon transposition and a high stability once established in the genome (Berg & Berg, 1983; DeBruijn & Lupski, 1984). Chromosome samples from transconjugants were not subjected to sufficient analysis to determine whether points (ii) and (iii) are true for Tn5 transposition in *M.capsulatus*. The presence of a 'hot-spot' for insertion of Tn5 can usually be determined by, either an unusually high frequency of one particular mutant being produced or by analysis of many chromosome samples from transconjugants. Not enough chromosome samples were analyzed to determine if a 'hot-spot' existed and as no auxotrophs were isolated the frequency of mutants could not be used, therefore, the possibility of a 'hot-spot' for the insertion of Tn5 in *M.capsulatus* cannot be ruled out.

The vector pJFF350 was found to be transferred by conjugation into *M.capsulatus* and the Omegon-kanamycin fragment expressed kanamycin resistance. The kanamycin resistance in this case was a derivative of Tn903. Analysis of the chromosome of several transconjugants found that the entire pJFF350 vector, or at least a substantial part of it, had been incorporated into the chromosome. This included the defective IS1 ends and the RP4-*mob* fragment. The disabled IS1 ends should mediate the transposition of the Omegon-kanamycin together with the *col*E1 replicon, but not the IS1 ends and the RP4-*mob* fragment.

PJFF350 was developed for use in *Pseudomonas putida*. When chromosome from *Ps putida* transconjugants was cut with *Eco*R1 and probed with the Omegon-kanamycin fragment, a single band showed homology. This band was in all cases larger than the 3.8 kb Omegon-kanamycin fragment, but of a variety of sizes, indicating loss of the *Eco*R1 restriction sites that occur just outside the Omegon kanamycin and the randomness of the insertion event (Fellay *et al.*, 1989). This type of analysis of *M.capsulatus* transconjugants revealed that, except for one transconjugant, a single hybridizing DNA fragment of identical size in each transconjugant around 3.8 kb, the size of the Omegon-kanamycin. This could have been due either to the insertion of the plasmid in a 'hot-spot' or the retention of the *Eco*R1 ends of Omegon-kanamycin due to insertion of DNA outside the Omegon-kanamycin from IS1 and RP4-*mob*. The latter situation was found to be the case, as further analysis showed that IS1 and RP4-*mob* DNA was present in the chromosome. The plasmid appeared to have inserted in four different ways: IS1 and RP4-*mob* present on a 1.5 kb fragment (chromosome K and M), IS1 and RP4-*mob* DNA present on a fragment of approximately 2.3 kb (chromosome B, D and L) and IS1 and RP4-*mob* DNA present on a fragment of approximately 1 kb (chromosome J), and finally chromosome C where 2 bands, one at 2.3 kb and one at approximately 23 kb appear to contain Omegon-kanamycin DNA, as well as a small band at 1.5 kb which contains IS1 and RP4-*mob* DNA. The

samples where the band containing the IS1-RP4-*mob* DNA occurs on a fragment smaller than 1.5 kb, the original size of this DNA in pJFF350, may indicate deletions having taken place (chromosome J). The samples were this fragment is larger than 1.5 kb may indicate loss of an *Eco*R1 restriction site, and therefore *M.capsulatus* chromosomal DNA is present in the restriction fragment as well (chromosome B, D, L). Chromosome M and J appear to be identical to the pJFF350 control and may therefore represent correct insertion of the whole plasmid with all *Eco*R1 restriction sites intact. Chromosome C is difficult to understand as the Omegon-kanamycin appears to occur twice in the chromosome on two different restriction fragments, a duplication of the Omegon-kanamycin must have taken place at some point, along with loss of the *Eco*R1 restriction sites. More samples would have to be analyzed to say whether these are the only four restriction patterns that occur.

A forerunner to pJFF350 was pJFF98 (Fellay *et al.*, 1988). This contains an intact copy of IS1 and was found to mediate the insertion of fragments external to the Omegon-kanamycin fragment in 70% of the insertion events. The modified ends of IS1 in pJFF350 were found to insert the entire plasmid in only 20% of the insertion events. The insertion of different parts of pJFF350 was determined by probing with the entire plasmid and with the IS1-RP4-*mob* fragment.

Fellay and colleagues suggested three possible reasons for the insertion of the entire plasmid in *Pseudomonas putida*; the formation of cointegrates, in which the plasmid pJFF350 flanked by two copies of Omegon-kanamycin is inserted, by low-level transposition using the ends of the disabled IS1, or by transposition from multimeric donor plasmids as suggested for IS50 (Saskawa and Berg, 1982).

All the *M.capsulatus* transconjugants analyzed showed insertion of IS1 and RP4-*mob* fragments. There must therefore exist the possibility of factors present in the cell that either induce the formation of cointegrates or multimeric plasmids or alternatively act in conjunction with the disabled IS1 ends to bring about this insertion.

Desomer *et al.* (1991) designed two vectors, pRF32 and pRF41, for insertional mutagenesis of *Rhodococcus fasciens*. They were designed for use in a method identical to that for pJFF350, except that they were transferred to the cell via electroporation. Integration of the vectors into the chromosome could be determined due to the acquisition of an antibiotic resistance. Mutant genes 'tagged' in this manner could then be isolated by virtue of a *col*E1 replicon attached to the antibiotic resistance. These vectors, unlike pJFF350, contained no known insertion sequences or transposon-like elements. There were three classes of integration event. The first, where more than one copy of pRF32 (or pRF41) had been integrated, and two others

where different amounts of DNA had been deleted from the plasmid. It was not known whether the deletion had occurred post or prior to integration. The insertion appeared to involve 3 to 4 bases shared by the target sites in the chromosome and the plasmids. It was also concluded that the integration of the plasmids was probably mediated by a host-encoded factor or factors (Desomer *et al.*, 1991.)

The integration of IS1 ends and RP4-*mob* fragment may be a disadvantage in attempts to mutagenize *M.capsulatus*, since the presence of these elements will possibly decrease the stability of any mutants isolated and complicate analysis of the mutants. There is however one possible advantage, the presence of the RP4-*mob* fragment in the chromosome opens up the possibility for the mobilization of the chromosome. Simon *et al.*(1983) actually developed a transposable *mob* fragment by loading the fragment onto Tn5, the transfer of this Tn5-*mob* into previously unmobilizable chromosome would enable it to be mobilized by RP4. This was successful with *Rhizobium meliloti* mega-plasmids and also increased the mobilization frequency of *E.coli* chromosome. The vector that was used in this case, pSUP201-1, was not successful in *M.capsulatus*. Whether this method will now be successful using pJFF350 remains to be seen.

The plasmids isolated were cut with *Eco*R1 and each contained the 3.8 kb Omegon kanamycin fragment plus extra DNA. This extra DNA was found to be chromosomal DNA. The plasmids obtained could possibly be used to attempt

complementation of mutants or as a gene bank for the isolation of genes. However, due to the insertion of the Omegon-kanamycin fragment with external *Eco*R1 sites intact, restriction of the chromosome with *Eco*R1 to produce the plasmids would leave the Omegon-kanamycin fragment free to religate to any other chromosome fragment and it would not be possible to isolate DNA surrounding the original point of insertion. The use of *Xho*1, which does not cut so frequently in the chromosome and not in pJFF350 at all, could mean the isolation of larger fragments of chromosome that do surround the original point of insertion. Although several gene banks have been produced for *M.capsulatus* DNA, this technique could yield the isolation of a mutated gene which could then be used for marker-exchange mutagenesis.

Both pSUP2021 and pJFF350 were used to attempt to isolate auxotrophs but both were unsuccessful. One reason for the lack of auxotroph isolation could be the media used for the isolation of transconjugants. This contained a complex carbon source, Proteose-Peptone at 0.02% w/v thought to be sufficient to support any auxotrophs. The 'normal growth' of *M.capsulatus* (Bath) is not inhibited by this concentration of Proteose-Peptone in NMS medium. It has also been found that *M.capsulatus* is not inhibited by individual amino acids present in the medium and possesses a broad specificity amino acid transport system (Eccleston & Kelly, 1972). There is however no precedent for the growth characteristics or nutritional requirements of cells of

M.capsulatus defective in the production of amino acids as no auxotrophs have ever been isolated (apart from a leaky p-amino benzoic acid auxotroph). It has however been shown with the methylotroph, *M.flagellatum*, that auxotrophs were obtained at a higher frequency if a specific auxotroph was selected for by the incorporation of a single amino acid at a high concentration to minimal medium (Tsygankov & Kazakova, 1987).

Another reason for the lack of auxotroph isolation is the possibility that there is a 'hot-spot' for insertion of Tn5. A 'hot-spot' for the insertion of pJFF350 may also be likely. For some reason it may be that insertion into any of the genes involved with amino acid metabolism causes cell death making it impossible to isolate auxotrophs, or there is the possibility that multiple pathways exist and there are enzymes which can compensate for the loss of another. This effect has been seen in *Desulfovibrio fructosovorans*. Rousset (1991) found that, following marker exchange mutagenesis that resulted in complete inactivation of the (NiFe) Hydrogenase (*hydN*) gene, one particular strain was still able to grow under H_2/sulphate growth conditions. They concluded that the observed hydrogenase activity was attributable to a second enzyme unmasked by the deletion of *hydN*. This enzyme activity was lower but sufficient to allow growth. It is also possible that the lack of *M.capsulatus* auxotroph isolation may merely have been due to too few transformants being tested.

The ability of pJFF350 to insert into the chromosome of *M.capsulatus* opened up the possibility of its use as a vector for marker-exchange mutagenesis. A vector would have to be developed based on pJFF350 but containing a fragment of mutated *glnA* only, unlike the pSD100 vector.

A problem with the other marker exchange vectors, pDC2 and pSD10, is that streptomycin is not the best marker for use in *M.capsulatus* as spontaneous resistance occurs. The presence of the RP4-*mob* fragment in pSD10 would increase the frequency of transfer which would help towards the isolation of a mutant as this transfer system has been shown to work with *M.capsulatus*, whereas, the *col*E1 system has not definitely been shown to work and is also far more complicated.

Neither pSUP2021 nor pJFF350 appear to be ideal vectors for random insertional mutagenesis of *M.capsulatus*, there are obviously a great deal of factors affecting the entry and insertion of foreign DNA into the *M.capsulatus* chromosome. Any mutant produced would have to undergo extensive analysis to ascertain exactly the molecular events that had occurred. It is possible that several systems affect the stability of foreign DNA in the cell. The vectors do still have possibilities for the development of marker exchange mutagenesis vectors.

A lot of problems have been found to occur with the insertion of foreign DNA into *M.capsulatus*, but it has been shown for the first time that it is possible to have

Tn5 insertion and subsequent expression in the chromosome of *M.capsulatus* and insertion of an Omegon fragment based element and its' expression.

RESULTS

ELECTROPORATION OF *M.CAPSULATUS*

3.3 ELECTROPORATION OF *M.CAPSULATUS*

The development of electroporation has enabled DNA to be transferred reliably and efficiently into many bacterial species which are recalcitrant to chemical methods of transformation. For some of these organisms it was possible to transfer DNA into the cell by conjugation. However, reliance on conjugation limits the range and type of vector that can be used and the requirement for a donor organism to be involved means that selection of transconjugants must also involve selection against the donor. No such complications are found with electroporation and often DNA from a variety of sources can be used, i.e, small-scale plasmid preparations and ligations, without the need for removal of the chemicals involved in these processes.

When developing an electroporation system for a Gram-negative organism, most workers have initially followed the method devised by Dower *et al.* (1988) for *E.coli*. However, many factors must be taken into account when developing a procedure, for example, the organism's morphology, physiology, the presence of extracellular structures, such as capsules, and the production of extracellular enzymes. So although the method of Dower *et al.*, (1988) for *E.coli* can be a starting point, many different conditions need to be tested to produce an efficient system of electroporation for a particular bacterial species. The recent development of 0.1 cm cuvettes

for electroporation has increased further the possible range of conditions.

3.3.1 Preparation of *M.capsulatus* for electroporation and procedures for recovery following electroporation.

The first step in the development of a procedure for the electroporation of *M.capsulatus* (Bath) was the preparation of cells for electroporation. The medium in which the cells are suspended for electroporation must fulfil two criteria; low ionic strength and it must have no adverse effects on the cells when the cell wall and membrane are damaged by the electric pulse. The final method chosen for the preparation of cells for electroporation is outlined in figure 3.3.1 and also in detail in section 2.2.7. This procedure is basically the same as that for *E.coli*, although initial volumes of cells used varied, depending on the source of the cells, i.e, from 250 ml flasks or a fermenter. Viable cell numbers were assessed prior and subsequent to this treatment by a dilution series plated on NMS. No drop in viable cell number was found following this procedure.

E.coli prepared in this manner for electroporation can be rapidly frozen by dry ice/ethanol and remain viable for at least 6 months at -70^{0}C. *M.capsulatus* cells were frozen in this manner and viable cell numbers monitored at intervals by a dilution series plated on NMS plates (Table 3.3.1).

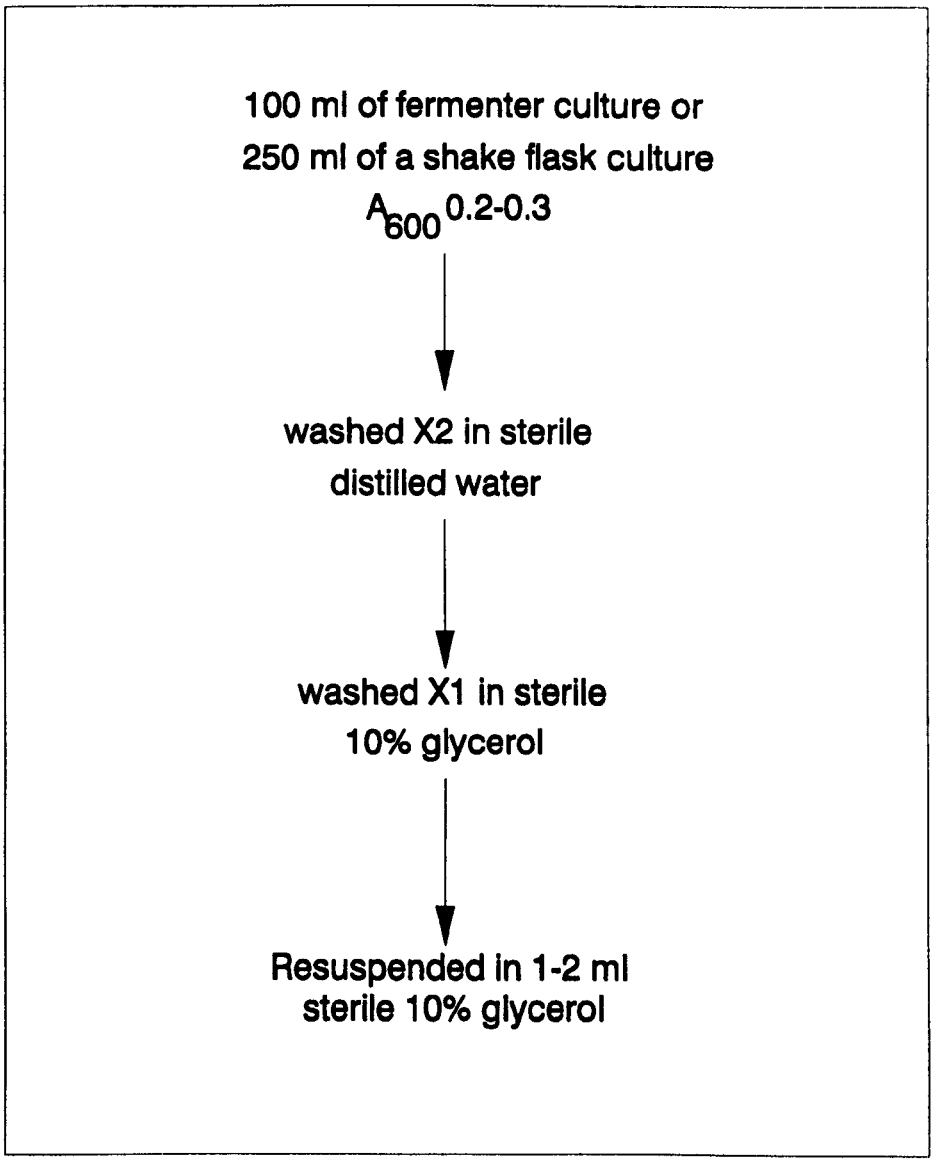


Figure 3.3.1: Preparation of M.capsulatus cells for electroporation

Time frozen	Percentage of original number remaining viable.(%)
1 week	100
1 month	73
6 months	18

Table 3.3.1

All electroporation experiments, except where indicated, used freshly prepared cells which had been resuspended in 10% glycerol.

During the process of electroporation the cell is presumed to be under great stress and, therefore, the medium and procedure for recovery of the cells is very important and can significantly affect the number of transformants obtained (Dower *et al.*, 1988). Initially 1 ml of NMS plus 0.02% Proteose-Peptone was added to the cells immediately after electroporation. The cells were then transferred to a sterile bijou. The bijou was sealed with a suba seal, 2 ml of air removed which was replaced by 3 ml of methane. The bijoux were shaken at 200 rpm at 45^0C overnight. Cells were then plated onto selective plates.

This method of recovery was assessed for its effect on *M.capsulatus* by checking the viable cell number before and after this treatment, when no electroporation had taken place. 40 μl of cells (10^9 per ml), prepared as in figure 3.3.1, were added to 960 μl of NMS plus 0.02% Proteose-Peptone in a bijou, a sample was taken and the viable cell number determined by a dilution series. The cells were then left shaking overnight at 200 rpm and 45^0C. A sample was taken the following day and the viable number

of cells calculated. There was a drop by a factor of 10 from 10^9 to 10^8 per ml. It was concluded that if this recovery procedure had this effect upon cells that had not been electroporated, then it would not be an adequate recovery procedure for cells that had been electroporated, therefore a new recovery procedure was devised.

Following electroporation, 960 μl of NMS plus 0.02% Proteose-Peptone were added to the cells, as in the previous method. The cells were then added to 50 ml of NMS plus 0.02% Proteose-Peptone in a 250 ml flask. This flask was sealed with a suba seal, 50 ml of air removed and replaced with 100 ml of methane. The flask was shaken at 200 rpm at 45^0C. This method allowed for better oxygen and methane transfer to the medium which would lead to efficient recovery of cells. Instead of plating the cells on selective medium, the selective antibiotic was added to the flask by syringe through the suba seal. The flask was kept shaking and samples could be removed at time intervals and plated onto selective medium or the whole flask contents could be centrifuged, concentrated to 1 ml and plated onto selective plates. Samples taken before and after 24 hours recovery by this procedure showed that there was no drop in viable numbers.

In all the experiments where the viable cell number was assessed, the procedure was to plate out 5 dilution series. A control treated in an identical manner to the experimental cultures, except that it had not been

electroporated nor undergone any of the treatments, was always included. The viable cell number of the cultures that had undergone electroporation or treatments could be compared to this control.

This procedure for recovery would be sufficient to allow detection of electro-transformants even if produced at a very low level.

The standard procedure for recovery and selection of electrotransformants was as follows: the cells were allowed to recover for a certain time period, either 24 or 48 hours, then kanamycin (50 μg/ml) added and the cells left to grow. Samples would be taken on subsequent days and plated onto selective media, NMS plus kanamycin (50 μg/ml). After 7 days, the entire flask was centrifuged, cells resuspended in 1 ml NMS and plated onto selective plates. For each experiment testing for the transfer of plasmids, duplicate cultures were used, to one of which kanamycin was added after 24 hours, the other after 48 hours.

3.3.2 The effect of alteration of electrical parameters

In studies on *E.coli*, alteration of electrical parameters has been found to be the most influential factor in the efficiency of the electro-transformation procedure (Dower *et al.*, 1988). The two important parameters are the field strength (f) and the pulse length or time constant (tc). These are determined by settings on the Biorad electroporation apparatus (electroporator and pulse

controller). The field strength is determined by the applied voltage (V) and the distance between the electrodes (d) (the width of the cuvette in which the cells are placed). The field strength is calculated by the following equation:

$$f = V/d$$

Table 3.3.2 gives field strengths for different applied voltages and two different cuvette sizes. The maximum field strength obtainable at the beginning of these studies was 12.5 kV/cm, but with the advent of the 0.1 cm cuvettes, this maximum was increased to 25 kV/cm. The apparatus was set at a capacitance of 25 μF for all experiments.

d (cm)	V (kV)	f (kV/cm)
0.2	0.5	2.5
0.2	1.0	5.0
0.2	1.5	7.5
0.2	2.0	10.0
0.2	2.5	12.5
0.1	0.5	5.0
0.1	1.0	10.0
0.1	1.5	15.0
0.1	2.0	20.0
0.1	2.5	25.0

Table 3.3.2

The other important electrical parameter, the time constant, is dependent on the size of the resistor (R) present in parallel with the cuvette. Table 3.3.3 gives approximate time constants at a set field strength of 12.5 kV/cm. The time 'constant' at any particular field stength

is variable due to the differing levels of salts in the medium.

R (Ω)	tc (Msec)
100	2.4
200	4.7
400	9.2
600	13.5
800	17.6

Table 3.3.3

One problem encountered with the alteration of the electrical parameters, is that of arcing. Arcing occurs when the current passes across the cuvette at some area of very low resistance/high conductivity such as salt residues, causing explosion of the medium and a large spark. Field strengths over 17 kV/cm and resistance settings over 600Ω had a tendency to produce arcing.

The effect on *M.capsulatus* of altering these electrical parameters was determined. The viable cell number after a 24 hour recovery period was assessed. An increase in field strength up to 17 kV/cm had no significant discernible effect on viable cell numbers. Increasing the time constant also had no discernible effect on the viable cell numbers (Table 3.3.4). If arcing did occur however, the result was significant cell death. A culture containing 10^8 cells per ml was reduced to 10^4 cells per ml.

f[1] Kv/cm	cells/ml*	tc[2] msec	cells/ml*
0	6.4×10^6	0	5.1×10^6
2.5	6.5×10^6	2.4	5.2×10^6
5.0	6.2×10^6	4.7	5.6×10^6
7.5	6.6×10^6	9.2	5.1×10^6
10.0	6.35×10^6	13.5	5.0×10^6
12.5	6.5×10^6	17.6	5.2×10^5
15.0	6.0×10^6		
17.0	6.1×10^6		
20.0	$1 \times 10^{6**}$		
25.0	$1.3 \times 10^{5**}$		

1. Time constant of approximately 2.4 msec while field strength altered.

2. Field strength of 12.5 kV/cm while time constant altered.

* Average of 5 electroporation experiments taken (each experimental result was an average of 5 dilution series)

** Due to arcing only 2 electroporation experiments were possible at these high field strengths.

Table 3.3.4

The next step was the attempt to transfer a variety of plasmids into *M.capsulatus* using electroporation. The plasmids used were a variety of sizes and types (Table 3.3.5).

Plasmid	size(Kb)	Selection
pVK100	23	Km
pDSK509	9.3	Km
pULB113	60	Km
pJFF350	5.2	Km
RP4	56	Km

Table 3.3.5

Plasmids were obtained from *E.coli* by the alkaline-lysis method followed by CsCl density gradient centrifugation (section 2.2.9). DNA concentrations were determined by absorbance at 260 nm and by visual estimation using agarose gel electrophoresis (section 2.2.12.7).

In each test 40 μl of *M.capsulatus* (concentration of at least 10^8 cells/ml) were mixed with 5 μl of DNA solution, containing approximately 1 μg of DNA, transferred to the cold cuvette and then pulsed. For all combinations of field strength and time constant used (Table 3.3.6) no electro-transformants were isolated. Duplicate cultures were allowed 24 and 48 hour recovery times before addition of kanamycin (50 μg/ml)

These experiments were performed using cells grown in continuous culture in a fermenter and cells grown in batch in 250 ml flasks (for growth conditions see section 2.2.1). It was found that the origin of the cells made no difference to the results, no electro-transformants were isolated.

f\tc	0	2.4	4.7	9.2	13.5	17.6
0	+	-	-	-	-	-
2.5	-	+	+	+	+	+
5.0	-	+	+	+	+	+
7.5	-	+	+	+	+	+
10.0	-	+	+	+	+	+
12.5	-	+	+	+	+	+
15.0	-	+	+	+	-	-
17.0	-	+	+	-	-	-
20.0	-	+	+	-	-	-
25.0	-	+	+	-	-	-

+ condition tested
- condition not tested or unable to be tested.

Table 3.3.6: The combinations of field strength (f) and time constant (tc) used to attempt to transfer plasmids in by electroporation.

3.3.3 Pretreatments of *M.capsulatus*

The use of pretreatment to facilitate the entry of plasmids to the cell during electroporation was attempted using several methods. In all these experiments, pVK100, pDSK509, and pJFF350 were the test plasmids.

3.3.3.1 Freeze-thawing of cells

M.capsulatus was prepared as in figure 3.3.1 then rapidly frozen in 100 μl aliquots in a dry ice/ethanol bath and stored at -70^{0}C. When cells were required they were thawed at room temperature then placed on ice prior to use. Cells that were used had either just been frozen or had been stored at -70^{0}C for no longer than a week. Cells were also put through a cycle of freeze-thawing, ie, thawed then frozen then thawed again, this was carried out for up to 3 cycles. Electroporation was carried out using pVK100, pJFF350 and pDSK509. None of these treatments produced any electro-transformants.

3.3.3.2 Treatment with Cetyl-trimethylammonium bromide (CTAB)

The effect on *M.capsulatus* of exposure to CTAB was determined by treating cultures of *M.capsulatus* with varying concentrations of CTAB for different lengths of time at room temperature. The cells were washed as for electroporation in sterile distilled water and then resuspended in a solution

of CTAB. Table 3.3.7 shows the effect of three concentrations of CTAB on viable cell numbers.

Concentration µg/ml	number of cells per ml
Exposure time = 15 minutes	
0	1.3×10^8
100	1×10^8
500	3×10^5
1000	1×10^4
Exposure time = 30 minutes	
0	1.1×10^8
100	4×10^7
500	5×10^4
1000	0

Table 3.3.7

Following treatment with CTAB, cells were washed in 10% glycerol. CTAB treatment had a detrimental effect on the cells and so cells were treated only with 100 µg/ml for 15 min and then the effect of electroporation determined. Table 3.3.8 shows the effect of increasing time constant at a field strength of 12.5 kV/cm on the viable cell number. The controls, no CTAB treatment and CTAB treatment but no electroporation, indicated that the CTAB treatment made the cells more susceptible to the electric pulse.

CTAB treatment made the cells more susceptible to death by electric pulse but no electro-transformants were isolated following this treatment.

tc (msec)	Cells/ml
4.7	6×10^7
9.2	6.2×10^7
13.5	1×10^6
17.6	3×10^4
Cont 1	6.1×10^7
Cont 2	6×10^7

Cont 1 = No CTAB treatment, but electroporation at a tc of 13.5 mSec.

Cont 2 = CTAB treatment but no electroporation

Table 3.3.8

3.3.3.3 Other treatments

Two other treatments were tried in an attempt to transfer plasmids to *M.capsulatus* by electroporation. These were treatment with lysozyme and increasing the number of pulses that the cell cultures received.

Cells were treated with 4 mg/ml lysozyme at 37^0C after undergoing 2 washes with distilled water and resuspension in 1 ml 10% glycerol. Two conditions were used: 15 min and 30 min. The effect of the treatment was assessed by treating the cells with a drop of 20% SDS and looking for clearing of the solution which indicated cell lysis had taken place and therefore the cell walls had been weakened. Cells treated for both 15 and 30 minutes were susceptible to SDS. Therefore cells that had been treated for varying amounts of time between 5 and 30 minutes were washed gently in 10% glycerol then electroporated with the three test

plasmids, pVK100, pDSK509 and pJFF350. No electro-transformants were isolated.

The second treatment was to increase the number of pulses that the cells received. The number of pulses at 12.5 kV/cm was varied from 1 to 8. No time period was left between each pulse. This procedure was carried out at different time constants (Table 3.3.9)

No. of pulses	R setting (Ω)		
	200	400	600
1	+	+	+
2	+	+	A
3	+	+	A
4	+	+	A
5	+	+	A
6	+	A	A
7	+	A	A
8	+	A	A

A = arcing occurred

Table 3.3.9

Electroporation under these conditions produced no electro-transformants.

3.3.4 Does plasmid enter the cells following electroporation?

There could be three possible reasons for the lack of success with the transfer of plasmids into *M.capsulatus* by electroporation: the lack of plasmid entry into the cell, the instability of the plasmid once it has entered the cell, or the non-recovery of transformants following electroporation.

The following experiment was designed to determine whether plasmid actually entered the *M.capsulatus* cell. The method is outlined in the Methods section 2.2.8 but a summary is shown in figure 3.3.2 as a flow diagram. The basic theory behind the method is that plasmid DNA that enters the cell following electroporation is thereafter protected from destruction by DNase external to the cell. The plasmid can subsequently be isolated and detected, even if present in very small amounts, by electroporation of the plasmid preparation into *E.coli* under optimum conditions.

Table 3.3.10 shows the results of this method using two plasmids, pVK100 and pJFF350. If pJFF350 was the initial plasmid used for the electroporation, this was the plasmid that was recovered from the final kanamycin resistant *E.coli* colonies obtained. The same was true if pVK100 was the initial plasmid, pVK100 was recovered from the resulting kanamycin resistant *E.coli*.

The results appear to indicate that plasmid enters *M.capsulatus*, and therefore the problem lies in the subsequent maintenance of the plasmid or the recovery of electro-transformants.

3.3.5 The use of plasmid isolated from *M.capsulatus*

The preceding experiment appeared to indicate that there was possibly a problem with maintenance of the plasmid. The plasmid could enter the cell by electroporation but was not maintained. All the plasmids that were used had

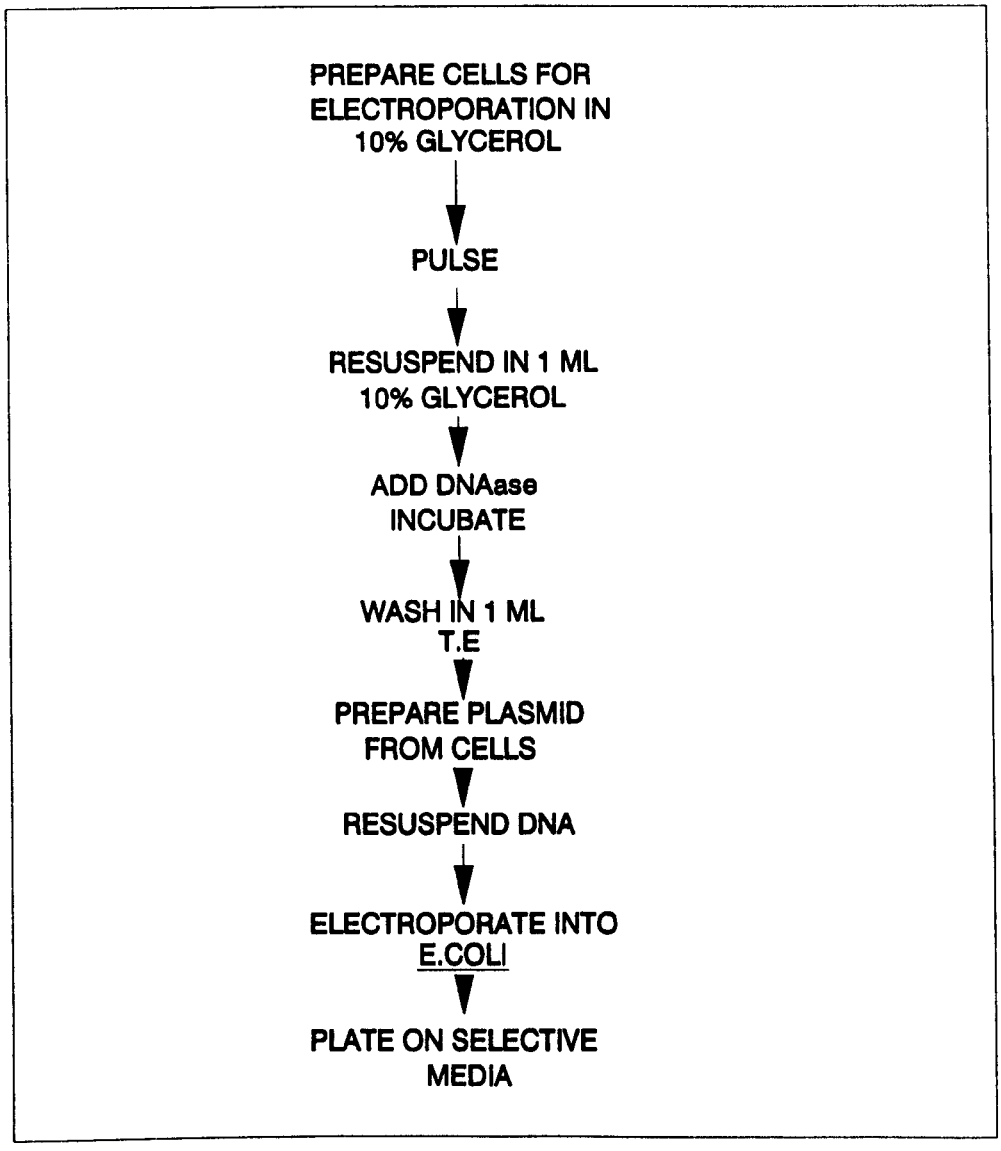


Figure 3.3.2: Does plasmid enter the cells of M.capsulatus following electroporation?

Control	Cell Type (E.c or M.c)	Pulse	DNAase	Plasmid	Colonies
A	M.c	No	No	Yes	No
B	M.c	Yes	No	No	No
C	M.c	No	No	No	No
D	M.c	No	Yes	Yes	No
1	E.c	No	No	Yes	No
2	E.c	Yes	No	No	No
3	E.c	No	No	No	No
4	E.c	No	Yes	Yes	No
5	E.c	Yes	Yes	Yes	Yes
6	No	No	No	Yes	No

M.c = *M.capsulatus*
E.c = *E.coli*

Explainations of what controls indicate

A and 1 = Plasmid DNA in solution around cells of *M.capsulatus* or *E.coli* does not enter the cell to produce transformants by some unknown method.

B and 2 = The cultures of cells used did not contain plasmids at the onset of the experiments.

C and 3 = The cultures of cells used did not contain plasmids at the onset of the experiments.

D and 4 = Plasmid DNA in solution, possibly attached to the cells was destroyed by the DNAase and not carried through the plasmid isolation step.

5 = Plasmid electroporated into *E.coli* could be subsequently isolated by the method and detected by re-electroporation into *E.coli*.

6 = Plasmid in solution was not carried through the method.

In conclusion, the only DNA that was eventually electroporated into *E.coli* was that which had entered the original cells by electroporation.

Table 3.3.10 - continued on next page-

pVK100 Cell Type E.c or M.c	R (Ω)	No. of colonies of *E.coli* (Average of 10 plates)
M.c	200	1
M.c	400	5
M.c	600	52
E.c	200	>100
E.c	400	>100
E.c	600	>62
pJFF350		
M.c	200	0
M.c	400	13
M.c	600	44
E.c	200	>100

M.c = *M.capsulatus*
E.c = *E.coli*

Table 3.3.10 Are plasmids pVK100 and pJFF350 transferred into *M.capsulatus* by electroporation.

been isolated from *E.coli*, and may therefore have been subject to restriction and modification systems in *M.capsulatus*. pVK100 and pDSK509 isolated from *M.capsulatus* (section 2.2.10) were used in electroporation experiments with a number of combinations of field strength and time constant. However, no electro-transformants were isolated.

3.3.6 Electrotransfer

Electrotransfer is the process by which plasmid is transferred between bacteria by an electric pulse being applied to a mixture of a plasmid-containing strain and a non-plasmid containing strain. This procedure was attempted with *M.capsulatus* and *E.coli* (DH1). The *E.coli* contained pDSK509 and was prepared for electroporation resuspended in 10% glycerol. The *M.capsulatus* contained no plasmid and was was prepared for electroporation resuspended in 10% glycerol. 20 μl of each culture was mixed in an eppendorf tube, then transferred to a cold 0.2 cm electroporation cuvette. The mixture was subjected to an electric pulse of 12.5 kV/cm (R=200 , 25 μF), then 0.9 ml of NMS plus 0.02% (w/v) Proteose-Peptone was added and the solution transferred to 50 ml of NMS in a 250 ml flask. The *M.capsulatus* was put through the normal recovery procedure and kanamycin (50 μg/ml) was added to the flask after 24 hours. After 4 days the culture was centrifuged and

resuspended in 1 ml NMS and plated onto NMS plus kanamycin (50 μg/ml). No transformants were isolated.

3.3.7 Discussion

To date a great many Gram-negative bacteria have been transformed by electroporation. The methods have been optimized by the alteration of many factors that play a role in electroporation. A survey of the literature reveals that there is great variance between conditions that give maximum transformation efficiency for different species of bacteria and even differences between strains (Vande Broeck *et al.*, 1989). One conclusion however that has been drawn by many workers is that the electrical parameters are of paramount importance and that alteration of many of the other conditions is merely 'fine-tuning' the system for maximum efficiency (Dower *et al.*, 1988; Miller *et al.*, 1988; Ueda *et al.*, 1991; Chassy *et al.*, 1988).

So far electroporation has been unsuccessful for *M.capsulatus*. The reasons for this failure are not totally clear. Theoretically, on a purely physical basis, all bacteria should be electrotransformable. The failure to electro-transform *M.capsulatus* could be due to one or several of the following reasons:

a) Electrical factors- non-permeabilization of the membrane to allow entry of the DNA

b) Choice of DNA/host restriction barriers

c) Inadequate recovery procedure

d) Physiological state of the cells

e) Medium for electroporation

a) Electrical factors

The electrical parameters are the strength of the electrical field and the duration of the pulse. Sufficient intensity of current must be applied to produce the optimum number of permeabilized areas for DNA entry. The intensity of current is a function of the field strength and time constant. Dower *et al.* (1988), working with *E.coli*, found that above a certain threshold field strength there was a reciprocal relationship between field strength and time constant, i.e, the efficiency of electroporation at 7 kV/cm and 20 msec was the same as that at 11 kV/cm and 5 msec.

In the process of developing a system of electroporation for an organism, the most important step is to determine the threshold field strength. From the literature surveyed, it appears that for Gram-negative organisms, although electroporation may not be very efficient at this threshold field strength, it does take place and it is then a case of optimizing the system by further alteration of the electrical factors or the adjustment of some other parameter.

The determining factors for the threshold field strength are the size and shape of the cell, the membrane

composition and the presence of surrounding structures such as capsules.

Theoretically spherical cells with small diameters should require greater field strengths than larger rod-shaped cells (Chassy *et al.*, 1988). On the strength of this theory it may be predicted that *M.capsulatus* would require a high field strength, being spherical with a diameter of only 1.0 μm (Green, 1992). For a long time the maximum field strength available was 12.5 kV/cm and this was unsuccessful, with the advent of the 0.1 cm cuvettes it was possible to acheive 25 kV/cm. This is an extremely high field strength which was not easy to obtain without arcing. Arcing is due normally to salt residues which would be thought to occur at random in a certain culture but since the same culture was used for the experiments at field strengths above 12.5 kV/cm, and arcing did not occur below 17 kV/cm the arcing may well have been due to rupture of the cells 'en masse' and thus creating a sudden increase in conductivity of the culture. It is extremely difficult to determine the effects occurring in the culture at these high field strengths as they have not been used for many organisms. Zealey *et al.*, (1988) went up to field strengths of 25 kV/cm to electroporate *Bordetella pertussis*. Although maximal transformation was obtained at this field strength some transformants were obtained at field strengths of 8 Kv/cm.

It has been observed with many organisms that the passage of DNA into the cell is most efficient when the

field strength also produces a certain percentage of cell death. For example the most efficient electroporation of *E.coli* is achieved when there is 50-75% cell death (Dower *et al.*, 1988). The lack of a high percentage of cell death at field strengths up to 17 kV/cm could have been indicative of a total lack of effect of these electric pulses on the cells. However, the death of a significant proportion of the cell population is not a prerequisite for efficient transformation of other organisms, e.g., *Bordetella pertussis* (Zealey *et al.*, 1988), *Camplylobacter jejuni* (Miller *et al.*, 1988), and *Yersinia* (Conchas & Carniel, 1990). If the problem of arcing could be dealt with perhaps by incorporating an extra wash in the preparation of the cells (if indeed the presence of salt is the problem) the higher field strengths might be used successfully.

The field strength can also be altered, in that multiple pulses can be administered to the culture. This method was successful for the facultative methylotroph *Methylobacterium extorquens* NR-2. This organism requires 10 pulses at 3 mSec and 10 kV/cm for maximal transformation (Ueda *et al.*, 1991). Increasing the number of pulses did not result in the isolation of electro-transformants for *M.capsulatus*. It was however impossible to deliver 10 pulses due to the tendency for arcing to occur. The apparatus used by Ueda *et al.* (1991) was different from the Bio-rad apparatus available for these experiments, the other apparatus was able to deliver several pulses at 0.5 sec

intervals. Increasing the number of pulses has been found not to significantly increase transformation efficiency for many organisms and is often detrimental to the efficiency, probably due to the rise in temperature of 15-25^{0}C caused by a single high voltage pulse (Chassy *et al.*, 1988)

The other electrical parameter, that of time constant (tc) can be altered but is thought not to have much effect until the threshold field strength has been reached. Despite various combinations of time constant and field strength no *M.capsulatus* electro-transformants were produced. The initial conclusion from these results would be that the threshold field strength had not been reached and not enough pores were being produced in the cell membrane to allow entry of the DNA. If this is indeed the case the logical next step is to treat the cell to increase the permeability of the cell.

The pretreatments attempted with *M.capsulatus* were designed to weaken the cell wall and although the treatments did appear to increase the cells susceptibility to lysis, (In the case of CTAB treatment to lysis by the electric pulse and in the case of lysozyme to lysis by SDS), they did not produce any electro-transformants. The cells appeared to survive the treatments, but it may be that cells that had been weakened sufficiently to allow DNA entry were not able to recover from damage by these substances. Detergents such as CTAB are very difficult to wash from the medium and trace amounts may have been present in the medium in which the

cells were electroporated which could have adversely affected the cells.

The use of lysozyme to inhibit the correct cross-linkages being formed in peptidoglycan and so weaken the cells, to make them osmotically sensitive, although unsuccessful in *M.capsulatus*, has been found to be successful in increasing the frequency of electroporation in the Gram-positive organism *Corynebacterium glutamicum* (Wolf *et al.*, 1989). The use of freeze-thaw cycles has also been found to be effective for this organism. If indeed the lack of success of electroporation for *M.capsulatus* is due to the inability of the DNA to enter the cells, there are several more treatments that could possibly be attempted, such as the addition of EDTA to the medium which has been shown to increase the efficiency of electroporation in *E.coli* (Cymbalyuk *et al.*, 1988), probably due to producing an increase in permeability of the external membrane. However, *M.capsulatus* cells are not difficult to lyse when treated with lysozyme during preparation of plasmid DNA, which would indicate that they do not have extraordinarily tough cell walls. Wirth *et al* (1989) found that the organisms that did not lyse with lysozyme during attempted plasmid preparation would not electroporate either and so concluded that there was definitely a relationship between susceptibility of the cell wall to lysis and ability to be electroporated.

It may be that the right combination of treatments with the correct field strength and time constant have not

been tried, particularly as many of the treatments were not carried out with the new 0.1 cm cuvettes. It must be pointed out that, although the conditions for maximal electro-transformation may be very specific for a particular organism there is usually a very wide window of conditions in which electro-transformants are obtained, it is unusual to not get even one electro-transformant.

The DNAase experiment to determine whether DNA did in fact enter the cells upon administration of the electric pulse could not determine for certain that DNA enters the cells. Increasing the time constant increasing the number of *E.coli* transformants produced in the last stage of the experiment, may have indicated that increasing the time constant increased the number of *M.capsulatus* containing plasmid. This result coupled with the fact that, it is very unusual to not get even one transformant, might indicate that it is not the electrical parameters that need to be altered but that some other factor is involved.

b) Choice of DNA

The plasmids chosen for all the electroporation experiments have all been shown to be transferred by conjugation into *M.capsulatus*. pVK100, RP4 and pDSK509 are all stably maintained and pJFF350 is able to transfer into the chromosome. All the plasmids contain genes which express kanamycin resistance factors at a high enough level to be able to cope with 50 μg/ml of kanamycin in the medium.

However, the majority of the experiments were carried out with DNA that had been prepared from *E.coli* and it has been well documented that the presence of a restriction/modification system in the host cell can significantly decrease the electroporation efficiency (Miller *et al.*, 1988; Hatterman & Stacey, 1990; Marcus *et al.*, 1990, Guerinot *et al.*, 1990) and in the case of *Aquaspirillum itersonii*, no transformants were obtained if the RP4 used was obtained from *E.coli* (Eden & Blakemore, 1991). Wirth *et al* (1989) however, using pKT231 prepared from *E.coli* HB101, were able to transform successfully a range of species of Gram-negative bacteria, both laboratory and recently isolated "wild-type" strains.

The mechanism by which DNA enters the cells by electroporation is presumed to be by passive diffusion (Dower *et al.*, 1988) through transient pores produced by passage of the electrical pulse and there is no involvement of membrane proteins, plasmid genes or chromosomal genes. Eden & Blakemore (1991) observed for *Aquaspirillum itersonii* that RP4 could be conjugally transferred into *A. itersonii* from *E.coli* and the plasmid was stably maintained with no observable alteration in the DNA. However, RP4 prepared from *E.coli* and electroporated into *A.itersonii* was not stably maintained. They concluded that this was an indication that the presence of conjugal transfer-related plasmid genes engender protection from restriction barriers. The model proposed for conjugal transfer of plasmids is the

transfer of a single strand of DNA which is then used as a template for production of the second strand of DNA once the plasmid is in the cell. The first strand is thought to not be subject to the restriction systems and the second strand produced by the host cell will be modified (Willetts & Wilkins, 1984). Plasmid entering the cell by electroporation is double-stranded-unmodified and therefore a target for the host restriction systems.

The susceptibility of plasmids to restriction in *M.capsulatus* could possibly be indicated by the results of the experiments designed to determine whether DNA does in fact enter the cell of *M.capsulatus* following the electric pulse. These results may indicate that plasmid does enter the cell where it is protected from the action of DNAase. If this is the case the barrier to successful electroporation of *M.capsulatus* may lie in the choice of plasmid and more success may be achieved if plasmid isolated from *M.capsulatus* were used.

PVK100 and pDSK509 obtained from *M.capsulatus* were used for a few experiments but the full range of conditions has yet to be tried. If a large enough quantity of plasmid is not obtainable from *M.capsulatus*, or as in the case for transposon mutagenesis vectors, unable to be obtained from *M.capsulatus*, the plasmid could be prepared in *E.coli* strains lacking at least one of the three modification systems. This approach has been successfully used for *Bradyrhizobium japonicum* (Guerinot *et al.*, 1990).

c) Recovery procedure

The recovery procedure is of great importance as the process of electroporation damages the cell and the conditions after the event must be adequate for the cell to be able to repair this damage. The recovery procedure for *E.coli* was based on the recovery procedure developed for chemical transformation. However, for *M.capsulatus* there is no such method upon which a recovery procedure could be based therefore, the ideal growth conditions for *M.capsulatus* must be considered as the starting point for the development of the recovery procedure. *M.capsulatus* requires oxygen and methane and its growth has also been shown to be stimulated by the presence of 0.02% w/v Proteose-Peptone in the medium. The final recovery procedure used for the cells would appear to contain all the correct factors for the adequate recovery of *M.capsulatus* following electroporation, but there could still be something lacking in the procedure. The selective antibiotic was added after 24 or 48 hours of recovery, this length of time was thought to be sufficient for cell recovery and expression of the antibiotic resistance genes on the plasmid.

d) Physiological state of the cell

In the majority of electroporation experiments the *M.capsulatus* cells were taken from a fermenter culture on continuous culture. This method would have produced a supply

of healthy cells not limited to the extent that growth would be in the stationary phase. The cells used from batch culture were grown to an OD_{600} of 0.2-0.3, mid-logarithmic stage of growth.

Although it was presumed that electroporation would be more efficient if cells at the most active stage of early to mid-logarithmic growth were used, this has not always been found to be the case. *E.coli* (Dower *et al.*, 1988), *Actinobacillus pleuropneumoniae* and *Methylobacterium extorquens* (Ueda *et al.*, 1991) were all most efficiently electroporated when in early to mid logarithmic growth. Wirth *et al.*(1989) in their study of 11 different genera of Gram-negative bacteria chose to grow them to mid-logarithmic growth. Other workers however, found that the growth phase of the cells did not effect the transformation of the cells (Miller *et al.*, 1988; Thomson & Flint, 1989; Smith *et al.*, 1990; Zealey *et al.*, 1988; Lalonde *et al.*, 1989). The type of growth medium of the cells prior to being prepared for electroporation has been found to be important in some organisms, for example *Campylobacter jejuni* can be transformed when grown on sheep blood agar or Mueller Hinton agar but not when grown in Brucella broth or Mueller Hinton broth (Miller *et al.*, 1988). The possibility of using cells of *M.capsulatus* grown on agar has not yet been explored.

e) Medium for electroporation

The 10% glycerol used for the resuspension of *M.capsulatus* did not appear to adversely affect the cells

and would not therefore be an obvious cause for the failure of electroporation, but, there are several other buffers that have been used for other organisms that could be tried. Buffers that could be used include ones that are phosphate and sucrose based.

There are a large number of factors that can be altered producing a wide variety of factor combinations. One possibility is to work as it where 'backwards', i.e, start with a culture that contains a plasmid, subject it to an electric pulse and look for plasmid curing. It has been shown by Heery *et al.* (1989) that *E.coli* cultures can be cured at a frequency of 80-90% under conditions that would normally produce efficient electroporation. This experiment would indicate whether or not pores where produced in the cells through which DNA could pass, be it in or out of the cell.

In conclusion, the results of numerous electroporation experiments would seem to indicate that the greatest barrier to successful electroporation of *M.capsulatus* is the restriction of the plasmid entering the cell. It is possible to obtain plasmid DNA in reasonable quantities from *M.capsulatus* (see section 3.1.4.2) that has been transferred in by conjugation, and this could be used for electroporation. However, the fact that the DNA has to be isolated from *M.capsulatus* restricts the variety of plasmids that can be used. However there is still the possibility of the isolation of plasmids from either *E.coli*

lacking certain modification systems or plasmid isolated from another organism, perhaps even isolated from another methanotroph.

RESULTS

ATTEMPTS TO MOBILIZE THE CHROMOSOME OF M.*CAPSULATUS*

3.4 ATTEMPTS TO MOBILIZE THE CHROMOSOME OF *M.CAPSULATUS*

Mobilization of the chromosome of methylotrophs by IncP and P1 broad host range plasmids has been discussed in detail in section 1.4. This genetic technique has enabled preliminary mapping of the chromosome of several methylotrophs and is a valuable tool in the elucidation of chromosomal organization. Although the plasmids R68.45 and pULB113 have been transferred into the methanotroph *Methylosinus trichosporium* (Warner *et al.*, 1980; Al-Taho & Warner, 1987) and RP4 into *Methylomonas albus* (McPheat *et al.*, 1987), the possibility that mobilization of the chromosome occurred was not looked at in detail. So far two broad host range plasmids that have chromosome mobilizing ability, RP4 and pULB113, have been transferred by conjugation into *M.capsulatus* (section 3.1), and the possibility that they have chromosome mobilizing ability in *M.capsulatus* was investigated. Two approaches to the problem were investigated. The plasmids used are indicated in table 3.4.1, along with their transfer frequencies per recipient.

Plasmid	Transfer frequency E.c to M.c	Transfer frequency M.c to E.c
RP4	4.3×10^{-8}	3.22×10^{-5}
pULB113	1.5×10^{-6}	5.3×10^{-4}
pSUP::Tn5B10	0	0
pSUP::Tn5B11	0	0
pSUP::TnB12	0	0

Table 3.4.1

3.4.1 RP4 and pULB113

pULB113 (Van Gijsegem & Toussaint, 1982) is a derivative of RP4 containing a defective mu phage (Figure 3.4.1). The bacteriophage is defective in all functions that cause cell death, but transposition into the chromosome can still occur, thus allowing random insertion of the 'phage into the chromosome. The 'phage DNA transposes into the chromosome producing a region of DNA homologous with the pULB113 present in the cell and the plasmid is able to integrate into the chromosome. The mobilization of pULB113 from the cell results also in mobilization of chromosome surrounding the plasmid.

pULB113 and RP4 were transferred to *M.capsulatus* by the filter mating technique. The cells from the filter (*E.coli* and *M.capsulatus*) were resuspended in 50 ml NMS plus 50μg/ml kanamycin and grown at 45^{0}C under conditions favourable for growth of *M.capsulatus*. It was assumed that the growth temperature of 45^{0}C and the lack of any carbon source other than methane would select against the *E.coli* present in the culture. After several days growth the entire culture was used in fiter mating experiments with several *E.coli* auxotrophs, using DH1 as a control to check for transfer. (Table 4.1.2).

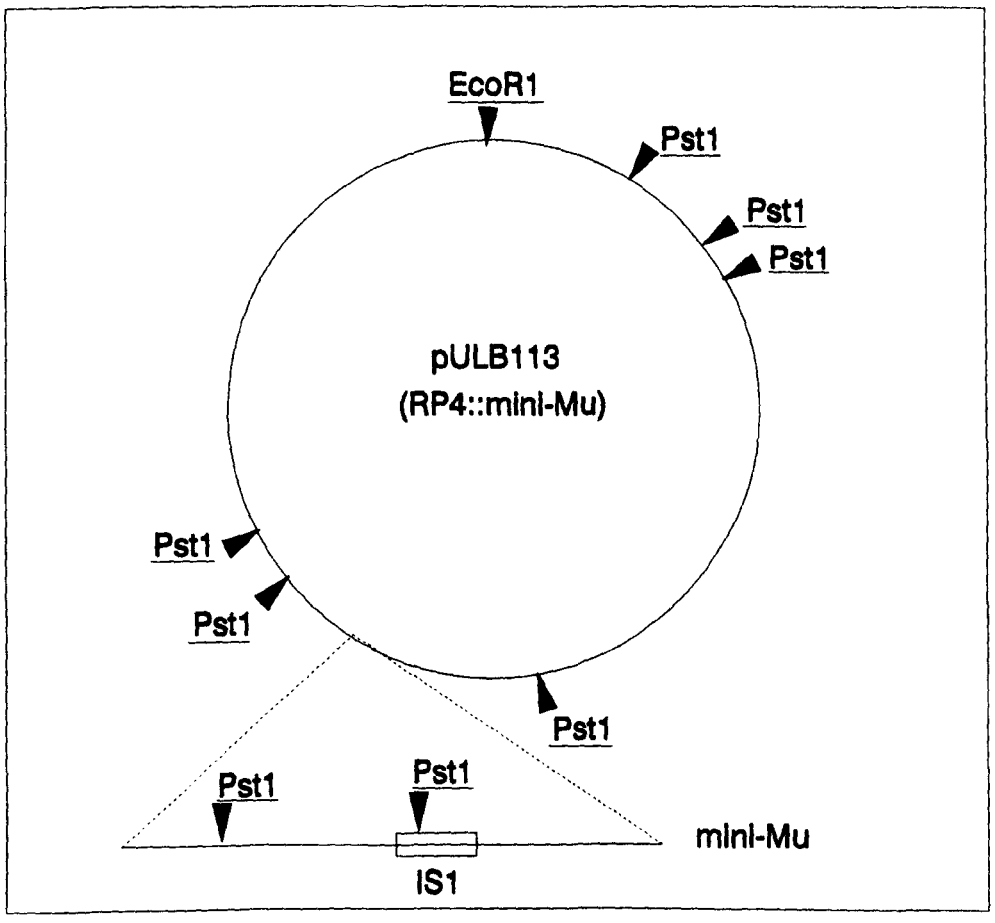

EcoR1
Pst1
Pst1
Pst1
pULB113
(RP4::mini-Mu)
Pst1
Pst1
Pst1
Pst1
Pst1
mini-Mu
IS1

Figure 3.4.1

E.coli	Amino acids required in M9 medium for growth					
	thr	leu	thi	pro	his	arg
9482	+	+	+	-	-	-
10241	-	-	-	+	+	-
11865	-	-	-	+	-	-
ET8045	-	-	-	-	-	+

Table 3.4.2

After 24 hours on the filter, cells were plated onto selective M9 minimal media plates containing the appropriate amino acid supplements at 0.02% (w/v), but omitting one amino acid required for growth of the auxotroph. The transconjugants grew on M9 medium containing all the supplements, +/- kanamycin, but if an amino acid supplement were omitted, no growth was obtained. The presence of kanamycin resistance indicated transfer of RP4 and pULB113, at the frequencies given in table 3.4.1 for DH1, but no complementation of any of the auxotrophic markers. Plasmid was prepared from several of the transconjugants and restriction digests carried out. Both RP4 and pULB113 isolated from the transconjugants contained no extra DNA nor did they appear to have any deletions or rearrangements in the plasmid DNA, and they expressed all their resistance markers in *E.coli*.

3.4.2 The production of *in-vitro* prime plasmids with RP4

This approach has been used successfully to mobilize the chromosome of *Proteus morganii* (Beck *et al.*, 1982), and also to increase the mobilization frequency for *E.coli* (Barth, 1979). The method is outlined in figure 3.4.2. Previous studies have chosen to use the *Hin*dIII site but in this study the *Bam*H1 site was the site for insertion of the chromosome fragments, since the *Hin*dIII site lies within the kanamycin resistance gene which is necessary to select for transconjugants of *M.capsulatus*. The *Bam*H1 cut RP4 was phosphatased and mixed in a ratio of 1 vector to 5 chromosome. 5 μl of the 12 μl ligation mix was electroporated into *E.coli* DH1. 10 ml of LB plus kanamycin (50 μg/ml) were added to the 1 ml electroporation mixture to select for transformants containing RP4. The mixture was left to grow overnight. The culture was assumed to contain a variety of RP4 plasmids containing *M.capsulatus* chromosomal fragments. From this culture the RP4 derivatives where transferred 'en masse' to *M.capsulatus* by filter-matings. Kanamycin resistant *M.capsulatus* resulting from conjugation with *E.coli* containing RP4 prime plasmids were treated as in section 3.4.1 and filter mated 'en masse' with several *E.coli* auxotrophs. No chromosome mobilization or complementation of auxotrophs was found.

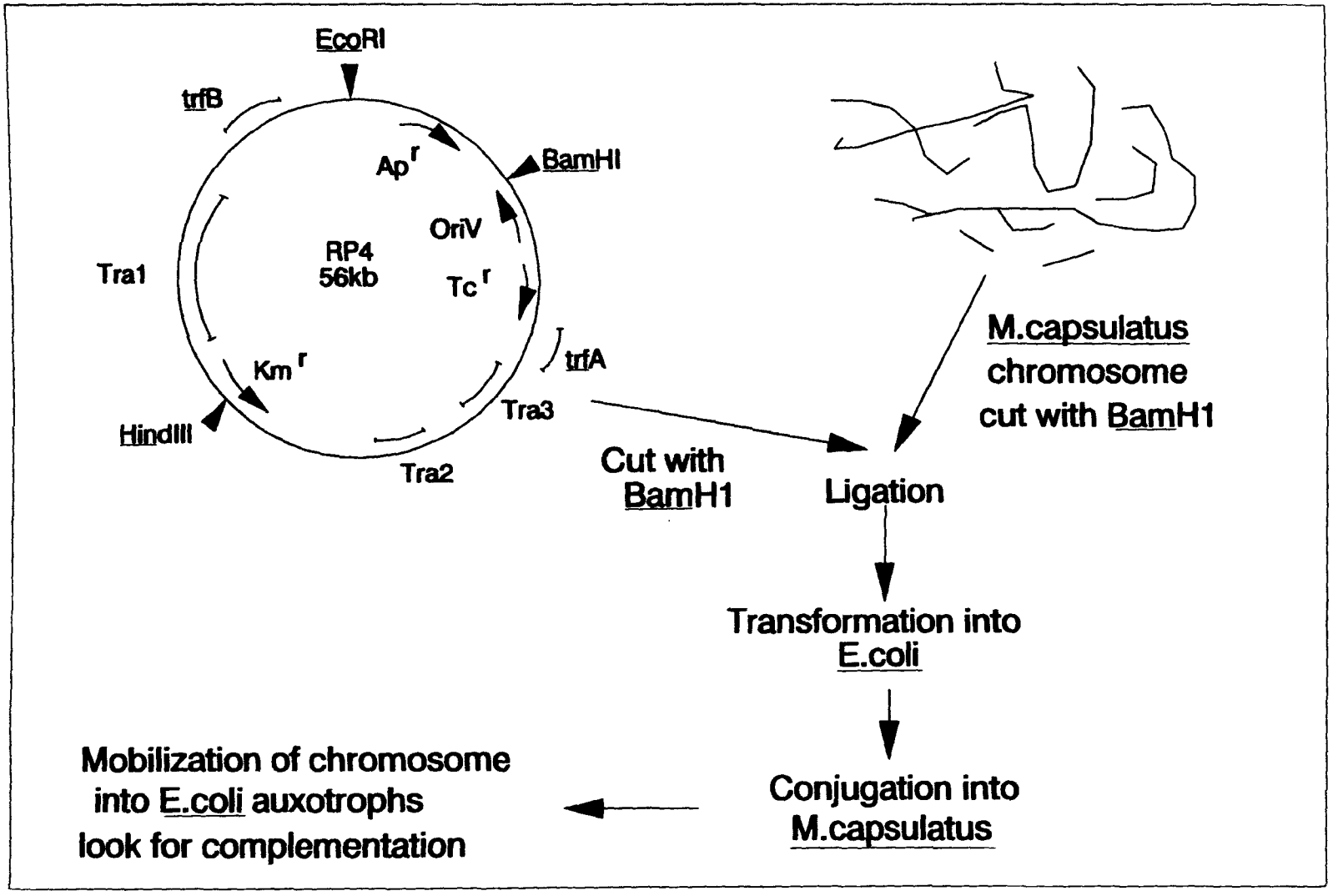


Figure 3.4.2: Production of in-vitro prime plasmids with RP4

100 µl of the *E.coli* mixture containing the RP4 derivatives were plated onto LB plus kanamycin (50 µg/ml) to select for RP4 containing *E.coli*. All the colonies analyzed (40 in total) contained RP4 or derivatives, 71% of the derivatives contained either one or two inserts, of 6.6 Kb or 5.0 Kb, or they contained both of these sized inserts. There was not a range of insert sizes.

3.4.3 The Use of a Transposable *mob* or *oriT* Region.

The final approach was the use of three plasmids designed by Simon *et al.*, (1983), pSUP::Tn5B10, B11 and B12. These plasmids all contained the *mob* (or *oriT*) region of RP4 on the transposon Tn5. The entire cassette was on a suicide vector derivative of pBR325, pSUP202. These plasmids could be transferred by conjugation to *M.capsulatus*, where the transposon would insert into the chromosome along with the *mob* region. This insertion could be selected for by the resistance determinant present on the Tn5, kanamycin in the case of B10 and gentamicin in the case of B11 and B12. There should now be a region on the *M.capsulatus* chromosome which could act as a region of homology with RP4 for mobilization by RP4 subsequently transferred into *M.capsulatus*. However, no transconjugants were obtained from conjugations with any of the plasmids.

3.4.4 Discussion

Two problems have been found with attempts to mobilize the chromosome of organisms such as methylotrophs and methanotrophs. The first is that the resulting plasmids which contain chromosomal DNA can be subject to restriction in the *E.coli* to which they are transferred. The possibility of restriction and modification is increased due to the duplication of either an insertion sequence or in the case of pULB113 the mini-mu sequence, part of the process of mobilization by these broad host range plasmids. The inserted chromosomal DNA is surrounded by these sequences and so is subject to excision and rearrangement in the *E.coli*. This problem can be avoided by the use of restriction deficient *E.coli* strain, such as the K-12 derivative DH1, as hosts. The *E.coli* used in this study were all restriction deficient which should have reduced this problem.

The second problem is that relying on complementation of *E.coli* auxotrophs for detection of chromosomal mobilization assumes that the mutations in *E.coli* can be complemented by genes from *M.capsulatus*. Although the *glnA* of *M.capsulatus* has been shown to complement a *glnA* mutant of *E.coli* (Cardy, 1988) one can not assume that this is always going to be the case. However, there is now a large number of mutants of the methylotroph *Methylobacterium* AM1 (Tatra & Goodwin, 1985), and *M.flagellatum* (Tsygankov *et al.*, 1990) and it would perhaps

be better to use these methylotrophs as hosts if transfer to them is possible since they may provide a better heterologous background in which to express methanotroph genes. There are currently no available methanotroph mutants which could be used.

The plasmids resulting from ligation of RP4 with chromosomal DNA fragments, of a range of sizes, contained only inserts of two sizes. There may be two possible causes, either the ligation conditions some how favoured these sizes, or the more likely explaination is that the initial transfer by electroporation was very inefficient resulting in a low number of transformed *E.coli*. During recovery RP4 containing the two insert sizes would have rapidly been transferred to a large number of *E.coli* present that did not contain RP4, thus producing a population containing a very limited type of RP4 prime plasmid.

The possibility of producing cosmid gene banks, which contain large amounts of *M.capsulatus* chromosomal DNA and which can then be used to *E.coli* complement mutants (section 1.4) has not yet been investigated. This method of chromosme mapping (section 1.4) does not rely upon mobilization of the chromosome by a plasmid and could perhaps be the way forward for mapping the *M.capsulatus* chromosome.

One possibility for the mobilization of the chromosme of *M.capsulatus*, that has already been discussed in section 3.2, is the transfer by pJFF350 of the RP4 *mob*

region into the chromosome. There is now a region in the chromosome which can be used for mobilization by RP4. Whether this will be successful has yet to be determined.

GENERAL OVERVIEW

4. GENERAL OVERVIEW

The biochemistry of methane oxidation in the methanotroph *Methylococcus capsulatus* (Bath) is perhaps the most highly characterized of all the methanotrophs, but further characterization at the genetic level is hampered by the lack of any genetic systems, such as those for mutagenesis and the study of gene expression. These studies were initiated to develop techniques for the transfer of heterologous and homologous DNA into *M.capsulatus*, and thereby leading to the development of a system for the production of mutants of *M.capsulatus*.

The development of a system for the transfer of DNA into *M.capsulatus* was initiated with the determination of suitable plasmids. *M.capsulatus* was found to exhibit resistance to a great many antibiotics and therefore, the plasmids that could be used were limited to broad-host range plasmids encoding kanamycin resistance.

A system was developed for the transfer of plasmids into *M.capsulatus* by a filter-mating system. Using the transfer of pVK100 in a triparental conjugation, with pRK2013 as the helper plasmid, as the test conjugation, it was found that transfer was most efficient if *M.capsulatus* was in early logarithmic growth and transfer was allowed to take place over a 24 hour time period at 37^{0}C. It was also possible to transfer RP4 and pULB113 from *M.capsulatus* to *E.coli*.

Several plasmids were transferred using the method, all of which relied upon the RP4 mobilization system and either the IncP or Q replication systems. The frequency of isolation of antibiotic resistant *M.capsulatus*, i.e, the frequency of transfer and maintenance of the plasmid in the cell, varied, even between plasmids that were very similar, and the possibility of a restriction/modification system effecting plasmid stability could not be ruled out. The frequency of transfer of the majority of plasmids, pDSK509 being the exception, was not high but sufficient for the transfer of vectors for the study of gene expression. Further development of these vectors will now be possible to produce ones for a variety of genetic analysis.

Several techniques used for *E.coli*, such as plasmid preparation, colony hybridization, have been altered for use with *M.capsulatus*.

Utilizing the filter-mating system it was possible to transfer several vectors capable of random mutagenesis, in other microorganisms, into *M.capsulatus*. Of these vectors, two showed insertion of antibiotic markers into the chromosome. pSUP2021 inserted Tn5 into the chromosome, however, no auxotrophs were isolated. Previous attempts to insert Tn5 into the chromosome of methanotrophs have been unsuccessful (Lidstrom *et al.*, 1984). The entire pJFF350 vector, or a large part of it, was found to insert into the chromosome as identified by the acquisition of kanamycin resistance due to insertion of a Tn903 derivative coupled

with an Omegon fragment, and hybridization studies of chromosome from kanamycin reistant colonies. Unfortunetly, the insertion of large amounts, or the entire pJFF350 vector, would make analysis of any mutants extremely difficult. No auxotrophs were isolated using this plasmid for random mutagenesis. The insertion of pJFF350 into the chromosome allowed the production of novel plasmids containing pJFF350 DNA and *M.capsulatus* chromosomal DNA. The use of *Xho*1 to restrict the chromosome would allow the possibility for the isolation of mutated chromosomal genes, and perhaps in future the use of these novel plasmids in complementation studies with any *M.capsulatus* mutants or mutants of any methanotroph or methylotroph.

The vector pJFF350 was used to develop the vector pSD100 in an attempt to produce a *glnA* mutant by marker-exchange mutagenesis. The vector was transferred by filter-mating and kanamycin resistant *M.capsulatus* were isolated, transfer of the vector into the chromosome was confirmed by hybridization studies, however, it was not clear what type of insertion event had occurred. Two other vectors, pDC2 and pSD10, both developed for marker exchange mutagenesis of *glnA*, were unsuccessful.

Electroporation, as a method for the transfer of plasmid DNA into *M.capsulatus*, was unsuccessful, despite the testing of a wide range of electrical conditions and pretreatments of the cells. The inability to isolate even

one transformant together with the results of the experiment to determine whether plasmid entered the cell, may indicate that the problem was plasmid instability Further tests on the use of electroporation should concentrate on plasmid isolated from *M.capsulatus*, or another methanotroph, or plasmid isolated from *E.coli* defective in several restriction systems.

No evidence of chromosome mobilization by RP4 or pULB113 was found but the transfer of RP4-*mob* to the chromosme by pJFF350 may lead to a method for the mobilization of the chromosome of *M.capsulatus*.

In conclusion these results indicate that *M.capsulatus* is not amenable to many of the genetic techniques used routinely for other Gram-negative organisms. There are as yet many unknown events surrounding the transfer of heterologous DNA into the organism. However, the studies outlined have highlighted several vectors and methods which, if perhaps not used for *M.capsulatus*, have potential for use for another methanotroph, one in which it is perhaps easier to isolate mutants

REFERENCES

5. REFERENCES

Allen, L.N. and Hanson, R.S. (1985) Construction of broad-host range cosmid cloning vectors: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. J. Bact. 161(3):955-962

Al-Taho, N.M. and Warner, P.J. (1987) Restoration of phenotype in *Escherichia coli* auxotrophs by pULB113-mediated mobilization from methylotrophic bacteria. FEMS Microbiol. Lett. 43:235-239

Al-Taho, N.M., Cornish, A., and Warner, P.J. (1990) Molecular cloning of the methanol dehydrogenase structural gene from *Methylosinus trichosporium* OB3b. Curr. Microbiol. 20:153-157

Anderson, D.J. and Lidstrom, M.E. (1988) The *moxFG* region encodes four polypeptides in the methanol-oxidising bacterium *Methylobacterium* sp. strain AM1. J. Bact. 170(5):2254-2262.

Anderson, D.J., Morris, J.C., Nunn, D.N., Anthony, C., and Lidstrom, M.E. (1990) Nucleotide sequence of the *Methylobacterium extorquens* AM1 *moxF* and *moxJ* genes involved in methanol oxidation. Gene 90:173-176.

Anthony, C. (1982) Methanol dehydrogenase, formaldehyde dehydrogenase and formate oxidation in, The biochemistry of the methylotrophs.(Anthony, C. ed.) pp 167-194

Anthony, C. (1986) Bacterial oxidation of methane and methanol. Adv. Microb. Physiol. 27:113-210

Augustin, J. and Gotz, F. (1990) Transformation of *Staphylococcus epidermidis* and other staphylococcal species with plasmid DNA by electroporation. FEMS Microbiol. Lett. 66:203-208.

Bagdasarian, M., Lutz, R., Ruckert, B.F., Franklin, C.H., Bagdasarian, M.M., Frey, J., and Timmis, K.N.(1981) Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene 16:237-247.

Bainbridge, B.W. (1983) The potential of methylotrophic bacteria: genetic approaches to improving bacteria of industrial interest in, Advances in Fermentation Conference Proceedings, Wheatland Journals, Ricksmansworth, England, pp 97-107

Bamforth, C.W. and Quayle, J.R. (1977) Hydroxypyruvate reductase activity in *Paracoccus denitrificans*. J. Gen. Micro. 101:259-267

Barth, P.T. (1979) Plasmid RP4, with *Escherichia coli* DNA inserted *in vitro*, mediates chromosomal transfer. Plasmid 2:130-136.

Bastien, C., Machlin, S., Zhang, Y., Donaldson, K., and Hanson, R.S. (1989) Organization of genes required for the oxidation of methanol to formaldehyde in three type II methylotrophs. App. Env. Micro. 55(32):3124-3130

Beck, Y., Coetzee, W.F., and Coetzee, J.N. (1982) *In vitro* constructed RP4-prime plasmids mediate orientated Mobilization of the *Proteus morganii* chromosome. J. Gen. Micro. 128:1163-1169

Belas, R., Erskine, D., and Flaherty, D. (1991) Transposon mutagenesis in *Proteus mirabilis*. J. Bact. 173(19):6289-6293.

Bennett, P.M., Grinstead, J., and Richmond, M.H. (1977) Transposition of TnA does not generate deletions. Mol. Gen. Genet. 154:205-211

Berg, D.E. and Berg, C.M. (1983) The prokaryote transposable element Tn5. Biotechnology 1:417-435

Beringer, J.E. (1974) R factor transfer in *Rhizobium leguminosarum*. J. Gen. Micro. 84:188-198

Beringer, J.E., Beynon, J.L. Buchanan-wollaston, A.V., and Johnston, A.W.B. (1978) Transfer of the drug-resistance transposon Tn5 to *Rhizobium*. Nature (London) 276:633-634

Best, D.J and Higgins, I.J. (1981) Methane-oxidizing activity and membrane morphology in a methanol-grown obligate methanotroph, *Methylosinus trichosporium* OB3b. J. Gen. Micro. 125 :73-84

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction for screening recombinant plasmid DNA. Nucleic Acids Research 1:1513-1523

Biville, F., Turlin, E., and Gasser, F. (1989) Cloning and genetic analysis of six pyrroloquinoline quinone biosynthesis genes in *Methylobacterium organophilum* DSM760. J. Gen. Micro. 135:2917-2929.

Biville, F., Turlin, E., and Gasser F. (1991) Mutants of *Escherichia coli* producing pyrrolquinoline quinone. J. Gen. Micro. 137:1775-1782

Bohanon, M.J., Bastien, C.A., Yoshida, R., and Hanson, R.S. (1988) Isolation of auxotrophic mutants of *Methylophilus methylotrophus* by modified-marker exchange. App. Env. Micro. 54(1):271-273.

Bonamy, C., Guyonvarch, A., Reyes, O., David F., and Leblon, G. (1990) Interspecies electro-transformation in corynebacteria. FEMS Microbiol. Lett. 66:263-270.

Bone, E.J. and Ellar, D.J. (1989) Transformation of *Bacillus thuringiensis* by electroporation. FEMS Microbiol. lett. 58:171-178.

Boulnois, G.J., Varley, J.M., Sharpe, G.S., and Francklin, F.C.H. (1985) Transposon donor plasmids based on Col Ib-P9 for use in *Pseudomonas putida* and a variety of other Gram-negative bacteria. Mol. Gen. Genet. 200:65-67

Brown, M.R.W. and Williams, P. (1985) The influence of environment on envelope properties affecting survival of bacteria in infections. Ann. Rev. Microbiol. 39:527-556

Brusseau, G.A., Tsien, H-G., Hanson, R.S., and Wackett, L.P. (1990) Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation 1:19-29

Buchanan-Wollaston, V., Passiatore, J.E., and Cannon, F. (1987) The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. Nature (London) 328:172-175

Burrows, K.J., Cornish, A., Scott, D., and Higgins, I.J.(1984) Substrate specificities of the soluble and particulate methane mono-oxygenases of *Methylosinus trichosporium* OB3b. J. Gen. Micro. 130:3327-3333

Byrom, D., (1984) Host-vector systems for *Methylophilus methylotrophus* in, Microbial Growth on C_1-compunds (R.L. Crawford and R.S. Hanson, eds.) American Society for Microbiology, Washington DC pp 221-223

Cadenas, R.F., Martin, J.F., and Gil, J.A.(1991) Construction and characterization of promoter-probe vectors for corynebacteria using the kanamycin-resistance reporter gene. Gene 98:117-121

Cannon, F.C., Riedel, G.E., and Ausubel, F.M. (1979) Overlapping sequences of *Klebsiella pneumoniae nif* DNA cloned and characterized. Mol. Gen. Genet. 174:59-66

Cardy, D.L.N. (1989) The molecular biology of ammonia assimilation in the obligate methane-oxidizing bacterium *Methylococcus capsulatus* (Bath), PhD Thesis, University of Warwick, Coventry, UK

Cardy, D.L.N. and Murrell, J.C. (1990) Cloning, sequencing and expression of the glutamine synthetase structural gene (*glnA*) from the obligate methanotroph *Methylococcus capsulatus* (Bath). J. Gen. Micro. 136:343-352

Cardy, D.L.N., Laidler, V. Salmond, G.P.C., and Murrell, J.C. (1991) Molecular analysis of the methane monooxygenase (MMO) gene cluster of *Methylosinus trichosporium* OB3b. Mol. Micro. 5(2):335-342

Chakrabarthy, A.M., Mylroie, J.R., Friello, D.A., and Vacca, J.G. (1975) Transformation of *Pseudomonas putida* and *Escherichia coli* with plasmid-linked drug resistance factor DNA. Proc. Natl. Acad. Sci. (USA) 72:3647-3651

Chassy, B.M., Mercenier, A., and Flickinger, J. (1988) Transformation of bacteria by electroporation. TIBTECH 6: 303-309.

Chistoserdov, A., Eremashvili, M., Mashko S., Lapidus, A., Skortsova, M., and Sterkin, V. (1987) Expression of human interferon F gene in obligate methylotroph *Methylobacillus flagellatum* KT and *Pseudomonas putida*. Mol. Genet. Microbiol. Virol. 8:36-42

Colby, J. and Zatman, L.J. (1973) Trimethylene metabolism in obligate and facultative methylotrophs. Biochem. J. 148:505-511

Colby, J. and Dalton, H. (1976) Some properties of a soluble methane monooxygenase from *Methylococcus capsulatus* strain Bath. Biochem. J. 157:495-497

Colby J. and Dalton H. (1979) Characterization of the second prosthetic group of the flavoenzyme NADH-acceptor reductase (component C) of the methane monooxygenase from *M.capsulatus* (Bath). Biochem. J. 177:903-908

Colby, J., Dalton, H., and Whittenbury, R. (1979) Biological and biochemical aspects of microbial growth on C_1 compounds. Ann. Rev. Microbiol. 33:481-517

Conchas, R.F. and Carniel, E. (1990) A highly efficient electroporation system for transformation of *Yersinia*. Gene 87:133-137.

Condon, C., FitzGerald, R.J., and O'Gara, F.(1991) Conjugation and heterologous gene expression in *Gluconobacter oxydans* ssp. *suboxydans*. FEMS Microbiol. Lett 80:173-178

Cruz-Rodz, A.L. and Gilmore, M.S. (1990) High efficiency introduction of plasmid DNA into glycine treated *Enterococcus faecalis* by electroporation. Mol. Gen. Genet. 224:152-154.

Curtiss, R., Caro, L.G., Allison, D.P., and Stallions, D.R. (1969) Early stages of conjugation in *Escherichia coli*. J. Bact. 100(2):1091-1104

Cymbalyuk, E.S., Chernomordik, L.V., Broude, N.E., and Chimadzhev, Y.A. (1988) Electro-stimulated transformation of *E.coli* cells pretreated by EDTA solution. FEBS Biochem. Lett. 234(1):203-207

Dalton, H and Whittenbury, R. (1976) The acetylene reduction technique as an assay for the nitrogenase activity in the methane oxidizing bacterium *Methylococcus capsulatus* (Bath). Arch. Microbiol. 109:147-151

Davies, S.L. and Whittenbury, R. (1970) Fine structure of methane and other hydrocarbon-utilizing bacteria. J. Gen. Micro. 61:227-232

DeBruijn, F.J., and Lupski, J.R. (1984) The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids - a review. Gene 27:131-149

Denarie, J., Rosenberg, C., Bergeron, B., Boucher, C., Michel, M., and De Bertalmer (1976) RP4: Mu plasmids: obtention, transfer by conjugation into *Klebsiella pneumoniae, Pseudomonas solanacearum* and *Rhizobium meliloti* and evaluation of potentials as a tool for *in vivo* genetic engineering of Gram-negative bacteria, in, DNA Insertions (Bukhari, A.J., Shapiro, J., and Adhya, S. eds.) Cold Spring harbor Laboratory N.Y.

Denhardt, D.T. (1966) A membrane filter technique for the detection of complimentary DNA. Biochem. Biophys. Res. Comm. 23:641-646

Desomer, J., Dhaese, P., and van Montagu, M. (1990) Transformation of *Rhodococcus fasciens* by high-voltage electroporation and development of *R.fasciens* cloning vectors. App. & Env. Micro. 56(9):2818-2825

Desomer, J., Crespi, M., and Van Montagu, M. (1991) Illegitimate integration of non-replicative vectors in the genome of *Rhodococcus fascians* upon electro-transformation as an insertional mutagenesis system. Mol. Micro. 5(9):2115-2124.

DeVries, G.E. (1986) Molecular biology of bacterial methanol oxidation. FEMS Microbial. Rev. 39:235-258

Dijkhuizen, L., Harder, W. de Boer, L., van Boven, A. Clement, W., Bron, S., and Venema, G. (1984) Genetic manipulation of the restricted facultative methylotroph *Hyphomicrobium* X by the R-plasmid-mediated introduction of the *Escherichia coli pdh* genes. Arch. Microbiol 139:311-318.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1980) Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci USA. 77:7347-7351

Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X-W., Finlay, D.R., Guiney D., and Helinski, D.R.(1985) Plasmid 13:149-153

Doronina, N.V., Govorukhina, N.I. Lysenko, A.M., and Trotsenko, Y.A.(1989) Analysis of DNA-DNA homology in obligately methylotrophic bacteria. Mikrobiologiya 57:629-633

Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988) High efficiency transformation of *E.coli* by high voltage electroporation. Nucleic Acids Research 13:6127-6144

Duine, J.A., Frank, J., and Dijkstra, M. (1982) Quinoproteins in the dissimilation of C_1 compounds in, The Biochemistry of the Methylotrophs (Anthony, C. ed.) pp.105-112

Dworkin, M. and Foster, J.W. (1956) Studies on *Pseudomonas methanica* (Sohgen) Nov. Comb. J. Bacteriol. 91:646-659

Eccleston, M. and Kelly, D.P.(1972) Competition among amino acids for incorporation into *Methylococcus capsulatus*. J. Gen. Micro. 72:303-314

Eden, P.A., and Blakemore, R. (1991) Electroporation and conjugal plasmid transfer to members of the genus *Aquaspirillum*. Arch. Microbiol. 155:449-452.

Ely, B. (1985) Vectors for transposon mutagenesis of non-enteric bacteria. Mol.Gen.Genet. 200:302-304

Fellay, R., Frey, J., and Krisch, H. (1988) Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of Gram-negative bacteria. Gene 52:147-154

Fellay, R., Krisch, H.M., Prentki, P., and Frey, J.(1989) Omegon-Km: a transposable element designed for *in vivo* insertional mutagenesis and cloning of genes in Gram-negative bacteria. Gene 76:215-226.

Fieldler, S. and Wirth, R. (1988) Transformation of bacteria with plasmid DNA by electroporation. Analytical Biochem. 170:38-44.

Figurski, D.H. and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. (USA) 76:1648-1652

Figurski, D.H., Young, C., Schreiner, H.C., Pohlman, R.F., Bechhofer, D.H., Prince, A.S., and D'Amico, T.F. (1985) Genetic interactions of broad-host range plasmid RK2: evidence for a complex replication regulon, in, Plasmids in Bacteria (Helinski, D.R., Cohen, S.N., Clewell, D.B., Jackson, D.A., and Hollaender A. eds.) Plenum Publishing Corp. New York pp 227-241

Foster, J.W. and Davis, R.H. (1966) A methane dependent coccus, with notes on classification and nomenclature of obligate, methane utilizing bacteria. J. Bact. 91:1924-1931

Fox, B.G., Froland, W.A. Dege, J.E., and Lipscomb, J.D.(1989) Methane monoxygenase from *Methylosinus trichosporium* OB3b. J. Biol. Chem. 264:10023-10033

Fox, B.G., Liu, Y. Dege, J.E.and Lipscomb, J.D. (1990) Complex formation between the protein components of methane monoxygenase from *Methylosinus trichosporium* OB3b. J. Biol. Chem. 266:540-550

Fox, B.G., Borneman, J.G., Wackett, L.P., and Lipscomb, J.D. (1990) Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry 29:6419-6427

Frey, J., Bagdasarian, M., Feiss, D., Franklin, F.C.H., and Deshusses, J.(1983) Stable cosmid vectors that enable the introduction of cloned fragments into a wide range of Gram-negative bacteria. Gene 24:299-308.

Frey, J. and Krisch, H.M. (1985) Omegon mutagenesis in Gram-negative bacteria: a selectable interposon which is strongly polar in a wide range of bacterial species. Gene 36:143-150

Frey, J. and Bagdasarian, M. (1989) The molecular biology of the IncQ plasmids in, Promiscuous Plasmids of Gram-negative Bacteria (Thomas, C., and Franklin, F.C.H., eds.) Academic Press, London pp. 79-94

Fulton, G.L., Nunn, D.N., and Lidstrom, M.E. (1984) Molecular cloning of a malyl coenzyme A lyase gene from *Pseudomonas* sp. strain AM1, a facultative methylotroph. J. Bact. 160(2):718-723.

Galchenko, V.F., Shishkina, V.N., Tyurin, V.S., and Trotsenko, Yu. A. (1975) Isolation of pure cultures of methanotrophic bacteria and their properties. Mikrobiologiya 44:844-850

Galchenko, V.F. (1977) New species of methanotrophic bacteria in, Microbial Growth on C_1-compounds (G.K. Skryabin *et al.*, eds.) Scientific Centre for Biological Research USSR Academy of Sciences, Pushchino p10

Galchenko, V.F. and Andreev, L.V. (1984) Taxonomy of obligate methanotrophs in microbial growth on C_1-compounds in Microbial Growth on C1-compounds (Crawford, R.A. and Hanson, R.S. eds.) American Society for Microbiology, Washington DC pp 221-223

Garfinkel, D.J., Simpson, R.B., Ream, L.W., White, F.F., Gordon, M.P., and Nester, E.W. (1981) Genetic analysis of crown gall: fine structure mapping of the T-DNA by site-directed mutagenesis. Cell 27:143-153

Gilchrist, A. and Smit, J. (1991) Transformation of freshwater and marine caulobacters by electroporation. J. Bact. 173 2:921-925.

Goncharoff, P., Saadi, S., Chang, C-H., Saltman, L.H., and Figurski, D.H.(1991) Structural, molecular, and genetic analysis of the *kil*A operon of broad-host-range plasmid RK2. J. Bacteriol. 173, 11 3463-3477.

Green, J. and Dalton, H. (1985) Protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). J. Biol. Chem. 260:15795-15801

Green, P.N. (1992) Taxonomy of methylotrophic bacteria in, Methane and Methanol Utilizers (Murrell, J.C. and Dalton, H., eds.) Plenum Publishing Corp. New York pp 23-85

Grunstein, M. and Hogness, D. (1975) Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. (USA) 72:3961-3965

Guerinot, M.L., Morisseau, B.A., and Klapatch, T. (1990) Electroporation of *Bradyrhizobium japonicum*. Mol. Gen. Genet. 221:287-290.

Guiney, D.G. and Lanka, E. (1989) Conjugative transfer of IncP plasmids in, Promiscuous Plasmids of Gram-negative Bacteria (Thomas, C.M., and Franklin, F.C.H., eds.) Academic Press, London pp. 27-56

Haas, D. and Holloway, B. W. (1976) R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol. Gen. Genet. 144:243-251

Haber, C.L., Allen, L.N., Zhao, S., and Hanson, R.S.(1983) Methylotrophic bacteria: biochemical diversity and genetics. Science 221:1147-1153.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166:557-580

Hanson, R.S. (1980) Ecology and diversity of methylotrophic organisms. Adv. Appl. Microbiol. 26:3-39

Haring, V., and Scherzinger, E. (1989) The replication proteins of IncQ plasmid RSF1010 in, Promiscuous Plasmids of Gram-negative Bacteria (Thomas, C., and Franklin, F.C.H., eds.) Academic Press, London pp. 95-124

Harms, N., de Vries, G.E., Maurer, K., Veltkamp, E., and Stouthamer, A.H. (1985) Isolation and characterization of *Paracoccus denitrificans* mutants with defects in the metabolism of one-carbon compounds. J. Bact. 164(3):1064-1070.

Harwood, J.H., Williams, E., and Bainbridge, B.W. (1972) Mutation of methane oxidizing bacterium *Methylococcus capsulatus*. J. Appl. Bact. 35(1):99-108.

Haselkorn, R. (1986) Organization of the genes for nitrogen fixation in photosynthetic bacteria and cyanobacteria. Ann. Rev. Microbiol. 40:525-547

Hatterman, D.R., and Stacey, G. (1990) Efficient DNA transformation of *Bradyrhizobium japonicum* by electroporation. App. & Env. Micro. 56(4):833-836.

Haynes, J.A. and Britz, M.L. (1990) The effect of growth conditions of *Corynebacterium glutamicum* on the transformation frequency obtained by electroporation. J. Gen. Micro. 136:255-263.

Heery, D.M., Powell, R. Gannon, F., and Dunican, L.K. (1989) Curing of a plasmid from *E.coli* using high-voltage electroporation. Nucleic Acids Research 17(23):10131

Heinemann, J.A. (1991) Genetics of gene transfer between species (review). Trends Genet. 7:181-185

Hennam, J.F., Cunningham, A.E., Sharpe, G.S., and Atherton, K.T. (1982) Expression of eukaryotic coding sequences in *Methylophilus methylotrophus*. Nature (London) 297:80-82.

Hennecke, H. (1981) Recombinant plasmids carrying nitrogen fixation genes from *Rhizobium japonicum*. Nature (London) 291:354-355

Hermans, J., Boschloo, J.G., and de Bont, J.A.M. (1990) Transformation of *Mycobacterium aurum* by electroporation: the use of glycine, lysozyme and isonicotinic acid hydrazide in enhancing transformation efficiency. FEMS Microbiol. lett. 72:221-224.

Higgins, I.J., Best, D.J., Hammond, R.C., and Scott, D. (1981) Methane-oxidizing microorganisms. Microbiological Reviews 45(4):556-590

Holmes, D.S. and Quigley, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114:193

Holo, H. and Nes, I.F. (1989) High-frequency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. App. & Env. Micro. 55(12):3119-3123.

Hou, C.T., Laskin, A.I., and Patel, R. (1978) Growth and polysaccharide production by *Methylocystis parvus* OBBP on methanol. Appl. & Env. Micro. 37:800-804

Houck, D., Hanners, J., Unkefer, C., Van Kleef, M., and Duine J. (1989) PQQ; Biosynthetic studies in *Methylobacterium* AM1 and *Hyphomicrobium X* using specific ^{13}C labelling and NMR. Ant. van Leeuw. 56:93-99

Itoh, Y. and Haas, D. (1985) Cloning vectors derived from the *Pseudomonas* plasmid pVS1. Gene 36:27-36

Jeyaseelan, K. and Guest, J.R. (1979) Transfer of antibiotic resistance to facultative methylotrophs with plasmid R68.45. FEMS Microbiol. Lett. 6:87-89

Jongen, W.M., Alink G.M., and Koeman J.H. (1978) Mutat. Res 56:245-248

Kado, C.I. and Liu, S.T. (1981) Rapid procedure for detection and isolation of large and small plasmids. J. Bact. 145(3):1365-1373

Kearney, P. and Holloway, B.W. (1987) Expression in *Escherichia coli* and *Pseudomonas aeruginosa* of *Methylophilus methylotrophus* AS1 genes carried on prime plasmids. FEMS Microbiol. Lett. 44:7-11

Keen, N.T., Tamaki, S., Kobayashi, D., and Trollinger, D.(1988) Improved broad-host range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191-197

Kim, Y.M. and Lidstrom, M.E. (1989) Plasmid analysis in pink facultative methylotrophic bacteria using a modified acetone-alkaline hydrolysis method. FEMS Microbiol. Letts. 60:125-130.

Kletsova, L.V., Chibisova, E.S., and Tsygankov, Y.D. (1988) Mutants of the obligate methylotroph *Methylobacillus flagellatum* KT defective in genes of the ribulose monophosphate cycle of formaldehyde fixation. Arch. Microbiol. 149:441-446.

Klug, G. and Drews, G. (1984) Construction of a gene bank of *Rhodopseudomonas capsulata* using a broad host range DNA cloning system. Arch. Microbiol. 139:319-325.

Knauf, V.C. and Nester, E.W. (1982). Wide host range cloning vectors: a cosmid clone bank of an *Agrobacterium* Ti plasmid. Plasmid 8:45-54.

Knight, D.E. and Scrutton, M.C. (1986) Gaining access to the cytosol: the technique and some applications of electropermeabilization. Biochem. J. 234:497-506.

Kues, U. and Stahl, U. (1989) Replication of plasmids in Gram-negative bacteria. Microbiol. Rev. 53(4):491-516

Kusaoke, H., Hayashi, Y. Kadowaki, Y., and Kimoto, H. (1989) Optimum conditions for electric pulse-mediated gene transfer to *Bacillus subtilis* cells. Agri. Biol. Chem. 53(9):2441-2446.

Lalonde, G., Miller, J.F., Tompkins, L.S., and O'Hanley, P. (1989) Transformation of *Actinobacillus pleuropneumoniae* and analysis of R factors by electroporation. Am. J. Vet. Res. 50(11):1957-1961.

Leak, D.J. and Dalton, H (1983) *In vivo* studies of primary alcohols, aldehydes and carboxylic acids as electron donors for the methane monooxygenase in a variety of methanotrophs. J. Gen. Micro. 129:3487-3497

Leemans, J., Langenakens, J., De Greve, H., Deblaere, R., Van Montagu, M., and Schell, J.(1982) Broad-host-range cloning vectors derived from the W-plasmid Sa. Gene 19:361-364.

Lejeune, P., Mergeay, M., Van Gijsegem, F., Faelen, M., Gerits, J., and Toussaint, A. (1983) Chromosome transfer and R-Prime plasmid formation mediated by plasmid pULB113 (RP4::Mini-Mu) in *Alcaligenes eutrophus* CH34 and *Pseudomonas fluorescens* 6.2. J. Bact. 155:1015-1026

Levering, P.R., Tiesma, L., Woldendorp, J.P., Steensma, M., and Dijkhuizen, L. (1987) Isolation and characterization of mutants of the facultative methylotroph *Arthrobacter* P1 blocked in one-carbon metabolism. Arch. Microbiol. 146:346-352.

Lidstrom-O'Connor, M.E., Fulton, G.L., and Wopat, A.E. (1983) *'Methylobacterium ethanolicum'*: a syntrophic association of two methylotrophic bacteria. J. Gen. Micro. 129:3139-3148.

Lidstrom, M.E. and Wopat, A.E. (1984) Plasmids in methanotrophic bacteria: isolation, characterization and DNA hybridization analysis. Arch. Microbiol. 140:27-33.

Lidstrom, M.E., Wopat, A.E., Nunn, D.N., and Toukdarian, A.E. (1984) Manipulation of methanotrophs in, Genetic Control of Environmental Pollutants (Omenn, G.S. and Hollaender, A., eds.), Plenum Publishing Corp. New York, pp273-279

Lidstrom, M., Nunn, D., Anderson, D., Stephens, R., and Haygood, M. (1987) Molecular biology of methanol oxidation, in Microbial Growth on C1 Compunds (H Van Verseveld and J. Duine, eds.), Nijhoff, Amsterdam, pp 246-254

Lidstrom, M.E. (1990) Genetics of carbon metabolism in methylotrophic bacteria. FEMS Micro. Reviews 87:431-436.

Lidstrom, M.E. (1992) The genetics and molecular biology of methanol-utilizing bacteria in, Methane and Methanol Utilizers (Murrell J.C., and Dalton H., eds.) Plenum Publishing Corp. pp 183-207

Luchansky, J.B., Muriana, P.M., and Klaenhammer, T.R. (1988) Application of electroporation for transfer of plasmid DNA to *Lactobacillus, Lactococcus, Leuconostoc, Listeria, Pediococcus, Bacillus, Staphylococcus, Enterococcus* and *Propionibacterium*. Mol. Micro. 2(5):637-646.

Lund, J., Woodland, M.P., and Dalton, H. (1985) Electron transfer reactions in the soluble methane monooxygenase of *Methylococcus capsulatus*(Bath). Eur. J. Biochem. 147:297-305

Lyon, B.R., Kearney, P.P., Sinclair, M.I., and Holloway, B.W. (1988) Comparative complementation mapping of *Methylophilus* spp. using cosmid gene libraries and prime plasmids. J. Gen. Micro. 134:123-132

Lynch, M.J., Wopat, A.E., and O'Connor, M.E. (1980) Characterization of two new facultative methanotrophs. Appl. Environ. Microbiol. 40:400-407

Machlin, S.M. and Hanson, R.S. (1988) Nucleotide sequence and transcriptional start site of the *Methylobacterium organophilum* XX methanol dehydrogenase structural gene. J. Bact. 170(10):4739-4747.

Machlin, S.M., Tam, P.E., Bastien, C.A., and Hanson, R.S. (1988) Genetic and physical analyses of *Methylobacterium organophilum* XX genes encoding methanol oxidation. J. Bact. 170(1):141-148

MacNeil, T., MacNeil, D., and Tyler, B. (1982) Fine structure deletion map and complementation analysis of the *glnA-glnL-glnG* region in *E.coli*. J. Bact. 150:1302-1313

Malashenko, Yu.R., Romanovskaya, V.A., and Kvasnikov, E.I. (1972) Taxonomy of bacteria utilizing gaseous hydrocarbons. Mikrobiologiya 49:871-879

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory

Marcus, H., Ketley, J.M., Kaper, J.B., and Holmes, R.K. (1990) Effects of DNase production, plasmid size, and restriction barriers on transformation of *Vibrio cholerae* by electroporation and osmotic shock. FEMS Microbiol. Lett. 68:149-154

Marmur, J. (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208-218

Marquardt, R., Wohner, G., and Winnacker, E.L. (1984) Properties of plasmids from *Methylomonas clara*. J. Biotechnology 1:317-330.

Mattanovich, D., Ruker, F., de Camara Machado, A., Laimer, M., Regner, F., Steinkeller, H., Himmler, G., and Katinger, H. (1989) Efficient transformation of *Agrobacterium* spp. by electroporation. J. Bact. 17(16):6747

Mazodier, P., Biville, F., Turlin, E., and Gasser, F. (1988) Localization of a pyrroloquinoline quinone biosynthesis gene near the methanol dehydrogenase structural gene in *Methylobacterium organophilum* DSM 760. J. Gen. Micro. 134:2513-2524.

Mazodier, P., Petter, R., and Thompson, C. (1989) Intergenic conjugation between *Escherichia coli* and *Streptomyces* species. J. Bact. 171(6):3583-3585

Mazur, B.J., Rice, D., and Haselkorn, R. (1980) Identification of blue-green algal nitrogen fixation genes by using heterologous DNA hybridization probes. Proc. Natl. Acad. Sci. USA 77:186-190

McIntyre, D.A. and Harlander, S.K. (1989) Improved electroporation efficiency of intact *Lactococcus lactis* subsp. *lactis* cells grown in defined media. App. & Env. Micro. 55(10):2621-2626.

McPheat, W.L., Mann, N.H., and Dalton, H. (1987a) Isolation of mutants of the obligate methanotroph *Methylomonas albus* defective in growth on methane. Arch. Microbiol. 148:40-43.

McPheat, W.L., Mann, N.H., and Dalton, H. (1987b) Transfer of broad host range plasmids to the type I obligate methanotroph *Methylomonas albus*. FEMS Microbiol. Lett. 41:185-188

Merrick, M.J. (1988) Regulation of nitrogen assimilation by bacteria in, SGM Symposium. 42, The Nitrogen and Sulphur Cycle (Cole, J.A. and Ferguson, S.J. eds.) Cambridge University Press pp 331-361

Mersereau, M., Pazour, G.J., and Das, A. (1990). Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene 90:149-151.

Metzler, T., Marquardt, R., Prave, P., and Winnacker, E.L. (1988) Characterization of a promoter from *Methylomonas clara*. Mol. Gen. Genet. 211:210-214.

Micheletti, P.A., Sment, K.A., and Konisky, J. (1991) Isolation of a co-enzyme M-auxotrophic mutant and transformation by electroporation in *Methanococcus voltae*. J.Bact. 173(11):3414-3418.

Miller, J.F., Dower, W.J., and Tompkins, L.S. (1988) High-voltage electroporation of bacteria: genetic transformation of *Campylobacter jejuni* with plasmid DNA. Proc. Natl. Acad. Sci. USA 85:856-860

Moore, A.T., Nayudu, M., and Holloway, B.W. (1983) Genetic mapping in *Methylophilus methylotrophus* AS1. J. Gen. Micro. 129:785-799.

Monteiro, M.J., Typas, M.A., Moffett, B.F., and Bainbridge, B.W. (1982) Isolation and characterization of a high molecular weight plasmid from the obligate methanol-utilizing bacterium *Methylomonas (Methanomonas) methylovora*. FEMS Microbiol. Lett. 15:235-237.

Mullens, I.A. and Dalton, H. (1987) Cloning of the gamma-subunit methane monoxygenase from *Methylococcus capsulatus*. Biotechnology 5:490-493.

Murrell, J.C. (1981) Nitrogen metabolism in the obligate methane-oxidizing bacteria, Ph.D Thesis, University of Warwick, Coventry, UK

Murrell, J.C. and Dalton, H. (1983) Nitrogen fixation in obligate methanotrophs. J. Gen. Micro. 129:3481-3486

Murrell, J.C. (1992) The genetics and molecular biology of obligate methane-oxidising bacteria in, Methane and Methanol Utilizers (Murrell, J.C., and Dalton, H., eds.) Plenum Publishing Corp. pp 115-149

Nesvera, J., Patek, M., Hochmannova, J., Chibisova, E. Serebrijski, I., Tsygankov, Y., and Netrusov, A. (1991) Transformation of a new Gram-positive methylotroph, *Brevibacterium methylicum*, by plasmid DNA. App. Micro. Biotech. 35:777-780

Nicolaidis, A.A. and Sargent, A.W. (1987) Isolation of methane monooxygenase deficient mutants from *Methylosinus trichosporium* OB3b using dichloromethane. FEMS Microbiol. Lett. 41:47-52.

Nunn, D.N. and Lidstrom, M.E. (1986a) Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. J. Bact. 166(2):581-590.

Nunn, D.N. and Lidstrom, M.E. (1986b) Phenotypic characterization of 10 methanol oxidation mutant classes in *Methylobacterium* sp. strain AM1. J. Bact. 166(2):591-597.

Nunn, D., and Anthony C. (1988a) The nucleotide sequence and deduced amino acid sequence of the genes for cytochrome cL and a hypothetical second subunit of the methanol dehydrogenase of *Methylobacterium* AM1. Nucl. Acid Res. 16:7722

Nunn, D., and Anthony, C. (1988b) The nucleotide sequence and deduced amino acid sequence of the cytochrome C_L gene of *Methylobacterium extorquens* AM1, a novel class of c-type cytochrome. Biochem. J. 256:673-676

Oakley, C.J and Murrell, J.C. (1988) *nif*H genes in the obligate methane oxidizing bacteria. FEMS Microbiol. Lett. 49:53-57.

Oakley, C.J. and Murrell, J.C. (1991) Cloning of nitrogenase structural genes from the obligate methanotroph *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. 78:121-126

O'Callaghan, D. and Charbit, A. (1990) High efficiency transformation of *Salmonella typhimurium* and *Salmonella typhi* by electroporation. Mol. Gen. Genet. 223:156-158.

Patel, R.W and Savas, J.C. (1987) Purification and properties of the hydroxylase component of methane monooxygenase. J. Bact. 169:2313-2317

Patt, T.E., Cole, G.C., Bland, J., and Hanson, R.S. (1974) Isolation of bacteria that grow on methane and organic compounds as sole sources of carbon and energy. J. Bact. 120:955-964

Pawlowski, K., Ratet, P., Schell, J., and de Bruijn, F.J. (1987) Cloning and characterization of *nifA* and *ntrC* genes of the stem nodulating bacterium ORS571, the nitrogen-fixing symbiont of *Sesbiana rostrata*. Mol. Gen. Genet. 206:207-216

Peteranderl, R., Schotts, E.B., and Wiegel, J. (1990) Stability of antibiotics under growth conditions for thermophilic anaerobes. App. Environ. Microbiol. 56:1981-1983

Pfau, J. and Youderian, P. (1990) Transferring plasmid DNA between different bacterial species with electroporation. Nucleic Acids Research 18(20):6165.

Pilkington, S.J., Salmond, G.P.C., Murrell, J.C., and Dalton, H. (1990) Identification of the gene encoding the regulatory protein B of soluble methane monooxygenase. FEMS Microbiol. Lett. 72:345-348

Prentki, P. and Krisch, H.M. (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene 29:303-313

Quayle, J.R. and Ferenci, T. (1978) Evolutionary aspects of autotrophy. Micro. Revs. 42(2):251-273

Ribbons, D.W. and Michalover, J.L. (1970) Methane oxidation by cell-free extracts of *Methylococcus capsulatus*. FEBS Biochem. Lett. 11:41-44

Romanovskaya, V.A., Malashenko, Yu.R, Grishchenko, N.I. (1980) Diagnosis of methane oxidizing bacteria by numerical methods based on cell fatty acid composition. Mikrobiologiya 49:969-975

Rousset, M., Dermoun, Z., Chippaux, M., and Belaich, J.P. (1991) Marker exchange mutagenesis of the *hydN* genes in *Desulfovibrio fructosovorans*. Mol. Micro. 5(7):1735-1740.

Rudd, J.W. and Taylor, C.D. (1980) Methane cycling in aquatic environments. Adv. Aquat. Microbiol. 2:77-150

Ruvkun, G.B. and Ausubel, F.M. (1981) A general method for site-directed mutagenesis in prokaryotes. Nature (London) 289:85-88.

Salisbury, S.A., Forrest, H.S., Cruise, W.D.T., and Kennard, O. (1979) A novel coenzyme from bacterial primary alcohol dehydrogenases. Nature (London) 280:843-844

Sasakewa, C and Berg, D.E. (1982) IS50 mediated inverse transposition: discrimination between the two ends of an IS element. J. Mol. Biol. 159:257-271

Schafer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A-H, and Puhler, A. (1990) High-frequency conjugal plasmid transfer from Gram-negative *Escherichia coli* to various Gram-positive coryneform bacteria. J. Bact. 172(3):1663-1666

Scholtz, R., Wackett, L.P., Cook, A.M., and Leisinger, T (1988) Dichloromethane dehalogenase with improved catalytic activity isolated from a fast-growing dichloromethane-utilizing bacterium. J. Bact. 170:5699-5704

Scholtz, P., Haring, V., Wittman-Liebold, B., Ashman, K., Bagdasarian, M., and Scherzinger, E. (1989) Complete nucleotide sequence and gene organization of the broad-host range plasmid RSF1010. Gene 75:271-288

Schwab, H., Saurugger, P.N., and Lafferty, R.M. (1983) Occurrence of deletion plasmids at high rates after conjugative transfer of the plasmids RP4 and RK2 from *Escherichia coli* to *Alcaligenes eutrophus* H16. Arch. Microbiol. 136:140-146.

Scott, P.T. and Rood, J.I. (1989) Electroporation-mediated transformation of lysostaphin-treated *Clostridium perfringens*. Gene 82:327-333

Serebrijski, I.G., Kazakova, S.M., and Tsygankov, Y.D. (1989) Construction of Hfr-like donors of the obligate methanol-oxidizing bacterium *Methylobacillus flagellatum* KT. FEMS Microbiol. Lett. 59:203-206.

Sharpe, G.S.,(1984). Broad host range cloning vectors for Gram-negative bacteria. Gene 29:93-102.

Shigekawa, E., and Dower, W.J. (1988) Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. Biotechniques 6:742-751

Shivarova, N., Forster, W., Jacob, H-E., and Grigorova, R. (1983) Microbiological implications of electric-field effects VII. Stimulation of plasmid transformation of *Bacillus cereus* protoplasts by electric-field pulses. Z Allg. Mikrobiol. 23:595-599

Simon, R., Priefer, U., and Puhler, A. (1983a) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Biotechnology 1: 784-791

Simon, R., Priefer, U., and Puhler, A.(1983b) Vector plasmids for *in-vivo* and *in-vitro* manipulations of Gram-negative bacteria in, The Molecular Genetics of the Bacteria-Plant Interaction (A. Puhler, ed.)

Simon, R., Quandt, J., and Klipp, W. (1989) New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. Gene 80:161-169

Smith, A.W. and Iglewski, B.H. (1989) Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Research 17(24):10509.

Smith, D.S. and Dalton, H.(1989) Solubilization of methane monoxygenase from *methylococcus capsulatus* (Bath). J. Biochem. 182:667-671

Smith C.J., Parker, A., and Rogers, M.B. (1990) Plasmid transformation of *Bacteroides* spp. by electroporation. Plasmid 24:100-109.

Sohngen, N.L. (1906) Uber Bakterien, welche mathen als kohlenstoffnafrung und energiequelle gebrauchen. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2, 15:513-517

Somkuti, G.A. and Steinberg, D.H. (1989) Electrotransformation of *Streptococcus sanguis* Challis. Curr. Micro. 19:91-95

Stainthorpe, A.C., Murrell, J.C., Salmond, G.P.C., Dalton H., and Lees, V. (1989) Molecular analysis of methane monoxygenase from *Methylococcus capsulatus* (Bath). Arch. Microbiol. 152:154-159.

Stainthorpe, A.C., Lees, V., Salmond, G.P.C., Dalton H., and Murrell, J.C. (1990) The methane monooxygenase gene cluster of *Methylococcus capsulatus* (Bath). Gene 91:27-34

Stanisch, V.A and Holloway, B.W. (1971) Chromosome transfer in *Pseudomonas aeruginosa* mediated by R factors. Genet. Res. 17:169-172

Stanley, S.H., Prior, S.D., Leak, D.J., and Dalton, H. (1983) Copper stress underlies the fundamental change in intracellular location of methane monooxygenase in methane-oxidising organisms: studies in batch and continuous cultures. Biotech. Lett. 5:487-492

Stephens, R.L., Haygood, M.G., and Lidstrom, M.E. (1988) Identification of putative methanol dehydrogenase (*moxF*) structural genes in methylotrophs and cloning of *moxF* genes from *Methylococcus capsulatus* (Bath) and *Methylomonas albus* BG8. J. Bact. 170(5):2063-2069.

Stirling, D.I. and Dalton, H.(1978) Purification and properties of an $NAD(P)^+$-linked formaldehyde dehydrogenase from *Methylococcus capsulatus* (Bath). J. Gen. Micro. 107:19-29

Stirling, D.I. and Dalton, H. (1979) Properties of the methane monooxygenase from *Methylosinus trichosporium* OB3b and evidence for its similarity to the enzyme from *M.capsulatus* (Bath). Eur. J. Biochem. 96:205-212

Stone, S. and Goodwin, P.M. (1989) Characterization and complementation of mutants of *Methylobacterium* AM1 which are defective in C-1 assimilation. J. Gen. Micro. 135:227-235.

Summers, D.K. and Withers, H.L. (1990) Electrotransfer: direct transfer of bacterial plasmid DNA by electroporation. Nucleic Acids Research 18(8):2192.

Szeto, W.W., Nixon, B.T., Ronson, C.W., and Ausubel, F.M. (1987) identification and characterization of the *Rhizobium meliloti ntrC* gene: *R.meliloti* has separate regulatory pathways for activation of nitrogen fixation genes in free-living and symbiotic cells. J. Bacteriol. 169:1423-1432

Tabor, S. and Richardson, C.C. (1985) A bacteriophage T7 RNA polymerase/promotor system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA 82:1074-1078

Tait, R.C., Close, T.J., Lundquist, R.C., Hagiya, M. Rodriguez, R.L., and Kado, C.I. (1983) Construction and characterization of a versatile broad-host-range DNA cloning system for Gram-nagative bacteria. Biotechnology 269-275.

Tatra, P.K. and Goodwin, P.M. (1983) R-plasmid mediated chromosome mobilization in the facultative methylotroph *Pseudomonas* AM1. J. Gen. Micro. 129:2629-2632.

Tatra, P.K. and Goodwin, P.M. (1985) Mapping of some genes involved in C-1 metabolism in the facultative methylotroph *Methylobacterium* sp. strain AM1 (*Pseudomonas* AM1). Arch. Microbiol. 143:169-177.

Taylor, S., Dalton, H., and Dow, C.S. (1980) Purification and initial characterization of ribulose 1 5-bisphosphate carboxylase from *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. 8:157-160

Thiel, T. and Poo, H. (1989) Transformation of a filamentous cyanobacterium by electroporation. J. Bact. 171(10):5743-5746.

Thomas, C.M and Smith, C.A. (1987) Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Ann. Rev. Microbiol. 41:77-101

Thomson, A.M. and Flint, H.J. (1989) Electroporation induced transformation of *Bacteroides ruminicola* and *Bacteroides uniformis* by plasmid DNA. FEMS Microbiol. Lett. 61:101-104.

Tikhonenko, A.S., Bespalova, I.A., Tyutikov, F.M., Martynkina, L.P., Galchenko, V.F., and Kriviskii, A.S. (1982) Lysogeny in methanotrophic bacteria. Mikrobiologiya 51:482-486

Tonge, G.M., Harrison, D.E.F., and Higgins, I.J. (1977) Purification and properties of the methane monooxygenase enzyme system from *Methylosinus trichosporium* OB3b. Biochem. J. 161:333-344

Toukdarian, A.E. and Lidstrom, M.E. (1984) Molecular construction and characterization of *nif* mutants of the obligate methanotroph *Methylosinus* sp. strain 6. J. Bact. 157(3):979-983.

Trevors, J.T. (1990) Electroporation and expression of plasmid pBR322 in *Klebsiella aerogenes* NCTC 418 and plasmid pRK2501 in *Pseudomonas putida* CYM 318. J. Basic Microbiol. 30(1):57-61.

Trieu-Cuot, P., Carlier, C., and Courvalin, P. (1988) Conjugative plasmid transfer from *Enterococcus faecalis* to *Escherichia coli*. J. Bact. 170(9):4388-4391

Tsuji, K., Tsien, H.C., Hanson, R.S., DePalma, S.R., Scholtz R., and LaRoche, S. (1990) 16S ribosomal RNA sequence analysis for determination of phylogenetic relationship among methylotrophs. J. Gen. Micro. 136:1-10.

Tsyganakov, Y.D. and Kazakova, S.M. (1987) Development of gene transfer systems in *Methylobacillus flagellatum* KT: Isolation of auxotrophic mutants. Arch. Microbiol. 149:112-119

Tsyganakov, Y.D., Kazakova S.M., and Serebrijski, I.G. (1990) Genetic mapping of the obligate methylotroph *Methylobacilluss flagellatum*: Characteristics of prime plasmids and mapping of the chromosome in time-of-entry units. J. Bact. 172(5):2747-2754

Tucker, W.T. and Pemberton, J.M. (1979) Conjugation and chromosome transfer in *Rhodopseudomonas sphaeroides* mediated by W and P group plasmids. FEMS Microbiol. Lett 5:173-176

Tyutikov, F.M., Bespalova, I.A., Rebentish, B.A., Aleksandrushkina, N.N., and Krivisky, A.S. (1980) Bacteriophages of Methanotrophic bacteria. J. Bact. 144(1):375-381

Ueda, S., Matsumoto, S., Shimizu S., and Yamane, T. (1991) Transformation of a methylotrophic bacterium, *Methylobacterium extorquens,* with a broad-host range plasmid by electroporation. App. Env. Micro. 57(4):924-926.

Urakami T. and Komagata, K. (1979) Cellular fatty acid composition and coenzyme Q system in Gram-negative methanol utilizing bacteria. J. Gen. Appl. Microbiol. 25:343-360

Van de Broeck, A., van Gool, A. and Vanderleyden, J. (1989) Electroporation of *Azospirillum brasilense* with plasmid DNA. FEMS Microbiol. Lett. 61:177-182.

Van Fliet, F., Siva, B., Van Montagu, M., and Schell, J. (1978) Transfer of RP4::Mu plasmids to *Agrobacterium tumefaciens*. Plasmid 1:446-455

Van Gijsegem, F. and Toussaint, A. (1982) Chromosome transfer and R-Prime Formation by an RP4::mini-Mu derivative in *Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae,* and *Proteus mirabilis*. Plasmid 7:30-44

Van Haute, E.H., Joo, M., Maes, G., Warner, M., Van Montagu, and Schell, J. (1983) Intergenic transfer and exchange recombination of restriction fragments cloned into pBR325: a novel strategy for the reversed genetics of the T_i plasmids of *Agrobacterium tumefaciens*. The EMBO Journal 2(3):411-417

Van Kleef, M.A.G., Dokter, P., Mulder, A.C., and Dulne, J.A. (1988) Detection of co-factor pyrrolquinoline quinone. Analytical Biochem. 162:143-149

Viljanen, P. and Boratynski, J.(1991) The susceptibility of conjugative resistance transfer in Gram-negative bacteria to physiochemical and biochemical agents. FEMS Micro. Rev. **88**:43-54

Waechter-Brulla, D. and Lidstrom, M.E. (1990) Localization of the putative promoter for the *moxF* gene of *Methylomonas* sp. A4 in, Abstracts of the Annual Meeting, American Society for Microbiology, Anaheim, CA, p 234

Walter, E.G., Thomas, C.M., Ibbotson, J.P., and Taylor, D.E. (1991) Transcriptional Analysis, Translational analysis, and sequence of the *kil*A-tellurite resistance region of plasmid RK2Te^{r}. J. Bact **173**(3):1111-1119.

Warner, P.J., Higgins, I.J., and Drozd, J.W. (1977) Examination of obligate and facultative methanotrophs for plasmid DNA. FEMS Microbiol. Lett. **1**:339-342

Warner, P.J., Higgins, I.J., and Drozd, J.W. (1980) Conjugative transfer of antibiotic resistance to methylotrophic bacteria. FEMS Microbiol. Lett. **7**:181-185

Whitta, S., Sinclair, M.I., and Holloway, B.W. (1985) Transposon mutagenesis in *Methylobacterium* AM1 (*Pseudomonas* AM1). J. Gen. Micro. **131**:1547-1551

Whittenbury, R. and Dalton, H. (1981) The methylotrophic bacteria in, The Prokaryotes. (Stolp *et al.*, eds.) Springer-Verlag, K.G. Berlin

Whittenbury, R. and Krieg, N.R. (1984) *Methylococcaceae* fam. nov. in Bergey's Manual of Determinative Bacteriology Vol. 1, Williams & Williams Baltimore pp 256-262

Whittenbury, R., Phillips, K.C., and Wilkinson, J.F. (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. J. Gen. Micro. **61**:205-218

Willetts, N. and Wilkins, B. (1984) Processing of plasmid DNA during bacterial conjugation. Microbiological reviews **48**(1):24-41

Williams, E. and Bainbridge, B.W. (1971) Genetic transformation in *Methylococcus capsulatus*. J. Appl. Bact. **34**(4):683-687.

Williams, E. and Bainbridge, B.W. (1976) Mutation repair mechanisms and transformation in the methane-utilizing bacterium *Methylococcus capsulatus* in, Genetics of Industrial Microorganisms (MacDonald, K.D., ed.) 2nd International Symposium, Academic Press, pp 313-321

Williams, E., Shimmin, M.A., and Bainbridge, B.W. (1977) Mutation in the obligate methylotrophs *Methylococcus capsulatus* and *Methylomonas albus*. FEMS Microbiol. Letts **2**:293-296.

Williams, E. and Shimmin, M.A. (1978) Radiation-induced filamentation in obligate methylotrophs. FEMS Microbiol. Lett. **4**:137-141

Windass, J.D., Worsey, M.J., Pioli, E.M., Pioli, D. Barth, P.T., Atherton, K.T., and Dart, E.C. (1980) Improved conversion of methanol to single-cell protein by *Methylophilus methylotrophus*. Nature (London) **287**:396-401.

Wirth, R., Friesenegger, A., and Fiedler, S.(1989) Transformation of various species of Gram-negative bacteria belonging to 11 different genera by electroporation. Mol. Gen. Genet. **216**:175-177.

Woodland, M.P. and Dalton, H. (1984) Purification and characterization of component A of the methane monooxygenase from *Methylococcus capsulatus* (Bath). J. Biol. Chem. **259**(1):53-59.

Wolf H., Puhler, A., and Neumann, E. (1989) Electrotransformation of intact and osmotically sensitive cells of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **30**:283-289.

Zealey, G., Dion, M., Loosmore, S. Yacoob, R., and Klein, M. (1988) High frequency transformation of *Bordetella* by electroporation. FEMS Microbiol. Lett. **56**:123-126.